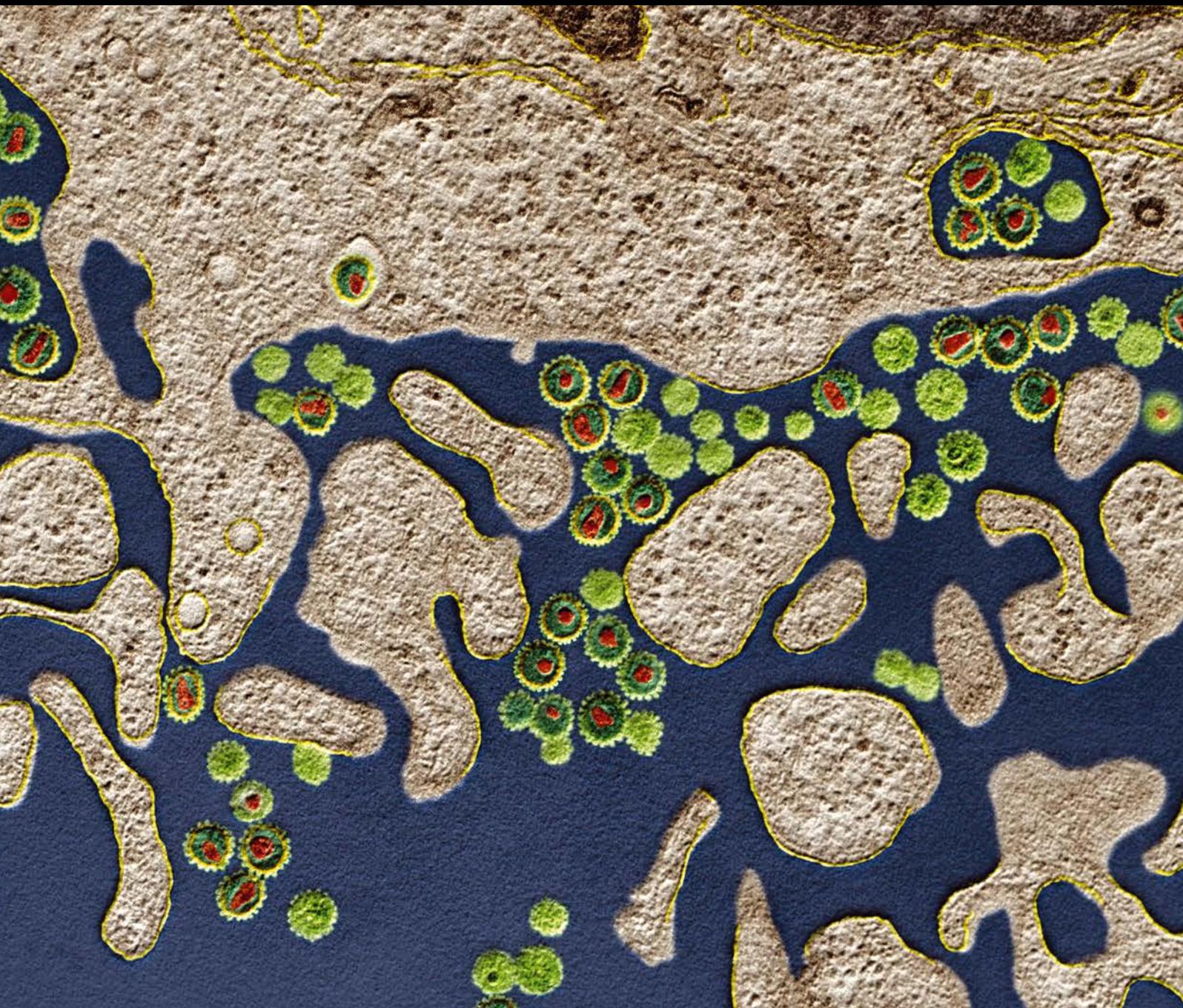


# Clinical and Experimental Immunomodulation 2016

Guest Editors: Lenin Pavón, Hugo Besedovsky, Oscar Bottasso, Marco A. Velasco-Velázquez, and Moisés E. Bauer





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**Clinical and Experimental  
Immunomodulation 2016**

Journal of Immunology Research

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Marco A. Velasco-Velázquez, and Moisés E. Bauer



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## Editorial

# Clinical and Experimental Immunomodulation 2016

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Inflammatory response (IR), which is crucial in injuries or infected anatomical regions, also generates systemic effects, regulating multiple physiological processes. Those effects depend on the concentration of soluble mediators like cytokines, chemokines, and other inflammatory molecules. For example, concentrations of soluble mediators around 10 nM are enough to induce a neuroendocrine response. The diverse systemic effects triggered by IR are plastic and continuously modified by fluctuations of circulatory levels of hormones, neurotransmitters, and mediators of inflammation. These feedback loops are possible by the constitutive expression of receptors for hormones, neurotransmitters, and cytokines on leukocytes, which modulate key cellular functions like proliferation, differentiation, and the secretion profile.

That is the reason whereby the constant research on clinical and experimental parameters that modulate is of great importance. This third special issue on clinical and experimental immunomodulation compiles a selection of high quality works on the field.

For the clinical immunomodulation research, we present the work of M. Żabińska et al., who analyzed quantitative changes of CD3+CD8+CD28-T cells and Foxp3 expression in patients with lupus nephritis. Flow cytometry data revealed increased CD3+CD8+CD28-T cells and a lack of Foxp3

expression on such cells, pointing out to a favoring role of these lymphocytes in the increased inflammation seen in active lupus patients. In a paper that focuses on the treatment of diseases in which TNF- $\alpha$  is pathological, M. P. Miranda-Hernández et al. compared the physicochemical and biological properties of a biosimilar etanercept along with its performance in rheumatoid arthritis (RA) patients. Their results showed a full correspondence of the primary and higher order structures between the biosimilar and the reference product. Despite being highly heterogeneous, both compounds exhibited no significant differences in the in vitro inhibition of apoptosis nor in the pharmacodynamic pattern seen in RA patients. I. Siloși et al. analyzed the serum concentrations of IL-13 and IL-17 in patients with early RA as well as the link of these cytokines with disease activity scores and levels of some autoantibodies. They found that high concentrations IL-13 and IL-17 correlate with disease activity. In the case of the groups with severe and moderate RA, the IL-13 concentrations were statistically higher than in the group of mild RA. Also, IL-17 concentrations increased proportionally with the disease activity. According to these findings, both cytokines have a great potential like RA biomarkers as well as for diagnostic applications.

W.-P. Lee et al. demonstrated that 1,25-dihydroxyvitamin D3 treatment in vitro of monocyte-derived DC results in

a semimature phenotype and anti-inflammatory cytokine profile as compared to conventional DC in both healthy controls and MS patients. Cryopreservation of DCs did not affect this profile. A. Gutiérrez-Hoya et al. demonstrate that G-CSF treatment in vivo induced pro- and anti-inflammatory T-cell profiles in healthy controls and in patients with graft-versus-host disease. In particular, they reported expansions of Tc1, Th1, Tc17, and CD8+IL-10+ T cells following treatment. S. Harm et al. tested different commercially available adsorbents for the effective removal of cytokines from plasma. This work has an important clinical significance in severe inflammation, sepsis, and septic shock, among other diseases with significant inflammatory component. Their report confirms the hypothesis that cytokine reduction in blood is required in order to reduce endothelial cell activation.

Finally, we must mention the interesting work by D. Ramírez-Ramírez et al., which shows that the production of specialized NK lymphoid lineages can be strengthened by dialyzable leukocyte extracts (DLE) from their early steps of differentiation. The study sheds some light on lymphopoiesis regulation by self-components and suggests that DLE may promote innate NK cell reconstitution during emergency conditions such as infection or malignant diseases.

In this issue we also present several works on experimental immunomodulation. The work by L. L. W. Ishikawa et al. demonstrated that pretreatment with several doses of proteoglycan (PG) partly protects BALB/c mice from the experimental arthritis induced by PG plus dimethyldioctadecyl ammonium bromide. Protection coincided with a lower production of IFN- $\gamma$  and IL-17 along with an increased release of IL-5 and IL-10 by PG-stimulated spleen cells. L. Zhang and C.-Q. Xia showed that transfer of UVB-treated immature DC from BALB/c induces tolerance in C3H mice. The underlying mechanisms were related to PD-1/PD-L1 interactions in tolerant mice and essential for controlling alloantigen-specific T cells. W. G. Bain et al. showed that macrophages from wild-type mice exposed to cigarette smoke for 5 weeks, followed by intratracheal instillation of *Pseudomonas aeruginosa* and 35–40% oxygen, exhibit improved survival and reduced lung CFUs and inflammation. Macrophages from these mice expressed less TNF- $\alpha$  and more scavenger receptors. This finding suggests that the protective role of low-dose oxygen may enhance macrophage phagocytosis.

In view of the influence of the major histocompatibility complex on the course of HBV infection, L. Wang et al. reviewed evidence that, in general terms, suggests that single nucleotide polymorphisms near certain HLA gene loci are linked to different infection outcomes as the spontaneous viral clearance or its persistence resulting in liver cirrhosis and hepatocellular carcinoma in chronic carriers as well as the efficacy of anti-HBV treatment and their specific vaccination. Since M1 and M2 macrophages may display antitumoral or protumoral activities, respectively, K. Chimal-Ramírez et al. performed a study wherein cell line-derived or primary monocytes were subjected to M1/M2 polarization procedures and to conditions for skewing monocytes to a protumoral function. Except for the fact that IDO enzyme and CD86 M1 markers correlated with M1 polarization,

protumor differentiation was not associated with a clear separation into M1 or M2 phenotypes. S. Cai et al. showed that low-dose *Lactobacillus rhamnosus* GG stimulates DC to induce greater Th1 polarization in T cells. This suggests an important immunomodulatory effect with a potentially relevant clinical effect, especially in antitumor therapy.

The study by R. Flores-Fernandez et al. demonstrated that PRL promotes self-reactivity by analyzing the effect of PRL in B-cell tolerance models employing WEHI-231 cells or immature B cells. PRL rescued WEHI-231 cells from cell death by decreasing the apoptosis induced by the cross-linking of the B-cell antigen receptor; a similar effect was found in immature B cells from lupus prone MRL/lpr mice. Y. Wang et al. analyzed the frequency and function of circulating T follicular helper (Tfh) cells in patients with psoriasis vulgaris as well as their presence in skin lesions. Results showed both increased frequency and activation of Tfh cells, correlating with disease severity as well as an increased presence of Tfh cells in affected skin. Given the role of lipopolysaccharide (LPS) in neurodegenerative diseases, Liu et al. carried out a study assessing the protective effects of epigallocatechin gallate (EGCG), the major component in green tea, on LPS-mediated inflammation and neurotoxicity. EGCG inhibited the LPS-mediated production of inflammatory cytokines by immune cells as well as the synthesis of reactive oxygen species in LPS-exposed neurons, highlighting the potential neuroprotective role of EGCG.

L. Paskova et al. compared the effects of a potential immunomodulator, natural polyphenol N-feruloylserotonin (N-f-5HT), with methotrexate (MTX), in the rat model of adjuvant-induced arthritis (AA). Both compounds reduced inflammation-associated surrogates and the transcription levels of TNF- $\alpha$  and iNOS in liver. Unlike MTX, N-f-5HT lowered IL-1 $\beta$  plasma levels and its mRNA expression in the liver and spleen of AA rats, implying a potential benefit of combined treatment with N-f-5HT and MTX in joint inflammation. M. Yuan et al. showed that CXCL1 serum levels arise in a lung cancer model induced by 3LL cell inoculation. Changes in CXCL1 contributed to tumor-associated neutrophils inhibition. In turn, this affects CD4+ and CD8+ T cells activation, allowing tumor growth. C. Chi et al. report that the compound “kaempferol 3-a-L-(4-O-acetyl) rhamnopyranoside-7-a-L-rhamnopyranoside (SA)” isolated from *D. crassirhizoma* has the capacity to stimulate the head kidney macrophages from the fish *Ctenopharyngodon idella*. This natural immunostimulant could be a potential substitute for antibiotics and chemicals in aquaculture practices.

In this issue we also present eight interesting reviews. J. S. Apostólico et al. compile, in a very interesting review, knowledge of the adjuvants commonly used in experimental and clinical settings with emphasis on their mechanisms of action. They also highlight the requirements for licensing new vaccine formulations. S. Xia et al. present a review about “inflamm-aging” in which they discuss studies of inflammation in old patients. The authors explore the concept of inflamm-aging with its pathological features and mechanisms. They also suggest therapeutic strategies that could be useful in Alzheimer’s disease, atherosclerosis, heart disease, type II diabetes, or cancer. W. Wan et al. review the

roles of HMGB1 in neuropathic pain. HMGB1 is an alarmin released by damaged tissues as well as by leukocytes. It may induce proinflammatory actions through binding in Toll-like receptors (TLRs) and RAGE and NMDA receptors; hence, it is considered as a therapeutic target for neuroinflammation.

R. Arreola et al. present an interesting review about dopamine (DA) features to modulate the immune response and generate changes in cellular phenotype and function. Leukocytes have the molecular machinery to synthesize and respond to this catecholamine but the expression of this machinery is dependent on the leukocyte type, the state of cellular activation, and the concentration and exposure time to DA. Some diseases present alterations in dopaminergic transmission in CNS and peripheral nervous system affecting the immune system modulation and causing the complications of the disease. In the review by E. A. Ivanova and A. N. Orekhov, the implications of abnormal monocyte activation and the acquisition of pro- or anti-inflammatory macrophage activities in disease states, like atherosclerosis or neoplasms, are discussed. Development of monocyte/macrophage activation tests may also be valuable in diagnostic or prognostic terms. I. L. Vladimirovna et al. review the properties and mechanisms whereby two distinct populations of immature cells, mesenchymal stem cells and myeloid derived suppressor cells, mediate immune regulation. The authors discuss cell similarities, discrepancies, and potential clinical applications.

Y. Zhou et al. contribute with an interesting meta-analysis about tolerogenic DCs and immunosuppressive (IS) therapies in multiple models of transplantation. They conclude that Toll-DC therapy significantly prolonged multiple allograft survival and further prolonged survival with IS. G. Hurtado-Alvarado et al. present a review about blood-brain barrier disruption observed in sleep-deprived rodents. The disruption could be associated with the increase of inflammatory mediators induced by sleep loss that are able to modify the expression of tight junction proteins in the brain microvasculature. The effect of these inflammatory molecules should be taken into account for the study of general consequences of sleep loss including the risk of developing neurological and neurodegenerative diseases.

## **Acknowledgments**

We thank all the authors of the contributions in this special issue. Lastly, we hope that our readers will find the present compilation enticing and enjoy its reading, as we have done.

*Lenin Pavón  
Hugo Besedosky  
Oscar Bottasso  
Marco A. Velasco-Velázquez  
Moisés E. Bauer*

## Research Article

# Role of CD8 Regulatory T Cells versus Tc1 and Tc17 Cells in the Development of Human Graft-versus-Host Disease

**Adriana Gutiérrez-Hoya,<sup>1,2,3</sup> Rubén López-Santiago,<sup>1</sup> Jorge Vela-Ojeda,<sup>4</sup> Laura Montiel-Cervantes,<sup>4</sup> Octavio Rodríguez-Cortés,<sup>5</sup> Víctor Rosales-García,<sup>6</sup> Vladimir Paredes-Cervantes,<sup>7</sup> Raúl Flores-Mejía,<sup>5</sup> Daniela Sandoval-Borrego,<sup>1</sup> and Martha Moreno-Lafont<sup>1</sup>**

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CD8<sup>+</sup> T cells that secrete proinflammatory cytokines play a central role in exacerbation of inflammation; however, a new subpopulation of CD8 regulatory T cells has recently been characterized. This study analyzes the prominent role of these different subpopulations in the development of graft-versus-host disease (GVHD). Samples from 8 healthy donors mobilized with Filgrastim® (G-CSF) and 18 patients who underwent allogeneic hematopoietic stem cell transplantation (HSCT) were evaluated by flow cytometry. Mobilization induced an increase in Tc1 ( $p < 0.01$ ), Th1 ( $p < 0.001$ ), Tc17 ( $p < 0.05$ ), and CD8<sup>+</sup>IL-10<sup>+</sup> cells ( $p < 0.05$ ), showing that G-CSF induces both pro- and anti-inflammatory profiles. Donor-patient correlation revealed a trend ( $p = 0.06$ ) toward the development of GVHD in patients who receive a high percentage of Tc1 cells. Patients with acute GVHD (aGVHD), either active or controlled, and patients without GVHD were evaluated; patients with active aGVHD had a higher percentage of Tc1 ( $p < 0.01$ ) and Tc17 ( $p < 0.05$ ) cells, as opposed to patients without GVHD in whom a higher percentage of CD8 Treg cells ( $p < 0.01$ ) was found. These findings indicate that the increase in Tc1 and Tc17 cells is associated with GVHD development, while regulatory CD8 T cells might have a protective role in this disease. These tests can be used to monitor and control GVHD.

## 1. Introduction

Graft-versus-host disease (GVHD) is one of the major causes of mortality after allogeneic hematopoietic stem cell transplantation (HSCT); it is induced by the inflammatory

immune response of donor cells against host tissues recognized as foreign. It is usually referred to as acute GVHD (aGVHD) when damage appears within the first 100 days after allogeneic HSCT and the main organs involved are the skin, liver, and gastrointestinal tract. The development of this

disease depends on diverse immunological characteristics of the patient and donor at the time of infusion [1–3].

A central aspect and a subject of evaluation in GVHD development is the role of cytokines. In this context, GVHD has been extensively associated with Th1-related cytokines (IFN $\gamma$ , IL-2, and IL-12) [4, 5] although these are not the only cytokines involved in inflammation. Recently, Th17-related cytokines (IL-17A and IL-17F) have been said to be prominent in solid organ rejection in murine models [6–9] and while their presence is not required for GVHD development, they contribute to exacerbation of this disease [8].

As a counterpart to inflammation and as part of homeostasis, a beneficial process known as immune regulation takes place. Research on this subject has focused on the study of regulatory T cells (Treg), in particular those that express the CD4<sup>+</sup>CD25<sup>high</sup>FoxP3<sup>+</sup> phenotype, which are able to control immune responses to alloantigens and are therefore potential targets for establishing tolerance in transplantation. This Treg subpopulation has been studied the most; however, other groups of cells with regulatory functions have been described, for example, the subsets CD8<sup>+</sup>,  $\gamma\delta$  T, NK, and NKT. This is the reason why many studies are now focusing on them in order to promote an immune tolerance status via the adoptive transfer of these cells [10, 11]. Within this context are CD8<sup>+</sup> Treg, initially described by Gershon and Kondo (1970) [12], the study of which was abandoned due to the lack of markers to characterize them and has recently been taken up in clinical studies that have established their role in diverse diseases such as experimental autoimmune encephalitis [13–15], colorectal cancer [16, 17], multiple myeloma [18, 19], multiple sclerosis [20], and ovarian carcinoma [21, 22]. These findings demonstrate the prominent immunosuppressive role of CD8<sup>+</sup> cells in control of autoimmunities and evasion of the immune response. These antecedents, together with CD8<sup>+</sup> Treg generation through continuous stimulation of the antigen [23] and involvement of these cells in GVHD control in murine models [24], denote the importance of the regulatory functions carried out by CD8<sup>+</sup> cells. Nevertheless, there are no studies on the role of CD8<sup>+</sup> Treg in GVHD development in humans, and findings regarding proinflammatory Tc17 cells are few and controversial.

The present study was designed to determine the utility of Tc1 and Tc17 cells, as opposed to CD8<sup>+</sup> Treg, as predictors of GVHD development and severity.

## 2. Materials and Methods

**2.1. Patients and Donors.** Eighteen human leukocyte antigen-(HLA-) identical sibling donors and their recipients with different hematologic disorders were studied. Half of the patients developed GVHD (55.5%). All individuals complied with the requirements to be included in the Stem Cell Transplantation Program at Centro Médico Nacional La Raza (IMSS-Mexico) and signed an informed consent before entering the study. The Hospital Ethical Committee approved the study, which was conducted according to the principles of the Declaration of Helsinki.

Blood samples were obtained on months 1, 2, 6, 9, and 12 after transplantations. All patients were clinically evaluated on a monthly basis for GVHD development.

Granulocyte-colony stimulating factor (G-CSF) (Filgrastim, Amgen-Roche, Thousand Oaks, CA) was subcutaneously administered to donors in daily doses of 16  $\mu$ g/kg for five days. Apheresis was performed on day 5 of G-CSF administration using a Cobe Spectra device. Patient characteristics are shown in Table 1. Patients with cytomegalovirus (CMV) infection were excluded from the study.

**2.2. Determination of Lymphocyte Subpopulations and Cytokines by Flow Cytometry.** Peripheral blood (PB) samples were obtained, stained for multiparametric flow cytometry, and fractionated in 4 Eppendorf tubes (A, B, C, and D) containing 500  $\mu$ L of blood each. The sample in tube A was unstimulated; tube B was stimulated with brefeldin A plus monensin; tube C was stimulated with 40 ng PMA plus 1  $\mu$ g ionomycin; tube D was stimulated with 40 ng of PMA and 1  $\mu$ g ionomycin plus brefeldin A and monensin. Samples were incubated at 37°C in a 5% CO<sub>2</sub> atmosphere for 6 h. The following antibodies were used for cell surface staining: mouse anti-human CD4 PE-*TexRed* or CD4 PerCP, CD8 PE-*TexRed*, CD8-PerCP, CD25-APC (BioLegend, San Diego, CA), CD8-FITC, CD8-PE, or CD3-PerCP (BD, San Jose, CA). Cells were incubated for 20 min at 4°C in staining buffer (PBS, 0.5% BSA, and 0.01% sodium azide). The cell suspensions were then washed, lysed, and permeabilized for intracellular staining with anti-human-TGF $\beta$ -FITC, Bcl-2-FITC, active caspase 3-FITC, Ki67-PE-Cy7, Ki67-PerCP, IFN $\gamma$ -APC, IL17-FITC, and IL17-PE-Cy7 (BioLegend). The tubes were incubated for 20 min at 4°C in the dark. Finally, the cells were washed, fixed with 1% paraformaldehyde, and analyzed in a flow cytometer (FACSARIA III, BD) (Figure 1).

**2.3. Statistical Analysis.** Differences between patients with and without GVHD were analyzed with a Kruskal-Wallis test followed by Dunn's multiple comparisons test, while differences between donors before and after administration of G-CSF were evaluated with a Mann-Whitney *U* test (GraphPad Prism v5.0). Kaplan-Meier curves were used to evaluate survival. *p* values <0.05 (\*), <0.01 (\*\*), and <0.001 (\*\*\*) were considered significant.

## 3. Results and Discussion

**3.1. G-CSF-Based Mobilization Induces Proinflammatory Cytokines but Not Cell Death.** To detect whether mobilization with G-CSF induces proinflammatory cytokines and/or cell death, the expression of IFN $\gamma$  and IL-17 as inflammation markers was determined, while Bcl-2 and active caspase-3 were used to evaluate the viability of CD4<sup>+</sup> and CD8<sup>+</sup> cells. Determinations were performed on two groups of healthy individuals: a control group (*n* = 6) and a group of G-CSF-mobilized donors (*n* = 8). Results show that mobilization induces an increase in the percentage of Th1 (*p*  $\leq$  0.001), Tc1 (*p*  $\leq$  0.01), and Tc17 (*p*  $\leq$  0.05) cells. There was a trend to increase in the percentage of Th17 cells (*p* = 0.08). It is worth noting that this increase was higher in type 1 cells.

TABLE 1: Patient characteristics.

Number of patient	Sex	Age (years)	Disease	Degree of GVHD
01	F	49	AML M2	I
02	F	32	NHL IIIB	I
03	F	19	MDS	IV
04	M	55	MDS	III
05	M	56	MDS	CMV
06	M	44	AA	0
07	M	35	AA	I
08	M	18	NHL	HSV
09	F	26	AA	I
10	M	39	AML M5	III
11	M	28	ALL L2	III
12	F	18	AML M5	IV
13	F	32	AA	0
14	F	37	AA	Multiple bacterial infections
15	M	44	AML M2	III
16	F	46	Biphenotypic leukemia	0
17	M	29	AA	0
18	M	44	AA	III

AA: aplastic anemia; AML: acute myeloid leukemia; MDS: hypoplastic myelodysplastic syndrome; NHL: non-Hodgkin lymphoma; ALL: acute lymphoblastic leukemia; GVHD: graft-versus-host disease; HSV: herpes simplex virus; CMV: cytomegalovirus.

To assess whether mobilization affects cell viability, active caspase-3 was determined as an apoptotic marker and Bcl-2 as an antiapoptotic marker. Results show that mobilization does not induce death on both CD4<sup>+</sup> and CD8<sup>+</sup> T cells (Figure 2).

GVHD is a process characterized by exacerbation of the inflammatory immune response and absence of immune regulation. In this context, CD8<sup>+</sup> cells play a fundamental role due to their rapid reconstitution after allogeneic HSCT, as well as the fact that they constitute the major population in the transplant recipients. For years these cells have been considered preeminently cytotoxic, having the capacity to secrete proinflammatory cytokines; however, recent data in murine models indicate the existence of a subpopulation of CD8<sup>+</sup> regulatory T cells with functional characteristics that may contribute to control of GVHD [25–27].

There is an evident need to evaluate the effect of mobilization on CD8<sup>+</sup> cells in donors and its correlation with GVHD development in patients, since prior reports indicate that G-CSF is able to induce diverse immunological profiles; for example, some investigators propose that mobilization promotes Th2 response while mitigating Th1 response [28, 29], while others report that mobilization in mice induces proinflammatory type 1 and type 17 cells [30]. Zhao et al., 2011, conclude that, in vivo human samples, G-CSF inhibits production of Th17 cells in bone marrow (BM) and PB grafts [31]. In our study a significant increase was observed in the percentage of proinflammatory Th1: CD4<sup>+</sup>IFN $\gamma$ <sup>+</sup> ( $p < 0.001$ ), Tc1: CD8<sup>+</sup>IFN $\gamma$ <sup>+</sup> ( $p < 0.01$ ), and Tc17: CD8<sup>+</sup>IL-17<sup>+</sup> ( $p < 0.05$ ) cells and this increase was not significant in Th17 cells ( $p = 0.08$ ). It is important to note that this increase was higher in Th1 and Tc1 cells (Figure 2). These results indicate

that mobilization with G-CSF induces proinflammatory cell types, and that this may affect patients who receive such cells.

*3.2. G-CSF-Based Mobilization Induces an Increase in the Percentage of Proliferating and IL-10-Positive CD8<sup>+</sup> Cells.* To find whether mobilization with G-CSF induces anti-inflammatory molecules and/or cell proliferation, the expression of the regulatory molecules TGF $\beta$ , IL-10, CD39, and CD73 was evaluated, and Ki-67 was determined in CD4<sup>+</sup> and CD8<sup>+</sup> cells from the control group ( $n = 6$ ) and the group of mobilized donors ( $n = 8$ ). Results indicate that mobilization induces an increase in the number of CD8<sup>+</sup>Ki-67<sup>+</sup> ( $p \leq 0.05$ ) and CD8<sup>+</sup>IL-10<sup>+</sup> ( $p \leq 0.01$ ) cells and that while the increase in CD8<sup>+</sup>CD73<sup>+</sup> cells is not significant, a trend was observed ( $p = 0.06$ ). However, no differences or tendencies were observed in regard to CD4<sup>+</sup> cells (Figure 3).

A significant increase was also observed in the percentage of CD8<sup>+</sup>IL-10<sup>+</sup> cells ( $p < 0.01$ ), indicating that a regulatory phenotype was induced (Figure 3). This is consistent with previous reports indicating that G-CSF induces an increase in the number of IL-10-positive cells, as well as other reports in which the number of CD4<sup>+</sup> Treg and some of their regulatory molecules also increased [32, 33]. It is worth noting that this increase was not induced in CD8<sup>+</sup>TGF $\beta$ <sup>+</sup> cells, a cytokine being widely recognized for its regulatory role [34]. Other molecules evaluated in the present study that are involved in immune regulation were CD39 and CD73 [35–37], but no significant differences were found. However, a tendency to increase was found in the percentage of CD8<sup>+</sup>CD73<sup>+</sup> cells ( $p = 0.06$ ) (Figure 3), which may indicate a Treg population since CD73 is a molecule which has been extensively described as a marker of CD4 Treg and is known

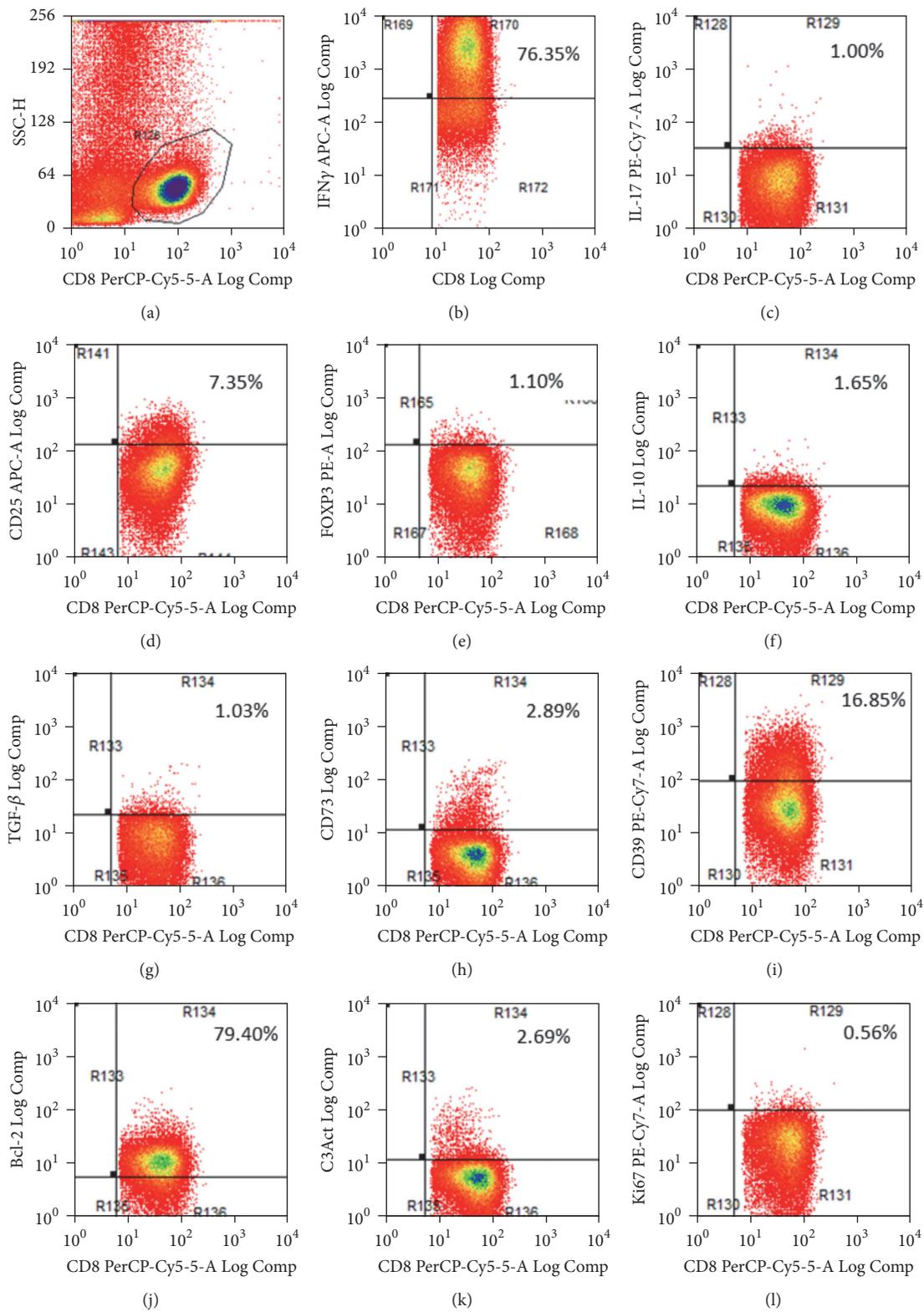


FIGURE 1: Characterization of CD8<sup>+</sup> cells by flow cytometry. (a) CD8<sup>+</sup> gating. (b)–(l) Determination of (b) IFN $\gamma$ ; (c) IL-17; (d) CD25; (e) FoxP3; (f) IL-10; (g) TGF $\beta$ ; (h) CD73; (i) CD39; (j) Bcl-2; (k) active caspase-3; and (l) Ki-67.

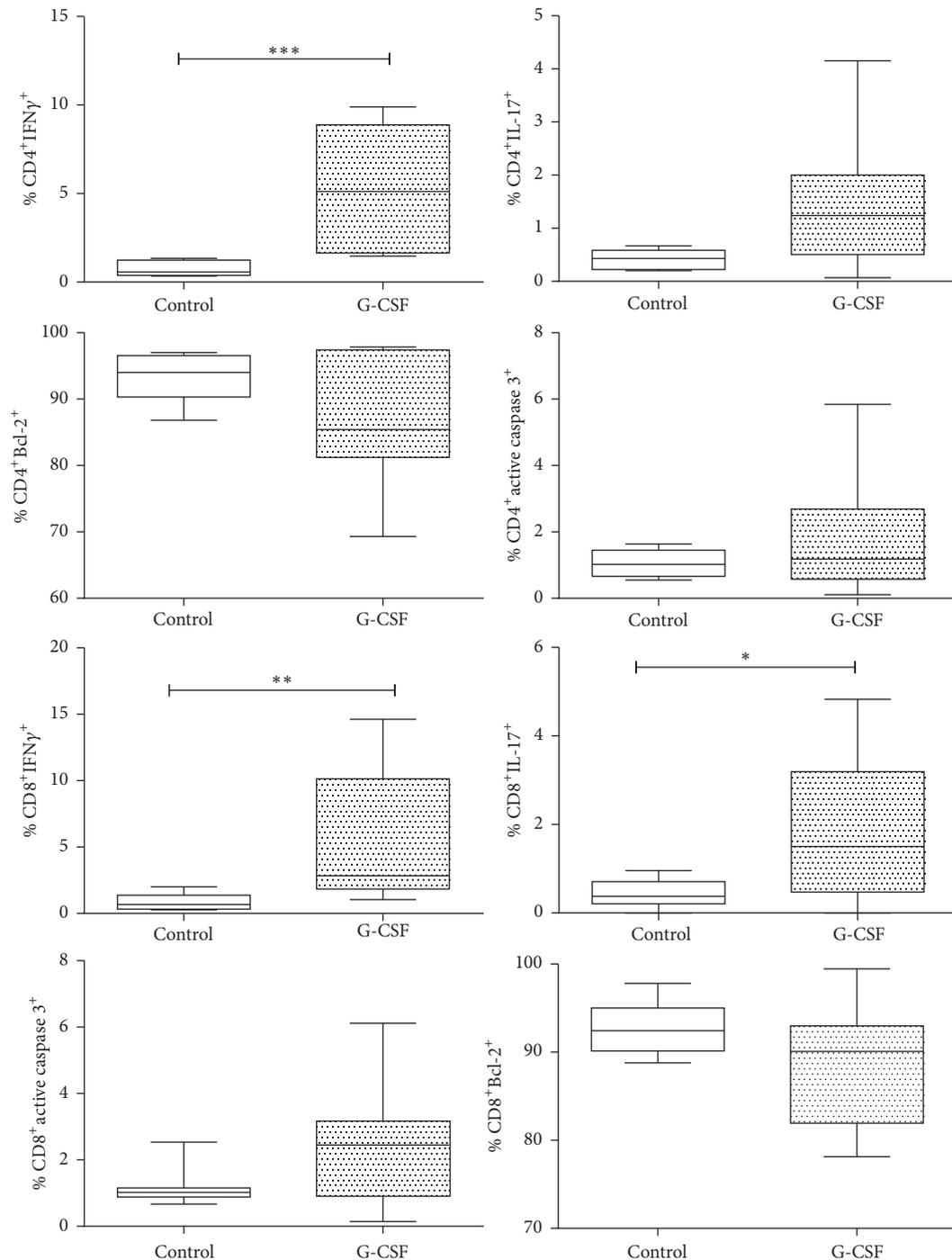


FIGURE 2: Cell viability and proinflammatory cytokines in healthy donors mobilized with G-CSF. Determination of IFN $\gamma$ , IL-17, Bcl-2, and active caspase-3 in peripheral blood CD4<sup>+</sup> and CD8<sup>+</sup> cells from a control group ( $n = 6$ ) and a group of G-CSF-mobilized donors ( $n = 8$ ). Box plots show population distribution and whiskers denote one standard deviation. A significant increase in the number of CD4<sup>+</sup>IFN $\gamma$ <sup>+</sup> (Th1), CD8<sup>+</sup>IFN $\gamma$ <sup>+</sup> (Tc1), and CD8<sup>+</sup>IL-17<sup>+</sup> (Tc17) cells is observed; this increase is not significant in CD4<sup>+</sup>IL-17<sup>+</sup> (Th17) cells, but a marked tendency is shown ( $p = 0.06$ ). \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ .

to carry out regulation via depletion of ATP in the medium in order to inhibit activation [35]. This piece of information is important since there are no reports of the effect induced by G-CSF on this molecule, and this indicates induction of a regulatory phenotype in response to mobilization.

**3.3. Donor-Patient Correlation.** Having seen that mobilization with G-CSF induces an increase in type 1 and type 17 cells, the next question was to determine whether the activation status of these cells influenced GVHD development in the patient. Two groups of patients, with and without

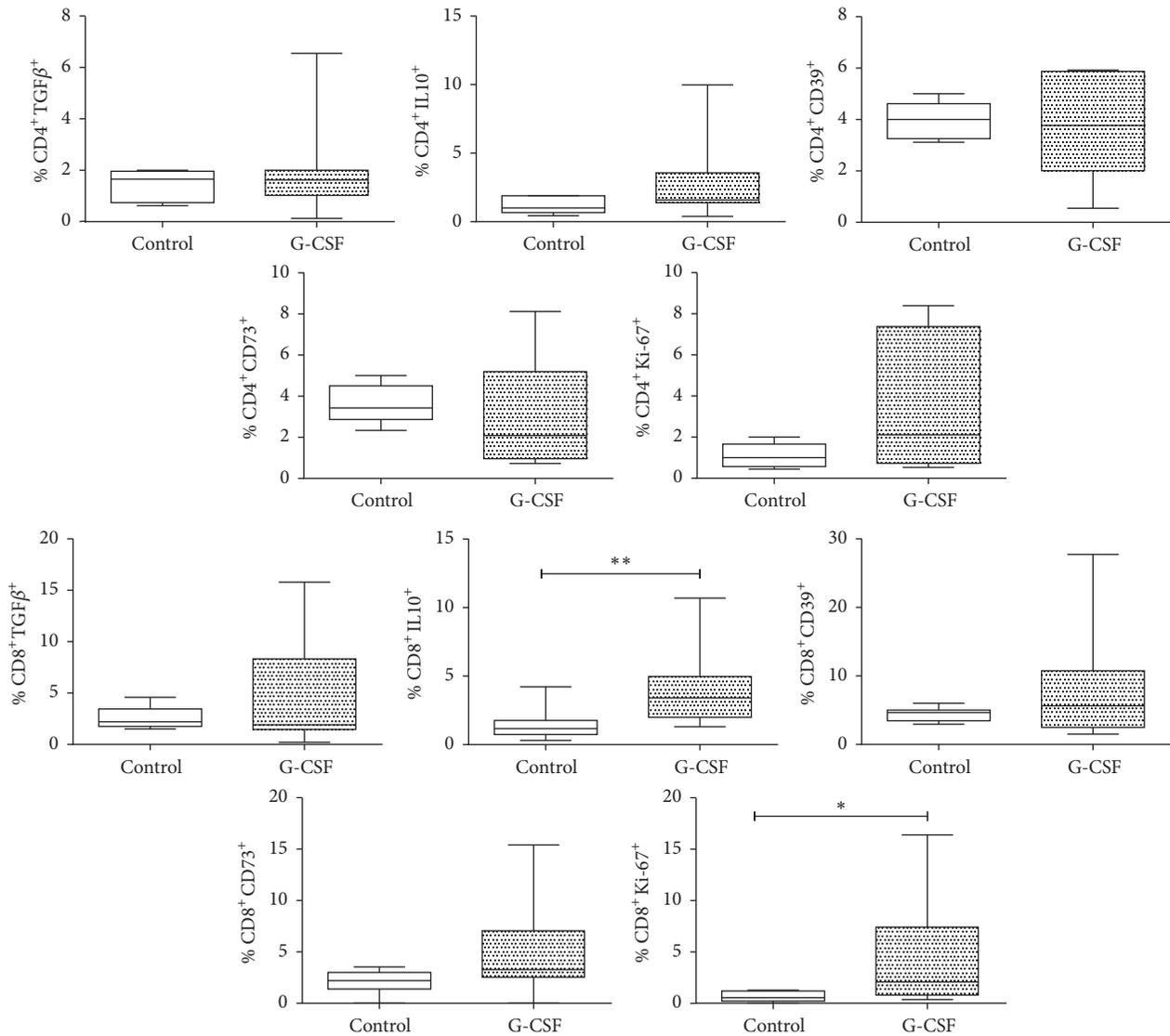


FIGURE 3: Cell viability and anti-inflammatory molecules in healthy donors mobilized with G-CSF. Determination of TGF $\beta$ , IL-10, CD39, CD73, and Ki-67 in peripheral blood CD4<sup>+</sup> and CD8<sup>+</sup> cells from a control group ( $n = 6$ ) and a group of G-CSF-mobilized donors ( $n = 8$ ). Box plots show population distribution and whiskers denote one standard deviation. A significant increase is observed in the number of CD8<sup>+</sup>IL-10<sup>+</sup> and CD8<sup>+</sup>Ki67<sup>+</sup> cells; the change in CD8<sup>+</sup>CD73<sup>+</sup> cells is not significant, but a marked tendency to increase is shown ( $p = 0.06$ ); no changes or tendencies are seen in marker expression in CD4<sup>+</sup> cells. \* $p < 0.05$ ; \*\* $p < 0.01$ .

GVHD, were tested for correlations with donor response to mobilization. The results obtained were not significant, but a trend ( $p = 0.06$ ) was observed in patients who receive a higher percentage of Th1 cells that are more susceptible to develop GVHD (Figure 4).

Knowing that G-CSF may polarize into proinflammatory and regulatory phenotypes and, even more notably, that type 1 and type 17 cells are induced, it was decided to examine the donor-patient correlation in order to determine if a link exists between the activation status of cells infused in the patient and GVHD development. Donor cell response was therefore tested for correlations with patients who developed aGVHD and those who did not. No significant differences were observed, but a tendency was noticed in the case

of CD8<sup>+</sup>IFN $\gamma$ <sup>+</sup> cells ( $p = 0.06$ ), suggesting that donors who respond to mobilization with a higher percentage of these cells are the donors of patients who develop aGVHD (Figure 4).

The following step was to follow up patients after allogeneic HSCT and to evaluate GVHD development. Three groups of patients were evaluated: patients with active GVHD, without GVHD, and with controlled GVHD. A wide panel of biomarkers was studied on CD8<sup>+</sup> cells of these patients, since this is one of the earlier cell populations to be reconstituted after allogeneic HSCT [38].

**3.4. CD8 Treg versus Tc1 and Tc17 Cells in Development and Severity of GVHD.** To evaluate the role of the cells

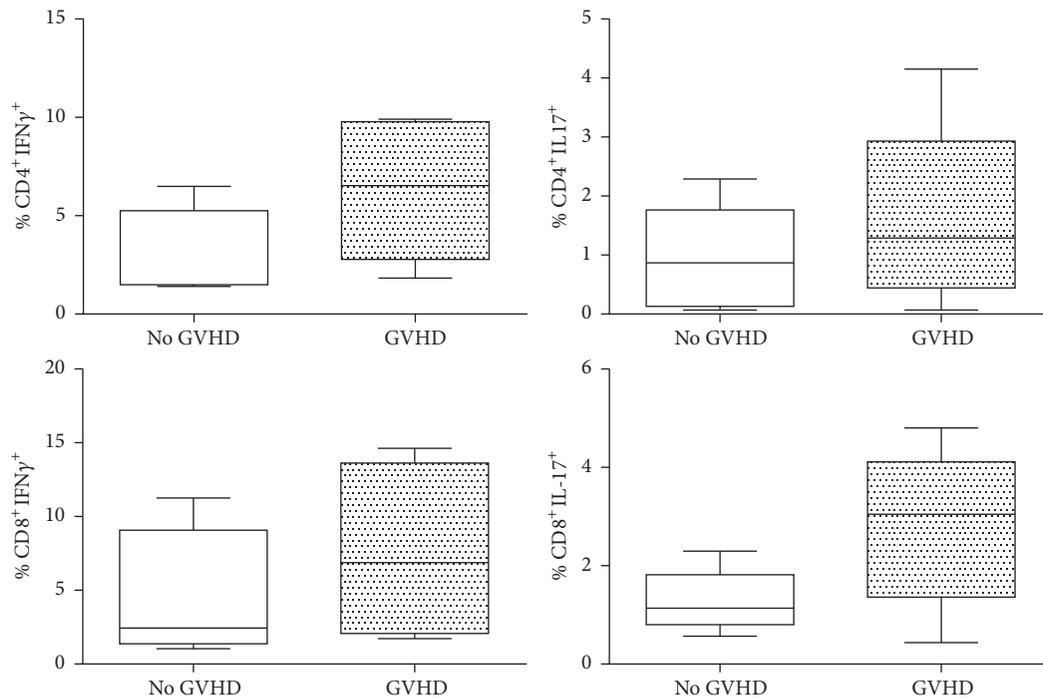


FIGURE 4: Correlation of patients with GVHD and without GVHD and percentage of type 1 and type 17 cells in the corresponding donor. No significant differences are seen, but a correlation ( $p = 0.06$ ) exists, which indicates that patients who develop GVHD are patients who received a higher percentage of Th1 cells ( $CD4^+IFN\gamma^+$ ).

that express proinflammatory or regulatory phenotypes in GVHD development, regulation-related markers ( $TGF\beta$ , IL-10, CD39, CD73, and FoxP3) and inflammation markers ( $IFN\gamma$ , IL-17, and CD25) were evaluated in patients without GVHD, as well as patients with grade III or IV GVHD during the active phase of the disease and once symptoms were controlled (controlled GVHD). Eighteen patients entered the study group; however, patients with CMV, aspergillus, and/or herpes zoster virus (HZV) infection were excluded. A higher percentage of unstimulated and stimulated Tc1 cells was observed in patients with active GVHD ( $p \leq 0.01$  and  $p \leq 0.05$ , resp.), compared to those without GVHD (Figure 5). On the other hand, Tc17 cells increased in patients with active and controlled GVHD, compared to patients without GVHD, but this increase was evidenced only after polyclonal activation with PMA and ionomycin. These results suggest that Tc1 and Tc17 cells play a prominent role in GVHD. A further aspect evaluated was the involvement of CD8 Treg in GVHD development. To this end, cells described as regulatory in other pathologies, that is,  $CD8^+CD39^+$ ,  $CD8^+TGF\beta^+$ ,  $CD8^+IL-10^+$ ,  $CD8^+CD25^+$ ,  $CD8^+CD73^+$ , and  $CD8^+FoxP3^+$  cells, were evaluated. No significant differences or tendencies were observed in the first four of these subsets. On the other hand, the number of  $CD8^+FoxP3^+$  cells increased in patients without GVHD compared to patients with active or controlled GVHD ( $p \leq 0.01$  and  $p \leq 0.05$ , resp.). Interestingly, other cells involved in regulation (i.e.,  $CD8^+CD39^+$ ) showed a tendency to increase in patients without GVHD, compared to patients with either active or controlled GVHD (Figure 5).

To evaluate induction of proinflammatory cytokines,  $IFN\gamma$  and IL-17 were determined. An increase in the percentage of Tc1 cells was found in patients with active GVHD compared to patients without GVHD, these differences occurred at basal levels and in response to polyclonal stimulation ( $p < 0.01$  and  $p < 0.05$ , resp.), their impact being greater at basal levels (Figure 5). This is consistent with diverse reports in human and murine models in which an increase in Th1 cells in PB and higher serum levels of  $IFN\gamma$  are evidenced in patients with GVHD [4, 5, 39, 40]. The role of Tc17 cells was also evaluated since these cells have recently been proposed to be implicated in inflammation and were linked to GVHD development and severity; however, results in human and murine models are contradictory [41–43]. In the present study, an increase in the percentage of Tc17 cells was found in patients with active GVHD compared to patients without GVHD ( $p < 0.05$ ). This is in agreement with data reported in murine models [8]; however, it is important to note that this increase was also found in patients with controlled GVHD compared to those without GVHD ( $p < 0.05$ ) and that in both cases the increase was evidenced only after polyclonal stimulation; this was unexpected. However, the fact that it was seen only after polyclonal activation suggests that once the disease is controlled, these cells do not receive damage signals that induce them to migrate to target organs and therefore are consequently found in PB. It should be made clear that these cells are preferentially found in the mucosae. Moreover, the group of Zhao et al. [44] found that the differentiation of cytokine-producing Tc1 and Tc17 cells may be the key step in the initiation of GVHD, whereas Th1 and Th17 cells are

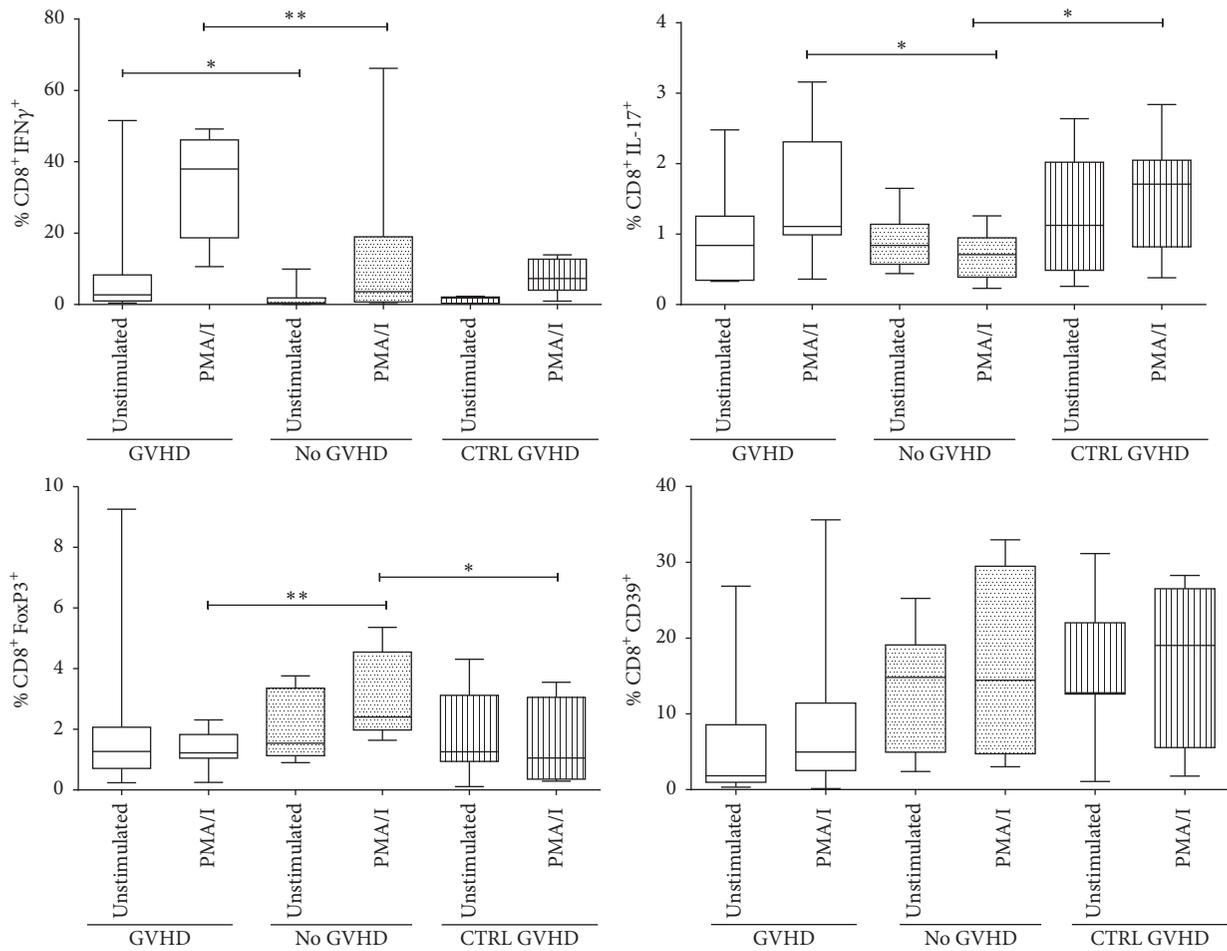


FIGURE 5: Determination of Tc1, Tc17, and CD8<sup>+</sup> Treg in patients without GVHD, with active GVHD, and with controlled GVHD. Patients with active GVHD show an increase in Tc1 and Tc17 cells, while patients without GVHD display an increase in CD8<sup>+</sup>FoxP3<sup>+</sup> cells compared to patients with active or controlled GVHD. \*  $p < 0.05$ ; \*\*  $p < 0.01$ .

considered to be a pathophysiological factor leading to the continuous aggravation of GVHD this antecedent support our findings.

The following aspect to be evaluated was regulatory molecules expression. A wide range of regulation-related markers was determined, revealing the prominent role of CD8 Treg. A decrease in the percentage of these cells occurred in patients with active GVHD compared to those without GVHD ( $p < 0.01$ ); a similar finding was observed in patients with controlled GVHD but with a lower level of significance ( $p < 0.05$ ), probably indicating reconstitution of this cell subpopulation (Figure 5). It is worth noting that there are no data on the role of these cells in GVHD development in humans; the only prior report is in murine models by Beres et al., 2012 [25], reporting this subpopulation as a possible regulator of GVHD development. On the other hand, Zheng et al. [45] found in a model of GVHD in humanized mice how the allogeneic-specific CD8 Treg controlled the development of GVHD in an allospecific manner by reducing alloreactive T cell proliferation, decreasing inflammatory cytokines as IFN $\gamma$ , IL-17, TNF $\alpha$ , IL-6, IL-2, and IL-1 $\beta$  as

well as chemokine secretion through a CTLA-4 dependent mechanism. Other reports on the role of CD8<sup>+</sup> Treg in malignant tumors associate this subset of Treg cells with poor prognosis as well as with the severity in multiple sclerosis [16, 21, 22, 46]. Other regulatory molecules were also evaluated such as TGF $\beta$ , IL-10, and CD73 but no significant differences were observed (data not shown), while a tendency to decrease was seen in the percentage of CD8<sup>+</sup>CD39<sup>+</sup> cells in patients with active GVHD, compared to those without GVHD. This may imply that this subset of T cells plays a prominent role in GVHD development. As far as today, the only reports are available in murine models and show that blocking CD73+ cells potentiates GVHD development [47], while another study shows that Treg from CD73 KO mice are less effective than WT Treg in suppression of GVHD [48]. However, there is no information in regard to the role of CD39 in GVHD development, and it was therefore decided to analyze these molecules. No significant differences were found, although a tendency to increase is observed in the percentage of CD8<sup>+</sup>CD39<sup>+</sup> cells in patients with active GVHD.



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## Competing Interests

The authors declare that they have no competing interests.

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## Research Article

# Early Differentiation of Human CD11c<sup>+</sup>NK Cells with $\gamma\delta$ T Cell Activation Properties Is Promoted by Dialyzable Leukocyte Extracts

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Reconstitution of the hematopoietic system during immune responses and immunological and neoplastic diseases or upon transplantation depends on the emergent differentiation of hematopoietic stem/progenitor cells within the bone marrow. Although in the last decade the use of dialyzable leukocyte extracts (DLE) as supportive therapy in both infectious and malignant settings has increased, its activity on the earliest stages of human hematopoietic development remains poorly understood. Here, we have examined the ability of DLE to promote replenishment of functional lymphoid lineages from CD34<sup>+</sup> cells. Our findings suggest that DLE increases their differentiation toward a conspicuous CD56<sup>+</sup>CD16<sup>+</sup>CD11c<sup>+</sup> NK-like cell population endowed with properties such as IFN $\gamma$  production, tumor cell cytotoxicity, and the capability of inducing  $\gamma\delta$  T lymphocyte proliferation. Of note, long-term coculture controlled systems showed the bystander effect of DLE-stromal cells by providing NK progenitors with signals to overproduce this cell subset. Thus, by direct effect on progenitor cells and through activation and remodeling of the supporting hematopoietic microenvironment, DLE may contribute a robust innate immune response by promoting the emerging lymphopoiesis of functional CD11c<sup>+</sup> NK cells in a partially TLR-related manner. Unraveling the identity and mechanisms of the involved DLE components may be fundamental to advance the NK cell-based therapy field.

## 1. Introduction

Emergency hematopoiesis defines the production of functional hematopoietic cells under nonhomeostatic,

proinflammatory, or biologically stressed conditions [1–4]. Blood cell production is a tightly regulated process that, after birth and throughout life, starts in a conspicuous hematopoietic stem cell (HSC) subset residing within the

bone marrow (BM). Our current understanding of how HSC early differentiation is governed by the microenvironment indicates that, besides the stromal cell components of the various hematopoietic niches, not only essential growth and differentiation factors but also microbes and their products can influence differentiation fate decisions [3, 5, 6]. Of note, emergency hematopoiesis is regulated at the stem and progenitor cell (HSPC) level, where conditions such as infection demand the expedited production and activation of innate immune cells to combat noxious extrinsic agents, and the resulting proinflammatory conditions can at the time regulate the earliest steps of the hematopoietic development in favor of the clearance of insulting cues and to further maintain homeostasis [1].

We have previously shown that pathogens and damaged tissue products and proinflammatory cytokines promote emergency hematopoiesis and alter patterns of early lymphoid differentiation in mouse and human [3–5, 7–10]. In mice, pathogen recognition through Toll like receptors (TLR) and the resulting cytokine release induce the expansion of HSC and instruct lineage differentiation fates so immediate innate cell development is guaranteed [6, 7]. In general, ligation of TLR2 and TLR4 on these seminal cells promotes redirection toward myeloid cell production, while the sole TLR9 stimulation of primitive common lymphoid progenitors (CLP) strikingly induces B cell differentiation blockage while development of dendritic cells (DC), plasmacytoid dendritic cells (pDC), and NK-related interferon killer dendritic cells (IKDC) is substantially enforced [5, 8]. In humans, most findings relate to strengthening of myeloid lineage cell production under emergent scenarios, whereas adjustments within the lymphoid branch of the hematopoiesis have been poorly addressed [2, 6]. According to what mouse research has shown, human multilymphoid progenitors (MLP) are capable of responding to TLR stimulation by producing dendritic cells, and our recent work suggests that primitive early lymphoid progenitor populations are also capable of microbial components discrimination through TLR, a mechanism that mostly facilitates their differentiation to innate lymphoid lineage cells. Of special interest, TLR9 ligation on adult BM progenitors promotes the quick development of NK lineage cells by using mechanisms that involve IL-15R upregulation [4, 9].

Thus, innate immune quick responses against viral threatening infections start in earlier developmental stages than we previously thought. Whether the actual TLR-emergent hematopoiesis contributes to innate immunity under pathological conditions and other biological stress settings, including malignant diseases, is a highly relevant topic under investigation [11]. Interestingly, not only conventional pathogen associated molecular patterns (PAMPs) but also the damage associated molecular patterns- (DAMPs-) like molecules can trigger innate immune sensors and PRR signals, including microRNAs, histones, fibronectin, and bacterial second messengers like di-GMP (reviewed in [3, 12]). Even though efficient therapeutic agents have been developed that improve infectious and malignant disease outcomes and increase the overall survival rates, the adjuvant effect of molecules capable of remodeling hematopoietic pathways should be taken into

consideration to change the prognosis of diseases. Thus, the possibility of having extensive means of replenishing innate cells opens additional venues for receptor-ligand axes of clinical significance.

Disruption of normal peripheral blood leukocytes results in the release of heterogeneous mixtures of peptides, among other complex molecules. Upon dialysis, the enriched mixture of low-molecular-weight polar and hydrophilic peptides (<10 kDa), named dialyzable leukocyte extracts (DLE), has shown a number of therapeutic and adjuvant properties through modulation of immune responses [13, 14]. Although the precise molecular mechanisms underlying its positive experimental and clinical effects are currently unknown, critical signaling pathways for survival and cellular activation states, including Toll like receptor (TLR) and NF $\kappa$ B, are apparently involved and often trigger the production of proinflammatory cytokines [14–18]. Of note, a recent investigation using *in vitro* controlled models of TLR-mediated proinflammation suggested the content of TLR-2 agonist ligands within DLE [15]. The exposure of human peripheral mononuclear cells to DLE induced the copious secretion of TNF $\alpha$  by the monocyte fraction. Whether this phenomenon is due to DAMPs or DAMP-like related peptides within DLE is still a matter in question.

Using an elegant mouse model of experimental tuberculosis, Fabre and colleagues demonstrated that the administration of DLE (formerly denominated transfer factor) evokes an efficient reconstitution of the cell-mediated immunity, concomitant with a substantial production of IFN $\alpha$ , IL-2, and iNOS, an immune protective profile provoking inhibition of bacterial proliferation [19, 20].

In contrast, inflammatory injuries of human ocular tissues where limbal epithelial stem cells giving rise to corneal epithelium are compromised have been shown to respond to DLE treatment by downregulating the secretion of IL-8 and IL-6 [18]. The same is true for atopic dermatitis, where DLE contribute the decrease of inflammatory cells and the severity of the disease [21]. Then, despite the proinflammatory potential induction of DLE, the net balance—induction or suppression—may depend on additional biological settings of the damaged tissues, where the dose of DLE would be absolutely crucial to get a beneficial result [20].

Resolution of herpes virus infections is remarkably benefited from the adjuvant effect of DLE treatment [13, 16]. In fact, a herpes murine model has become a powerful biological assay to test the functional activity of DLE [22]. Of special interest, a direct effect on viral replication or infected target cell viability could not be recorded. Instead, its protective effects correlated with serum cytokine levels and, most likely, with changes in the cellular immune system. Accordingly, as cell-mediated immunity plays a central role in controlling viral-infected and tumor cells, and the capability of DLE of strengthening the cellular immunity has been suggested from several studies, DLE is considered as a presumptive instrument with adjuvant potential for treating virally induced cancer [13]. Moreover, a growing list of viral, parasitic, or fungal infections, as well as acute and chronic diseases,

including immunodeficiencies, malignancies, allergies, and autoimmune disorders, seems to favorably respond to DLE.

However, a more comprehensive understanding of its biological mechanisms is yet needed and will be benefited from less heterogeneous and highly controlled extracts. DLE Transferon, a blood product licensed for clinical use, has been shown to exhibit batch-to-batch reproducibility and relatively high homogeneity when ultra-performance liquid chromatography (SE-UPLC) is used to characterize its content [14]. Due to the putative TLR agonist elements within DLE and their biological capability of inducing proinflammatory microenvironments, here we sought to examine the DLE's contribution to innate immune replenishment through emergent hematopoiesis. By using *in vitro* functional assays and controlled early differentiation culture systems, our findings define a powerful route to promote development of a unique CD11c<sup>+</sup> NK cell subset with immune-surveillance capacity.

## 2. Materials and Methods

**2.1. Sample Collection and Progenitor Cells Isolation.** Umbilical cord blood (UCB) samples were obtained from normal full-term neonates upon mothers' written informed consent, while adult bone marrow (ABM) was collected from healthy adult donors who entered orthopedic surgery and according to institutional guidelines. Mononuclear cells (MNCs) were prepared by Ficoll-Paque Plus (GE Healthcare Bioscience) gradient centrifugation and preserved at  $-80^{\circ}\text{C}$  until use. All procedures were approved by the Ethics and Scientific Committee of Health Research at IMSS (R-2006-3602-16).

**2.2. Isolation of Cell Populations and Flow Cytometry.** CD34<sup>+</sup> cells from ABM and UCB were enriched using the Human CD34 Progenitor Cell Isolation Kit (Miltenyi Biotec) according to manufacturer instructions and our previous reports [9]. After staining with PE-conjugated antineurolineage markers (CD3, CD8, TCR, CD56, CD14, CD11b, CD20, CD19, and CD235a), anti-CD34-APC, anti-CD38-FITC, and anti-CD45RA-PE-TxR conjugated antibodies, primitive cell populations were highly purified by multicolor flow cytometry using a FACSAria sorter (BD Biosciences). Hematopoietic stem cells (HSC) were separated as Lin<sup>-</sup>CD34<sup>+</sup>CD38<sup>-</sup>CD45RA<sup>-</sup>, while multipotent progenitors (MPP) were sorted as Lin<sup>-</sup>CD34<sup>+</sup>CD38<sup>+</sup>CD45RA<sup>-</sup>, and early lymphoid progenitors (ELP) as Lin<sup>-</sup>CD34<sup>+</sup>CD38<sup>+</sup>CD45RA<sup>+</sup>, as described [9]. Upon harvesting from culture, anti-CD56-PE, anti-CD11c-FITC, and anti-CD16-APC (BD Biosciences) were used to evaluate innate cell production in lymphoid lineage conditions. NK cells were identified by flow cytometry in a FACSCanto II equipment (BD Biosciences) as CD56<sup>+</sup>CD11c<sup>+</sup> or CD56<sup>+</sup>CD16<sup>+</sup>CD11c<sup>+</sup> cells, while dendritic cells (DC) were detected as CD56<sup>-</sup>CD11c<sup>+</sup>. Analysis of flow cytometry data was performed using the FlowJo 10 software (TreeStar Inc., USA).

**2.3. Dialyzable Leukocyte Extracts (DLE Transferon) Stimulation.** DLE Transferon is manufactured by UDIMEB at

GMP facilities in the National School of Biological Sciences, National Polytechnic Institute (IPN), as described [14, 15]. Transferon is registered by Mexican Health Authorities as a drug and commercialized nationally. Briefly, DLE were prepared from packed blood peripheral leukocytes from healthy donors. Cells were disrupted by 5 cycles of freezing and thawing followed by dialysis against water using Spectra/Pore membranes with a "cut-off" of 12 kDa (Spectrum Labs, USA). The quality control of Transferon comprised (A) endotoxin content, quantified using the Endosafe-Portable Test (Charles River Laboratories, Charleston, SC, USA) according to the manufacturer's instructions (the specification for endotoxin was established in Mexican Pharmacopeia, Section MGA-0316 ( $\leq 4.0$  EU/mL)); (B) microbiological tests, according to Mexican Pharmacopeia, Section MGA-0571; and (C) physicochemical characterization by a validated ultra-performance liquid chromatography (UPLC) method that analyzes molecular weights and the time of retention of the main peaks compared with those of an internal batch pattern. Peptide content per final dose was measured by bicinchoninic acid (BCA) method using the Pierce BCA kit (Thermo Fisher Scientific, Waltham MA, USA) according to the manufacturer's instructions [22]. Batch-to-batch reproducibility is consistently analyzed by SE-UPLC chromatographic profiles, while IFN $\gamma$  production from DLE-stimulated Jurkat cells is quantified as a biological activity test [14].

Enriched CD34<sup>+</sup> cells from UCB and ABM were cultured for 24 hours in  $\alpha$ -MEM medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin. DLE Transferon was used for cell culture stimulation at 5  $\mu\text{g}/\text{mL}$ .

**2.4. Stromal Cell Cocultures.** MS-5 stromal cells were grown in presence or not of DLE Transferon 24 hours before coculturing with CD34<sup>+</sup> HSPC. On the other hand, and upon 24 hours of DLE prestimulation, HSPC (including the whole enriched CD34<sup>+</sup> fraction or Lin<sup>-</sup>CD34<sup>+</sup>CD38<sup>-</sup>CD45RA<sup>-</sup> HSC, Lin<sup>-</sup>CD34<sup>+</sup>CD38<sup>+</sup>CD45RA<sup>-</sup> MPP, or Lin<sup>-</sup>CD34<sup>+</sup>CD38<sup>+</sup>CD45RA<sup>+</sup> ELP) were centrifuged to remove the medium and cocultured in the presence of MS-5 stromal cells for 30 more days. Cocultures were performed using  $\alpha$ -modified essential medium ( $\alpha$ -MEM) (GIBCO) supplemented with 10% fetal bovine serum and 100 U/mL penicillin and 100 mg/mL streptomycin. Lymphoid lineage cytokines and growth factors were contained throughout coculture: 1 ng/mL Flt3-L (FL), 2 ng/mL SCF, 5 ng/mL IL-7, and 10 ng/mL IL-15 (Preprotech). Coculture systems were incubated at  $37^{\circ}\text{C}$  in a humidified atmosphere of 5% CO<sub>2</sub>.

**2.5. BrdU Incorporation Assay.** Enriched CD34<sup>+</sup> cells from UCB and ABM were cultured for 72 hours with 5  $\mu\text{g}/\text{mL}$  of DLE Transferon and bromo-2'-deoxyuridine (BrdU) to a final concentration of 10  $\mu\text{M}$ , in  $\alpha$ -MEM serum-free medium supplemented with lymphoid cytokines. After stimulation, cells were stained for the identification of CD34<sup>+</sup> cell progenitors by multicolor flow cytometry followed by intracellular staining with a monoclonal antibody to BrdU according to an established protocol (BrdU flow kit, BD Biosciences).

Cells were analyzed on a FACSCanto II flow cytometer (BD Biosciences).

**2.6. Inhibition of TLR Signaling Pathway.** UCB CD34<sup>+</sup> cells were pretreated with 20  $\mu$ M of MyD88 control or inhibitory peptides (Imgenex IMG-200501) for two hours, followed by 2x washing and staining with 5  $\mu$ M Cell Trace Violet (CTV) dye. DLE Transferon was included or not at 5  $\mu$ g/mL in a 72 h culture. Harvested cells were analyzed by flow cytometry for cell division numbers. The dilution of fluorescence intensity was estimated using the application for cell proliferation from the FlowJo 7.6.2 software.

To investigate the influence of TLR signals in the DLE-mediated NK-like cell differentiation, UCB CD34<sup>+</sup> cells were pretreated with 20  $\mu$ M of MyD88 control or inhibitory peptides (Imgenex IMG-200501) for 24 hours, followed by 2x washing and coculturing on MS-5 stromal cells for 15 more days. Cocultures were performed using  $\alpha$ -modified essential medium ( $\alpha$ -MEM) (GIBCO) supplemented with 10% fetal bovine serum and 100 U/mL penicillin and 100 mg/mL streptomycin (as described above in Section 2.4).

**2.7. Cytotoxicity Assay.** Natural killer cytolytic activity was evaluated using a fluorescence-based assay, as described [9, 23]. This assay uses the dye carboxyfluorescein succinimidyl ester (CFSE) to distinguish target from effector cells, and the DNA intercalating dye 7-aminoactinomycin D (7-AAD) (BD Pharmingen) for dead/live cells distinction. Briefly, the myeloid leukemia K562 cell line (ATCC) was kept in log phase growth in RPMI 1640 + GlutaMAX supplemented with 10% of fetal calf serum (FCS). K562 cells were stained with 5  $\mu$ M carboxyfluorescein succinimidyl ester (CFSE). *In vitro* differentiated NK cells from DLE-cultures were enumerated by flow cytometry and cocultured with CFSE-labeled K562 cells, according to an effector: target ratio curve. A relation of 10 CD56<sup>+</sup> cells per each K562 cell was used. IL-2 (40 ng/mL) (Preprotech) was added to induce NK cell activation, followed by 4 hours of incubation at 37°C. Cells were washed and 7-AAD (BD Pharmingen) was incorporated into the cell suspension. Multiparametric flow cytometry (FacsCanto II, BD Biosciences) was conducted to determine functionality.

**2.8. Intracellular Detection of IFN-Gamma.** To investigate the capability of DLE-newly derived NK-like cells of producing IFN-gamma (IFN $\gamma$ ) upon stimulation, an IFN $\gamma$  assay was performed. After 30-day coculture, the produced cells were stimulated with IL-12 (10 ng/mL) and IL-18 (50 ng/mL) for 12 hours. NK-like cells were harvested and Brefeldin A (BFA, 10  $\mu$ g/mL) was added for 4 hours to inhibit protein intracellular transport. After BFA blockage, cells were carefully washed, followed by incubation with anti-CD56-APC, anti-CD11c-FITC (BD Biosciences), and anti-IFN $\gamma$ -PE (Biolegend) conjugated antibodies to perform extra- and intracellular staining, respectively. A FACSCanto II equipment was used for multiparametric flow cytometric analysis.

**2.9. Proliferation Induction Assay of  $\gamma\delta$  T Lymphocytes.** Gam-madelta T peripheral blood lymphocytes (TCR $\gamma\delta$ -1) from healthy donors were enriched by the  $\gamma\delta$  T Lymphocyte Isolation Kit (MiltenyiBiotec, IgG1 clone 11F2) according to manufacturer instructions, followed by staining with 5  $\mu$ M Cell Trace Violet (CTV). Simultaneously, newly differentiated CD11c<sup>+</sup>CD56<sup>+</sup> NK cells from DLE-stimulated 30-day cocultures were harvested and purified by flow cytometry sorting in BD FACSaria equipment. Postsort cell purity was investigated by using the anti-human TCR  $\gamma/\delta$  mouse IgG1 antibody clone B1. The purified  $\gamma\delta$  T cells were then cocultured with the NK cell population of interest, at a ratio of 2:1 CD56<sup>+</sup>CD11c<sup>+</sup> NK :  $\gamma\delta$  T lymphocyte. Supplemented  $\alpha$ -MEM medium with 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin was used.  $\gamma\delta$  T cells proliferation was assessed at 72 hours by dilution of CTV (within the Pacific Blue channel).

**2.10. Statistics.** The Prism software, version 5.01 (GraphPad, USA), was used for statistical analysis. Comparisons between groups were performed with either the unpaired *t*-test or the one-way ANOVA. *P* values were 2-tailed and considered significant if less than 0.05.

### 3. Results and Discussion

**3.1. Human Hematopoietic Stem/Progenitors Cells Are Promoted by Dialyzable Leukocyte Extracts (DLE) to Differentiate into CD11c<sup>+</sup> NK-Like Cells.** Our previous research on mouse and human early hematopoietic fate redirection in response to extrinsic PRR agonists prompted us to investigate whether this phenomenon may occur upon exposure to DLE Transferon. To first determine sensitivity of primitive populations to DLE components, we performed BrdU incorporation analyses on CD34<sup>+</sup> cell fractions from cord blood and adult bone marrow. Notably, we recorded significant increases of proliferative cell frequencies from both sources after 72 h of DLE stimulation (Figure 1). Furthermore, bone marrow cell stimulation under lymphoid or myeloid culture conditions revealed that adult lymphoid progenitors preferentially expand in response to DLE (Supplemental Figure 1 in Supplementary Material available online at <http://dx.doi.org/10.1155/2016/4097642>).

Natural Killer (NK) cells constitute a granular innate immune cell type comprising 3–20% of the human peripheral blood mononuclear cell (PBMC) lymphocyte fraction, which function as major players in innate responses by producing cytokines and chemokines and exerting cytolytic activity against virus-infected or tumor cells [24–26]. Here, DLE stimulation of CB CD34<sup>+</sup> cells rapidly influenced early cell fate decisions and accelerated the differentiation toward a special population of CD56<sup>+</sup>CD11c<sup>+</sup> NK cell (Figure 2). Although final yields per input progenitor were not indicative of higher net production when the full CD34 compartment is considered, substantially discrepant CD56<sup>high</sup>CD11c<sup>+</sup> cell frequencies suggest the speeding up of such specialized cells (Figures 2(a) and 2(b)). In line with the observations in the context of TLR signaling [9], cells harboring early lymphoid

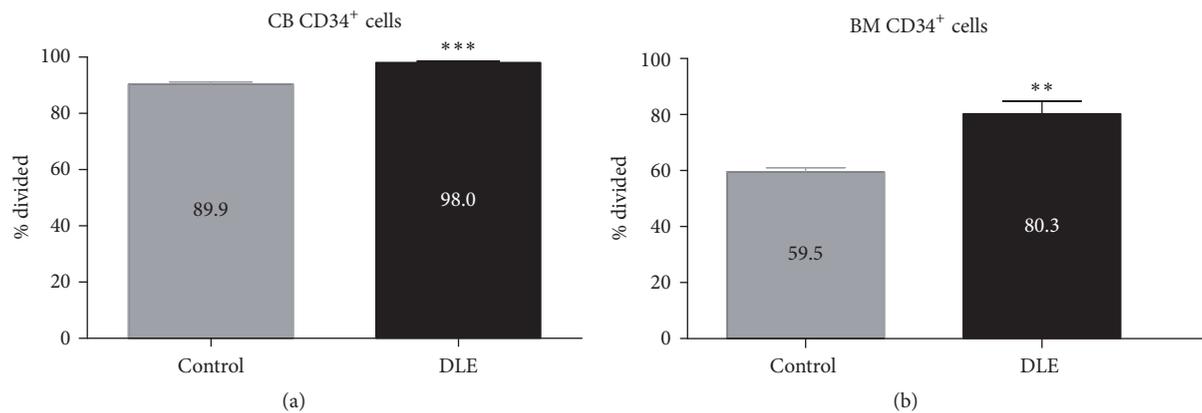


FIGURE 1: Dialyzable leukocyte extracts (DLE) promote proliferation of primitive hematopoietic CD34<sup>+</sup> cells from neonatal and adult sources. CD34<sup>+</sup> cells from adult bone marrow (ABM) and umbilical cord blood (UCB) were enriched and cultured for 72 hours with DLE Transferrin and BrdU. Cells were stained for the identification of CD34<sup>+</sup> cell progenitors followed by intracellular staining of BrdU. Multiparametric flow cytometry was performed in a FACSCanto II and the cell frequencies of BrdU<sup>+</sup> cells calculated. Data are representative of 3 independent experiments. \* $P < 0.05$ ; \*\* $P < 0.01$ .

progenitor phenotype were the major producers of DLE-newly differentiated NK cells (Figure 2(c)).

The identity of CD11c<sup>+</sup> NK cells has been a matter in question and a debate point for years. The various studies from DeMatteo have indicated that CD11c expression defines a distinct subset of murine liver NK cells that respond to adenoviral infection by producing IFN- $\gamma$  in a TLR9-IL-12-IL-18-dependent way [27]. Moreover, the same NK dendritic cells (NKDC) phenotype was found to be multifunctional cells with pleiotropic functions, including the IFN $\gamma$ -mediated response against bacterial infections and malignant processes, and the migration to central nervous system for viral clearance [28–32]. Belonging to the Hardy Fraction A of the mouse B cell developmental pathway, the discrete B220<sup>+</sup>CD11c<sup>+</sup>NK1.1<sup>+</sup> NK subset seemed to meet the biological properties of NKDC, that is, dependency on IL-15 and ability of producing highest amounts of IFN $\gamma$  [33]. Of relevance, two simultaneous reports recently revealed the “hybrid” phenotypic and functional characteristics of DC and NK within a novel subset of interferon-producing killer dendritic cell (IKDC) population, endowed with strong cytotoxic and antitumor activities, and antigen presenting competence [34–37]. IKDCs share some properties with other innate lymphoid cells, but their development is unique and is most efficiently generated from L-selectin<sup>+</sup> BM progenitors, while common lymphoid progenitors (CLP) are the most effective source of conventional NK cells [36]. Even though a IKDC subset has not been formally described in humans, expression of CD11c in NK lineage cells is induced by combination of IL-15 and inflammatory cytokines and distinguishes a conspicuous cell subtype participating in immune regulation of chronic diseases [38].

Our studies were then extended to CD11c<sup>+</sup> dendritic cells. Starting from the HSC fraction, DLE stimulation could efficiently trigger their production (Supplemental Figure 2), emphasizing its potential to aim at innate responses but suggesting the divergent origin of DLE-derived NK and

DC. Accordingly, animals treated with DLE have showed increased NK cell frequencies and numbers, among other hematopoietic cells [19, 20].

Due to their fundamental position in viral and tumor immune-surveillance, extensive efforts have been achieved to define experimental conditions to expand NK cells for adoptive immunotherapy [24]. Our data suggest a preliminary strategy to produce high numbers of these cells and, most of all, to robust the *in vivo* production by remodeling central hematopoiesis. Of note, inhibition of MyD88 adaptor molecule reduced proliferation and NK-differentiation potentials of DLE-stimulated CD34<sup>+</sup> cells, suggesting the partial contribution of TLR signaling pathway in this emergent phenomenon (Supplemental Figure 3).

**3.2. DLE-Associated Microenvironmental Activation Contributes the Emergent Production of CD11c<sup>+</sup> NK Cells.** Despite the wealth of information relevant to transcription factors- and phenotype-associated biological functions of NK lineage cells, we still need to understand the role of environmental niches in their early differentiation under steady-state and pathological conditions [24]. It is well known that they are generated and perform their critical functions in the context of hematopoietic niches within the bone marrow [24, 39] or in extramedullary tissues, respectively. Indeed, besides the identified developmental niches, an immune response niche, an inflammatory or stress niche, a tumor niche, and a pregnancy niche have been proposed to regulate the biology of NK cells. Such niches are presumably controlled by nonhematopoietic cells providing signals triggered from adhesion molecules, chemokines, and cytokines/growth factors [39–41]. Generation of NK cells requires IL-15 and Fms-like tyrosine kinase 3 ligand, but not IL-7. Strikingly, the CXCR4/CXCL12 axis conformed by CXCL12-abundant reticular (CAR) mesenchymal cells and NK precursors might function as the major niche element and is crucial for NK

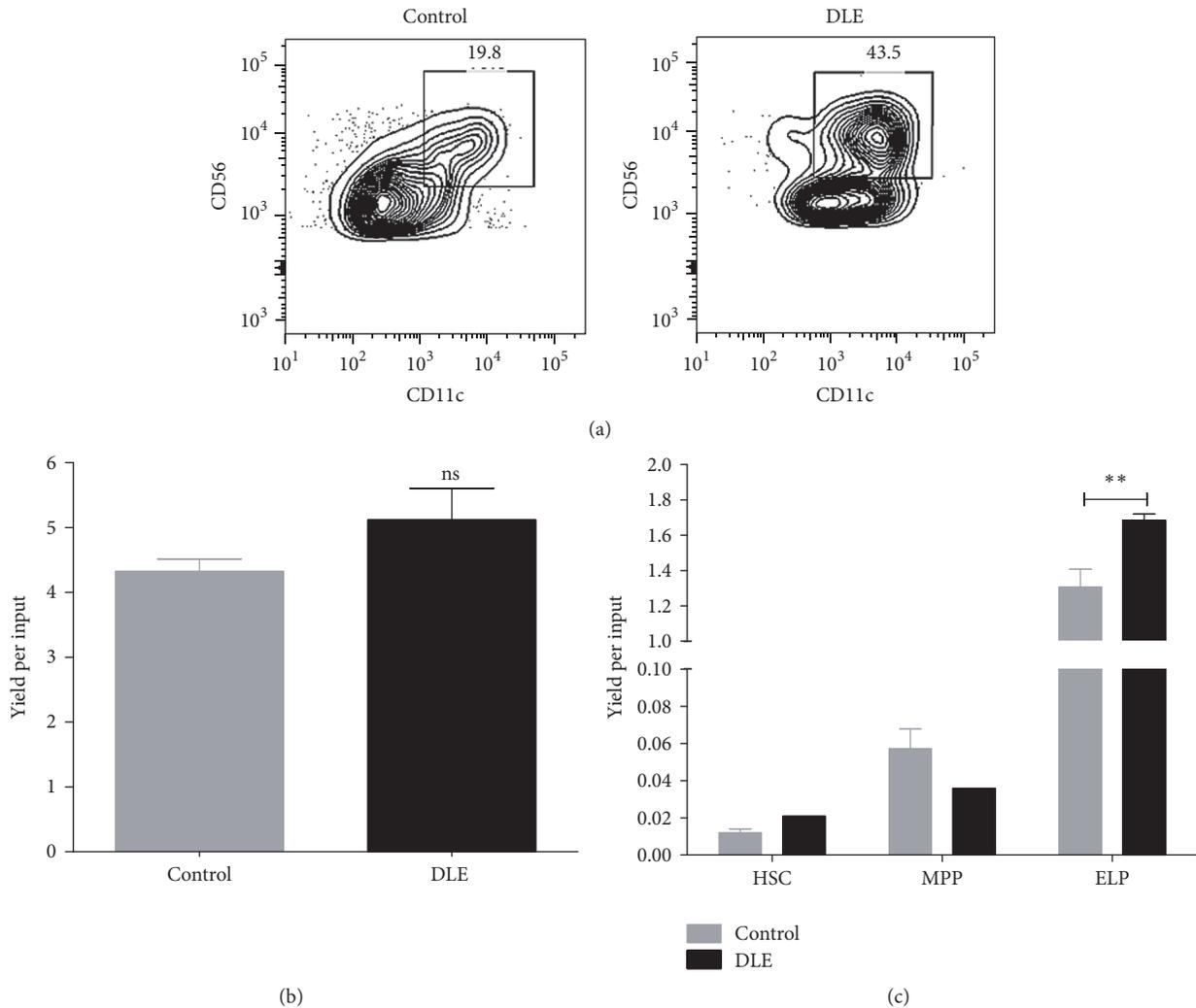


FIGURE 2: CD56<sup>+</sup>CD11c<sup>+</sup> NK-like cells expeditiously develop from early lymphoid progenitors upon DLE stimulation. Upon 24 hours of DLE Transferon stimulation, the whole fraction of CD34<sup>+</sup> cells (a and b) or purified primitive cells, including hematopoietic stem cells (HSC), multipotent progenitors (MPP), and early lymphoid progenitors (ELP) (a), were cocultured with MS-5 stromal cells for 30 days under lymphoid lineage conditions. CD56<sup>+</sup>CD11c<sup>+</sup> NK-like cell production was investigated by flow cytometry and reported as cell frequencies (a) or yield per input progenitor (b and c). For comparison, unstimulated cultures were considered as control conditions. DLE, dialyzable leukocyte extracts Transferon. \*\*  $P < 0.01$ .

development within BM [39, 41]. Moreover, production of IL-15 is the highest in CAR cells.

Our data confirm that microenvironment cues affect the development and biological roles of distinct NK subsets and suggest a substantial positive effect of DLE on stromal cell activation that, in turn, may promote CD11c<sup>+</sup>NK and DC expeditious differentiation (Figure 3). The additive differentiation effect by stromal cells may result from the action of cytokines and NK growth factors. Certainly, a multiplex type assay has shown the differential production of lymphoid factors and VEGF (our preliminary unpublished observations).

Two main subtypes of human NK cells are recognized: CD56<sup>low</sup>CD16<sup>+</sup> and CD56<sup>high</sup>CD16<sup>+/-</sup>. While the abundant peripheral CD56<sup>low</sup>CD16<sup>+</sup> NK cells are phenotypically classified as the most cytotoxic ones, the less copious

CD56<sup>high</sup>CD16<sup>+/-</sup> subset has been shown to be endowed with noticeable cytokine production capability. Their activation is mediated by the net balance between activating and inhibitory receptor engagement signals and in response to cytokines such as IL-2, IL-12, IL-15, and IL-18 [25]. Among activating molecules displayed by the CD56<sup>low</sup>CD16<sup>+</sup> NK cells, CD16 plays a critical role in targeting antibody Fc-bound cells and executing antibody dependent cell-mediated cytotoxicity (ADCC). Expression of granzyme B and perforin is also high compared to the CD56<sup>high</sup> counterpart. Predominantly in tissues and secondary lymphoid organs, the CD56<sup>high</sup>CD16<sup>+/-</sup> subset is responsible for production of IFN $\gamma$ , TNF $\alpha$ , GM-CSF, and RANTES after activation.

The recent reevaluation of functional properties of both cell categories has changed the binary paradigm of NK

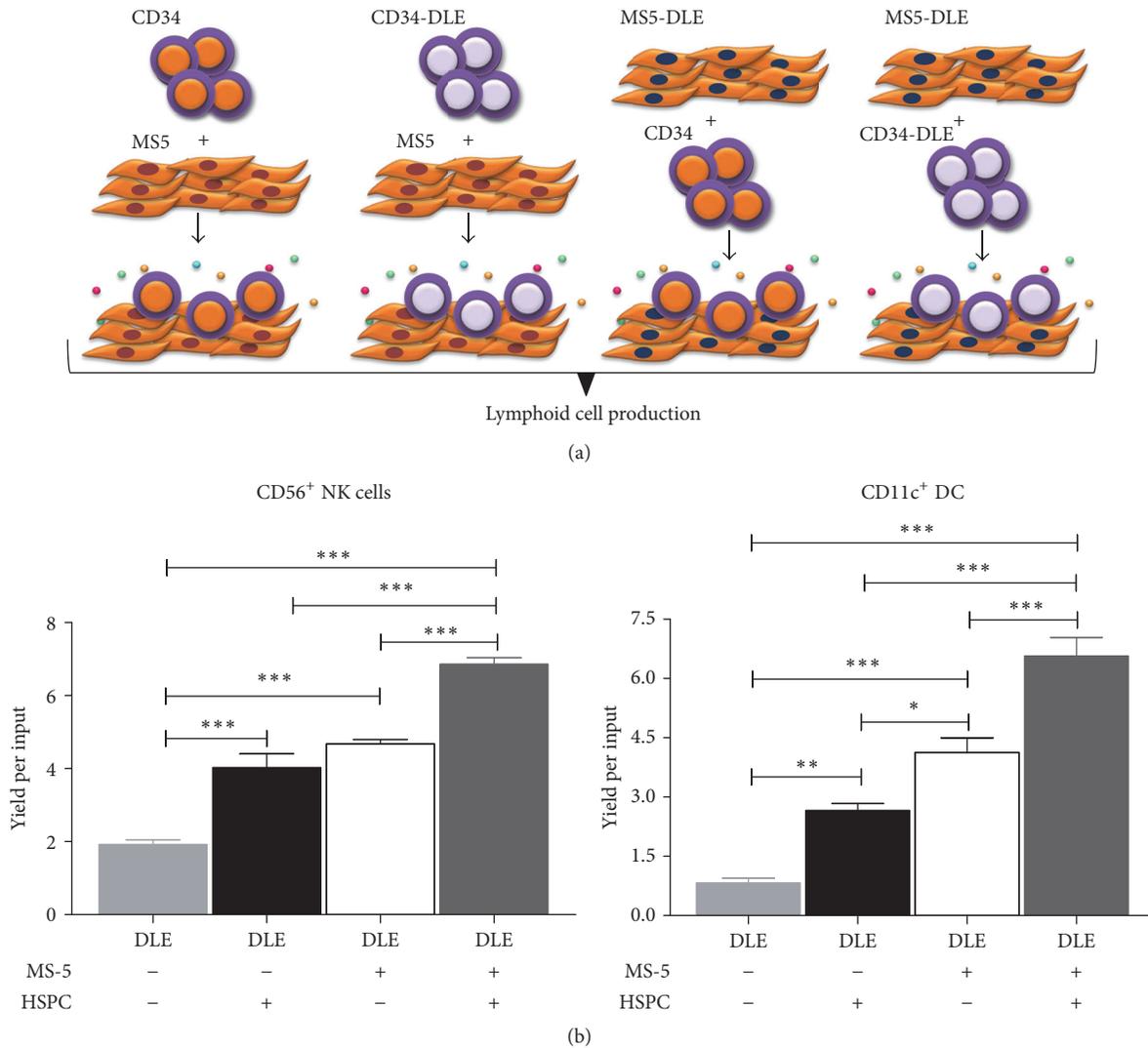


FIGURE 3: Stromal cell activation by DLE increases the emergent innate immune cell differentiation. MS-5 stromal cells were exposed or not to DLE Transferron 24 hours before coculturing with CD34<sup>+</sup> hematopoietic stem and progenitor cells (HSPC) that were pretreated 24 hours with DLE Transferron (a). All conditions were placed in lymphoid lineage cocultures for 30 days followed by flow cytometry analyses of CD56<sup>+</sup> NK cells and CD11c<sup>+</sup> DC production. Yields per input progenitor were tabulated to show the positive contribution of activated stromal cells to innate differentiation (b). DLE, dialyzable leukocyte extracts Transferron. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

biology, providing clear evidence of the rapid and simultaneous effector activities exerted by the same CD56<sup>low</sup>CD16<sup>+</sup> NK cell subset [25]. Thus, a prompt and abundant IFN $\gamma$  production overlaps with their cytolytic ability and may lead to amplification of macrophage and DC-mediated responses.

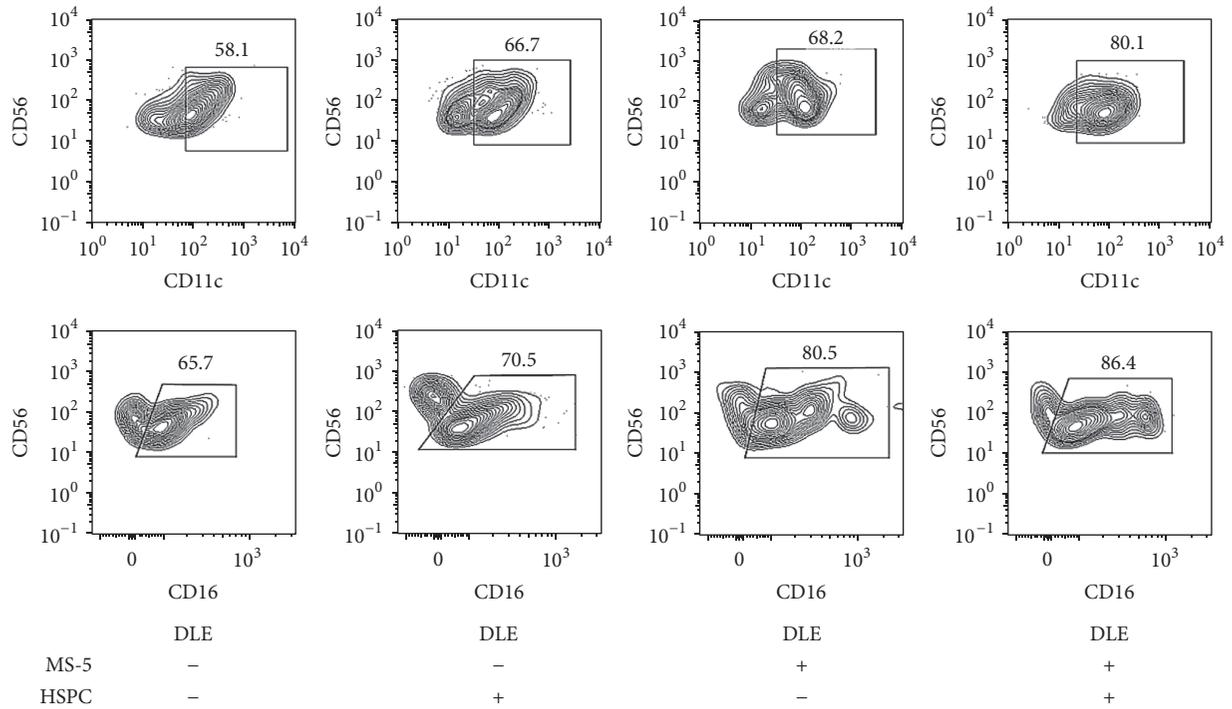
Interestingly, we have now found that most DLE-newly produced cells expressed CD16 and that microenvironmental prestimulation induces the density of this molecule (Figure 4).

**3.3. DLE-Derived CD11c<sup>+</sup> NK Cells May Function As Antiviral and Antitumoral Cell Mediators.** The functional competence of NK cells produced from DLE-stimulated lymphoid progenitors was tested, demonstrating a very efficient

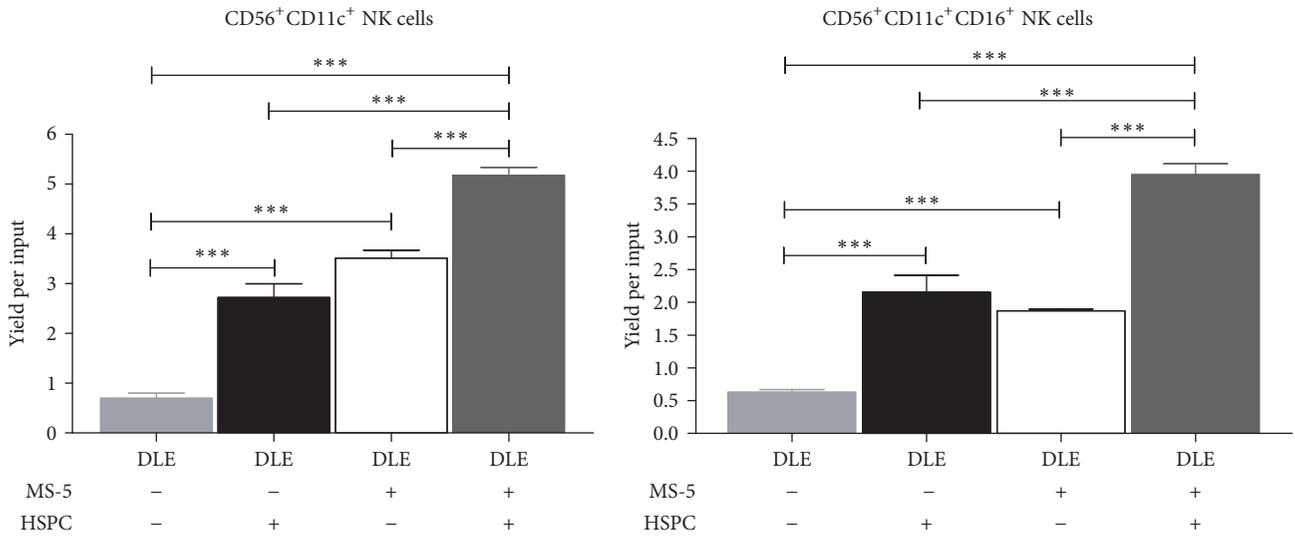
cytolytic potential when a nonradioactive method was used (Figure 5(a)). We also observed a copious production of IFN $\gamma$  only from the specialized CD11c<sup>+</sup> NK population (Figure 5(b)), which is highly relevant to antitumoral and antimicrobial immune protection as this proinflammatory cytokine is pivotal in initiating Th1 immune responses against pathogens and tumors [30, 31].

Thus, these data suggest that early progenitors are promoted to differentiate toward both cytotoxic and regulatory cells in response to DLE. As mentioned before, the CD16<sup>+</sup> NK subset may contain both biological properties [25].

Recently, CD56<sup>hi</sup>CD11c<sup>+</sup> human NK-like cells were shown to induce the proliferation of  $\gamma\delta$  T cells in an IL-18 dependent manner [42, 43]. Because V $\gamma$ 2V $\delta$ 2 T lymphocytes are key components of the innate immune system



(a)



(b)

FIGURE 4: Preferential production of functional CD11c<sup>+</sup>CD16<sup>+</sup> NK-like cells promoted by hematopoietic-stromal cell communication responding to DLE. MS-5 stromal cells were exposed or not to DLE Transferron 24 hours before coculturing with DLE Transferron-pretreated CD34<sup>+</sup> hematopoietic stem and progenitor cells (HSPC), as described in Figure 3(a). All conditions were placed in lymphoid lineage cocultures for 30 days followed by flow cytometry analyses, where the indicated gates were used to discriminate CD56<sup>+</sup>CD11c<sup>+</sup> and CD56<sup>+</sup>CD11c<sup>+</sup>CD16<sup>+</sup> NK cell frequencies (a). Yields per input progenitor were tabulated to record the significant variations by stromal or hematopoietic cells exposure to DLE Transferron (b). \*\*\*  $P < 0.001$ .

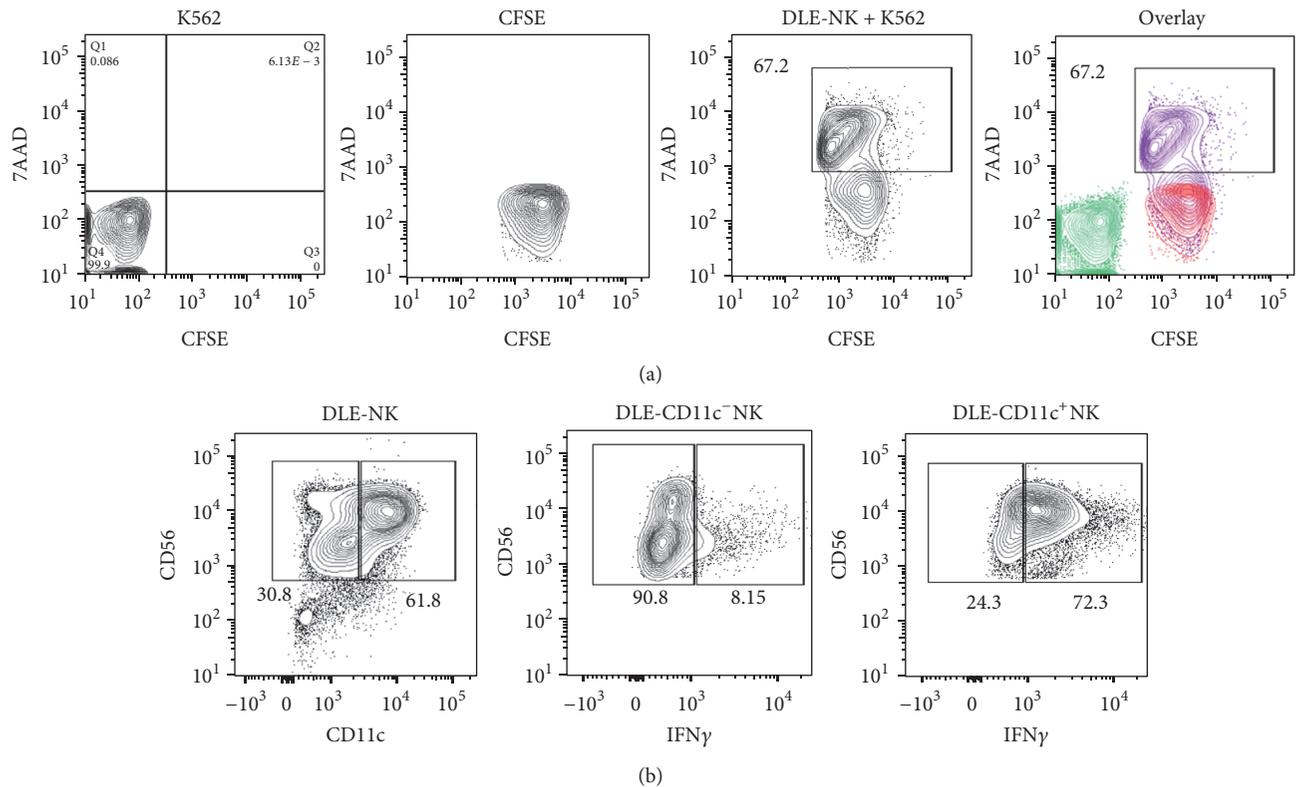


FIGURE 5: Effector functions of CD11c<sup>+</sup> NK cells differentiated from DLE-induced lymphoid progenitor cells include tumor cytotoxicity and IFN-gamma production. NK cytotoxicity function was evaluated on CFSE+ K562 tumor cells as target of NK-like cells derived from DLE stimulation (DLE-NK) (a). We used 7-aminoactinomycin D (7-AAD) to evidence the frequency of CFSE<sup>+</sup>7-AAD<sup>+</sup> killed target cells. Production of IFN-gamma by CD11c<sup>-</sup>CD56<sup>+</sup> and CD11c<sup>+</sup>CD56<sup>+</sup> NK cells was detected by intracellular staining (b).

that function against infections and human tumor cells, we explore the faculty of DLE-NK cells of activating them. Of note, dilution of the dye CTV was significantly apparent when  $\gamma\delta$  T cells were cultured with CD11c<sup>+</sup>NK that are produced upon stimulation with DLE. Surprisingly, neither unstimulated  $\gamma\delta$  T cells nor DLE-directly stimulated  $\gamma\delta$  T cells got activated (Figures 6 and 7). Activation of  $\gamma\delta$  T cells can be achieved by a number of microorganisms and phosphoantigens and does not require antigen processing, but some atypical costimulatory molecules such as NKG2D play crucial roles in activating them to kill neoplastic or pathogen-infected cells [44].

These innate cells function through a variety of mechanisms, including secretion of cytokines and chemokines, dendritic cell activation, macrophage recruitment, cytolytic activity, and antigen presentation [45]. Their contribution to immune-surveillance in a major histocompatibility complex-(MHC-) unrestricted way, by their capability of producing IFN $\gamma$  and TNF $\alpha$  and via an NK-like pathway, has positioned them as attractive targets for immunotherapy strategies [45–48].

Taken together, our study provides a rational explanation for the adjuvant contribution of DLE to the observed effective immune responses against viral and malignant diseases. DLE

could play an inductor role in the NK lineage emergent hematopoiesis, suggesting the need of elucidating central mechanisms that govern DLE-associated tissue regeneration and give an insight into novel cooperative drug activities.

#### 4. Concluding Remarks

While remarkable progress has long been recorded in identifying targets for cellular-based immunotherapeutic strategies to improve acute and chronic disease outcomes, it is becoming clear that definition of extrinsic factors promoting innate cell differentiation processes from the earliest developmental stages may change our vision of immunomodulation. Our findings suggest, for the first time, that, in response to dialyzable leukocyte extracts (DLE) Transferon, human stem and progenitor cells contribute to the emergent production of a special subset of innate CD11c<sup>+</sup> NK cells. Of note, this DLE-derived NK cell population is endowed with properties such as IFN $\gamma$  production, tumor cell cytotoxicity, and the capability of inducing  $\gamma\delta$  T lymphocyte proliferation that may, in turn, function as coadjuvant component of innate immune responses against virus-infected or tumor cells.

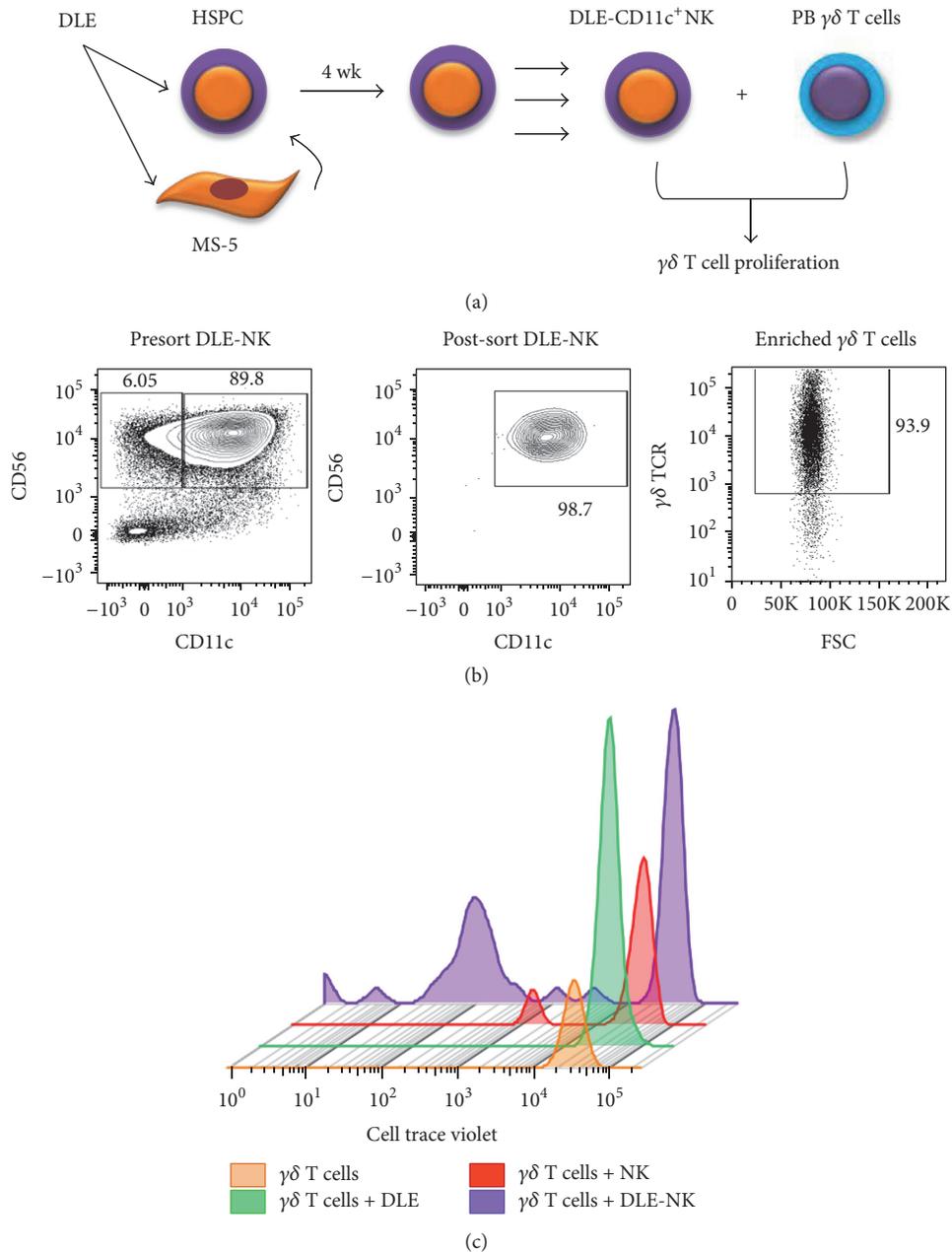


FIGURE 6: Activation of  $\gamma\delta$  T cells as a result of DLE-associated CD11c<sup>+</sup> NK cell emergent production.  $\gamma\delta$  T cells from healthy donors were cocultured with the DLE-derived CD11c<sup>+</sup> NK cell at a ratio of 2 : 1 CD56<sup>+</sup>CD11c<sup>+</sup> NK :  $\gamma\delta$  T cell (a). Both NK and  $\gamma\delta$  T cell populations were highly purified by flow cytometry sorting before setting the proliferation assay (b).  $\gamma\delta$  T lymphocyte proliferation was assessed at 72 hours by dilution of Cell Trace Violet (CTV) dye (c).

## Competing Interests

Sonia Mayra Pérez-Tapia is employee or has been compensated for her work at “UDIMEB,” the producer of dialyzable leukocyte extracts Transferon™. Dalia Ramírez-Ramírez, Eduardo Vadillo, Lourdes Andrea Arriaga-Pizano, Héctor Mayani, Marco Antonio Velasco-Velázquez, and Rosana Pelayo declare that they have no conflict of interests.

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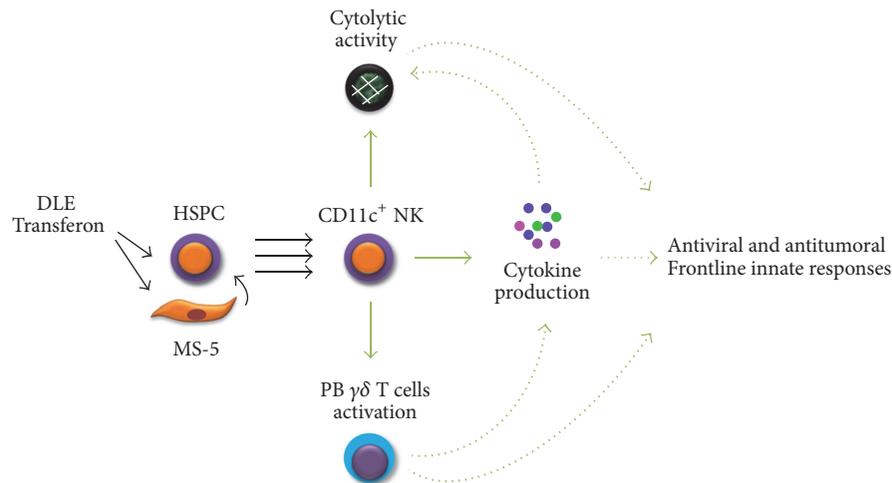


FIGURE 7: Emergent CD11c<sup>+</sup> NK cell production contributing the adjuvant immune-surveillance effects of DLE Transferron: a proposed model. Dialyzable leukocyte extracts Transferron promote the early differentiation of functional CD11c<sup>+</sup> NK cells endowed with the capabilities of tumor cell cytotoxicity, IFN $\gamma$  production, and  $\gamma\delta$  T lymphocyte proliferation induction, which may in turn contribute to innate immune responses against virus-infected or tumor cells. The potential involved mechanisms require further investigation.

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## Review Article

# Immunomodulatory Effects Mediated by Dopamine

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Dopamine (DA), a neurotransmitter in the central nervous system (CNS), has modulatory functions at the systemic level. The peripheral and central nervous systems have independent dopaminergic system (DAS) that share mechanisms and molecular machinery. In the past century, experimental evidence has accumulated on the proteins knowledge that is involved in the synthesis, reuptake, and transportation of DA in leukocytes and the differential expression of the D1-like (D1R and D5R) and D2-like receptors (D2R, D3R, and D4R). The expression of these components depends on the state of cellular activation and the concentration and time of exposure to DA. Receptors that are expressed in leukocytes are linked to signaling pathways that are mediated by changes in cAMP concentration, which in turn triggers changes in phenotype and cellular function. According to the leukocyte lineage, the effects of DA are associated with such processes as respiratory burst, cytokine and antibody secretion, chemotaxis, apoptosis, and cytotoxicity. In clinical conditions such as schizophrenia, Parkinson disease, Tourette syndrome, and multiple sclerosis (MS), there are evident alterations during immune responses in leukocytes, in which changes in DA receptor density have been observed. Several groups have proposed that these findings are useful in establishing clinical status and clinical markers.

## 1. Introduction

Dopamine (DA) is a monoamine that is best known for its neurotransmitter function, and like other neurotransmitters, its effects are not limited to the central nervous system (CNS). Several studies support the notion that DA is a coregulator of the immune system (IS) [1–7], tissues and

organs, such as adipose tissue and kidney [8, 9]. Alterations in the DAS have been associated with many health problems, including high blood pressure [10], psychiatric disorders (e.g., schizophrenia), and neurodegenerative diseases (e.g., Parkinson disease).

Based on the involvement of DA in behavioral and cognitive processes, many studies have focused on the nervous

system [11–14], describing the general mechanisms, physiological issues, and signaling pathways of the DAS [15, 16]. The existence of DA in the bloodstream suggests the presence of the dopaminergic components that modulate functions in the immune system [17], as in other systems [18]. Studies on monoamines, such as serotonin, DA and its derivatives, and neuropeptides, have become increasingly significant since the 1980s, given their neuroimmunoregulatory functions [19–22].

The CNS and immune system are the main adaptive systems, participating in continuous and functional crosstalk to ensure homeostasis. DA and other catecholamines, such as noradrenaline, function as neuroimmunotransmitters in the sympathetic-adrenergic terminals of the autonomic nervous system, which innervates the primary and secondary lymphoid organs—in addition to the direct local effects that nonsynaptic varicosity secretions have on immune cells [1, 2, 23, 24].

This review focuses on the function of the DAS in the immune system and the function of DA as an immunoregulatory molecule and on the communication between the CNS and IS, based mainly on studies in human cells. We also discuss the clinical aspects of disturbances in the DAS in mental disorders, such as schizophrenia, Parkinson disease, and other clinical conditions that are related to cancer, viral infections, and autoimmunity.

## 2. The Early History of DA and Its Receptors

DA (3-hydroxytyramine; 3,4-dihydroxyphenethylamine;  $C_8H_{11}NO_2$ ) was first synthesized in 1910 [25–27]. The initial experiments on DA, in the same year, evaluated its biological effects as a weak sympathomimetic [26, 28]. After nearly 30 years, in 1938, Peter Holtz and colleagues identified L-DOPA decarboxylase in mammals, which uses L-DOPA as a substrate to obtain DA. One year later, Hermann Blaschko in 1939 postulated the biosynthetic pathway of catecholamines, which remains valid and places DA as a precursor of adrenaline and noradrenaline [29].

In subsequent years, observations of small concentrations of DA in several peripheral tissues were reported. Curiously, the name “dopamine” was not adopted until 1952, when a shorter name was proposed by Henry Dale [29]. In the 1950s, the participation of DA in biological processes became recognized, in addition to it being a precursor of adrenaline and noradrenaline, with significant physiological function in the mammalian brain. Arvid Carlsson and colleagues (1957–1959) found that DA has a fundamental function and unique distribution throughout the brain and other tissues [30, 31]. Bertler and Rosengren (Carlsson’s students) reported that DA was present in the brains of all of the mammals that they studied but its distribution in the brain differed [32].

This difference, combined with results from other studies that used reserpine, an inhibitor of chromaffin granule amine transporter and synaptic vesicular amine transporter [33, 34], and L-DOPA prompted speculation that DA was involved in the modulation of motor function. Early reports on the distribution of dopamine in animals and humans showed that DA exists primarily in the caudate nucleus in significant

amounts [32, 35–37]. At the beginning of the 1960s, the initial studies on Parkinson disease were performed using human tissue from autopsies, demonstrating the absence of DA in the striatum [38].

The idea of providing L-DOPA to patients with Parkinson disease and psychotic disorders arose soon thereafter, leading to the first clinical trials on L-DOPA to mitigate Parkinsonian symptoms [39]. After an extended trial period, L-DOPA was commercialized in 1973 with benserazide, a DOPA decarboxylase inhibitor [40–43].

Moreover, several studies already reported the relevance of DA as a modulator of motor function [45]; the biochemical study of DA receptors (DRs) in the CNS began with Greengard’s research, just like the discovery that DA stimulates adenylyl cyclase in the cervical sympathetic ganglia and rat caudate nucleus [46, 47]. The results on stimulation with DA led to the classification of two types of receptors for the second messenger cAMP: stimulatory (alpha-type) and inhibitory (beta-type) [48, 49]. cAMP function depends on the coupling of its receptor (DR) to the heterotrimeric G proteins  $G\alpha$ -s/olf and  $G\alpha$ -i/o. The subtypes of receptors are the *D1-like subtype (D1-like)*, which includes D1R and D5R, and the *D2-like subtype (D2-like)*, comprising D2R receptor longer, D2R receptor short, D3R, and D4R [16]. Another important component of the DAS is sodium-dependent dopamine transporter (DAT; gene *SLC6A3*), which was cloned from rat and cow by several groups in 1991 [50–53] and in humans in 1992 [54].

Arvid Carlsson, Paul Greengard, and Eric Kandel were awarded the Nobel Prize in Physiology or Medicine in 2000 for their work on signal transduction in the nervous system [55]. The function of DA as a neurotransmitter precedes its importance in the immune system, based on the many processes in which it participates in the CNS. However, the history of DA and its functions in the IS and other tissues has recently begun.

## 3. Dopaminergic System

The DAS is a vast protein assembly that synthesizes, releases, senses, and metabolizes DA in various cell types in mammals. It also modulates a vast set of neuronal processes. Several examples of brain functions in which DA participates are cognition, motor control, mood, reward systems, pain perception, and sexual behavior [11, 12].

The function of DA outside of the nervous system has only recently been studied. For instance, DA mediates *stem cell-mediated dental repair* with platelets, regulates salt excretion by the kidney, and modulates blood pressure [56, 57]. DA is unable to cross the blood-brain barrier; thus, signaling in the neuronal DAS should be independent of that of the DAS in peripheral systems [11].

**3.1. DA Concentrations in the Peripheral Region Outside of the CNS.** DA in peripheral systems originates from the nervous system and mesenteric region. The concentration of DA in peripheral plasma in humans is approximately 0.1 nmol/L (0.1 pmol/mL) and is derived primarily from sympathetic noradrenergic nerve fibers. This concentration can vary by

up to nearly 50-fold for derivatives, such as DA sulfate, after ingestion of a standard meal and according to the region of the circulatory system [58].

The concentration of DA has been assessed in the mesenteric region in samples from abdominal surgeries for gastric adenocarcinoma and pancreatic neoplasm. Abdominal DA concentrations in arterial plasma (samples from the radial and hepatic arteries) and venous plasma (from the right hepatic and portal veins) are approximately 0.312 pmol/mL and 0.937 pmol/mL, respectively (estimated from Figure 1 of Eisenhofer 1997). In addition, by immunoreactivity, tyrosine hydroxylase has been detected in human gastrointestinal mucosa, as have its catalytic activity and the presence of DRs [59, 60]. The concentration of DA and its metabolites in plasma of the portal vein with respect to arterial plasma has demonstrated greater production of DA in the mesenteric organs (12 nmol/min), representing approximately 50% of the DA that is produced in the human body [59].

**3.2. Dopaminergic System Expressed in Various Tissues and Leukocytes.** The physiological mechanisms of the cell signaling and pharmacology of DRs and DA metabolism have been described extensively in the murine CNS [15, 61]. The mechanisms and protein components of this system are likely to be shared between CNS cells and all other tissues in mammals. However, the genes of the DAS in each tissue type are differently expressed differentially, and the protein components must be specifically modulated, based on the function of the cell type. Thus, the genes and proteins of the DAS, such as DRs and DAT, are expressed in a wide range of tissues (e.g., adipose tissue, perivascular adipose tissue, kidney, heart, pituitary, the gastrointestinal tract, and pancreatic beta cells) that regulate processes that differ from those that the CNS governs (e.g., blood pressure regulation, sight process regulation in the retina, vascular permeability regulation in the epithelium, and insulin release) [9, 10, 13, 18, 62–68]. However, many of these studies have been performed primarily in murine models and cell lines (human and murine), potentially creating variations in the results.

The DAS has been observed in murine immune cells [69–73] and human platelets [74]. Recent studies have described its effects on the activation and proliferation of certain cells [75]. The expression of all DRs has been studied in all types of human leukocytes (Table 1). Although their mRNA and protein levels vary between human cell lines, DRs expression is lowest in T lymphocytes and monocytes among all leukocytes, whereas B lymphocyte and NK cell membranes bear the highest levels.

**3.3. Metabolic Pathways of DA.** In the CNS, DA is synthesized through an anabolic pathway that is shared with other catecholamines, such as L-noradrenaline (NE) and adrenaline (E) [61]. Catecholamine metabolism serves as a source of intracellular ROS (*reactive oxygen species*) production, which occurs in Parkinson disease, along with mitochondrial dysfunction [138]. The catecholamine pathway has been proposed to be particularly crucial in the reduction-oxidation (REDOX) homeostasis in cells. It might mediate

the overproduction of ROS in neurons, which can compromise the integrity of dopaminergic cells [61]. The effects of DA metabolism in leukocytes, the modulation of the REDOX balance, and the function of DA in mitochondria remain poorly documented [24].

In other tissues, such as the murine kidney, study of the relationship between DRs, oxidative stress, and the REDOX balance has provided information on high blood pressure [10]. The degradation of catecholamines, particularly DA, generates subproducts at low abundance, the concentrations of which depend strongly on the tissue and cell type [61].

A recent mathematical model of DA metabolism in Parkinson disease shows a certain degree of predictability with respect to pharmacological and genetic changes. The authors of this model propose its application *in silico* in the search for molecular approximations that allow the imbalance in DA contents to be restored and changes in oxidative stress to be detected. This model is a preliminary effort, and the authors have commented on future developments and extensions [139]. It would be desirable for such extensions to include peripheral DA systems—for example, studying the metabolism of adrenergic-sympathetic terminals in lymphoid organs and determining their predictive value in human immune system cells.

**3.3.1. Anabolism.** The classical pathway of DA synthesis begins with the production of L-tyrosine from L-phenylalanine by phenylalanine 4-hydroxylase (PAH) (EC: 1.14.16.1; PAH gene). DA is synthesized in the catecholamine pathway (Figure 1), the first enzymatic step of which is the transformation of L-tyrosine into L-DOPA by tyrosine 3-hydroxylase (TH) (EC: 1.14.16.2; TH gene). Both enzymes use molecular oxygen and tetrahydrobiopterin as cofactors of oxidation, rendering them two strongly regulated enzymes. Next, L-DOPA is converted to DA by DOPA decarboxylase (DDC) (EC: 4.1.1.28; DDC gene). DA is the precursor of L-noradrenaline, mediated by DA beta-hydroxylase DBH (EC: 1.14.17.1; DBH gene). Finally, L-noradrenaline is converted into adrenaline by phenylethanolamine N-methyltransferase (PNMTase) (EC: 2.1.1.28; PNMT gene) [61, 111].

Two alternative pathways of DA synthesis have been identified in the rat brain and human hepatic microsomes. In the first mechanism, DDC transforms L-phenylalanine into phenylethylamine, which in turn is converted into tyramine by PAH; in the second pathway, DDC uses L-tyrosine to produce tyramine. In both cases, tyramine is converted into DA by the cytochrome p-450 CYP2D6 isoform (EC: 1.14.14.1; CYP2, CYP2D genes) [140, 141].

The enzymatic activity of TH and PAH in human leukocytes has been detected since the 1980s [112–114]. In addition, the expression and immunodetection of TH in human and mouse leukocytes have been widely reported [77, 79, 83, 106, 107]. On the other hand, DDC expression in human cells has also been documented [79, 109, 110] (Table 1).

In human lymphocytes, the presence and synthesis of certain catecholamines, such as L-DOPA and noradrenaline, the synthesis of which appears to be linked to cholinergic stimulation, have been measured, but they are differentially synthesized between B and T lymphocytes; L-DOPA exists



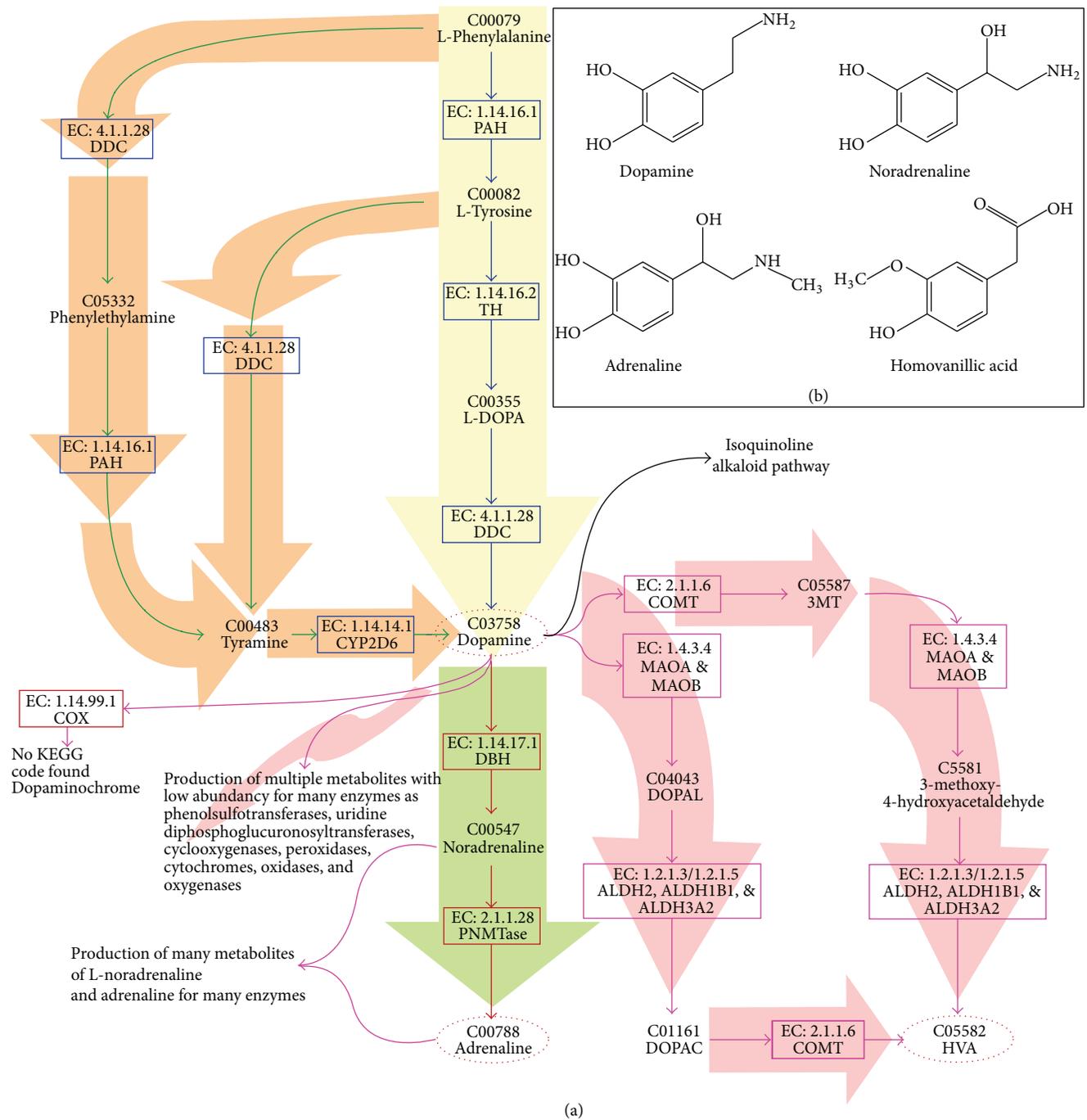


FIGURE I: Metabolic pathways associated with DA. The metabolic pathways branching from the catabolism of L-phenylalanine are shown. (a) The dark blue arrows (over light yellow branches) indicate the DA generation pathway, and the red arrows (over light green branches) represent the noradrenaline and adrenaline pathway. Green arrows (over orange branches) show the alternative synthesis pathways to dopamine. The magenta arrows (over pink branches) show dopamine catabolism; the right side shows the normal pathways converging on HVA; the left side shows the catabolic pathways when dopamine concentrations are high in the cytoplasm. Many enzymes can transform dopamine and metabolites, such as COX enzymes (cyclooxygenases), producing dopaminochrome and dopamine quinone [44]. Other enzymes can produce sulfur and glucuronide derivatives. (b) The inset shows the most important products (circled with dotted lines) of metabolic pathways associated with DA. The compounds in these pathways are denoted by Kyoto Encyclopedia of Genes and Genomes (KEGG) code (<http://www.genome.jp/kegg/>). Enzymes with their classification codes (EC, <http://www.chem.qmul.ac.uk/iubmb/enzyme/>) and UNIPROT gene names are shown in squares.

in both cell types, but noradrenaline is only found in T cells [142]. The incubation of human lymphocytes with L-tyrosine and acetylcholine increases L-DOPA and DA levels. In human and murine lymphocytes, incubation with L-tyrosine and L-DOPA increases L-DOPA, DA, and noradrenaline [122, 142, 143].

**3.3.2. Catabolism.** In neurons, DA is recovered from the *synaptic cleft* by DAT and accumulates in the cytosol, where it is carried to synaptic storage vesicles by synaptic vesicular amine transporter (VAT2). The excess of DA in the neuronal cytosol is degraded by an enzymatic set (Figure 1), comprising monoamine oxidases (MAO-A and MAO-B) (EC: 1.4.3.4; genes *MAOA* and *MAOB*), catechol o-methyltransferase (COMT) (EC: 2.1.1.6; gene *COMT*), aldehyde dehydrogenases (ALDHs) (EC: 1.2.1.3/1.2.1.5; gene type *ALDHs*), alcohol dehydrogenases (ADHs) (EC: 1.1.1.1; gene *ADH*), and aldehyde reductases (ARs) (EC: 1.1.1.21; gene type *AKR*).

Using DA, COMT produces 3-methoxytyramine (3MT), which MAO-A and MAO-B acquire to produce 3-methoxy-4-hydroxyacetaldehyde, which is then used by ALDH to generate *homovanillic acid* (HVA). Alternatively, MAO-A and MAO-B can act on DA to synthesize 3,4-dihydroxyphenylacetaldehyde (DOPAL), which is then transformed into 3,4-dihydroxyphenylacetate (DOPAC) by ALDH. DOPAC is converted to HVA by COMT. DOPAC and HVA are the final principal metabolites of the degradation pathway of DA [61]. However, other subproducts have been observed in the CNS, based on the activity of phenolsulfotransferases (PSTs) and uridine diphosphoglucuronosyltransferases (PAPS), which produce other *derivatives* with sulfate and glucuronic acid, respectively [144–146]. Cyclooxygenases, peroxidases, cytochromes, oxidases, and oxygenases can also oxidize DA. For instance, prostaglandin H synthase (COX) (EC: 1.14.99.1) produces prostaglandin H using DA as a cofactor; subsequently, DA is transformed to generate dopaminochrome. Other examples of low-abundance metabolites that are derived from the catabolism of DA, the functions of which have not been examined, are discussed in Sulzer 1999 and Muñoz 2012 [147, 148].

Other compounds have been detected by spontaneous oxidation of the catechol group of DA and L-DOPA by ROS and amino acids that are derived from cysteine. These compounds are the corresponding quinones, which are associated with oxidative stress, such as DOPA quinone, dopamine quinone, and 6-hydroxydopamine quinone. Beginning with these compounds, a series of oxidation steps with ROS and cysteines occur to generate thioester derivatives and cysteine adducts [44, 61, 147, 149]. These events regarding the catabolism and oxidation of DA and other catecholamines demonstrate that the classical enzymatic degradation pathway of DA must be tightly regulated under regular conditions to produce DOPAC and HVA (waste products). Otherwise, the excess of DA and its metabolites in the cytosol can lead to the formation of cumulative compounds, such as neuromelanin, in the lysosomes and cause severe damage to cells. In Parkinson disease, excess production and accumulation of DA and its catabolites in the cytosol can effect mitochondrial dysfunction, oxidative stress, the formation of neurotoxic

$\alpha$ -synuclein protofibrils, and impairments in protein degradation, which mediate the neurodegeneration of dopaminergic neurons in Parkinson disease [61, 148].

In the mesenteric system (the gastrointestinal tract, spleen, and pancreas), DOPA, DA, and DOPAC are metabolized in the kidneys, plasma, and primarily liver, increasing HVA levels in the portal vein [59]. DOPAC in the bloodstream originates primarily from sympathetic nerve endings and is the precursor of HVA through COMT activity [150].

Generally, the rise in certain metabolites from DA degradation might indicate that cells are damaged by oxidative stress, because when physiological DA concentrations are surpassed, the degradation catabolites (HVA, DOPAL, DOPAC, and 3MT) begin to generate reactive secondary catabolites through spontaneous oxidation by chemical interaction with ROS. Further, when the concentrations of all DA derivatives climb, other enzymes can use them as substrates to generate additional metabolites.

**3.4. Dopamine Receptors: DRs.** Most studies on DRs have been conducted in the CNS, but many reports in other tissues are being published. The human DAS comprises at least six membrane DRs: D1R, D2RL, D2RS, D3R, D4R, and D5R. D2DR has four isoforms that are generated by differential mRNA splicing and have varying functions and sizes. Whereas the two longer isoforms are 443 and 445 amino acids, the short isoforms have 29 and 31 fewer amino acids. The number of DRD3 and DRD4 isoforms has not been determined and is poorly documented in humans and animals [95, 151–155]. In addition, these isoforms have not been linked to any differential functions, unlike DRD2 isoforms.

Human lymphocytes express *DRD5* and two of its pseudogenes,  $\psi$ *D5DR-1* and  $\psi$ *D5DR-2*, which are transcriptionally competent and the functions of which are unknown [101, 119, 120]. However, the pseudogenes peptides are likely to generate truncated and nonfunctional receptors [120].  $\psi$ *D5DR-1* and  $\psi$ *D5DR-2* are segmental duplicated chromosomal regions that are 95% identical to *DRD5* that cover part of the transcribed region of *DRD5* [120, 121, 156] (Table 2).

Human peripheral blood lymphocytes also express *DRD3* and *DRD4* [95, 99, 101, 151, 152]. Although *DRD1* and *DRD2* were initially believed not to be expressed in these cells [94, 102, 157], recent studies showed that *DRD2* is expressed in T and B lymphocytes, whereas *DRD1* does not appear to be expressed in any leukocyte [88, 97]. By flow cytometry, all DRs, except D1R, are differentially expressed in nearly all human leukocyte cell lineages. The most recent studies show that *DRD1* and D1R are expressed in stimulated T lymphocytes and are linked to the negative regulation of the immune response [76]. In particular, D4R expression is low in all leukocyte subtypes, except NK cells. T lymphocytes and monocytes contain low amounts of all DRs, followed by neutrophils, which primarily express D3R and D5R but at lower levels. B lymphocytes and NK cells have the highest levels of DR [88]. Further, other techniques, such as radioligand binding assay (RBA), have been used to document DR expression in the membranes of immune system cells [94, 158, 159]. D1R, D2R, D3R, and D5R have been observed by

TABLE 2: Dopaminergic system protein components expressed in immune system.

Receptors of DRs	Genes names	References
D(1) receptor: D1R* (old name D1A receptor) D1-like subtype	<i>DRD1*</i>	[75–87]
D(2) receptor longer: D2R D2-like subtype	<i>DRD2</i>	[75, 76, 78–80, 82–93]
D(3) receptor: D3R D2-like subtype	<i>DRD3</i>	[75, 78, 79, 82–90, 92–98]
D(4) receptor: D4R D2-like subtype	<i>DRD4</i>	[79, 82–88, 92–94, 96, 98–100]
D(5) receptor: D5R (old name D1B receptor) D1-like subtype	<i>DRD5</i>	[75, 78, 79, 82–88, 90, 92, 93, 98, 101, 102]
Sodium-dependent dopamine transporter: DAT	<i>SLC6A3</i> (synonyms: <i>DAT1</i> )	[78, 79, 91, 103–105, 115, 116]
Protein components shared with other monoamines systems		
Chromaffin granule amine transporter: VAT1	<i>SLC18A1</i> isoform 2 (synonyms: <i>VMAT1</i> )	[83, 103, 117, 118]
Synaptic vesicular amine transporter: VAT2	<i>SLC18A2</i> (synonyms: <i>VMAT2</i> )	[75, 79, 83, 103, 116]
Protein components with unknown functions		
Chromaffin granule amine transporter: VAT1 $\Delta$ 15 <sup>↔</sup>	<i>SLC18A1</i> isoform 2 (synonyms: <i>VMAT1</i> $\Delta$ 15)	[117, 118]
<i>Pseudogene</i> D(5) receptor <sup>†</sup>	$\psi$ <i>DRD5-1</i>	[101, 119–121]
<i>Pseudogene</i> D(5) receptor <sup>†</sup>	$\psi$ <i>DRD5-2</i>	[101, 119–121]

\*This gene expresses low levels of mRNA and translates low quantities of the protein.

†Unknown peptide product but produces mRNA with unknown functions.

↔This protein cannot transport serotonin.

immunodetection in mouse bone marrow-derived dendritic cells (BMDCs) [75].

**3.4.1. DRs Are G Protein-Coupled Receptors (GPCR).** DRs belong to a superfamily of membrane proteins, called the *G protein-coupled receptor (GPCR) family of class A seven-transmembrane domain receptors (7TM)* [160, 161]. Dopaminergic GPCRs transmit signals toward two transducer-coupled systems: one using heterotrimeric G protein activation and the other using noncanonical G protein-independent,  $\beta$ -arrestin-dependent mechanisms. The heterotrimeric G protein complex comprises three subunits ( $G\alpha$ ,  $G\beta$ , and  $G\gamma$ ) that are coupled to the C-terminal end of dopaminergic GPCRs in the inner cell membrane. GPCRs and heterotrimeric G proteins conform two large coupled systems: the DR system and the dopaminergic signal transduction system. The receptor system is formed by a homodimer and sometimes a heterodimer, such as D1R/D2R. Thus, when a signal is received, it is transmitted to the intracellular region through the C-terminus of the receptor, which is coupled to heterotrimeric G protein systems. G proteins initiate signaling cascades by separating the  $G\alpha$  and  $G\beta/G\gamma$  subunits [16, 162].

DRs are functionally classified into the *D1-like* (D1R and D5R stimulatory receptors) and *D2-like* subtypes (D2RL, D2RS, D3R, and D4R inhibitory receptors), based on their

ability to stimulate the formation or inhibition of cAMP [48, 49]. The stimulation or inhibition of adenylate cyclases (ACs) (EC: 4.6.1.1) depends on the type of receptor that is coupled to heterotrimeric G proteins. Thus, D1R binds to the  $G\alpha_{s/olf}$  subunit; D5R binds to  $G\alpha_s$ , D2RL, D2RS, or D3R; and D4R binds to  $G\alpha_{i/o}$ . Nevertheless, reports on the putative D1R/D2R heterodimer and D1R and D5R receptors indicate the activation of complexes with the  $G\alpha_{q11}$  subunit, which acts on phospholipase C (PLC) signaling [16].

The activation of heterotrimeric G proteins is complex, because even if a DR has been classified by AC activation or inhibition, the specific proteins that constitute the heterotrimeric G protein complex are not accurately defined. This is evident when we consider the number of genes that encode for the  $G\alpha$ ,  $G\beta$ , and  $G\gamma$  subunits. In the human genome, 21  $G\alpha$  subunits are encoded by 16 genes, six  $G\beta$  subunits are encoded by five genes, and 12  $G\gamma$  subunits are encoded by 12 genes. The variations that arise by *splicing* increase the diversity of heterotrimers [163, 164]. The potential combinations of heterotrimeric G protein complexes suggest a delicate initiation of the signaling mechanism that is coupled to transduction in DR systems. In perspective, further study is needed to determine the specific subunits that form the trimeric complexes that are associated with AC activation or inhibition through the various DRs. These

data might help us understand the complex network of interactions that regulate dopaminergic signals in the immune system and its relationship with the CNS.

The complexity of these interactions increases if we also consider that heterotrimeric G proteins are divided into two complementary signaling systems. For D2R in striatal medium spine neurons, the activation of G protein releases the  $G\alpha_i$  subunit (AC activators) and the  $G\beta/\gamma$  subunits, which initiate the PLC activation cascade [165].

The other transducer system that is coupled to DRs is the noncanonical G protein-independent,  $\beta$ -arrestin-dependent mechanism, which, although studied less extensively, is just as important. D2R mediates the activation of the multifunctional adapter protein  $\beta$ -arrestin 2 ( $\beta$ Arr2) with phosphatase A2 (PP2A), which has slower and more persistent effects than the G protein system. Moreover, signals from G protein and  $\beta$ Arr2-PP2A have different physiological purposes, demonstrating that the DAS modulates signals by space and time [15, 166]. Further, D2R activation induces a signaling complex that comprises AKT1, PP2A, and  $\beta$ -arrestin 2 and downregulates PKA activity [167].

**3.5. DA Transporter DAT and Synaptic Vesicular Transporters VAT1 and VAT2.** The DAS has a plasma membrane-specific DA transporter (*SLC6A3* gene), called sodium-dependent dopamine transporter (DAT). Other transporters, such as chromaffin granule amine transporter (VAT1 protein; *SLC18A1/VMAT1* gene) and synaptic vesicular amine transporter (VAT2 protein; *SLC18A2/VMAT2* gene), participate as well. DAT and VAT2 have not been reported to have functional isoforms, but there are at least two isoforms of VAT1 (*VMAT1* and *VMAT1 $\Delta$ 15*) [117, 118].

VAT1 and VAT2, studied primarily in the brain, are general cytoplasmic amine transporter proteins that reside in the internal vesicular membranes of mammals [34, 168–172]. *SLC18A1/VMAT1* is preferentially expressed in neuroendocrine cells, whereas *SLC18A2/VMAT2* is mainly expressed in CNS cells [173]. *VAT1 $\Delta$ 15* has been observed in small amounts in the reticulum membrane and is unable to transport serotonin [118]. The function of this protein is yet unknown.

DA is captured and transported to the interior of the cell by DAT, a  $\text{Na}^+/\text{Cl}^-$ -dependent DA transporter. Also, SERT (serotonin transporter) is able to take DA to the inside of the cell, though at low rates [115, 173, 174]. In neuroendocrine and endocrine cells, once DA and other monoamines (serotonin, histamine, and norepinephrine) are found in the cytoplasm, they are stored in secretory vesicles by VAT1 and VAT2 through a capture system that is facilitated by a proton gradient that is generated by a vesicular protein, proton ATPase [175–178]. This vesicular confinement modulates monoamine degradation and reuse through a secretory system by exocytosis [61, 173].

DAT and VAT2 transporters are present in the membranes of human peripheral blood lymphocytes [103, 116], and the function and expression of DAT have been verified in leukocytes [69, 91, 104, 105, 179, 180]. However, few studies have examined the expression and function of *SLC18A1/VMAT1* and *SLC18A2/VMAT1* genes and their

protein products, VAT1 and VAT2, in immune cells in rodent models or human cells. This oversight represents an area of interest because mental and mood disorders have been linked to gene polymorphisms [181, 182] and because they might affect the function of the immune system.

Recently, the crystal structure of human DA D3R in complex with a D2R/D3R-specific antagonist, eticlopride, was solved at 2.89 Å resolution and deposited into Protein Data Bank under ID 3PBL [183]. This structure has guided the search for new drugs (agonists and antagonists) against DRs using *in silico* techniques (molecular dynamics simulation and homology modeling) and the creation and redesign of new chemical libraries [184]. The combination of these tools can help discover new molecules with potential use as drugs that are specific and selective for each type of DR [185].

#### 4. The Effects of DA on Leukocytes

The immune response is regulated by cytokines, hormones, and neurotransmitters; this regulation is possible because leukocytes have receptors for each one of these soluble factors (Table 3). DA is a neuroregulatory and immunoregulatory molecule that has significant effects on cells that are involved in the immune response. In leukocytes, there is a balance between internal DA, synthesized by DDC, and DA that is transported from blood plasma into the cell through DAT, the latter of which is the primary source of DA in leukocytes [186]. The protein components of the DAS are differentially expressed by leukocyte subtype and the state of cellular activation. One effect of DA is the regulation of leukocytes during activation and function in the immune response. These biological phenomena have recently begun to be examined in various cell types, such as granulocytes, monocytes/macrophages, dendritic cells, and lymphocytes. In this section, we will focus on the evidence showing the relationship of leukocytes with DA and how this catecholamine can regulate leukocyte populations.

**4.1. Hematopoiesis.** Bone marrow (BM) is innervated by autonomic sympathoadrenergic efferent nerve fibers, in which the local microenvironment is critical in the maintenance of hematopoietic stem cells (HSCs). Stem cells are characterized by their capacity for self-renewal throughout the life of an individual and respond to signals that are generated in the microenvironment and identified by cell surface markers, such as CD34 and CD38 [187]. Communication between the CNS and hematopoietic process is known as the “brain-bone-blood triad” and is mediated by many molecules, including such neurotransmitters as DA [188].

Maestroni and colleagues reported the first study on the functions of monoamines in hematopoiesis, performing chemical sympathectomy with 6-hydroxydopamine (6-OHDA) and observing a significantly higher peripheral blood leukocyte count in mice that underwent transplantation with BM [189].

Subsequently, Spiegel et al. demonstrated the expression of D3R and D5R in human CD34<sup>+</sup> cells by flow cytometry. The more primitive CD34<sup>+</sup>CD38<sup>lo</sup> cells had higher levels of D3R and D5R. However, D3R and D5R expression was

TABLE 3: The DRs effect on cytokine production.

Effect on cytokine production	Cellular types and stimuli	Receptors involved	References
↑ IL-6 <sup>††</sup> ↑ CCL2 <sup>††</sup>	HMDM + DA	D3R, D4R*	[79]
↑ IL-6 <sup>†</sup> ↑ CCL2 <sup>††</sup> ↑ CXCL8 <sup>††</sup> ↑ IL10 <sup>††</sup> ↓ TNF-α <sup>††</sup>	HMDM + DA + LPS	D3R, D4R*	[79]
↑ IFN-γ <sup>†</sup> ↓ IL-10 <sup>†</sup> ↓ IL-4 <sup>†</sup>	Human activated T cells + quinpirole	D3R	[97]
↑ IFN-γ <sup>†</sup> ↓ IL-10 <sup>†</sup> ↓ IL-4 <sup>†</sup>	Human activated CD4 T cells + quinpirole	D3R	[97]
↑ IFN-γ <sup>†</sup>	Human activated CD8 T cells + quinpirole	D3R	[97]
↑ IFN-γ <sup>†</sup>	Rats T cells + L-DOPA + carbidopa	D3R	[97]
↓ IL-12 <sup>†</sup> ↓ IL-23 <sup>†</sup>	D5RKO mice, mature bone marrow-derived dendritic cells + LPS	D5R absence	[75]
↓ IFN-γ <sup>†</sup> ↓ IL-2 <sup>†</sup> ↓ IL-4 <sup>†</sup>	Anti-CD3-stimulated human T cells + DA Anti-CD3-stimulated human T cells + SCH-23390 Anti-CD3-stimulated human T cells + clozapine	Not measured D1R/D5R D4R	[89]
↓ IL-10 <sup>††</sup> ↓ TGF-β <sup>††</sup>	Human CD4 <sup>+</sup> CD25 <sup>+</sup> regulatory T cells: (a) + reserpine + L-741626 (b) + reserpine + U-99194A (c) + reserpine + L-741741	(a) D2R, D3R, D4R (b) D3R (c) D4R	[83]
↓ IL-2 <sup>†</sup> ↓ IFN-γ <sup>†</sup> ↓ IL-6 <sup>†</sup> ↓ IL-2 <sup>†</sup> ↓ IFN-γ <sup>†</sup> ↓ IL-6 <sup>†</sup>	Mouse activated lymphocytes + L-DOPA Mouse activated lymphocytes + L-DOPA Mouse activated lymphocytes + L-DOPA Mouse activated lymphocytes + DA Mouse activated lymphocytes + DA Mouse activated lymphocytes + DA	Not measured	[122]
↑ IL-4 <sup>†</sup> ↑ IL-5 <sup>†</sup>	Naive CD4 <sup>+</sup> T cells + DA	D1R/D5R	[123]
↓ IFN-γ <sup>†</sup>	Human resting NK cells + DA	D5R	[92]
↑ IFN-γ <sup>††</sup> ↓ IFN-γ <sup>†</sup>	Human rIL-2 activated NK cells + DA Human rIL-2 activated NK cells + SKF-38393	D5R D5R	[92]
↑ IL-17 <sup>†</sup> ↓ IFN-γ <sup>†</sup> ↓ IL-17 <sup>†</sup> ↑ IFN-γ <sup>†</sup>	Immature human Mo-Dc + L75066 Immature human Mo-Dc + L75066 Immature human Mo-Dc + SHC23390 Immature human Mo-Dc + SHC23390	D2-like D2-like D1R/D5R D1R/D5R	[124]
↑ IL-10 <sup>††</sup> ↑ TNF-α <sup>††</sup>	T cells + DA T cells + DA	D2R, D1-like D3R, D1-like	[90]
↓ IL-2 <sup>†</sup>	Anti-CD3/CD28 stimulated T lymphocytes + PD168,077	D4R	[100]
↓ IL-2 <sup>†</sup> ↓ IL-10 <sup>†</sup>	Anti-CD3/CD28 activated Treg lymphocytes + DA Anti-CD3 + IL-2 activated Treg lymphocytes + DA	Not measured	[84]
↓ IL-2 <sup>††</sup>	Anti-CD3/CD28 activated T lymphocytes CD8 <sup>+</sup> + DA	Not measured	[98]
↓ IFN-γ <sup>†</sup> ↓ IL-4-γ <sup>†</sup>	Lymphocytes + DA	Not measured	[20, 125]
↓ IFN-γ <sup>†</sup> ↓ IFN-γ <sup>†</sup> ↑ IL-4-γ <sup>†</sup>	T cell (mesenteric lymph nodes) + ConA + SKF38393 T cell (mesenteric lymph nodes) + ConA + quinpirole T cell (mesenteric lymph nodes) + ConA + quinpirole	D1-like D2-like D2-like	[87]

TABLE 3: Continued.

Effect on cytokine production	Cellular types and stimuli	Receptors involved	References
↑ TNF- $\alpha$ <sup>†</sup>	Macrophages DAT <sup>-/-</sup> mice + LPS		
↑ IL-10 <sup>†</sup>	Macrophages DAT <sup>-/-</sup> mice + LPS		
↓ IFN- $\gamma$ <sup>†</sup>	Splenocytes DAT <sup>-/-</sup> mice activated	DAT absence	[126]
↓ IL-10 <sup>†</sup>	Splenocytes DAT <sup>-/-</sup> mice activated		
↑ IgG <sup>†</sup>	Serum DAT <sup>-/-</sup> mice		

<sup>‡</sup>Expression.

<sup>†</sup>Protein.

\*D3R and D4R were only detected by western blot.

lower in the more differentiated CD3<sup>+</sup>CD38<sup>hi</sup> cells, and positive correlations existed between DA receptor and an increase in the migration of CD34<sup>+</sup> cells cord blood that were pretreated with GM-CSF. Their results showed that DA is a chemoattractant that enhances the migration of immature CD34<sup>+</sup> cells [190]. As discussed, the function of DA in hematopoiesis, mediated by D3R and D5R, might be related to the elevation in circulating CD3<sup>+</sup> and CD4<sup>+</sup> lymphocytes, as suggested by the association of polymorphisms in *DRD1* and *DRD5* with these cell counts [191].

Additional work of the function of DA demonstrated the amelioration of neutropenia and the restoration of the number of colony-forming unit-granulocyte macrophage (CFU-GM) colonies in the bone marrow of mice that were treated with 5-fluorouracil (5FU). These results are consistent with reports that have indicated that DA can be used safely as an antiangiogenic drug for malignant tumors [192] (see Section 5).

**4.2. Granulocytes.** Granulocytes are fundamental immune cells, based on their abundance and rapid activation in the presence of foreign elements. Granulocytes contain granules in their cytoplasm that harbor various inflammatory and antimicrobial mediators that effect their defensive activities. Depending on the content of their granules, these cells are classified in eosinophils, basophils, and neutrophils [193].

Eosinophil function in immunity is related to the response against parasites [193]. These cells have a higher density of D3R and D5R receptors and a low density of D2R and D4R, whereas DIR is not detected [88]. No study has reported the effects of DA on eosinophils. Similarly, it is unknown whether basophils, involved in the allergic response, express DRs or respond to DA.

Neutrophils are the most abundant leukocyte population and have a significant function at the beginning of an inflammatory response [193]. These cells contain intracellular catecholamines, such as DA, epinephrine (E), and norepinephrine (NE), and several of their metabolites, such as DOPAC, 3MT, HVA, DL-3,4-dihydroxyphenylglycol (DHPG), and metanephrine (MET). Further, neutrophils synthesize and degrade such amines. *In vitro*, incubation with  $\alpha$ -methyl-*p*-tyrosine, an inhibitor of TH, reduces the intracellular concentration of DA, NE, and its metabolite, DHPG. Similarly, reserpine, a VAT inhibitor, lowers intracellular concentrations of DA and NE, and desipramine, an inhibitor of NE transporter (NET), decreases intracellular

NE concentrations. These findings implicate the existence of catecholamine storage and catecholamine reuptake mechanisms in neutrophils [194].

Neutrophils express D3R and D5R DA receptors and, at lower densities, D2R and D4R [88], which allows DA to modulate neutrophil function. Neutrophils (from peripheral blood) that are incubated with DA reduce their phagocytic activity dose-dependently, just like the production of oxygen reactive species and chemotaxis, with the maximum effect at 100 ng/mL [195]. Further, at 10  $\mu$ Mol/L and 100  $\mu$ Mol/L, DA influences the viability of neutrophils from healthy volunteers and patients with Systemic Inflammatory Response Syndrome (SIRS) inducing apoptosis after 12 h of incubation in healthy volunteers and 6 h in SIRS patients, whereas respiratory burst activity remains undisturbed [196].

DA also reduces the density of the adhesion molecules CD11b (Mac-1) and CD18 in neutrophils, decreasing endothelial adhesion. Even in neutrophils that have been stimulated with LPS or TNF- $\alpha$ , DA (10 nM and 100 nM) slows transendothelial migration and impedes chemoattraction by IL-8 versus cells that are stimulated in the absence of DA [197]. In that regard, neutrophils that are activated with FMLP (N-formyl-methionyl-leucyl-phenylalanine) and incubated with pharmacological concentrations of DA (261 nM) increase their density of CD62l (L-selectin) and decrease the density of CD11b. In the same report, DA at physiological concentrations (0.26 nM) had no effect on CD62l or CD11b [198].

**4.3. Monocytes/Macrophages.** Monocytes are found in peripheral blood, and on entering tissues, they complete their differentiation into macrophages. Macrophages have high phagocytic capacity toward microorganisms and dead cells, secrete large amounts of cytokines, and present antigen in the context of MHC II [193]. According to McKeena and colleagues, human monocytes bear a higher density of D2R and D3R compared with D4R and D5R [88]. There are few reports about the effects of DA on monocytes; resting peripheral blood CD14<sup>+</sup> monocytes express *DRD4* but not other DA receptors [86]. Also, human CD14<sup>+</sup> monocytes from peripheral blood and the U937 cell line (premonocytes) harbor intracellular DA, E, and NE, and CD14<sup>+</sup> monocytes also contain some metabolites, such as 3MT, DHPG, and MET [199].

DA modulates the phenotype and function of monocytes/macrophages. An *in vitro* study in chicken macrophages

demonstrated that high DA concentrations (1–5  $\mu\text{g}/\text{mL}$ ) are cytotoxic, causing up to 53% of cells to die. Incubation with DA at 0.1 and 0.5  $\mu\text{g}/\text{mL}$  for 1 hour improved their phagocytic activity, but extended exposure to DA (3 h) lowered this activity, although the percentage of Fc receptor-positive macrophages increased due to DA [200]. Haskó and colleagues demonstrated in LPS-activated peritoneal macrophages of Swiss mice that D2R stimulation using agonists (bromocriptine and quinpirole) and antagonists (sulpiride) lowered TNF- $\alpha$  and nitric oxide (NO) secretion. In contrast, D1R stimulation with the antagonist SCH23390 only downregulated NO production compared with controls [201].

In an elegant study, Gómez and colleagues reported that macrophages from guinea pigs that were immunized *in vivo* for 7 days using DA agonists (bromocriptine, leuprolide, and pergolide) or DA antagonists (chlorpromazine, SCH23390, metoclopramide, sulpiride, veralipride, alizapride, and cisapride) and primary macrophage cultures from guinea pigs that were stimulated *in vitro* with these drugs had increased Fc $\gamma$  receptors expression. The DA agonists improved the clearance of IgG-sensitized RBCs (*in vitro* recognition of IgG-sensitized RBCs by splenic macrophages) and enhanced the membrane expression of Fc $\gamma$  receptors in macrophages; in contrast, the DA antagonists impaired macrophage Fc $\gamma$  receptor expression. Thus, the disturbance in Fc $\gamma$  receptor expression is more extensive when D1R is stimulated and less so on activation of D2R [202].

Bergquist and colleagues examined whether the binding of NF- $\kappa$ B to DNA was inhibited by DA in nontransformed human peripheral blood monocytes and transformed human monocyte cell lines that were activated with LPS (10 ng/mL). Cell proliferation declined at 10  $\mu\text{M}$  DA in peripheral blood monocytes and at 100  $\mu\text{M}$  in the monocyte cell line after 24 h; however, the low concentrations (1  $\mu\text{M}$  and 0.1  $\mu\text{M}$ ) had no effects. They also observed that DA suppressed LPS-mediated activation of NF- $\kappa$ B and LPS-induced binding of NF- $\kappa$ B to the TNF- $\alpha$  promoter dose-dependently—an effect that might be attributed to the inhibition of NF- $\kappa$ B translocation from the cytoplasm to nucleus by DA [203]. In 2002, Haskó and colleagues showed that DA has anti-inflammatory effects by binding to its receptors and through other mechanisms. Using the J774.1 cell line and C57Bl/6 mice peritoneal macrophages that were stimulated with LPS (10  $\mu\text{g}/\text{mL}$ ) and DA (0.01  $\mu\text{M}$ –100  $\mu\text{M}$ ), they observed that IL-12 p40 secretion and mRNA decreased dose-dependently, whereas IL-10 secretion was increased. These effects were not caused by DR stimulation (addition of the DR antagonists SCH23390 and raclopride did not inhibit the effects) but by the stimulation of  $\beta$ -adrenergic receptors as determined by the addition of the  $\beta$ -adrenergic antagonist propranolol, which had partial inhibitory activity [204].

Human monocyte-derived macrophages (HMDMs) from healthy donors express the *SLC6A3/DAT*, *SLC18A2/VMAT2*, *TH*, *DDC*, *DRD1* [79–81], *DRD2*, *DRD3*, *DRD4*, and *DRD5* genes and the D1R, D2R, D3R, D4R, DAT, VAT2, TH, and DDC proteins on the cell surface and in the cytoplasm, suggesting that these cells contain the machinery for synthesis, reuptake, and response to DA [79]. Another report showed

that DA modulates cytokine secretion in HMDMs with and without LPS stimulation; LPS-activated HMDMs that were stimulated with elevated concentrations of DA (2  $\mu\text{M}$  and 20  $\mu\text{M}$ ) increased IL-6, CCL2, CXCL8, and IL-10 secretion while TNF- $\alpha$  secretion declined. Conversely, lower DA concentrations (20 nM and 200 nM) affected only TNF- $\alpha$ , IL-6, and CCL2 secretion and upregulated IL-10, albeit insignificantly. In cells without LPS stimulation, 2  $\mu\text{M}$  and 20  $\mu\text{M}$  DA enhanced IL-6 and CCL2 secretion. These findings suggest that macrophages develop differential responses, depending on the microenvironment (inflammatory or homeostatic), that are modulated by DA [79].

**4.3.1. DA Receptor Roll in HIV Infection in Macrophages.** DA also has effects on macrophages with regard to D2R-mediated HIV replication. This effect has been observed in HMDMs of healthy donors that have been infected *in vitro* with the HIV<sub>ADA</sub> and HIV<sub>YU2</sub> strains [80] and in Jurkat cells (T-cell-derived line) that have been transfected with the HIV proviral genome—an effect that is mediated by the activation of NF- $\kappa$ B [205]. D2R activation by the agonist quinpirole stimulates ERK1 by phosphorylation and increases HIV replication dose-dependently compared with unstimulated infected cells and cells stimulated with the D1R agonist SKF82958 [80].

In macrophages, the entry of HIV via CD4 and CCR5 in the plasma membrane depends on gp120 binding [206], and an increase in the density of CCR5 and CD4 enhances HIV infectivity [207]. Incubation of HMDMs with IL-4 and IL-10 upregulates CCR5 and CD4, accelerating the infection [207, 208]. Similarly, a recent study showed that DA facilitates HIV entry through CCR5 and that TAK779, a CCR5 inhibitor, impedes viral entry [209]. These data suggest that entry of the virus requires the activation of DRs and is inhibited by a global DR antagonist, such as flupenthixol, through effects that do not depend on viral concentration.

The average percentage of infected HMDMs rises with high concentrations of DA—between  $10^{-5}$  and  $10^{-8}$  M—dose-dependently, with a “steep threshold” at approximately  $10^{-8}$  M. This finding confirms that CCR5 and DA-mediated DRs activation are necessary for viral entry in HMDMs. Specifically, *D1-like* and *D2-like* are the receptors that are activated and involved in HIV entry, indicating that there is a common pathway of activation that depends on Ca<sup>2+</sup> mobilization [209]. Methamphetamine enables infection in HMDMs and increases the activity of viral reverse transcriptase and CCR5 density, thus downregulating IFN- $\alpha$  and STAT1 protein expression. STAT1 is a signal transducer and transcriptional activator that mediates cellular responses to interferons, cytokines, and growth factors. However, the D1R antagonists SCH23390 and SKF83566c block HIV<sub>Bal</sub> ineffectiveness [81]. These data suggest the participation of dopamine receptors during macrophage infection by HIV which might have clinical applicability but demands more clinical trials.

**4.4. Dendritic Cells.** Dendritic cells (DCs) are the most efficient antigen-presenting cells of the immune system, with key functions in the induction of adaptive immune responses,

immune tolerance, and the modulation of immune responses [193]. *Human monocyte-derived dendritic cells* (Mo-DCs) express the *D1-like* and *D2-like* receptors in the membrane, of which the *D2-like* receptors predominate functionally [123, 124]. DCs synthesize DA and store it in vesicles near the cell membrane, as observed in Mo-DCs, in which DA synthesis and storage increase when intracellular cAMP levels rise. Further, Mo-DCs liberate DA during their interaction with naive CD4<sup>+</sup> T lymphocytes, which promotes polarization toward the Th2 phenotype; antagonism of Mo-DCs *D2-like* receptors with sulpiride and nemonapride raises cAMP levels, releasing DA and influencing naive CD4<sup>+</sup> T lymphocytes, for example, by increasing the Th2/Th1 ratio (through the IL-5:IFN- $\gamma$  relationship), upregulating CCR4 (a Th2-type receptor), and decreasing CXCR3 (a Th1-type receptor) expression [123].

DA also has effects on murine bone marrow-derived dendritic cells (BMDCs), which express the molecular components that are needed to respond to, synthesize, store, and degrade DA. BMDCs bear D1R, D2R, D3R, and D5R on the membrane and vary their expression profile according to their state of activation: mature (stimulated by LPS) and immature (without stimulus). In the mature state, the intracellular enzyme TH, low levels of *Scl18a2/Vmat2* mRNA, *Slc6a4/Sert*, *maoa*, and *maob* have been observed without detectable *Slc6a3/dat*, *Slc6a2/Net1* (NE transporter), or *Dbh* mRNA. The same results have been obtained for immature cells, but there was more *Scl18a2/Vmat2* mRNA [75]. Although BMDCs do not express DAT in the membrane or have quantifiable mRNA levels, SERT is present, and this transporter can carry DA at a low velocity [174].

In BMDCs, D5R appears to participate in maturation and regulate signaling pathways and cytokine release, thus contributing to the activation and proliferation of CD4<sup>+</sup> T lymphocytes. BMDC activation with LPS significantly decreases the density of D5R in the membrane. On stimulation with SKF38393, a selective D1R/D2R agonist, the phosphorylation of ERK1/2 increases. Notably, D5R is linked to IL-12 and IL-23 production; it has been observed that mature *Drd5* knockout (*Drd5KO*) cells express less *Il-23a* (IL-23) and secrete less IL-12. These reports implicate an autocrine regulatory mechanism during cell maturation in which the release of DA and D5R activation selectively promote the secretion of certain regulatory cytokines. Similarly, CD4<sup>+</sup> T lymphocytes significantly decrease IL-2 secretion and proliferation when they are activated and incubated using *Drd5KO* cells versus WT cells. This effect is not observed in CD8<sup>+</sup> T lymphocytes, suggesting that D5R expression in DCs facilitates the strong priming of CD4<sup>+</sup> T lymphocytes [75].

**4.5. Lymphocytes.** Lymphocytes are primordial cells of the adaptive immune response that recognize antigens in their molecular context. Depending on their ligands, lymphocytes have many subpopulations with a wide variety of functions; these cells can modulate, regulate, and coordinate the activities of other leukocyte populations through cytokine secretion and at the same time, lymphocytes can respond to circulatory levels of cytokines, hormones, and neurotransmitters [193].

**4.5.1. Synthesis of Dopamine and Receptors.** In the late 20th century, lymphocytes were demonstrated to have the metabolic ability to synthesize catecholamines and their metabolites; they also release and recapture these molecules, responding to them by expressing catecholamine receptors. The initial reports on DA and DOPAC in lymphocytes were based on cerebrospinal fluid and human T and B lymphocyte cultures; these studies reported that intracellular catecholamine concentrations and the inhibition of TH with  $\alpha$ -methyl-*p*-tyrosine downregulated intracellular catecholamines, which rose on exposure of the cells to DA [20]. Later, the presence of DA, L-DOPA, and NE in lymphocytes was confirmed by electrospray ionization mass spectrometry [210].

Another study reported that human peripheral lymphocytes exhibit intracellular DA, L-DOPA, and NE at concentrations that are detectable by HPLC and that T lymphocytes only contain L-DOPA and NE. *In vitro*, T lymphocytes that have been incubated with L-tyrosine ( $5 \times 10^{-5}$  M) or L-DOPA ( $10^{-8}$  M– $10^{-5}$  M) increase their intracellular NE concentrations. This finding suggests the presence of catecholamine synthesis pathways in these cells [143]. Marino and colleagues noted that PBMCs contain DA, NE, E, and metabolites, such as DOPAC, 3MT, HVA, DHPG, MET, and VMA. This group reported that the addition of  $\alpha$ -methyl-*p*-tyrosine decreased DA and NE concentrations intracellularly and in the medium; further, incubation with desipramine or GBR12909, 2 catecholamine reuptake inhibitors, significantly increases DA and NE levels in the medium, indicating the existence of an active recapture mechanism [116].

In the late 1990s, a study in rats confirmed that lymph node, splenic, and thymic lymphocytes contain intracellular catecholamines (DA, NE, and E), observing *TH* mRNA and protein in these cells. Also, the authors found that ConA-activated (concanavalin A) ( $5 \mu\text{g/mL}$ ) lymphocytes had higher intracellular catecholamine and TH concentrations than unstimulated cells [108, 211]. CD4<sup>+</sup> CD25<sup>-</sup> T<sub>H</sub> lymphocytes and CD4<sup>+</sup> CD25<sup>+</sup> T<sub>reg</sub> were proven to express TH and intracellular L-DOPA, DA, E, and NE and some catecholamines metabolites. Further, incubation of CD4<sup>+</sup> CD25<sup>+</sup> T<sub>reg</sub> with reserpine (an inhibitor of VAT1) downregulated intracellular catecholamine concentrations, whereas concentrations in the medium rose [83].

The first report on DAT in peripheral blood lymphocytes was performed using several techniques. By radiobinding assay (RBA) using a specific ligand for DAT, [<sup>3</sup>H]-GBR12935, it demonstrated specific binding in the membrane of human lymphocytes. By western blot, DAT, VAT1, and VAT2 were expressed, whereas DA and VAT2 showed immunoreactivity in cytoplasmic areas, corresponding to vacuoles, by immunofluorescence. Finally, DAT and VAT1 were detected in the plasma membrane and cytoplasm, respectively [103]. Later, Marazziti and colleagues confirmed DAT expression by RBA using [<sup>3</sup>H]-WIN35428 in human lymphocytes, 1 of the most DAT-selective radioligands. This group observed [<sup>3</sup>H]-DA reuptake, suggesting the presence of a DA reuptake mechanism [179]. In addition, Ferrari and colleagues reported that DA modulates its synthesis in human T and

B lymphocytes from peripheral blood through PKC activation using 12-O-tetradecanoylphorbol-13-acetate (TPA) at 100 ng/mL. This activity increases *TH* mRNA levels and intracellular catecholamines—effects that are significantly inhibited with DA (1  $\mu$ M) and SKF30393, a D1-like agonist, demonstrating that the stimulation of D1-like receptors impedes catecholamine synthesis [77].

The search for DA receptors in lymphocyte has been a difficult task. Unstimulated human lymphocytes express D2R, D3R, D4R, and D5R, but the activation of these cells modifies the expression of the receptors. In 1991, the initial data on specific dopamine binding sites in human lymphocytes were obtained using [<sup>3</sup>H]-DA, the binding of which declines considerably in the presence of such substances as cocaine and other inhibitors of biogenic amine uptake [69]. Subsequently, D5R was detected in the membranes of human lymphocytes by RBA using the dopaminergic antagonist [<sup>3</sup>H]-SCH23390, as were 3 *DRD5* mRNA sequences (by RT-PCR) and the transcription of 2 pseudogenes [101]. Similarly, D3R expression was observed in the membrane using the specific ligand [<sup>3</sup>H]-7-hydroxy-N,N-di-*n*-propyl-2-aminotetralin ([<sup>3</sup>H]-7-OH-DPAT) [95, 212], as were its full-length mRNA sequence and a shorter variant transcript, generated by alternative splicing [95, 151].

Another study with [<sup>3</sup>H]-sulpiride in human lymphocytes by RBA characterized the D2-like receptors in the membrane and their similarities with D2R and D4R and, to a lesser extent, D3R [158]. Conversely, the expression of D1-like receptors was described in 2 studies by RBA using [<sup>3</sup>H]-SCH23390 [73, 102] and immunocytochemistry [102], in which the expression of D5R, but not D1R, was demonstrated. Other studies on D4R in human lymphocytes detected it by RT-PCR [99, 100] and by RBA using [<sup>3</sup>H]-clozapine [159] and western blot [100].

In 1998, Ricci and colleagues measured the expression in membrane-bound D2-like receptors by RBA and immunocytochemistry with greater precision. They observed that the ligand [<sup>3</sup>H]-7-OH-DPAT is not specific for D3R, because, on incubation with anti-D3R and anti-D4R, the interaction decreases 53% and 32%, respectively, whereas anti-D2R has no effect [96]. Another study measured D3R and D4R by RBA using the radioligands [<sup>3</sup>H]-7-OH-DPAT, [<sup>3</sup>H]-spiperone, and [<sup>3</sup>H]-nemonapride and immunocytochemistry [94]. Cosentino and colleagues showed that intracellular catecholamines fall when TH is inhibited with  $\alpha$ -methyl-*p*-tyrosine and reserpine in primary human PBMC cultures; reserpine also decreases catecholamine concentrations in the medium. This study also demonstrated that cell lines from hematopoietic precursors, such as NALM-B (pre-B), Jurkat (T lymphoblastoid), and U937 (promonocytic), synthesize catecholamines, because their intracellular DA, E, and NE decrease on incubation with  $\alpha$ -methyl-*p*-tyrosine and reserpine [199].

In 2002, McKenna et al. performed flow cytometry to confirm the membrane expression of the three D2-like receptors and D5R and the absence of D1R [88]. The existence of D5R in the membrane was later confirmed by RBA using the radioligand [<sup>3</sup>H]-SCH23390. By RT-PCR, expression

of *DRD2*, *DRD3*, *DRD4*, and *DRD5*—but not *DRD1*—was observed [93]. DA receptors and DAT, with the exception of D4R, were detected in B lymphocytes and in several malignant B cell lines; also, this report showed higher *DRD1* and *DRD2* transcript levels versus *DRD3* and *DRD4* [78]. In 2014, by 5-color flow cytometry, Kustrumovic and colleagues showed that naive T CD4<sup>+</sup> T lymphocytes, central memory CD4<sup>+</sup> T lymphocytes (TCM), and effector memory CD4<sup>+</sup> T lymphocytes (TEM) expressed D1R, D2R, D3R, D4R, and D5R in the membrane at various densities between subpopulations. In CD4<sup>+</sup> T lymphocytes that were stimulated with anti-CD3/CD28 (0.01–0.1  $\mu$ g/mL) for 48 hours, the density of the five DA receptors in the membrane was altered after activation, with increases in the D1-like (71% to 84%) and D2-like (55% to 97%) receptors. With regard to the levels of DRs, the frequency of these receptors is higher in apoptotic cells than in viable T lymphocytes. However, the stimulation of viable T lymphocytes increases receptor density to similar levels as in apoptotic lymphocytes [85].

**4.5.2. Effects of Dopamine in Lymphocytes.** The immunomodulatory effects of DA have significant relevance in understanding the relationship between the immune system and CNS. Reports in rodent and human lymphocytes have found that DA receptors in PBMCs are functional and activate signaling cascades that change the phenotype and function of lymphocytes. Some groups have reported the effects of DA on cytokine secretion, cell adhesion, and chemotaxis in human and rodent lymphocytes.

In 2001, Levite and colleagues suggested the importance of DA in integrin-mediated cellular trafficking and extravasation of human T lymphocytes in the brain and periphery, based on findings that 7-hydroxy-DPAT (a D3R agonist), bromocriptine, and pergolide (D2R agonists) activate T lymphocytes, upregulating  $\beta$ -integrins expression (mainly  $\alpha$ 4 $\beta$ 1 and  $\alpha$ 5 $\beta$ 1) and increasing adhesion to fibronectin (FN) [213]. In 2004, Cosentino and colleagues showed that, at various doses, DA has opposing effects on oxidative metabolism and apoptosis in human lymphocytes. At low doses (0.1–5  $\mu$ M), DA decreases the concentration of reactive oxygen species (ROS) and inhibits apoptosis through stimulation of D1-like receptors. However, at high concentrations (100–500  $\mu$ M), DA increases intracellular ROS levels and lymphocyte apoptosis [24]. In 2005, Besser and colleagues demonstrated that human resting T lymphocytes express D2R, D3R, and D5R on their membrane and that their stimulation upregulates the secretion and expression of cytokines, such as TNF- $\alpha$  and IL-10. Stimulation with DA between 10<sup>-4</sup> M and 10<sup>-7</sup> M increased TNF- $\alpha$  secretion through D3R and D1-like receptors stimulation, whereas IL-10 secretion was mediated by D2R and D1-like receptors stimulation; in contrast, concentrations of DA between 10<sup>-9</sup> M and 10<sup>-14</sup> M did not have any discernible effects [90].

Watanabe and colleagues showed that CD8<sup>+</sup> T lymphocytes selectively express D3R and that its stimulation mediates chemotaxis and CD8<sup>+</sup> T lymphocyte adhesion. DA and its agonist, 7-OH-DPAT (100 nM), increase CD45RA<sup>+</sup> CD8<sup>+</sup> naive T lymphocyte chemotaxis, whereas the combination of DA and other chemokines enhances chemotaxis in CD45RA<sup>+</sup>

CD4<sup>+</sup> and CD45RA<sup>+</sup> CD8<sup>+</sup> T lymphocytes. Stimulation with only CCL19 (10 nM), CCL21 (10 nM), and CXCL12 (0.1 nM) significantly induces the migration of CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes, but the addition of DA (1 nM) induces the selective migration of CD45RA<sup>+</sup> CD8<sup>+</sup> T lymphocytes. In addition, D3R stimulation induces the adhesion of CD45RA<sup>+</sup> CD8<sup>+</sup> T and CD45RO<sup>+</sup> CD8<sup>+</sup> T lymphocytes to FN [86]. Conversely, Strell and colleagues showed that human CD8<sup>+</sup> T lymphocytes expressed D3R and D4R and, to a lower extent, D5R. They also found that these receptors were downregulated on cellular activation, with the exception of D5R. Stimulation with DA (1  $\mu$ M) decreased the activation of these cells by anti-CD3/CD28, decreasing the expression and secretion of IL-2 by reducing ERK1/ERK2 phosphorylation. This stimulation also increased I $\kappa$ B, which lowers NF- $\kappa$ B phosphorylation levels. This effect prevents the creation of an autocrine IL-2 loop, which is needed for optimal activation of T lymphocytes [98].

With regard to animal models, Kipnis and colleagues showed that DA regulates the adhesion and chemotaxis of mouse Treg lymphocytes by stimulating D1-like receptors via ERK. CTLA-4 expression decreases in Treg lymphocytes that are incubated with DA or SKF38393, as does IL-10 secretion at concentrations of 10<sup>-5</sup> M. These effects are attributed to ERK phosphorylation. Finally, this group found that DA affects the adhesion and chemotaxis of Treg lymphocytes; adhesion to SPG (extracellular matrix proteins that are associated with injured tissues) decreases dose-dependently (10<sup>-9</sup>–10<sup>-5</sup> M), and DA downregulates the receptor for CCR-4, affecting the migration of these cells toward macrophage-derived chemokines (MDCs) [84].

Watanabe and colleagues showed by *in vivo* assays that intraperitoneal administration of DA or 7-OH-DPAT to mice selectively attracts CD44<sup>low</sup> CD8<sup>+</sup> T lymphocytes, effecting their accumulation in the peritoneal cavity. They also reported that DA mediates the homing of naive CD8<sup>+</sup> T lymphocytes toward secondary lymphoid tissues through D3R, because U99194A, a D3R antagonist, reduces the number of CD44<sup>low</sup> CD8<sup>+</sup> T lymphocytes in the inguinal lymph nodes. Similarly, *in vitro* stimulation of LL2 pre-B lymphocytes with DA or 7-OH-DPAT (100 nM) increases calcium efflux and elicits a selective chemotactic response for CD44<sup>low</sup> CD8<sup>+</sup> T lymphocytes [86].

Other reports have shown that DA modulates the activation, proliferation, and differentiation of lymphocytes in humans and rodents. In human cells, Bergquist and colleagues showed that ConA-activated peripheral blood lymphocytes stimulated *in vitro* with DA (10  $\mu$ M and 100  $\mu$ M) slow their proliferation, differentiation, and synthesis of IFN- $\gamma$  dose-dependently, with complete inhibition reached at 500  $\mu$ M. Further, incubation with DA from 10  $\mu$ M to 500  $\mu$ M completely inhibits the production of antibodies in pokeweed mitogen- (PWM-) stimulated B lymphocytes [20]. This group also reported that human lymphocytes (activated with ConA or PWM) stimulated with DA (10  $\mu$ M and 100  $\mu$ M) produce less IL-4 but experience 2.8-fold greater apoptosis; apoptotic markers, such as Bcl/Bax and Fas/FasL, are also upregulated [125].

In 2001, Saha and colleagues demonstrated that high DA concentrations in serum affect the proliferation of CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes and the cytotoxicity of lymphocyte activated killer T cells (LAK-Ts) from patients with lung carcinoma and healthy subjects. The patients had high DA concentrations in plasma compared with healthy subjects (48.6  $\pm$  5.1 pg/mL and 10.2  $\pm$  0.9 pg/mL, resp.). *In vitro*, this high concentration (48 pg/mL) slowed the proliferation of CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes from patients and healthy donors, which did not occur at physiological concentrations (10 pg/mL). Similarly, LAK-Ts from patients and healthy subjects had less cytotoxic activity, attributed to D1R stimulation due to the rise in intracellular cAMP [214]. Further, the group performed the same experiments in CD4<sup>+</sup>, CD8<sup>+</sup>, and cytotoxic T lymphocytes from patients with uncoping stress and healthy subjects, showing that these patients also had high plasma DA concentrations (46.6  $\pm$  3.9 pg/mL) versus the latter (10  $\pm$  2.7 pg/mL). The effect of high DA concentration was the same as in patients with lung carcinoma [215].

Ghosh and colleagues reported that DA also affects TCR-mediated signaling in T lymphocytes. Incubation with DA at 3–5 ng/mL for 1 to 3 days inhibited the proliferation and secretion of IL-2, IFN- $\gamma$ , and IL-4 in anti-CD3-activated T lymphocytes, due to D2R and D3R stimulation. They also showed that DA downregulates the nonreceptor tyrosine kinases Lck and Fyn, which are important in TCR signaling; this effect can decrease lymphocyte activation and cytokine secretion [89]. In 2006, Sarkar and colleagues observed that D4R stimulation in human T lymphocytes induces quiescence by upregulating lung Krüppel-like factor 2 through inhibition of ERK1/ERK2 phosphorylation. T lymphocytes that were activated with anti-CD3/CD28 and a D4R-specific agonist (PD168,077 or APT724 at 1  $\mu$ M) experienced less proliferation, which was not observed in unstimulated T lymphocytes. Similarly, cells that were activated for 24 hours with PD168,077 (1  $\mu$ M) had equal levels of IL-2 secretion and CD69 and CD25 expression as in nonactivated lymphocytes. PD168,077 also prevented a decline in KLF2 expression, a transcription factor that regulates quiescence and inhibition of ERK1/ERK2 phosphorylation [100].

Studies in rodent cells *in vivo* and *in vitro* have demonstrated the effects of DA on lymphocyte activation, proliferation, and differentiation, such as Tsao et al., who observed *in vivo* that DA governs splenocyte proliferation, based on the intravenous administration of agonists for D1-like (SKF38393) and D2R (LY171555) (1, 5, and 10  $\mu$ g/Kg) in BALB/cByJ mice. They showed that agonists enhance LPS- or ConA-induced splenocyte proliferation; however, intraperitoneal administration of the neurotoxin MPTP (20  $\mu$ g/kg) reduced endogenous DA levels and suppressed proliferation [216]. Carr and colleagues determined that chronic administration of L-DOPA (126 mg/Kg every 5 days) or L-DOPA combined with domperidone, an agonist of D2R, affects proliferation and cytokine secretion in mouse splenocytes. This group demonstrated that L-DOPA increases the proliferation of spleen cells that have been stimulated with ConA or anti-CD3 by approximately 2.2-fold [217].

In 2011, a study reported that stimulation of *D1-like* receptors suppresses ovalbumin antigen-induced neutrophilic airway inflammation in OVA TCR-transgenic DO11.10 mice. These mice were nebulized with OVA or LPS and received a *D1-like* antagonist (SCH23390) by oral administration before OVA administration. SCH23390 significantly inhibited OVA-induced neutrophilic airway inflammation, due primarily to its ability to halt the infiltration of neutrophils, macrophages, and lymphocytes. IL-17 and IL-22 synthesis and infiltration of Th17 cells in the lung were also lower. Conversely, IL-23 production was suppressed in DC11c APCs in response to LPS/anti-CD40 [218].

*In vitro*, Cook-Mills and colleagues demonstrated that DA (10  $\mu$ M and 100  $\mu$ M) and NE inhibit the activation of splenic lymphocytes from BALB/c mice. However, this effect is not blocked by adrenergic or dopaminergic antagonists, suggesting that the inhibitory effect is mediated by other lymphocyte receptors [219], such as serotonin [7], hormone, and cytokine receptors [193]. According to Josefsson and colleagues, mouse splenocytes produce catecholamines and are stimulated by DA, L-DOPA, and NE, which are enhanced by L-tyrosine and inhibited by TH inhibitors. L-DOPA and DA (0–500  $\mu$ M) dose-dependently suppress mitogen-induced proliferation and differentiation of mouse splenocytes, even with short treatment times. Moreover, L-DOPA and DA (500  $\mu$ M) also inhibit IL-2, IL-6, and IFN- $\gamma$  synthesis and IgG and IgM secretion [122].

Bergquist and colleagues reported that, at high concentrations (100–500  $\mu$ M), DA inhibits the proliferation of T lymphocytes and the secretion of IL-2, IL-6, and IFN- $\gamma$  at 500 nM [125]. Tsao et al. demonstrated that DA promotes the proliferation of splenocytes from BALB/cByJ mice in response to LPS or ConA [216]. Carr and colleagues showed that mouse splenocytes (BALB/c) that were treated with L-DOPA secreted less IFN- $\gamma$  but produced the same amount of IL-4 [217]. Huang and colleagues found that T lymphocytes from mouse mesenteric lymph nodes, which express the five DA receptors, are polarized toward the Th2 phenotype when *D2-like* receptors are stimulated, suggesting that this effect involves the cAMP-CREB pathway. In the same lymphocytes, stimulation with the *D1-like* agonist SKF38393 reduced only IFN- $\gamma$  secretion; in contrast, quinpirole, a *D2-like* agonist, enhanced IFN- $\gamma$  and IL-4 secretion and decreased proliferation and cAMP and phosphorylated CREB content [87].

DA also has indirect effects on the phenotype and function of lymphocytes. This catecholamine first modulates the function of its target cell, which in turn affects the function and phenotype of the lymphocyte with which it interacts; these effects on human cells have been described by other groups.

In 2007, Cosentino and colleagues demonstrated that DA is released by Treg, allowing them to regulate their activity. However, when Treg do not release DA, they experience autoregulation and lose the ability to suppress Teff proliferation. CD4<sup>+</sup>CD25<sup>-</sup> Teff and CD4<sup>+</sup>CD25<sup>+</sup> Treg from healthy donors express the five DA receptors. Treg have detectable mRNA levels of *SCL18A1/VMAT1*, *SCL18A2/VMAT2*, *DRD2*, *DRD3*, *DRD4*, and *DRD5*, whereas

Teff only express *SCL18A1/VMAT1* and *SCL18A2/VMAT2*. When DA release is inhibited by reserpine (1  $\mu$ M) in Treg/Teff cultures, Treg diminish their mRNA levels and secretion of IL-10 and TGF- $\beta$  through stimulation of *D1-like* receptors. These events significantly reduce the proliferation of CD3/CD28-activated T lymphocytes but do not affect the secretion of TNF- $\alpha$  and IFN- $\gamma$  [83]. Nakano and colleagues demonstrated that *D2-like* antagonists induce the differentiation of Th17 cells (*in vitro*), mediated by DCs, using a mixed lymphocyte reaction (MLR) between *human monocyte-derived dendritic cells* (Mo-DCs) and naive CD4<sup>+</sup> T lymphocytes. These antagonists increased the secretion of IL-17 in 16-hour cultures of Mo-DCs and CD4<sup>+</sup> T lymphocytes that were activated with anti-CD3/CD28; in contrast, *D1-like* antagonists (SCH23390, SKF83566, and LE300) decreased IL-17 levels [124].

A 2004 study by Kipnis et al. in mice showed that Treg from splenic lymph nodes had lower regulatory activity on stimulation of *D1-like* receptors through ERK activation. Moreover, the incubation of Treg/Teff with DA (10<sup>-7</sup> M) increased Teff proliferation 2-fold, which was also observed in T lymphocytes that were activated with anti-CD3 and IL-2. This group also found that the negative regulatory activity of Treg on Teff proliferation is inhibited by genistein, a MEK and ERK inhibitor. This inhibition was also observed with the ERK inhibitor PD98059 [84].

Mori and colleagues reported that *D1-like* receptors mediate immediate and late-phase skin reactions by promoting Th2 differentiation and mast cell degranulation. In *in vivo* Th1-type contact hypersensitivity and Th2-type atopic dermatitis models, they observed that SCH23390 does not affect Th1-type contact hypersensitivity but suppresses immediate-type (ITRs) and late-phase reactions (LPRs) in the atopic dermatitis model. In addition, SCH23390-treated mice had higher *IFNG* and lower *IL-2* mRNA levels in the ear skin versus untreated mice. This report also used bone marrow-derived mast cells (BMMCs), fetal skin-derived cultured mast cells (FSMCs), and naive Th2 splenic lymphocytes as *in vitro* models. Using these models, the group demonstrated that mast cells and CD4<sup>+</sup> T lymphocytes have *D1-like* receptors and that DA increases mast cell degranulation and Th2 cell differentiation; both of these activities were abrogated by SCH23390. In T lymphocytes, the ratio of *IL-4/IFNG* mRNA rose on addition of DA. Also, DA increased the release of  $\beta$ -hexosaminidase from BMMCs dose-dependently; this effect was also observed when *D1-like* receptors were stimulated through IgE-triggered Fc $\epsilon$ R1 [220].

**4.5.3. Other Approaches.** Ilani and colleagues hypothesized that dopaminergic activation of blasts (cells that cross the blood-brain barrier) induces the Th1 phenotype and effects changes in membrane surface markers. The authors suggest that these alterations are transferred from blasts to peripheral resting T lymphocytes by neurotransmitter-mediated brain regulation of peripheral T lymphocytes. In this work, blast formation was induced from peripheral blood lymphocytes of healthy donors (cells that have been activated with mitogen and IL-2 that express VLA-4 on their membrane); these blasts were incubated with the D2R/D3R agonist quinpirole

( $10^{-5}$  M– $10^{-7}$  M) for 8 hours. Quinpirole downregulates *IL-4* and *IL-10* but increases *IFNG* expression but has no effects on resting T lymphocytes.

Further, this group noted differential responses between  $CD4^+$  and  $CD8^+$  blasts.  $CD4^+$  blasts had lower *IL-4* and *IL-10* mRNA levels, but *IFNG* mRNA rose. In contrast,  $CD8^+$  blasts only upregulated *IFNG* mRNA. Differences were also noted in adhesion molecules between T lymphocytes and blasts—unchanged in the former during quinpirole stimulation but with the latter upregulating *IL-2RA* (CD25) mRNA and decreasing *CXCR3* levels.

In resting T lymphocytes, incubation with blast supernatant for 24 hours without quinpirole increased *IL-4* and *IL-10* mRNA levels, whereas that with quinpirole upregulated only *IFNG*. These findings support the hypothesis that blasts that are stimulated with DA in the CNS trigger the phenotype implantation in peripheral blood resting T lymphocytes [97].

**4.6. NK Cells.** NK cells constitute less than 10% of all circulating lymphocytes and have significant functions in the immune response against viruses, intracellular bacteria and in tumor cell destruction [193]. Studies on the effects of DA on NK cells were initially performed in rodents and demonstrated that DA has antitumor effects against *Ehrlich ascites carcinoma* cells [221]; in Swiss mice with transplantable Ehrlich ascites carcinoma, cancer cells are controlled through increased splenic NK cells [222]. Other studies in NK cells from the spleens of APO-SUS rats with a hyperdopaminergic phenotype [223] and mice with the *slc6a3/Dat knockout* phenotype [126] reported a decline in NK cell activity and dampened *mitogen-induced cytokine responses*. Another study has reported that mouse splenic NK cells express the 5 DA receptors in their membrane and that the stimulation of D1-like receptors with SKF38393 increases the density of D1R and D5R; SKF38393 also improves the cytotoxic response against YAC-1 lymphoma cells (Moloney leukemia virus-induced mouse lymphoma) through cAMP-PKA-CREB signaling, but stimulation of D2-like receptors with quinpirole impairs NK lymphocyte function [82].

There are differences in the phenotype and function of human and murine NK cells—human NK cells express membrane D2R, D3R, D4R, and D5R but lack D1R [88, 92]. Without activation, human NK cells have no disturbances in phenotype or function when they are exposed to DA; however, stimulation with high concentrations of rIL-2 ( $\approx 200$  IU) for 5 days induces the overexpression of D5R. At concentrations between  $10^{-9}$  M and  $10^{-18}$  M, DA reduces NK cell division and inhibits IFN- $\alpha$  secretion dose-dependently, wherein D5R signaling is compromised [92].

## 5. Clinical Implications of the Effects on the Dopaminergic System

Central and peripheral DA can directly or indirectly regulate the immune system in several pathological conditions, such as neurodegenerative (Parkinson disease, Alzheimer disease, and Lesch-Nyhan syndrome), psychiatric (schizophrenia), and immune diseases (multiple sclerosis, encephalomyelitis,

and rheumatoid arthritis, among others), and conditions that have an addictive component, such as alcoholism.

In terms of a physiopathological perspective, disturbances in the levels of central DA affect the function of lymphocytes, because DA is supplied by the sympathetic nervous system to primary and secondary lymphoid tissues, modulating a wide range of immune activities, such as the regulation of innate immune and adaptive responses [224]. Several studies have reported disturbances in central DA production under pathological conditions, the most common of which is Parkinson disease, characterized by the selective destruction of dopaminergic neurons in the substantia nigra. However, recent studies indicate that DA production and the expression of dopaminergic receptors are dysregulated in other neurodegenerative and autoimmune diseases, which significantly impacts the immune response.

The expression of dopaminergic receptors in lymphocytes from patients with neurodegenerative and autoimmune diseases has recently been proposed as a diagnostic biomarker and a marker of pathological severity, because the variations in the density of DA receptors on lymphocytes are usually similar to what is observed in the brain. In this section, we catalog the evidence on variations in the expression of DRs on lymphocytes in various pathologies (Table 4).

**5.1. Neurodegenerative Diseases.** Changes in activation of the immune response in patients and in experimental models of neurodegenerative diseases have been described and implicated in their pathogenesis. Abnormalities in the number and function of circulating lymphocytes are linked to an increase in the production of proinflammatory mediators. The evidence in this section strongly suggests that the DAS participates in the modulation of the immune response.

**5.1.1. Parkinson Disease.** Parkinson disease is a neurodegenerative pathology that is characterized by the dysfunction and degeneration of dopaminergic neurons in the substantia nigra, neuroinflammation, and motor disturbances. In animal models in which the selective loss of dopaminergic neurons from the substantia nigra is induced by systematic administration of methyl-4-phenyl-1,2,3,6 tetrahydropyridine (MPTP), significant changes in the immune response have been observed. For example, proinflammatory cytokines, such as IFN- $\gamma$ , IL-2, IL-17, and IL-22, are upregulated in the spleen and mesenteric lymphatic ganglia; T-bet, a fundamental transcription factor in Th1 differentiation, is downregulated in T cells; and the expression of Foxp3, a transcription factor that induces the development and maintains the function of Treg, rises [225].

Regulation by the central DAS modifies peripheral immune functions. For instance, MPTP-induced depletion of DA in the striatum promotes tumor growth, which is associated with dysfunctional cytotoxic activity in T lymphocytes and NK cells [2, 226]. In this regard, NK cell activity wanes on injury to the nucleus accumbens in animal models [227].

Some studies propose that the activation of circulating lymphocytes is able to regulate the neurodegenerative events

TABLE 4: Pathology-associated dopaminergic protein expression in immune cells.

Pathology/condition	DA receptors and cell types involved	Reference
Parkinson disease Patients/MPTP mice	↓ D3R in lymphocytes	[127]
Lesch-Nyhan disease	↑ D5R in lymphocytes	[128]
Multiple sclerosis	↓ D5R in lymphocytes	[129]
Multiple sclerosis treatment with IFN- $\beta$	↑ D5R in lymphocytes ↓ D2R in lymphocytes	[129, 130]
Schizophrenia	↑ D4R in T CD8 <sup>+</sup> and T CD4 <sup>+</sup> lymphocytes ↑ D4R in T CD8 <sup>+</sup> lymphocytes ↓ D2R in T CD4 <sup>+</sup> lymphocytes	[131]
Rheumatoid arthritis type II collagen-induced arthritis (mice)	↑ D2R in lymphocytes	[132]
Rheumatoid arthritis and osteoarthritis	↓ D5R in B cells ↑ D2R & D3R in B cells	[133]
Systemic lupus erythematosus	↑ D4R in lymphocytes ↓ D2R in lymphocytes	[134]
Alcohol dependence syndrome	↑ D4R in lymphocytes	[135]
Alcohol withdrawal	↑ D1R in lymphocytes	[136]
Computer game addicts	↓ D5R in lymphocytes	[137]

in the substantia nigra in Parkinson disease. The stimulation of D3R in CD4<sup>+</sup> T lymphocytes decreases their synthesis of IL-4 and IL-10 while promoting IFN- $\gamma$  production. Thus, D3R is a relevant target in the physiopathology of Parkinson disease. DRD3-deficient mice that have been treated with MPTP are susceptible to neurodegenerative events in the substantia nigra after receiving CD4<sup>+</sup> T lymphocytes from MPTP-treated wild-type mice [127].

Similarly, *Drd3*KO mice are resistant to MPTP-induced neurodegeneration but become susceptible on transfer of CD4<sup>+</sup> T lymphocytes from MPTP-treated wild-type mice. However, they are not prone to MPTP-induced neurodegeneration when they receive CD4<sup>+</sup> T lymphocytes from D3R-deficient mice [127]. *DRD3* expression in lymphocytes is reduced in Parkinson disease patients and correlates with disease severity, possibly due to changes in D3R density in other lymphocyte populations. These data show that D3R in T lymphocytes favors the activation and acquisition of the Th1 phenotype, suggesting that D3R in CD4<sup>+</sup> T lymphocytes has an important function in the physiopathology of the murine model of Parkinson disease [127].

**5.1.2. Other Neurodegenerative Diseases.** Patients with a likely diagnosis of Alzheimer disease have a low density of D2-like receptors on lymphocytes. This decrease is also observed in postmortem samples of brains from Alzheimer disease patients [228]. However, Cosentino and colleagues did not observe any differences in the mRNA levels of DRs in lymphocytes in patients with a probable diagnosis of Alzheimer disease [229]. These controversial results might be attributed to the statistical parameters, such as sample size (number of participants), age, gender, and the presumptive diagnosis. With regard to the DAS, the lymphocytes of patients with

probable Alzheimer disease experience an increase in the immunoreactivity of dopamine  $\beta$ -hydroxylase [230]; nevertheless, more studies are needed to determine the function and effects of DA on lymphocytes in the physiopathology of Alzheimer disease.

Studies have reported changes in the expression of DRs in lymphocytes in various pathologies of the CNS. Lesch-Nyhan syndrome is a neurogenetic disorder that is caused by the complete deficiency of hypoxanthine-guanine phosphoribosyltransferase, which effects severe motor disturbances, predominantly dystonia (numbness) and occasionally chorea (involuntary movements); these secondary symptoms are related to disturbances in the production of DA in the CNS. Lesch-Nyhan syndrome patients have higher levels of *DRD5* in lymphocytes, rendering it a potential biomarker for the diagnosis of this disease and prompting the use of L-DOPA in Lesch-Nyhan patients as an alternative treatment [128].

## 5.2. Psychiatric Disorders

**5.2.1. Schizophrenia.** Studies on disturbances in the DAS in schizophrenia patients have reported changes in the expression of DA receptors. In the 1980s, a study examined the binding of [<sup>3</sup>H]-spiperone, a specific dopaminergic antagonist, to peripheral blood lymphocytes from healthy volunteers, 27 patients with acute schizophrenia under no treatment, and 16 psychiatric patients as a control group by RBA. The study did not find any differences in binding parameters between the healthy and psychiatric control groups, whereas the binding of [<sup>3</sup>H]-spiperone increased significantly in lymphocytes from schizophrenia patients with a slight decrease in affinity [70].

At the beginning of the 21st century, Kwak and colleagues performed an 8-week longitudinal study to measure D3R and D5R expression in peripheral blood lymphocytes from 44 patients who had been treated pharmacologically for over three years, 15 drug-naive schizophrenic patients, 28 drug-free patients, and healthy controls. *DRD3* mRNA in drug-naive patients climbed significantly compared with medicated patients and healthy controls, and *DRD5* mRNA was considerably higher only versus medicated patients. In drug-free and drug-naive patients, the expression of the receptors rose two weeks after antipsychotic treatment was begun, decreasing at 8 weeks of treatment. When the drug-naive and drug-free patients were divided into two groups by *DRD3* expression before the treatment, those with higher *DRD3* levels presented with more severe psychiatric symptoms [231]. In the same year, Ilani and colleagues observed a significant increase in *DRD3*, but not *DRD4*, mRNA in peripheral blood lymphocytes from 14 schizophrenic patients who were not under medication with respect to healthy subjects. This rise was not affected by treatment with typical or atypical antipsychotic drugs. Based on these data, the group implicated *DRD3* as an identification and tracking marker [232].

In 2006, Boneberg and colleagues measured DA receptor (D1R–D4R) expression in neutrophils, monocytes, B lymphocytes, NK cells, and CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes from 10 schizophrenic patients, reporting a significant increase in *DRD3* mRNA in T lymphocytes and downregulation of *DRD4* mRNA in T CD4<sup>+</sup> lymphocytes compared with healthy volunteers [233]. In contrast, Vogel and colleagues reported a decrease in *DRD3* mRNA in peripheral blood leukocytes from 13 schizophrenic patients and 11 patients with bipolar disorder, subdivided as follows: drug-naive (never having ingested antipsychotics), drug-free (without any treatment for at least four weeks), and drug-treated (under pharmacological treatment). The schizophrenic drug-naive and drug-free patients had significantly less *DRD3* mRNA compared with healthy subjects. However, patients who were under treatment had higher *DRD3* mRNA levels during the six weeks of treatment; consequently, these levels were similar to those in healthy controls [234].

Another study attempted to identify schizophrenia markers in peripheral blood lymphocytes from 13 drug-naive/drug-free patients. In a microarray analysis, *DRD2* and inwardly rectifying potassium channel (Kir2.3) were overexpressed compared with healthy subjects; this effect was confirmed, based on the elevated mRNA levels of both genes. The group suggested using these genes to predict schizophrenia [235]. Urhan-Kucuk and colleagues studied 55 schizophrenia patients and 51 healthy subjects to determine whether *DRD3* expression in peripheral blood lymphocytes could be used as a marker of disease. They noted no significant difference in *DRD3* mRNA levels between schizophrenic patients and healthy subjects. However, across schizophrenia subtypes (residual, disorganized, and paranoid), these levels differed between the disorganized and paranoid subtypes and between disorganized schizophrenia and healthy subjects, prompting the authors to conclude that *DRD3* mRNA could

be used as a peripheral marker of schizophrenia subtype [236].

Recently, Brito-Melo and colleagues used flow cytometry to measure membrane expression of D2R, D4R, and serotonin receptors in CD4<sup>+</sup> and CD8<sup>+</sup> peripheral blood T lymphocytes from schizophrenic patients who had been treated pharmacologically for ten years. They correlated these levels with several clinimetric scales: Brief Psychiatric Rating (BPRS), Positive and Negative Syndrome (PANSS), and Involuntary Movement (AIMS). The group observed significant overexpression of D4R in CD8<sup>+</sup> and CD4<sup>+</sup> T lymphocytes from schizophrenic patients and upregulation of D2R in CD8<sup>+</sup> T lymphocytes; in contrast, D2R levels were lower in CD4<sup>+</sup> T lymphocytes. Further, BPRS and PANSS scores correlated with CD8<sup>+</sup>D2R<sup>+</sup> lymphocyte levels, and AIMS scores were positively associated with CD4<sup>+</sup>D2R<sup>+</sup> T lymphocytes levels and inversely related to CD4<sup>+</sup>D4R<sup>+</sup> T levels [131].

In 2013, Liu and colleagues measured *DRD2* and *SLC6A3/DAT* expression in peripheral blood leukocytes from 25 patients with acute schizophrenia, 27 patients with chronic schizophrenia, and healthy subjects to determine whether their mRNA levels correlated with PANSS scores. There was no significant difference in *DRD2*, but *SLC6A3/DAT* was higher in patients with chronic schizophrenia compared with healthy subjects. In addition, they noted a correlation between *DRD2* mRNA levels and positive scores on the PANSS—but only in acute schizophrenia patients [91].

Another study analyzed the *DRD3*, *DRD2*, and *DARPP-32* (dopamine and cyclic adenosine 3',5'-monophosphate-regulated phosphoprotein-32) mRNA levels in peripheral blood lymphocytes from healthy subjects, patients with an unspecified psychotic disorder, and patients with schizophrenia/schizophreniform disorder, examining the relationship between these genes and the psychopathological state of the patients. The study demonstrated that *DRD3* mRNA in T lymphocytes differed considerably between the three groups but that *DRD2* and *DARPP-32* levels were similar. Further, *DRD3* expression correlated with the excitement factor on the PANSS in patients with schizophrenia/schizophreniform disorder. According to the authors, *DRD3* mRNA levels can be used as a diagnostic marker to differentiate patients with early psychosis from healthy controls [237].

### 5.3. Autoimmune Diseases

**5.3.1. Multiple Sclerosis.** Multiple sclerosis (MS) is the most common immune-mediated demyelinating disease of the CNS. This condition causes disability in 2.3 million people worldwide. In MS, myelin-reactive CD4<sup>+</sup> Th lymphocytes enter the CNS, where they interact with resident cells, promoting inflammation, demyelination, and neurodegeneration [238]. Th cell subsets that are involved in the pathogenesis of MS include Th1 cells, which secrete the proinflammatory cytokines TNF- $\alpha$  and IFN- $\gamma$ , and Th17 lymphocytes, which produce IL-17 [239].

The typical treatment for MS is IFN- $\beta$ , which induces the production of DA and other catecholamines in human lymphocyte cultures [240]. DA downregulates IL-17 and

IFN- $\gamma$  production by PBMCs in patients with relapsing-remitting MS and healthy controls, strengthening the evidence of the potential benefit of dopaminergic agents in MS [3].

In untreated patients, the expression and activity of D1-like receptors (but perhaps not D2-like receptors) in circulating PBMCs are tempered [238], although untreated MS patients express less *DRD5* mRNA and protein without an increase in D3R [129, 130]. Immunomodulatory drugs, such as IFN- $\beta$ , restore the functional responsiveness of DRs on lymphocytes. Moreover, IFN- $\beta$  therapy appears to shift the balance of DRs in lymphocytes from predominantly D2-like in the cells of untreated patients toward primarily D1-like. D1-like receptors mediate most of the dopamine-dependent inhibition of human T lymphocyte proliferation and cytotoxicity, whereas D2-like receptors induce T lymphocyte proliferation and adhesion. Upregulation of D1-like receptors is thus expected to be beneficial in MS [129, 130, 238, 241].

Functional dysregulation of Treg contributes to disease pathogenesis and activity in autoimmune mouse models of the CNS and in patients with MS. Thus, the use of DR agonists in MS might suppress Treg via D1-like receptors, with detrimental effects [238]. Notably, treatment with IFN- $\beta$  downregulates D1-like receptors on Treg and impedes the ability of DA to inhibit Treg function [3].

These findings suggest that the dopaminergic pathways in circulating lymphocytes have relevant immunomodulatory functions in the pathology of MS, impacting the development of drugs for patients with MS—DR agonists have beneficial effects as an add-on to immunomodulatory treatments with such agents as IFN- $\beta$ , and they might act preferentially on D1-like rather than D2-like receptors [238].

**5.3.2. Encephalomyelitis.** Experimental autoimmune encephalomyelitis (EAE) is an experimental model of human MS. Balkowiec-iskta and colleagues showed that injury to the dopaminergic system modulates the clinical course and inflammatory reaction during EAE; this group studied the effects of dopamine depletion with 1-methyl-4-phenyl-1,2,3,4-tetrahydropyridine (MPTP) in C57BL mice with EAE (induced by the MOG 35–55 peptide). They found that MPTP decreased striatal DA levels, and the mean number of inflammatory cells in the spinal cord infiltrate was significantly higher in MPTP + MOG 35–55-treated versus MOG 35–55-treated mice. The mortality rate in mice with a dysfunctional dopaminergic system was lower than in MOG 35–55-treated mice. Also, *Il1b* mRNA was significantly upregulated in the MPTP + MOG 35–55 group, correlating with clinical progression of the disease; this IL-1 $\beta$  increase could be responsible for these changes, causing a more severe course of EAE [242].

Nakano et al. showed that antagonizing D1-like receptors suppresses IL-17 production and prevents EAE in SJL/J mice—animals that were treated with L750667 (a D2-like antagonist) developed hyperacute EAE, the progression of which was accelerated and quickly resulted in death. Conversely, mice that were administered SCH23390 (a D1-like antagonist) did not present with any clinical symptoms. Also, splenocytes from EAE mice that were treated with

SCH23390 for 30 days produced less IL-17, whereas IFN- $\gamma$  levels rose. The transfection of BMDCs from SJL/J mice that were treated with antagonists *in vitro* into SJL/J mice affected the same clinical incidence of EAE. These results suggest that D1-like receptor antagonists ameliorate EAE, an effect that is accompanied by an increase in IFN- $\gamma$  and the suppression of IL-17 in antigen-specific T lymphocytes [124]. In 2012, Prado and colleagues demonstrated in C57BL/6 mice with EAE *in vivo* that D5R-deficient DCs that were transferred prophylactically into wild-type mice mitigated the severity of EAE. Further, mice into which D5R-deficient DCs were transferred experienced a significant reduction in the percentage of Th17 cells that infiltrated the CNS compared with animals that received wild-type DCs, whereas the percentage of Th1 lymphocytes remained in similar levels [75].

**5.3.3. Rheumatoid Arthritis.** Rheumatoid arthritis (RA) is a chronic inflammatory disease that is characterized by pannus tissue, consisting of synovial fibroblasts (SFs), macrophages, and lymphocytes. The inflammatory milieu in the joint activates resident SFs and transforms them [243]; in particular, SFs increase their expression of D1R and D5R [244]. RA has a high predominance of Th1 and Th17 lymphocytes; further, DA localizes to DCs in the synovial tissue of RA patients and significantly increases its levels in this fluid [244]. In RA patients, DCs release DA during antigen presentation to naive CD4<sup>+</sup> T lymphocytes [245], which raises IL-6-dependent IL-17 production via D1-like receptors, in response to T lymphocyte activation by anti-CD3 and anti-CD28 [244].

The involvement of D2R in RA has been demonstrated in several murine models. Its activation mitigates clinical symptoms, and *Drd2* knockout (*Drd2KO*) mice develop severe symptoms of RA; D2R antagonists induce the accumulation of IL-17<sup>+</sup> and IL-6<sup>+</sup> T cells in synovial fluid, exacerbating the inflammatory process. Similarly, RA patients express low levels of *DRD2* in lymphocytes, which is linked to disease severity [132]. Based on this evidence, D2R agonists have been proposed to be therapeutic agents for RA.

D5R levels in B lymphocytes from RA and osteoarthritis patients are lower than in healthy volunteers, whereas those of D2R and D3R are higher [133]; Nakano and colleagues have suggested that DA that is released by DCs activates the IL-6-Th17 axis, aggravating synovial inflammation in RA [246]. Thus, clinical protocols have been developed in which clinical researchers should use D2R agonists, such as bromocriptine and cabergoline, to lower prolactin synthesis and secretion by infiltrating synovial fibroblasts and lymphocytes in patients with RA—the improvement in RA activity might be attributed to a significant decrease in the secretion of prolactin by immune cells [247, 248], although these results are not conclusive.

**5.3.4. Systemic Lupus Erythematosus.** Systemic lupus erythematosus is an autoimmune disease that is characterized by the dysfunction of several organs, including the liver and brain, due to a dysregulated immune system. In PBMCs of systemic lupus erythematosus patients, *DRD2* is downregulated, whereas *DRD4* increases compared with a control group. The

decrease in D2R levels might be associated with the reduction in the function and numbers of Treg cells in this pathology [134].

#### 5.4. Miscellaneous Clinical Implications

**5.4.1. Glomerulonephritis.** Glomerulonephritis encompasses a range of immune-mediated disorders that cause inflammation in the glomerulus and other compartments of the kidney [249]. DA mediates the control of renal sodium excretion, and DRs have been detected in various regions of the nephron—it has been reported that DA is synthesized in the renal proximal tubules. A defect in renal DA receptor function and DA production has been suggested to accelerate the pathogenesis of hypertension [250]. Conversely, in a brain-dead model in rats, the administration of DA reduced monocyte infiltration in renal tissue, indicating that DA has a direct anti-inflammatory effect that is mediated by D1-like and D2-like receptors stimulation [251].

In another study by Hoeger et al. using the same brain-dead model in rats, the changes in cytokine and chemokine expression were measured to determine the mechanism by which DA lowers renal inflammation. This study evaluated the expression of IL-6, IL-10, macrophage chemoattractant protein 1 (MCP-1), and cytokine-induced neutrophil chemoattractant 1 (CINC-1), a rat homolog of IL-8. No significant changes were observed in IL-6, IL-10, and MCP-1, but CINC-1 was significantly downregulated in the brain-dead animal group that was treated with DA compared with controls, implicating this change as an anti-inflammatory mechanism that is induced by DA during renal inflammation [252]. Kapper et al. also demonstrated that DA dose-dependently inhibits the production of the chemokines Gro- $\alpha$ , ENA-78, and IL-8 in proximal tubular epithelial cells (PTECs) [253]. Collectively, these findings support the function of DA as an immunomodulator in glomerulonephritis.

**5.4.2. Cancer and Angiogenesis.** The dopaminergic system has garnered significant interest in angiogenesis and tumor immunity. Endothelial cells express components of the dopaminergic system; thus, DA governs angiogenesis, prompting an examination of the molecular mechanisms that are associated with modulation of tumor immunity, its mechanisms of control, and the link between tumor immunity and angiogenesis [214, 254, 255].

In a murine model, the antitumor effects of DA on Ehrlich ascites carcinoma cells already have been reported [221]. This inhibitory effect suppresses the growth of cancer cells by increasing the number of peripheral large granular lymphocytes (LGLs) and the activity of NK cells. Even in healthy mice (without Ehrlich cells) that have been treated with DA, these effects are also observed [222].

The low incidence of certain types of cancer in schizophrenic patients, in contrast with the high incidence in patients with Parkinson disease, reflects the inhibitory effects of DA on cancer cell growth. This hypothesis is based on the finding that schizophrenic patients express a hyperdopaminergic system, whereas Parkinson patients are hypodopaminergic. However, it is unknown if disruptions

in the dopaminergic system in the CNS contribute to the development of tumor angiogenesis outside of the CNS [256–260]. However, the effects of DA on schizophrenic patients might be linked to medication with DR antagonists, suggesting that the development of specific cancers is DR-dependent [261].

Conversely, DA selectively inhibits vascular permeability and the angiogenic activity of VEGF. This inhibitory effect is mediated by the activation of D2R and the induction of the endocytosis of VEGFR-2 [262, 263]. Further, at low pharmacological doses, DA delays tumor angiogenesis by inhibiting VEGFR-2 phosphorylation in endothelial cells that express D2R, as reported in rat malignant gastric tumors (adenocarcinoma type) and xenotransplanted human gastric cancers in mice. In malignant tumors of the stomach from humans and rats, endogenous DA and TH enzyme are absent compared with normal tissue [264].

Another study demonstrated that DA acts through D2R to inhibit the proliferation of gastric cancer cells that have been induced by insulin-like growth factor receptor-I (IGF-IR). This inhibition is mediated by the upregulation of Krüppel-like factor 4 (KLF4) [265]. Also, the ablation of peripheral dopaminergic nerves increases angiogenesis, density, and microvascular permeability, permitting the growth of malignant tumors in mice [266]. Recent reports have demonstrated that the administration of DA stabilizes and normalizes tumor blood vessels by acting on pericytes and endothelial cells, primarily by activation of D2R [254, 267]. Thus, DA has been proposed to be a safe antiangiogenic drug for the control of tumor progression [192].

The relationship between DA, transformed cells, and the immune system is unknown, but it is likely that a link exists with mechanisms of the identification and elimination of abnormal cells, because DA inhibits tumor angiogenesis and stimulates tumor immunity; nevertheless, more studies are needed to examine this issue.

**5.4.3. Diabetes Mellitus.** The involvement and significance of DA as a neurotransmitter and immunomodulator have been studied, but its effects on glucose homeostasis and pancreatic  $\beta$ -cell function are unknown [268]. Diabetes mellitus is a group of metabolic diseases that are characterized by hyperglycemia, resulting from defects in insulin secretion, insulin activity, or both. Chronic hyperglycemia in diabetes is associated with long-term damage, dysfunction, and failure of organs, especially the eyes, kidneys, nerve fibers, heart, and blood vessels [269].

DA and its derivatives can act directly in pancreas; the networks between the CNS and pancreatic islets are based on the central vagal connection through the parhypothalamic ventricular nucleus [270]. The pancreas expresses DA receptors, depending on cell type; mRNA of the five DA receptors is expressed in  $\beta$ -cells [271], but only D1R, D2R, and D4R proteins have been detected in these cells [271–273].

Insulin production depends on the concentration of DA [274]. In humans, neuroleptic drugs cause hyperinsulinemia in normal subjects and are associated with diabetes in psychiatric patients [268]. DA and its agonists modulate  $\beta$ -cell activity, but it is unknown whether DA promotes or

inhibits insulin secretion [275]. The activation of D2R receptor inhibits glucose-stimulated insulin secretion in isolated islets from rodents and  $\beta$ -cell lines [271], and another study in which D2R was knocked out in the INS-1 832/13 mammalian cell line reported an increase in insulin secretion [276]. García-Tornadú et al., using a global D2R knockout mouse, showed that the disruption in D2R impairs insulin secretion and causes glucose intolerance [277].

One regulatory mechanism that explains the effects of DA states that DA in pancreatic  $\beta$ -cells involves a dopaminergic negative feedback loop that regulates insulin secretion from human and murine pancreatic islets. According to this model, DA is stored in  $\beta$ -cells and is cosecreted with insulin; this endogenous DA acts in an autocrine/paracrine manner on insulin-secreting  $\beta$ -cells that express D2-like receptors [67, 278].

The effects of DA on the immune system in this disease are not well elucidated. One study showed that the D1-like antagonist SCH-23390 has a preventive effect on diabetes mellitus that occurs naturally in NOD mice. In this *in vivo* model, Th17 lymphocytes mediate the development of diabetes in NOD mice. The islet infiltrates appear to be composed of mononuclear cells that are positive for IL-23R (a specific Th17 marker), but SCH-23390 significantly decreases the levels of infiltrating cells. This group reported that the antagonism of D1-like receptors suppresses IL-17 production and prevents naturally occurring diabetes in NOD mice, accompanied by an increase in IFN- $\gamma$  production [279].

Diabetes mellitus is a disease with an important inflammatory component; understanding the regulation of DA in the immune system and the significance of the inflammatory condition in the development of this disease will require a more detailed examination of the regulation of DA in this condition with regard to immunity.

**5.5. Addictions.** The function of the D1R and D2R receptors in the brain is linked to the reward system and might be affected by extended drug use. Several reports indicate that the expression of DRs in immune system cells is altered during addiction. For instance, in patients who suffer from alcohol dependence, *DRD1* mRNA levels in lymphocytes are higher compared with healthy controls [136]. Other studies have demonstrated that in alcohol dependence syndrome patients, *DRD4* is upregulated in peripheral lymphocytes versus a control group [135].

Similarly, opioid addicts experience an increase in *DRD3* expression in circulating lymphocytes compared with healthy controls [280]. On the other hand, a chronic rise in D1R might be a risk factor that predisposes individuals to some type of addiction allowing one to diagnose the severity of the addictive condition [281]. The expression of DA receptors in the immune system might be a valuable biomarker of the risk for pathologies and addictive conducts; thus, it has also been measured in computer game addicts, in whom *DRD5* levels in circulating lymphocytes are lower than in control subjects [137]. Notably, the personality of volunteers has been correlated with the expression of the *DRD3* and *DRD4* receptors [282]. These findings suggest that DA receptor expression in the periphery constitutes a significant

link between neurobiological processes and immune system function.

## 6. Conclusions

Based on the activity of DA as a neurotransmitter, studies on the DAS have focused primarily on the CNS. Recently, however, copious experimental evidence indicates that the DAS has important physiological functions in the immune system. The human DAS is complex and comprises many elements; leukocytes have frequently been demonstrated to synthesize, release, perform reuptake of, and metabolize DA. In certain cases, as in Treg cells, DA is released to elicit autocrine effects, implicating the existence of a peripheral DAS that is independent of the CNS.

In this review, we have discussed the immunomodulatory effects of DA by activating DRs, which are differentially expressed in leukocytes, depending on cell type, activation state, DA concentration, and duration of exposure to DA. These effects regulate many cellular processes, such as cell activation, cell adhesion, proliferation, respiratory burst, chemotaxis, apoptosis, cytotoxicity, and cytokine and antibody secretion, and several changes at the phenotypic level and function in certain cell types. All of this activity is linked to the intracellular concentration of cAMP and the activation status of second messengers and transcription factors.

The information that we have here is based on existing reports, but the effects of DA on the immune system require further characterization. The peripheral DAS is dysregulated in patients with psychiatric disorders, neurodegenerative diseases, and other conditions, such as multiple sclerosis. However, more research is needed to demonstrate that peripheral disturbances in the DAS are equivalent to those in the CNS, which will facilitate the identification and characterization of new peripheral biomarkers for diagnostic purposes and the evaluation of the therapeutic efficacy of pharmacological treatments in these illnesses.

## Competing Interests

The authors declare no competing interests with regard to this paper.

## Authors' Contributions

Rodrigo Arreola, Samantha Alvarez-Herrera, Gilberto Pérez-Sánchez, Enrique Becerril-Villanueva, Carlos Cruz-Fuentes, Enrique Octavio Flores-Gutierrez, María Eugenia Garcés-Alvarez, Dora Luz de la Cruz-Aguilera, Emilio Medina-Rivero, Gabriela Hurtado-Alvarado, Saray Quintero-Fabián, and Lenin Pavón drafted the paper. All authors reviewed the paper and approved the final version.

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## Research Article

# Activated Circulating T Follicular Helper Cells Are Associated with Disease Severity in Patients with Psoriasis

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Circulating T follicular helper (cTfh) cells are known to be involved in numerous immune-mediated diseases, but their pathological role in psoriasis is less fully investigated. Herein, we aimed to identify whether cTfh cells contributed to the pathogenesis of psoriasis. The frequency and function of cTfh cells were compared between patients with psoriasis vulgaris and healthy controls, and the infiltration of Tfh cells was detected between lesional and nonlesional skin tissues of psoriasis patients. Moreover, the dynamic change of cTfh cells before and after acitretin treatment was evaluated. Our results showed both increased frequency and activation (indicated by higher expression of ICOS, PD-1, HLA-DR, and Ki-67 and increased production of IL-21, IL-17, and IFN- $\gamma$ ) of cTfh cells in psoriasis patients. Compared with nonlesional skin tissues of psoriasis patients, the number of infiltrated Tfh cells was significantly increased in psoriasis lesions. In addition, positive correlations between the percentage of cTfh, functional markers on cTfh cells in peripheral blood and disease severity were noted. Furthermore, the frequency of cTfh cells and the levels of cytokines secreted by cTfh cells were all significantly decreased after 1-month treatment.

## 1. Introduction

Despite years of efforts to develop new agents, the recurrence rate and adverse effects related to long-term agent use remain significant in patients with psoriasis [1]. After decades' study, it is generally accepted that psoriasis is a T cell-mediated disease [2]. In addition, though it was previously assumed that Th1 and Th2 cells were the major cells contributing to the development of psoriasis, increasing evidence in recent years showed that Th17 cells played a vital role in the pathogenesis of psoriasis [3]. Clinical trials have reported that the biological agents targeting IL-17, such as secukinumab, brodalumab, and ixekizumab, are effective and safe in patients with psoriasis vulgaris [4]. Except for Th17, Th9 and Th22 cells, which have a close interaction with Th17 [5, 6], were also found increased in psoriasis lesions [7, 8]. Therefore, these newly discovered T cell subsets and their interactions

possibly provide a new insight into the molecular mechanism of psoriasis.

T follicular helper (Tfh) cells, which are located in the germinal centers (GCs), are another specialized subset of CD4<sup>+</sup> T cells. High expression levels of chemokine CXC receptor 5 (CXCR5), inducible T cell costimulator (ICOS), programmed cell death protein-1 (PD-1), and the transcription factors B cell lymphoma 6 (Bcl-6) are distinctive characteristics of Tfh cells [9]. The function of Tfh cells has been found to be closely related to several factors, such as the expression of ICOS and PD-1 and the secretion of IL-21 [10, 11]. Recently, circulating T follicular help (cTfh) cells have been found in human blood, which are considered counterparts to Tfh cells in GCs. cTfh cells share similar markers and have familiar function with Tfh cells in GCs [12]. Many reports have shown that the dysregulated behavior of cTfh cells contributes to numerous autoimmune diseases

TABLE 1: Clinical characteristics of participants.

Group	Patients with psoriasis vulgaris (PV)	Healthy controls (HC)	P
	n = 32	n = 13	
Age (years)	47.22 ± 10.35	42.47 ± 11.21	>0.05
Gender (F/M)	19/17	9/8	>0.05
Disease duration (year)	4.55 ± 2.97	—	—
PASI scores (n = 32)	16.33 ± 8.06	—	—
Mild (n = 8)	7.17 ± 2.09	—	—
Moderate (n = 15)	14.80 ± 2.75	—	—
Severe (n = 9)	25.32 ± 6.61	—	—

and infectious diseases, such as systemic lupus erythematosus (SLE) [13], rheumatoid arthritis [14], pulmonary tuberculosis [15], and human immunodeficiency virus (HIV) infection [16]. However, the role of cTfh cells in psoriasis remains lacking, and it is still unclear whether the function of cTfh cells is changed in psoriasis.

To address these issues, we comprehensively explored the frequency, phenotype, and function of CXCR5<sup>+</sup>CD4<sup>+</sup> cTfh cells in patients with psoriasis vulgaris (PV). In addition, we also investigated the infiltration of Tfh cells in lesional and nonlesional skin tissues of psoriasis patients. To further understand the role of cTfh cells in the pathogenesis of psoriasis, we analyzed the frequency and function of cTfh cells in patients with psoriasis vulgaris before and after 1-month treatment.

## 2. Materials and Methods

**2.1. Study Subjects.** Blood samples were obtained from 32 patients with psoriasis vulgaris and 13 healthy donors. The diagnosis of psoriasis vulgaris was based on the established criteria of Nestle et al. [17]. Psoriasis area severity index (PASI) score was used to assess the disease activity in psoriasis [18]. Coexisting other autoimmune diseases, systemic diseases, or active infections were excluded in all of these subjects. In addition, no patients had received any systemic therapy for at least one month before enrolment. After enrolment, 24 of the 32 patients with moderate to severe psoriasis received 20 mg of oral acitretin (Fangxi, Chongqing Huapont Pharm. Co., Ltd.) once daily and topical therapy of calcipotriol ointment (Daivonex, LEO Laboratories Limited, Ireland) and mometasone furoate cream (Eloson, Shanghai Xianlingbaoya Pharm. Co., Ltd.) once daily for 4 weeks. Lesional and nonlesional skin tissues were obtained from 10 patients with psoriasis vulgaris. Normal skin tissues were collected from 5 healthy individuals. The study protocol was approved by China-Japan Friendship Hospital Research Ethics Committee, and written informed consent was obtained from each participant. The clinical background of the patients was shown in Table 1.

**2.2. Fluorescence Antibodies and Flow Cytometry.** The frequencies of CXCR5<sup>+</sup>CD4<sup>+</sup> cTfh cells, as well as the functional molecules of cTfh cells, in 32 psoriasis patients and 13 healthy controls were detected by flow cytometry. Peridinin chlorophyll protein- (PerCP-) conjugated anti-CD4

and anti-CD8, fluorescein isothiocyanate- (FITC-) conjugated anti-CXCR5, phycoerythrin- (PE-) conjugated anti-ICOS and anti-HLA-DR, and allophycocyanin- (APC-) conjugated anti-IL-10, anti-IFN- $\gamma$ , and Ki-67 were purchased from BD Bioscience and BD Pharmingen (San Diego, CA, USA). APC-conjugated anti-PD-1, APC/cy7-conjugated anti-CD3, and PE-conjugated anti-IL-21 were purchased from Biolegend (San Diego, CA, USA). PE-conjugated anti-IL-17A was purchased from eBioscience (San Diego, CA, USA). To explore the frequency and function of cTfh cells, one sample of peripheral blood (200  $\mu$ L) was incubated for 30 min with anti-CD4-PerCP, anti-CXCR5-FITC, anti-PD-1-APC, and anti-ICOS-PE. In addition, anti-CD4-PerCP, anti-CXCR5-FITC, anti-Ki-67-APC, and anti-HLA-DR-PE were added to another sample of freshly heparinized blood sample for 30 min. Then the cells were washed and analyzed by flow cytometry. For intracellular cytokines staining, 300  $\mu$ L heparinized peripheral blood and 700  $\mu$ L RPMI1640 medium supplemented with 10% fetal calf serum were incubated for 6 hours with phorbol 12-myristate 13-acetate (PMA, 300 ng/mL, Sigma-Aldrich, St. Louis, MO) and ionomycin (1  $\mu$ g/mL, Sigma-Aldrich, St. Louis, MO). Then, these cells were stained with anti-CD8-PerCP, anti-CD3-APC/Cy7, and anti-CXCR5-FITC for 20 min at room temperature. Subsequently, these cells were fixed and permeabilized for 50 min at 4°C with eBioscience Fixation/Permeabilization Buffer (eBioscience, San Diego, CA, USA). The samples were then incubated with anti-IL-21-PE and anti-IFN- $\gamma$ -APC or stained with anti-IL-17A-PE and anti-IL-10-APC for 20 min at room temperature. Finally, these cells were fixed in 1% paraformaldehyde and acquired by a multicolor flow cytometry. FlowJo software (Tritar, USA) was used to analyze the data.

**2.3. Immunohistochemistry.** Lesional and nonlesional skin tissues of 10 psoriasis patients and healthy skin tissues from 5 healthy donors were, respectively, collected. These tissues were analyzed by using immunochemical staining with anti-CD4 (Zhongshan Goldenbridge Biotech, Beijing, China) and anti-CXCR5 (Abcam, Cambridge, UK). Paraffin-embedded, formalin-fixed skin tissue was cut into 5  $\mu$ m sections and placed on polylysine-coated slides. Antigen retrieval was achieved via pressure cooking for 10 min in citrate buffer (pH 6.0). Sections were incubated with anti-CD4 and anti-CXCR5 and then incubated with biotinylated goat-rabbit antibody (Zhongshan Goldenbridge Biotech, Beijing,

China). The avidin-biotin-peroxidase system with 3-amino-9-ethylcarbazole (AEC) (brown color) or vector blue (blue color) as substrates was used to perform double staining. CXCR5 and CD4 double positive cells in the skin tissues were identified as Tfh cells. The number of Tfh cells was evaluated quantitatively by 2 independent observers from 3 representative fields (200x). In addition, to further determine Tfh cells in psoriasis lesions, a dual label immunofluorescence technology was performed. The skin tissues were incubated overnight with primary antibodies, including mouse anti-human CD4 (diluted 1:10, Abcam; Cambridge, UK) and rabbit anti-human CXCR5 (diluted 1:10; Abcam, Cambridge, UK). Then slides were incubated with secondary antibodies (rabbit anti-mouse FITC-conjugated IgG Ab, rhodamine-conjugated goat anti-rabbit IgG Ab, and DAPI for nucleic acid staining) for 45 minutes at room temperature.

**2.4. Statistical Analysis.** Statistical analysis was performed using SPSS 20.0 software (SPSS, Chicago, IL, USA) and the data was presented as the mean values  $\pm$  standard deviation. The statistical difference between the 2 groups was evaluated with a Mann-Whitney *U* test, whereas the statistical difference between the same individual across patient group was determined by Wilcoxon matched pairs test. Partial correlation was used to analyze the correlation of cTfh frequency and PASI score. Spearman's correlation was used to analyze the association between the other variables. For all tests,  $P < 0.05$  was considered to be significant.

### 3. Results

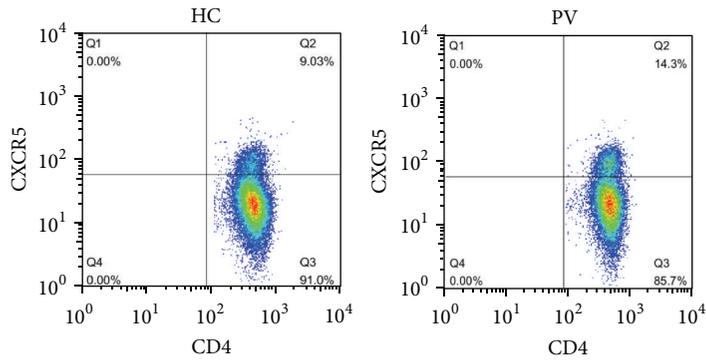
**3.1. cTfh Cells Are Significantly Increased in Patients with Psoriasis Vulgaris.** As shown in Figure 1(a), the frequency of cTfh cells was significantly increased in patients with psoriasis vulgaris compared with healthy individuals ( $14.55 \pm 2.67\%$  versus  $10.29 \pm 1.63\%$ ;  $P < 0.0001$ ). In addition, ICOS and PD-1 are two important surface markers on cTfh cells and have critical roles in the differentiation of cTfh cells. Thus, we investigated the expression of these makers in psoriasis. Our data showed that the levels of ICOS and PD-1 expression on cTfh cells were positively correlated with the percentage of cTfh cells (Figure 1(b),  $r = 0.44$  and  $P = 0.01$ ; Figure 1(c),  $r = 0.40$  and  $P = 0.02$ , resp.). To further investigate whether cTfh cells were activated in psoriasis, the expression of HLA-DR and Ki-67 on cTfh cells were detected. Our results demonstrated that there were higher levels of HLA-DR and Ki-67 expression on cTfh cells in patients with psoriasis vulgaris (Figure 1(d),  $2.01 \pm 1.27\%$  versus  $1.10 \pm 0.76\%$ ;  $P = 0.015$ ; Figure 1(e),  $1.90 \pm 1.34\%$  versus  $1.03 \pm 0.58\%$ ;  $P = 0.038$ , resp.).

Little information is available on the characteristics of Tfh cells infiltrating in psoriasis lesions. Thus, the numbers of Tfh cells in lesional and nonlesional skin tissues of psoriasis patients were first investigated by immunohistochemical double staining in our study. As shown in Figure 2(a), there were no Tfh cells ( $CD4^+$  and  $CXCR5^+$  double positive cells) in healthy donor skin tissue. In contrast, we detected an extensive infiltration of Tfh cells in psoriasis lesions. The

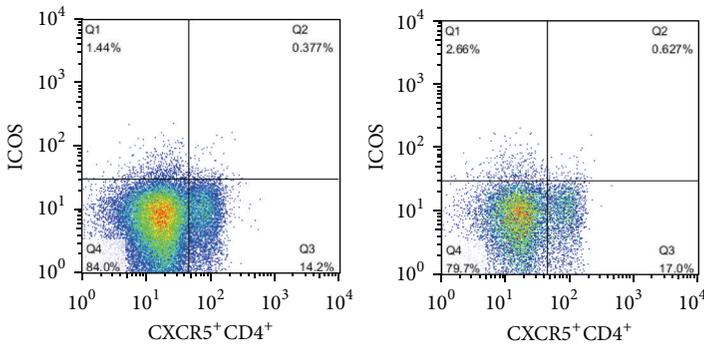
number of Tfh cells in psoriasis lesions was significantly higher than that in nonlesional skin tissues of psoriasis (Figure 2(b),  $5.6 \pm 3$  versus  $2.3 \pm 1.2$ ;  $P = 0.005$ ). However, our results demonstrated that although the number of Tfh cells was significantly increased in psoriasis lesions, there was no significant correlation between the number of infiltrating Tfh cells and PASI score in psoriasis (Figure 2(c),  $r = 0.17$  and  $P = 0.63$ ). Double-staining immunofluorescence further identified the higher infiltration of  $CXCR5^+ CD4^+$  T cells in lesions of psoriasis patients (Figure 2(d)).

**3.2. cTfh Cells Produce Higher Levels of Cytokines in Patients with Psoriasis Vulgaris.** Previous studies have reported that numerous cytokines, especially IL-21, have crucial effects on Tfh cell function. As described above, the frequency of cTfh cells was increased in psoriasis. However, it is unclear whether the function of cTfh cells is changed in patients with psoriasis vulgaris. To answer this question, we detected the levels of cytokines, including IL-21, IFN- $\gamma$ , IL-17, and IL-10, secreted by cTfh cells. In our study, the frequency of  $IL-21^+ CXCR5^+ CD4^+$  T cells was significantly higher in psoriasis patients than in healthy controls (Figure 3(b),  $7.83 \pm 3.94\%$  versus  $3.76 \pm 1.46\%$ ;  $P = 0.0003$ ). Additionally, the levels of IL-17 and IFN- $\gamma$  secreted by cTfh cells were also significantly increased in patients with psoriasis vulgaris compared with healthy individuals (Figure 3(b),  $3.60 \pm 1.54\%$  versus  $2.56 \pm 0.70\%$ ;  $P = 0.025$ ;  $12.42 \pm 6.45\%$  versus  $7.97 \pm 3.24\%$ ;  $P = 0.033$ , resp.). However, the secretion of IL-10 by cTfh cells showed no significant difference between psoriasis patients and healthy controls (Figure 3(b),  $0.48 \pm 0.27\%$  versus  $0.38 \pm 0.11\%$ ;  $P = 0.425$ ). Furthermore, our data represented that the IL-21 production of cTfh cells had positive correlation with the percentage of cTfh cells (Figure 3(c),  $r = 0.41$  and  $P = 0.02$ ).

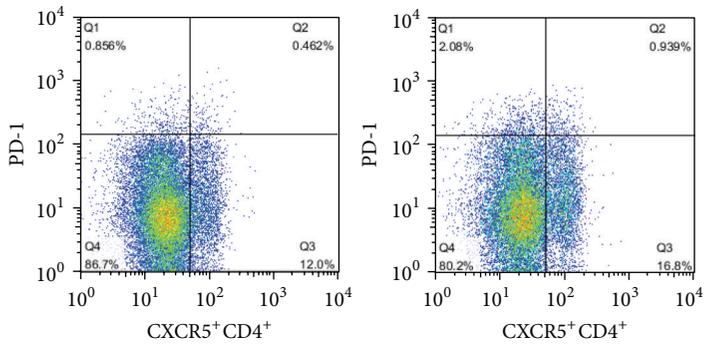
**3.3. Higher Frequency of cTfh Cells Was Positively Associated with Disease Severity in Psoriasis.** Next, we want to investigate whether cTfh cells associate with disease severity in psoriasis, assessed by PASI score. However, it is generally accepted that aging affects the function of immune system. To address this question, we used partial correlation to analyze the relationship of the frequency of cTfh cells and PASI score in psoriasis. Our results showed that when the age or disease duration was considered as control variables, positive relationship was found between the frequency of cTfh cells and PASI score (Tables 2(a) and 2(b),  $P = 0.014$ ;  $P = 0.029$ , resp.). Furthermore, we also investigated the correlations between PASI score and the levels of functional markers observed on cTfh cells, including ICOS, PD-1, IL-21, IL-17, IFN- $\gamma$ , and IL-10. Our results demonstrated that these functional markers except IL-10 positively correlated with PASI score in psoriasis (Figure 4,  $r = 0.43$  and  $P = 0.01$ ;  $r = 0.36$  and  $P = 0.04$ ;  $r = 0.35$  and  $P = 0.047$ ;  $r = 0.42$  and  $P = 0.017$ ;  $r = 0.42$  and  $P = 0.045$ ;  $r = 0.046$  and  $P = 0.80$ , resp.). Thus, our results implied that cTfh cells played an important role in the pathogenesis of psoriasis and may be a biomarker for evaluating the disease severity.



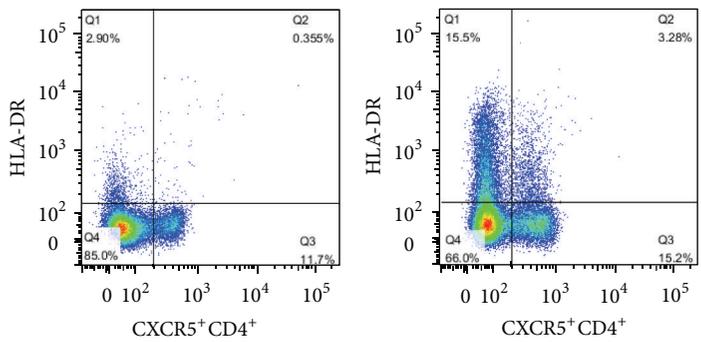
(a)



(b)



(c)



(d)

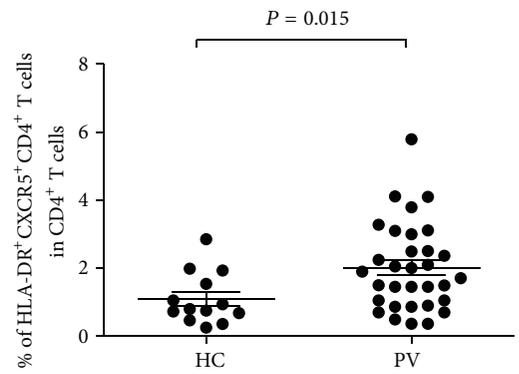
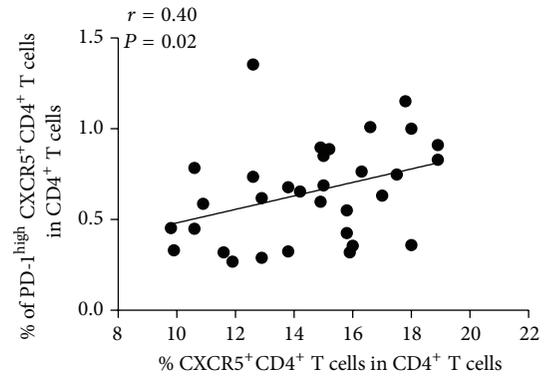
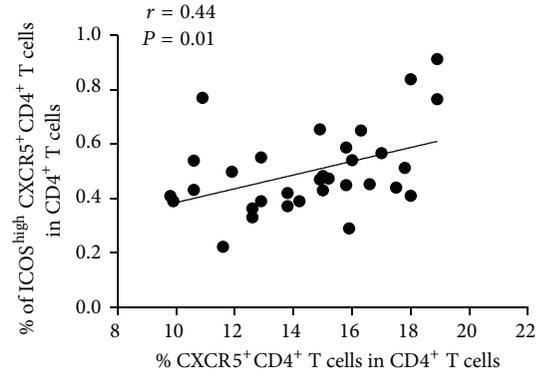
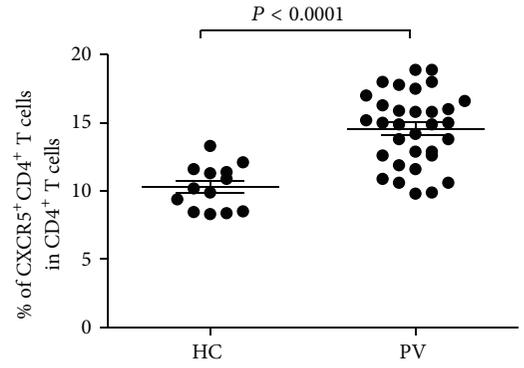


FIGURE 1: Continued.

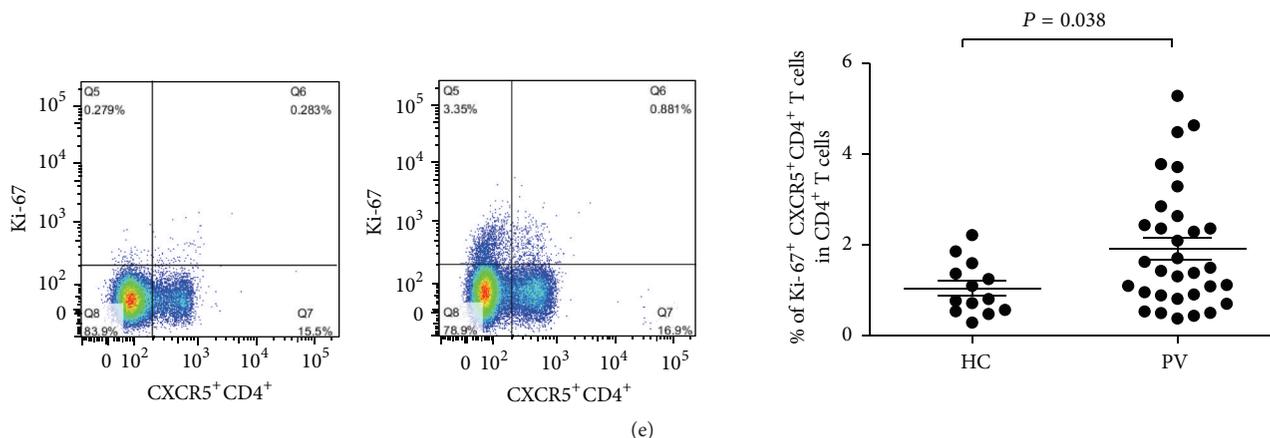


FIGURE 1: Increased frequency of circulating CXCR5<sup>+</sup>CD4<sup>+</sup> Tfh (cTfh) cells in patients with psoriasis vulgaris. (a) Comparison of the percentages of cTfh cells in patients with psoriasis vulgaris (PV) and healthy controls (HC). The cTfh cell frequency in patients with psoriasis vulgaris is significantly higher than in healthy controls. (b) ICOS expression on cTfh cells in patients with psoriasis vulgaris and healthy controls. Compared with healthy controls, ICOS expression on cTfh cells is significantly increased and has a positive correlation with the frequency of cTfh cells in psoriasis patients. (c) The levels of PD-1 on cTfh cells in patients with psoriasis vulgaris and healthy controls. The frequency of PD-1<sup>high</sup>CXCR5<sup>+</sup>CD4<sup>+</sup>/CD4<sup>+</sup> T cells is statistically increased and associated with cTfh cell frequency in psoriasis patients. Each dot represents one participant. *P* values are shown. (d) The expression of HLA-DR on cTfh cells in patients with psoriasis vulgaris and healthy controls. The expression of HLA-DR on cTfh cells was significantly higher in psoriasis patients than in healthy controls. (e) Ki-67 expression on cTfh cells in patients with psoriasis vulgaris and healthy controls. Compared with healthy controls, there was a higher level of Ki-67 expression on cTfh cells in psoriasis patients.

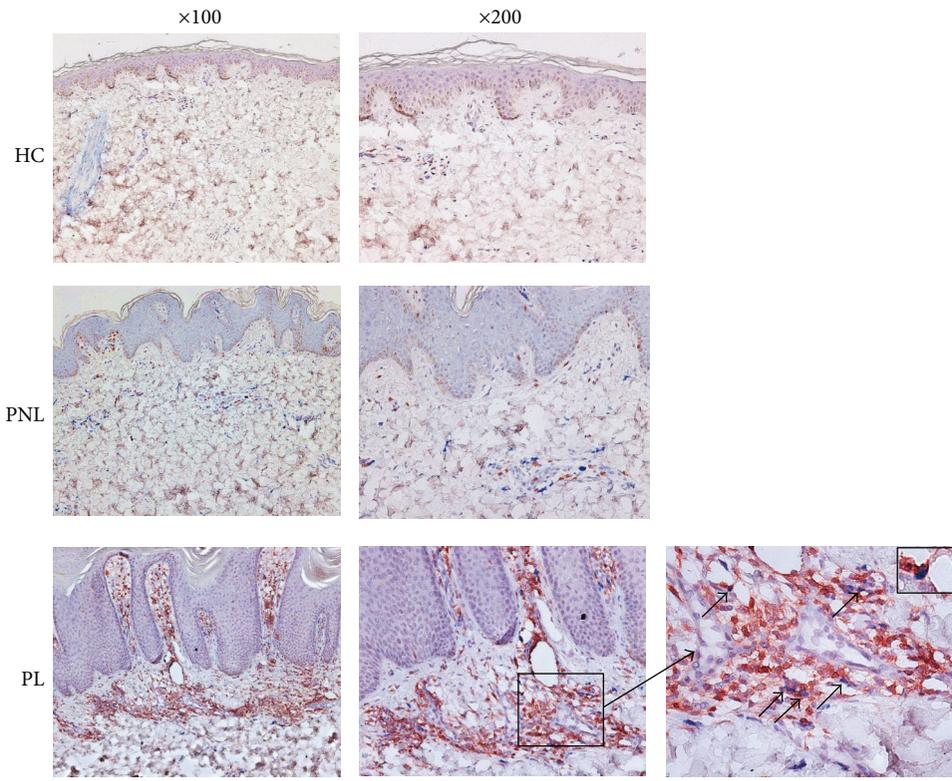
**3.4. The Frequency of cTfh Cells Was Significantly Decreased after Treatment.** Acitretin is a classic agent for the treatment of psoriasis. Previous studies have shown that acitretin was effective in patients with psoriasis. To further understand the role of cTfh cells in psoriasis, we investigated the function and frequency of cTfh cells in 24 patients with moderate to severe plaque psoriasis before and after 1-month treatment with acitretin and a topical therapy. All subjects did not receive any immunosuppressive agents during follow-up period. Our results demonstrated that the frequency of cTfh cells was significantly decreased in patients after 1-month treatment (Figure 5(a),  $15.02 \pm 2.63\%$  versus  $12.38 \pm 2.88\%$ ;  $P = 0.004$ ). In addition, there was also significant decline in the secretion of IL-21, IL-17, and IFN- $\gamma$  by cTfh cells after treatment (Figures 5(b), 5(c), and 5(d),  $8.35 \pm 4.04\%$  versus  $6.73 \pm 2.88\%$ ;  $P = 0.04$ ;  $3.80 \pm 1.62\%$  versus  $2.84 \pm 1.30\%$ ;  $P = 0.017$ ;  $13.31 \pm 6.76\%$  versus  $10.08 \pm 4.92\%$ ;  $P = 0.027$ , resp.), suggesting that cTfh cells are related to disease severity and may be a potential therapeutic target in psoriasis.

#### 4. Discussion

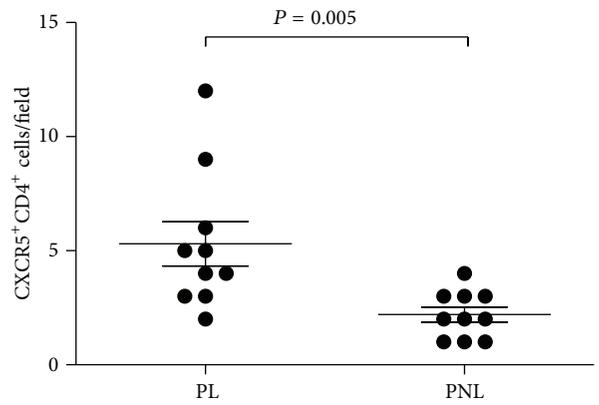
CD4<sup>+</sup> T cells are critical component of immune system and have been demonstrated to play a central role in the pathogenesis of psoriasis [19]. Naïve CD4<sup>+</sup> T cells can differentiate into distinct lineages driven by different cytokines in the environments, and these subsets have specialized functions [20]. The newly found subpopulations of CD4<sup>+</sup> T cells, such as Th17, Th22, and regulatory T cells, have enriched our understanding of the immune state of psoriasis [21, 22]. cTfh cells are recently discovered CD4<sup>+</sup> T cells subset and have been found to be associated with the development of many

diseases [23]. However, the researches on the role of cTfh cells in psoriasis are less. In the present study, we not only investigated the frequency of cTfh cells but also further explored the function of cTfh cells in patients with psoriasis vulgaris. Furthermore, the histological characteristics of Tfh cells in psoriasis lesions were firstly analyzed by our group. Finally, to further confirm the role of cTfh cells in psoriasis in vivo and to shed light on the corresponding therapeutic significance, we also firstly observed the dynamic changes of cTfh cell frequency and function in patients before and after treatment.

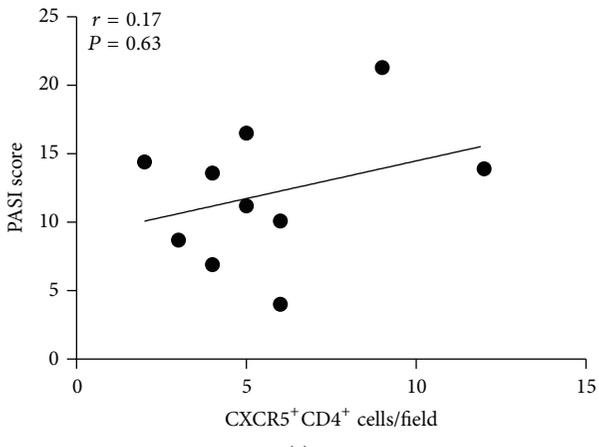
In clinical trials, cTfh cells have been shown to be increased in psoriasis [24]. Similarly, our study also demonstrated that the frequency of cTfh cells was higher in psoriasis patients than in healthy controls. Most importantly, we first observed that the number of Tfh cells was dramatically increased in psoriasis lesions compared with nonlesional skin tissues of psoriasis patients. However, although the number of infiltrated Tfh cells in lesions was increased in psoriasis, it was not correlated with PASI score in our study. This was possibly due to the limited sample size with only 10 psoriasis lesions collected in our study. We also investigated the CD19<sup>+</sup> B cells in lesions by immunohistochemistry. Unfortunately, the results showed there were very few CD19<sup>+</sup> B cells infiltrating in psoriasis lesions and no significant difference was found between lesional and nonlesional skin tissues of psoriasis patients (data not shown), and no ectopic GCs were observed in psoriasis lesions. Thus, additional psoriasis lesions need to be recruited to confirm whether Tfh cells participate in the pathogenesis through forming ectopic GCs in psoriasis lesions. Caruso et al. [25] have shown that IL-21 was highly expressed in the skin of individuals with psoriasis and stimulated human keratinocytes to proliferate. Higher levels



(a)

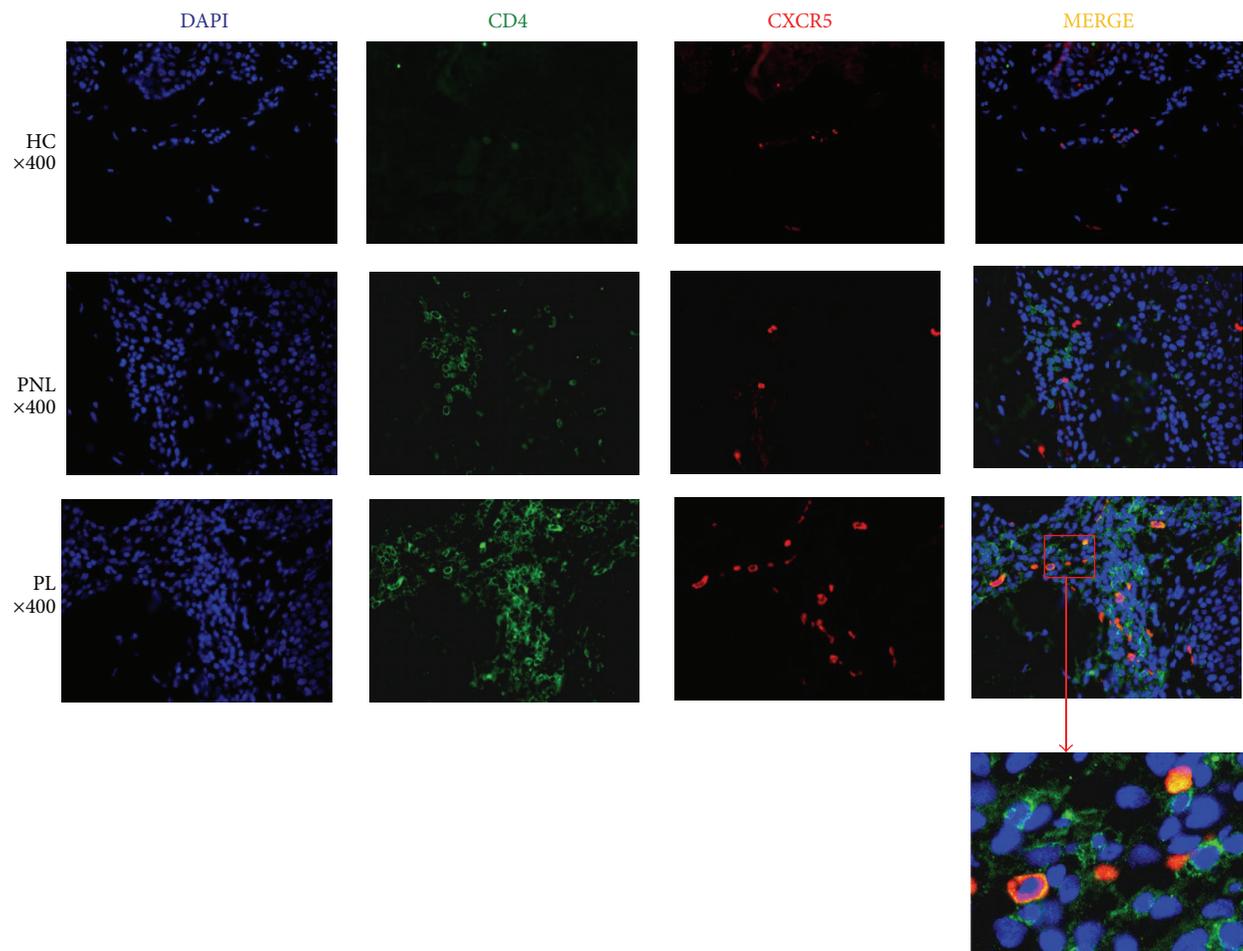


(b)



(c)

FIGURE 2: Continued.



(d)

FIGURE 2: Higher infiltration of Tfh cells in psoriasis lesions. (a) Representative immunohistochemical staining of Tfh in psoriasis lesions (PL), nonlesional skin tissues of psoriasis patients (PNL), and normal skin tissues of healthy controls (HC). Tfh cells are double stained for CD4 (brown, on cell membrane) and CXCR5 (blue, on cell membrane). There are no Tfh cells infiltrated in skin tissues of healthy controls. (b) The number of Tfh cells in psoriasis lesions is significantly increased compared with nonlesional skin tissues of psoriasis patients, which was evaluated quantitatively by 2 independent observers from 3 representative fields (200x). (c) The number of infiltrated Tfh cells in psoriasis lesions is not associated with disease severity, as assessed by psoriasis area severity index (PASI) score. *P* value is shown. (d) Costaining of CD4 and CXCR5 in skin tissues from psoriasis patients and healthy controls by immunofluorescence. Representative staining shows CD4 (green) and CXCR5 (red) cells. DAPI was used to counterstain nuclear DNA (blue). The 2-color merged panels were shown with colocalization visible in yellow. Representative staining at an original magnification of 400x is shown.

of IL-17 and IFN- $\gamma$  were also found in psoriasis lesions and correlated with disease severity [26, 27]. Our results showed that cTfh cells were activated and secreted increased levels of IL-21, IL-17, and IFN- $\gamma$  in peripheral blood of psoriasis (described below). We speculate that Tfh cells are also activated in lesions and secrete higher levels of inflammatory cytokines to participate in the development of psoriasis lesions. However, we need further investigation to confirm that Tfh cells are activated in psoriasis lesions. Wang et al. [28] have reported that the increased frequency of cTfh cells played vital roles in the pathogenesis of primary biliary cirrhosis (PBC). A higher frequency of cTfh cells was also found in patients with Henoch-Schönlein purpura [29]. These researches suggest that higher level of cTfh cells contributes to the activation of immune system. In addition, recent studies have reported that cTfh cells could be subdivided into several

populations with different phenotypes, which had specialized role in immune system [30]. Different with previous study, Le Coz et al. [31] found that the frequency of cTfh cells showed no substantially difference between SLE patients and healthy individuals, but the subsets of cTfh cells were significantly different. Thus, our knowledge about cTfh cells is still in primary stage, and further studies need to clarify their phenotypic characteristics and functions in psoriasis.

Next, we investigate whether the function of cTfh cells is changed in psoriasis, which has not been studied in psoriasis. The molecular ICOS expression on Tfh cells is essential for Tfh cells generation. ICOS deficiency could induce a severe reduction of CXCR5<sup>+</sup>CD4<sup>+</sup> T cells in germinal center [32]. In addition, ICOS-mediated signals could also promote Bcl-6 expression, a central regulator in Tfh cells development and function [10]. PD-1, another important receptor, acts as a key

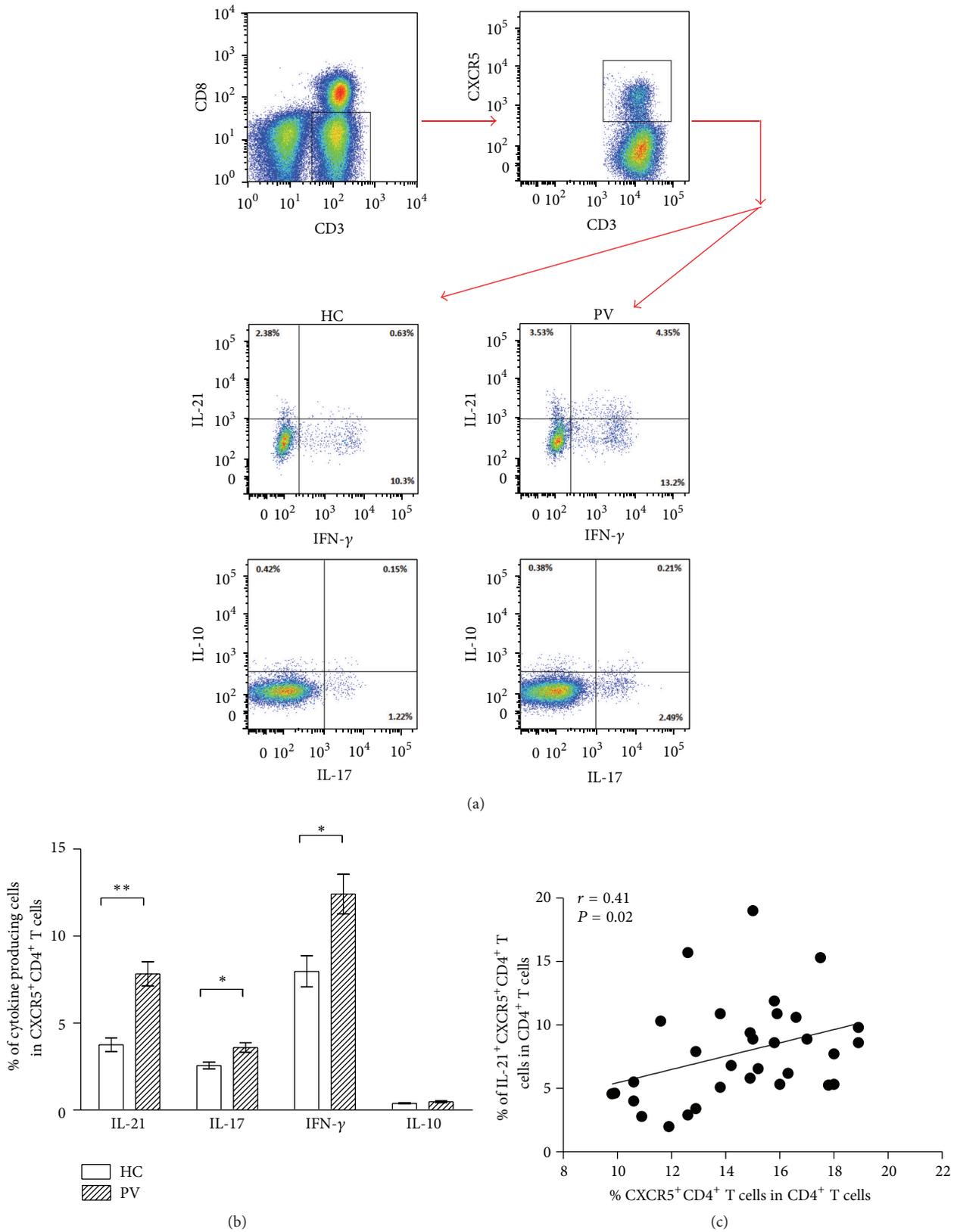


FIGURE 3: Cytokine production by cTfh cells in patients with psoriasis vulgaris and healthy controls. (a) Represent profiles of IL-21, IFN- $\gamma$ , IL-17, and IL-10 secreted by cTfh cells in psoriasis patients and healthy controls. (b) cTfh cells produce more IL-21, IFN- $\gamma$ , and IL-17 in psoriasis patients than in healthy controls, whereas the secretion of IL-10 shows no significant difference between two groups. \* $P < 0.05$ ; \*\* $P < 0.01$ . (c) The higher expression of IL-21 on cTfh cells is positively correlated with the frequency of cTfh cells in patients with psoriasis vulgaris ( $P < 0.05$ ).

TABLE 2: (a) Age as the control variable. (b) Disease duration as the control variable.

(a)

Control variables		cTfh cell frequency	PASI score	Age
cTfh cell frequency	Correlation	1.000	.410	-.152
	Significance (2-tailed)	.	.020	.406
	df	0	30	30
-None <sup>a</sup> PASI score	Correlation	.410	1.000	.131
	Significance (2-tailed)	.020	.	.474
	df	30	0	30
Age	Correlation	-.152	.131	1.000
	Significance (2-tailed)	.406	.474	.
	df	30	30	0
Age	Correlation	1.000	.439	
	Significance (2-tailed)	.	.014	
	df	0	29	
PASI score	Correlation	.439	1.000	
	Significance (2-tailed)	.014	.	
	df	29	0	

<sup>a</sup>Cells contain zero-order (Pearson) correlations.

“.” means there is no correlation between cTfh cell frequency and cTfh cell frequency, PASI score and PASI score, and disease duration and disease duration. All the results are created by SPSS software.

(b)

Control variables		cTfh cell frequency	PASI score	Disease duration
cTfh cell frequency	Correlation	1.000	.410	.233
	Significance (2-tailed)	.	.020	.200
	df	0	30	30
-None <sup>a</sup> PASI score	Correlation	.410	1.000	.135
	Significance (2-tailed)	.020	.	.463
	df	30	0	30
Disease duration	Correlation	.233	.135	1.000
	Significance (2-tailed)	.200	.463	.
	df	30	30	0
Disease duration	Correlation	1.000	.393	
	Significance (2-tailed)	.	.029	
	df	0	29	
PASI score	Correlation	.393	1.000	
	Significance (2-tailed)	.029	.	
	df	29	0	

<sup>a</sup>Cells contain zero-order (Pearson) correlations.

“.” means there is no correlation between cTfh cell frequency and cTfh cell frequency, PASI score and PASI score, and disease duration and disease duration. All the results are created by SPSS software.

role in the activity of Tfh cells [16]. HLA-DR is a marker of T cell activation, and Ki-67 is a marker of T cell proliferation. Besides the surface receptors, cytokines secreted by Tfh cells are also important functional markers. IL-21 has been shown to be the key cytokine of Tfh cells, which can stimulate B cell proliferation and class switching [33]. Moreover, it has been shown that Tfh cells have the capacity to produce IL-17 and IFN- $\gamma$  [34]. IL-17-producing Tfh cells have been shown to be involved in driving autoimmune responses [35]. Recent studies reported that the activated cTfh cells not only upregulated the expression of ICOS and IL-21 but

also produce high level of IL-10 [36]. Thus, we detected the expression of ICOS, PD-1, HLA-DR, and Ki-67 on cTfh cells and secretion of IL-21, IL-17, IFN- $\gamma$ , and IL-10 by cTfh cells in patients with psoriasis vulgaris. We found that the increasing levels of ICOS and PD-1 expression on cTfh cells had positive correlations with cTfh cell frequency, furthering the importance of ICOS and PD-1 for the generation of cTfh cells. Our data also showed that the expression of HLA-DR and Ki-67 on cTfh cells were significantly increased in psoriasis patients. The higher expression of HLA-DR indicated that cTfh cells were activated in psoriasis patients,

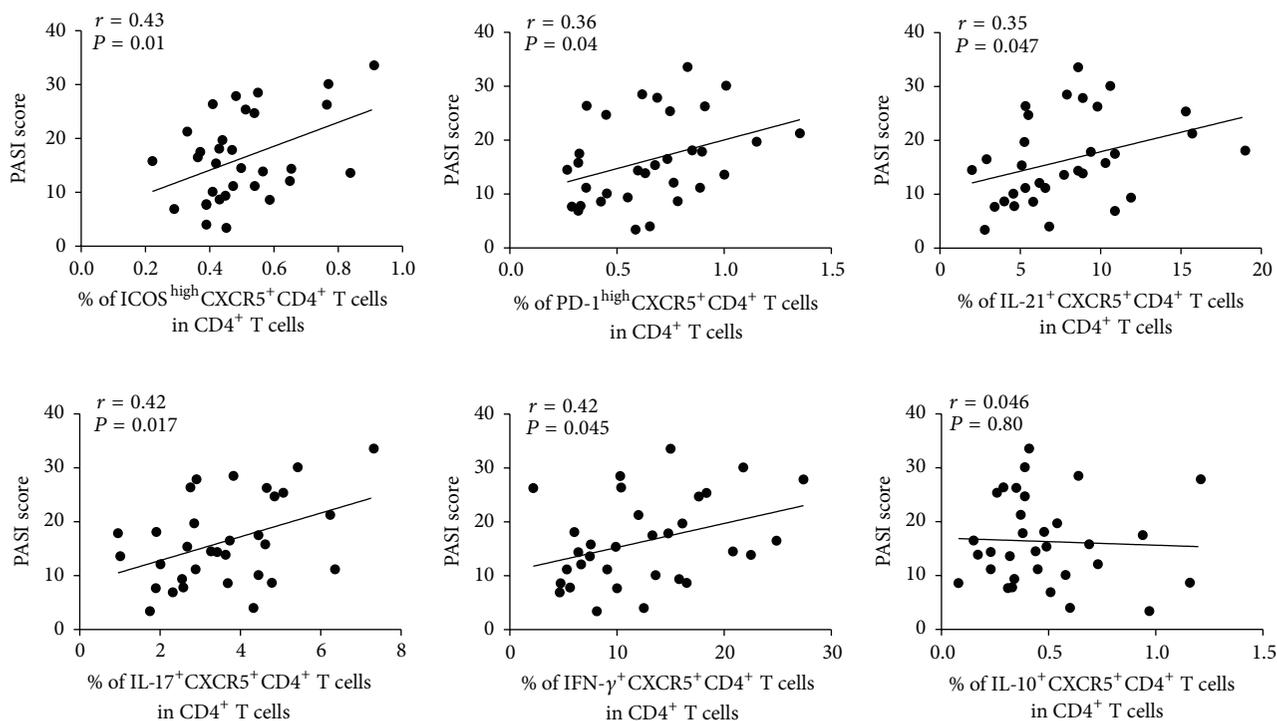


FIGURE 4: Correlations between the levels of functional markers on cTfh cells and PASI score. The ICOS and PD-1 expression on cTfh cells have positive correlations with PASI score. The positive relationships are also found between the levels of IL-21, IL-17, and IFN- $\gamma$  secreted by cTfh cells and PASI score. However, the IL-10 secreted by cTfh cells has no relationship with PASI score in psoriasis patients. Correlative coefficients and  $P$  values are shown.

and the increased level of Ki-67 suggested that cTfh cells were not quiescent cells in psoriasis patients. In addition, the levels of IL-21, IL-17, and IFN- $\gamma$  secreted by cTfh cells were all statistically increased in psoriasis patients as well, which was further proved that cTfh cells were activated in psoriasis. Niu et al. [24] found the serum level of IL-21 was also increased in psoriasis and had positive correlations with cTfh frequency and disease severity. In PBC patients, the levels of IL-21, IL-17, and IFN- $\gamma$  secreted by cTfh cells were all increased [28]. Jia et al. [37] represented that cTfh cell secreted lower levels of IL-21, IL-17, and IFN- $\gamma$  in patients with hepatocellular carcinoma. Thus, either the activated or impaired function of cTfh cells contributes to the development of diseases. Taken together, we may speculate that the currently observed molecules indicating the function of cTfh cells are closely related to the pathogenesis in psoriasis.

Previous study found that the increased frequency of cTfh cells was correlated with disease severity in psoriasis [24]. However, normal aging contributes to a decline in the function of the immune system at molecular level and cellular level [38]. The expression of numerous molecules required for Tfh cell differentiation and maintenance is dysregulated with age including ICOS, IL-21, and IL-12 [39]. Thus, if we want to determine whether cTfh cell frequency may be a biomarker of disease severity in psoriasis, we need to control the variables of age and disease duration. Our results showed that when the age or disease duration was considered as control variable, the percentage of cTfh cells was statistically correlated with disease severity in psoriasis. Furthermore, the

levels of functional markers observed on cTfh cells except IL-10 have positive correlations with PASI score as well. Thus, our results confirmed that cTfh cell could be a biomarker to assess the disease severity.

To further confirm the role of cTfh cells in psoriasis and evaluate the corresponding therapeutic value, we observed these cells in psoriasis patients before and after treatment. Interestingly, after 1-month treatment with acitretin and a topical therapy, the frequency of cTfh cells was significantly decreased, accompanied with the decreased production of IL-21, IL-17, and IFN- $\gamma$ . Previous studies demonstrated that acitretin could improve clinical symptoms of psoriasis through suppression of Th17 cells [40, 41]. Our data suggested that cTfh may also be a target of acitretin in psoriasis, thus possibly highlighted a potential therapeutic target in psoriasis.

## 5. Conclusion

In summary, this study first not only detects Tfh cells in psoriasis lesions and function of cTfh cells in psoriasis patients but also confirms the increased frequency and its positive correlation with disease severity in psoriasis patients. cTfh cells are activated in psoriasis, expressing high levels of ICOS, PD-1, HLA-DR, and Ki-67 and secreting increased levels of IL-21, IL-17, and IFN- $\gamma$ . In addition, the frequency of cTfh cells and the production of cytokines are observed to be significantly decreased after 1-month treatment. Taken together, these findings indicate that activated cTfh cells

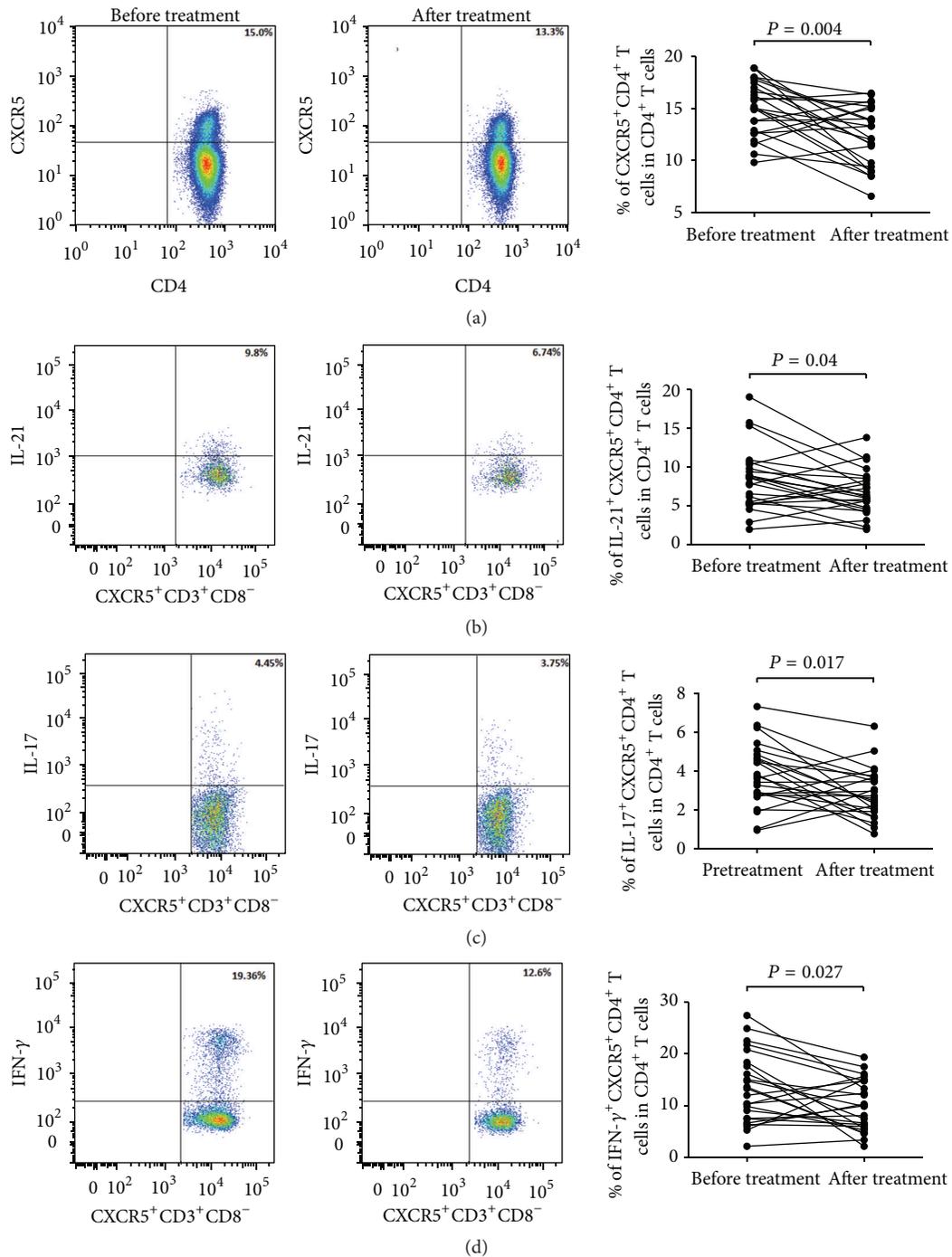


FIGURE 5: The frequency and cytokine production of cTfh cells before and after treatment. (a) After 1-month treatment, with alleviation of disease severity, the frequency of cTfh cells is significantly decreased in psoriasis patients. (b, c, and d) The levels of IL-21, IL-17, and IFN- $\gamma$  produced by cTfh are all lower than those before treatment. *P* values are shown.

contribute to the pathogenesis of psoriasis and may be a potential therapeutic target in psoriasis.

### Competing Interests

The authors declare that they have no competing interests.

### Acknowledgments

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## Review Article

# Blood-Brain Barrier Disruption Induced by Chronic Sleep Loss: Low-Grade Inflammation May Be the Link

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Sleep is a vital phenomenon related to immunomodulation at the central and peripheral level. Sleep deficient in duration and/or quality is a common problem in the modern society and is considered a risk factor to develop neurodegenerative diseases. Sleep loss in rodents induces blood-brain barrier disruption and the underlying mechanism is still unknown. Several reports indicate that sleep loss induces a systemic low-grade inflammation characterized by the release of several molecules, such as cytokines, chemokines, and acute-phase proteins; all of them may promote changes in cellular components of the blood-brain barrier, particularly on brain endothelial cells. In the present review we discuss the role of inflammatory mediators that increase during sleep loss and their association with general disturbances in peripheral endothelium and epithelium and how those inflammatory mediators may alter the blood-brain barrier. Finally, this manuscript proposes a hypothetical mechanism by which sleep loss may induce blood-brain barrier disruption, emphasizing the regulatory effect of inflammatory molecules on tight junction proteins.

## 1. Introduction

Almost all of our knowledge about the effect of inflammatory events on blood-brain barrier is related to chronic diseases or acute events, in which exacerbated responses to pathogens are present. The role of low-grade inflammation in the generation or exacerbation of neuropathologies is recently explored because several conditions such as obesity and diabetes concur with this inflammatory status during long-term periods and, perhaps, it may be related to systemic and central comorbidities. Most, if not all, pathologies are associated with sleep disturbances. Sleep loss *per se*, including sleep deprivation, sleep restriction, or sleep fragmentation (see Table 1 for a full differentiation between the concepts), generates a pathogen-independent low-grade inflammatory status. Here, we will review (1) the inflammatory mediators that increase during periods of sleep loss and their association with general disturbances in peripheral endothelium and epithelium and (2) how those inflammatory mediators might

alter the blood-brain barrier during sleep loss. With the evidence presented in this review, we propose a hypothetical mechanism by which sleep restriction could induce blood-brain barrier disruption, emphasizing the effect of inflammatory molecules on tight junction maintenance.

## 2. Sleep Loss as an Inflammatory Event

Sleep is one of the most widely observed phenomena in mammals and is recognized to play a vital regulatory role in a number of physiological and psychological systems [1, 2]. The paramount role of sleep in the physiology of animal models and humans is evident by the effects of sleep loss. Serious physiological consequences of sleep loss include decreased neurogenesis, cognitive dysfunction (deficits in learning, memory, and decision-making), metabolic alterations, cardiovascular diseases, immune disturbances, and blood-brain barrier disruption [1–8]. Both chronic and acute sleep loss associate with energy balance disturbances [9] and changes

TABLE 1: Sleep loss procedures.

	Human procedures	Duration	Animal models	Duration
Sleep deprivation (SD)	(i) Shift working [117] (ii) Voluntary SD [117]	(i) Several days (ii) 12–90 h	(i) Modified multiple platform method (REM SD) [118] (ii) Gentle SD (total SD) [119] (iii) Disk-over-water method (total and selective SD) [120]	(i) 3–96 h (ii) 3–96 h (iii) 3–96 h
Sleep restriction (SR)	(i) Voluntarily SR [117]	(i) 3–5 h	(i) Modified multiple platform method [7] (ii) Rotating bar at the bottom of the house-cage [8]	(i) 20 h of SD plus 4 h of daily sleep recovery (ii) 18 h of SD plus 6 h of daily sleep recovery
Sleep fragmentation (SF)	(i) Obstructive apnoea patients [117] (ii) The elderly [117]	(i) Several days (ii) Several days	(i) Gentle manipulation coupled to EEG recording [119] (ii) Disk-over-water method [120]	(i) 1 to several days (ii) 1 to several days

Sleep deprivation consists of sleep loss without sleep opportunity along a short period; sleep restriction consists of a reduction in total sleep time with short periods of sleep opportunity; and sleep fragmentation consists of multiple awakenings during sleep time.

in cellular and humoral immunity [10, 11]; however, the direct mechanism by which sleep induces a low-grade inflammatory status is unclear. Experimental research has demonstrated that acute and chronic sleep loss result in impairments in the immune response, characterized by deficits in the cellular component (both in number and in function) and increased levels of proinflammatory mediators, such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ), IL-6, IL-17A, and C-reactive protein (CRP) (for details of the cytokine levels related to varying periods of sleep loss see [12]). In addition to immune-derived inflammatory mediators, sleep loss also increases the levels of other inflammatory molecules such as cyclooxygenase-2 (COX-2) [8], nitric oxide synthase (NOS), endothelin-1 (ET-1), vascular endothelial growth factor (VEGF), and insulin-like growth factor-1 (IGF-1) [8, 13].

The major aim of this review is to discuss the role of low-grade inflammation in the blood-brain barrier disruption induced by sleep loss; nevertheless, because endothelial cells form the blood-brain barrier we considered it relevant also to discuss the effect of sleep loss on peripheral endothelial and epithelial cells as early markers of inflammation.

### 3. Peripheral Endothelial and Epithelial Disturbances Induced by Sleep Loss

Endothelial and epithelial cells form protecting barriers in the central nervous system but also in the periphery. Several pathological states are known to target peripheral epithelial and/or endothelial barriers; therefore the knowledge of regulatory mechanisms in those peripheral barriers may contribute to improving the understanding of central barriers. Among the pathologies affecting body barriers, those involving infections and also diabetes, cardiovascular diseases, psoriasis, and cancer are associated with sleep disturbances [14–16]. Here, we present evidence regarding the disrupting effect of sleep loss on peripheral epithelial and endothelial cells.

**3.1. Humans.** When fluid compartmentalization goes awry, homeostasis is altered and the possibility exists of induction of inflammation by microorganism invasion and even of tumor microenvironment induction [14]. In humans sleep restriction increases sympathetic activity and, concomitantly, causes endothelial dysfunction at the venous level [17]; the effect may be mediated via endothelin-1 (ET-1) because ET-1-mediated vasoconstriction is greater in adults with short sleep duration (less than 7 h per night) than in those with normal sleep duration (7–9 h per night) [18]. ET-1 is the most potent vasoconstrictor peptide released by the endothelium. The link between sleep restriction and increased ET-1 activity is not clear, but the role of the inflammatory status induced by sleep loss may partially explain this association. In this way, inflammatory cytokines, insulin, and epinephrine altered during sleep loss have each been shown to increase ET-1 in hypertensive subjects [19]. The cytokines that may increase in sleep-deprived humans (e.g., TNF- $\alpha$ , IL-1 $\beta$ , and IL-6) raise arterial vascular tone via endothelin receptors [20]. Several

reports indicate that sleep loss induces vascular alterations related to inflammatory markers (for a review see [21]). Some studies have tried to clarify the underlying mechanism; for instance, sleep deprivation in humans induced magnesium deficiency [22], which produces arterial constriction, and is a possible cause of myocardial damage [22]. Other barriers are not yet studied in sleep-deprived or sleep-restricted humans, but some studies indicate that sleep deficiency alters skin conductance [23].

**3.2. Animal Models.** Animal models currently used in sleep research include those that model shift work by totally sleep depriving rodents; human sleep deficiency by sleep restricting; and sleep loss-associated with pathologies, such as obstructive apnoea, by promoting sleep fragmentation. Contrary to the human studies, in the case of animal models, several studies have identified negative effects of sleep loss on peripheral endothelia and epithelia. For instance, sleep fragmentation in mice (20 weeks) induces vascular endothelial dysfunction and mild blood pressure increases. Those physiological effects are accompanied by morphological vessel changes characterized by elastic fiber disruption and disorganization, increased recruitment of inflammatory cells to the vessel wall, and increased plasma levels of IL-6 [24]. In rats, total sleep deprivation reduces endothelial-dependent cutaneous vasodilation. This endothelial dysfunction is independent of blood pressure and sympathetic activity but is associated with changes in NOS and COX pathways [25].

The effect of sleep loss on physical barriers such as the intestinal barrier or blood-testis barrier is not reported; however, gut bacteria are present in blood after sleep deprivation [26] and both sleep-deprived and sleep-restricted rats exhibit lower sperm viabilities associated with an increase in endothelial NOS expression [27]. Those data suggest that sleep loss also might alter the physiology of the above-mentioned barriers with the ensuing tissue damage.

### 4. Blood-Brain Barrier Impairment Induced by Sleep Loss

We reported for the first time that sleep restriction induces blood-brain barrier hyperpermeability in rats [7]. We used a procedure consisting of 20-hour sleep deprivation plus 4 hours of sleep opportunity during 10 consecutive days; because a reduction in total sleep time is observed, it is named sleep restriction. In our conditions, rapid eye movement (REM) sleep is fully suppressed and non-REM sleep is 30% reduced since the first day of sleep restriction. In those conditions we showed a widespread breakdown of the blood-brain barrier [7]. We described that brief periods of sleep opportunity (40 to 120 minutes) induced a progressive recovery of blood-brain barrier permeability to Evans blue (>60 000 Da) in the majority of brain regions studied, with exception of the hippocampus and cerebellum [7]. We also observed that in the hippocampus the number of pinocytotic vesicles increased threefold. In a subsequent study, mice were subjected to sleep restriction for 6 days in a rotatory bar for 12 hours per day. Sleep restriction by this method induced REM

sleep loss in the first 3 days with partial REM sleep recovery afterwards; at the end of the 6th day of sleep restriction, there was 13.3% increase of wakefulness, 10.2% reduction of non-REM sleep, and 2.1% reduction of REM sleep [8]. Under these conditions, increased blood-brain barrier permeability to sodium fluorescein, a low molecular-weight tracer, was observed; sleep recovery by 24 hours fully reverted the effect. In the same way, sleep restriction decreased the mRNA levels of the tight junction proteins claudin-5, zonula occludens-2 (ZO-2), and occludin [8]. In the first study [7] a yoked control was included to avoid any potential confounding effects of stress on blood-brain barrier permeability; rats were placed on large platforms during the same period of time as sleep-restricted subjects and despite being in the same stressful conditions as the sleep-restricted subjects they have a fully functional blood-brain barrier [7]. The second study did not include a yoked control, a newly developed sleep deprivation method was used that involves a rotating bar at the bottom of the house-cage with random changes of direction; this method may certainly be stressful to the rodents due to the presence of forced exercise; however, our recent results replicate their findings (Hurtado-Alvarado et al. personal communication). Therefore, the evidence of changes in the blood-brain barrier integrity induced by sleep loss is substantial and inflammatory molecules appear to play a key role in the mechanism subjacent to this phenomenon.

## 5. Role of Inflammatory Mediators Released during Sleep Loss in Blood-Brain Barrier Physiology

The increase in the levels of inflammatory mediators during chronic sleep loss may be related to blood-brain barrier disruption because several previous reports show that *per se* those inflammatory molecules affect the integrity of the blood-brain barrier (see Table 2 for a summary).

### 5.1. Proinflammatory Cytokines Involved in Sleep and Blood-Brain Barrier Modulation

**5.1.1. Tumor Necrosis Factor- $\alpha$ .** Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) is a protein synthesized mainly by monocytes and macrophages that plays an essential role in the initial activation of the immune system. In the central nervous system TNF- $\alpha$  is a multipotent cytokine produced by neurons, glia, and microvascular endothelial cells that is implicated in several physiological events, such as memory consolidation and sleep regulation. TNF- $\alpha$  is also a potent regulator of blood-brain barrier permeability. The role of TNF- $\alpha$  as an inducer of blood-brain barrier disruption includes its overexpression in microglia, astrocytes, and microvascular endothelial cells [28].

Several reports indicate that sleep loss increases the plasma and brain levels of TNF- $\alpha$  [29–33], the mRNA expression of TNF- $\alpha$  in the brain [33, 34], the spontaneous production of TNF- $\alpha$  in lymphocytes [35], and the mRNA expression of TNF- $\alpha$  in peritoneal and epididymal adipose tissue [36, 37]. Despite the fact that the changes in TNF- $\alpha$

induced by sleep loss are 2 to 5 times higher compared to rats sleeping *ad libitum*, the levels are below those reported in the case of infectious diseases; however, the chronic exposure to this inflammatory mediator may underlie the sleep-induced blood-brain barrier dysfunction.

The effect of TNF- $\alpha$  in endothelial cells is well studied. *In vivo* and *in vitro* studies report an increase in the permeability of microvascular endothelial cells after the administration of TNF- $\alpha$  in both animal models and human cell lines [38–41]. Nonetheless, the TNF- $\alpha$  levels used in those studies are 100,000 times higher compared to concentrations reported under sleep loss conditions. The lower dose of TNF- $\alpha$  used in *in vitro* studies (1 ng/mL) results in a transendothelial electric resistance (TEER) reduction at 60 minutes after treatment with TEER recovery at 210 minutes after administration, which is similar to the results observed using higher doses of TNF- $\alpha$  (50, 100 ng/mL), suggesting that the effect mediated by TNF- $\alpha$  receptors is saturable [42].

While we can infer that peripheral changes mediate the main effect of TNF- $\alpha$  on blood-brain barrier, we must not ignore the fact that TNF- $\alpha$  levels also increase in the brain. In this way, it is known that after the administration of TNF- $\alpha$  (250 ng) in the lateral ventricle an increase in the transport from cerebrospinal fluid (CSF) to blood of  $^{125}$ I-human serum albumin is observed in rats, which demonstrates that TNF- $\alpha$  promotes the clearance of macromolecules from the CSF to the venous blood [43]. Taking into consideration that the restorative function of non-REM sleep may be a consequence of the enhanced removal of waste products accumulated in the awaking brain via the glymphatic system [44], the TNF- $\alpha$  increase during sleep loss may contribute to the clearance of toxins by efflux of potentially neurotoxic waste products via the blood-brain barrier. Interestingly, in the brain, sleep restriction increases the mRNA expression of TNF- $\alpha$  in a region-dependent manner in the mouse [45], suggesting that if TNF- $\alpha$  regulates the microvascular brain endothelial cells from inside the brain, it may do it in specific areas, such as the somatosensory and frontal cortices, which indicates that blood-brain barrier regulation by inflammatory molecules is heterogeneous (a finding reported by us in the case of blood-brain barrier changes induced by sleep loss and recovery; see [7]).

Another example of TNF- $\alpha$  role in blood-brain barrier regulation during peripheral inflammation occurs after the induction of acute pancreatitis in rats, where an increase in TNF- $\alpha$  levels is observed as early as 6 hours after pancreatitis induction and at the same time increases the blood-brain barrier permeability to sodium fluorescein (365 Da) in the hippocampus and cerebellum as well as to Evans blue in the hippocampus, basal nuclei, and cerebellum. In the case of the low molecular-weight tracer the normal blood-brain barrier permeability reestablishes at 24 hours after induction, while, for Evans blue, reestablishment occurs 48 hours after induction [46]. We also observed region-dependent effects of sleep loss and recovery on blood-brain barrier integrity; for instance, in the cerebellum the hyperpermeability remained even after sleep opportunity periods of 40–120 minutes; meanwhile the cortex recovered the normal blood-brain barrier permeability at the same time points [7]. Therefore,

TABLE 2: Inflammatory mediators released during sleep loss that may potentially regulate blood-brain barrier integrity.

Inflammatory mediator	General changes during sleep loss	General effects on blood-brain barrier
TNF- $\alpha$	↑ circulating levels in human and rodents [29–32]	↑ blood-brain barrier permeability in <i>in vivo</i> and <i>in vitro</i> models (rodent and human brain endothelial cells) [38–40]
	↑ mRNA expression in mice brain [33]	↑ efflux of albumin from brain to blood [43]
		↓ ZO-1 expression [103]
IL-1 $\beta$	↑ circulating levels in human and rodents [3, 4, 29, 49]	↑ MMP-9 protein expression [106]
	↑ mRNA expression in mice brain [45]	↑ blood-brain barrier permeability in <i>in vivo</i> and <i>in vitro</i> models (rodent and human brain endothelial cells) [42, 54]
		↓ TEER of primary cultures of brain endothelial cells and human brain endothelial cells [42, 54]
IL-6	↑ circulating levels in human after chronic sleep loss [64, 65]	↑ production of PGE and COX [57]
	↓ circulating levels in humans [62]	↓ ZO-1 expression [103]
	↑ circulating levels during sleep recovery in humans [69]	
IL-17A	↑ mRNA expression in human PBMC [50, 67]	
	↑ circulating levels in rodents [29]	
	↑ mRNA expression in human PBMC [50]	
CRP	↑ circulating levels in humans and rodents [4, 8, 50, 67, 81–83]	↑ blood-brain barrier permeability in <i>in vivo</i> and <i>in vitro</i> models (rodent and human brain endothelial cells) [78, 79]
		↑ blood-brain barrier permeability in <i>in vivo</i> and <i>in vitro</i> models (rodent and human brain endothelial cells) [85]
		↑ ROS production in brain endothelial cells [86]

TNF: tumor necrosis factor; IL: interleukin; CRP: C-reactive protein; ZO: zonula occludens; MMP-9: matrix metalloproteinase-9; PBMC: peripheral blood mononuclear cells; ROS: reactive oxygen species; COX: cyclooxygenase; and TEER: transendothelial electric resistance.

the cerebellum could be considered as a highly susceptible region to inflammatory mediators such as TNF- $\alpha$  [47] in comparison with other brain regions (e.g., the hippocampus and cortex). The differential distribution of TNF- $\alpha$  receptors in the brain may explain why TNF- $\alpha$  regulates blood-brain barrier function in a region-dependent manner; however, it is also possible that other molecules may have synergistic effects with TNF- $\alpha$  to regulate blood-brain barrier physiology.

**5.1.2. Interleukin-1 Beta.** IL-1 $\beta$  is the prototypical signal molecule for neuroimmune communication. Classically, phagocytic cells in response to inflammatory stimuli release IL-1 $\beta$ ; in the brain IL-1 $\beta$  activates the regions involved in the generation of hyperthermia [48]. Similar to the effect of TNF- $\alpha$ , IL-1 $\beta$  administration promotes sleep in mammals [1] and sleep deprivation has been shown to increase serum IL-1 $\beta$  levels both in humans and in animal models [3, 4, 29, 49]. In addition, sleep loss induces IL-1 $\beta$  gene expression in the brain [34, 45], cardiac muscle, and adipose tissue [36] and on phytohaemagglutinin (PHA) activated peripheral blood mononuclear cells (PBMC) [50]. In the case of the brain, several reports indicate that the expression of the IL-1 receptor-1 (IL-1R1) in endothelial cells is high in the pre-optic area, subfornical organ, and supraoptic hypothalamus, while a lesser expression is found in the paraventricular hypothalamus, cerebral cortex, nucleus of the solitary tract, ventrolateral medulla, trigeminal and hypoglossal motor nuclei, and the area postrema [51–53].

In *in vitro* models of blood-brain barrier, IL-1 $\beta$  (in doses of 5, 100, and 1000 ng/mL) decreases the TEER similar to the levels observed after TNF- $\alpha$  administration [42, 54]. IL-1 $\beta$  also promotes the release of IL-6 and prostaglandin E (PGE<sub>2</sub>) in rat brain endothelial cells [55]. Likely, *in vivo* studies have shown that IL-1 induces sickness behaviour mediated by endothelial IL-1R1 activation in rats [56]; the probable mechanism may be the induction of COX-2 in brain endothelial cells after IL-1R1 activation with the concomitant increase in the synthesis of PGE<sub>2</sub> [57].

IL-1 $\beta$  may have a key role in blood-brain barrier dysfunction during sleep loss because it has been reported that sleep loss increases IL-1 $\beta$  gene expression in the cerebral cortex, hippocampus, and basal forebrain [45]. In addition, IL-1 $\beta$  released from activated microglia increases blood-brain barrier permeability; this effect may depend on the suppression of astrocyte-derived signals that maintain blood-brain barrier integrity (e.g., sonic hedgehog, SHH) [58]. IL-1 $\beta$  action on blood-brain barrier may induce the expression of other inflammatory mediators produced by microglia and astroglia. For instance, the lack of IL-1R1 specifically in endothelial cells precluded the brain increase of IL-1 $\beta$ , TNF- $\alpha$ , and IL-6 in stressed rats despite the presence of reactive microglia [59, 60], which places IL-1 $\beta$  and its receptor on endothelial cells as central mediators of brain inflammatory responses. Hence, the role of IL-1 $\beta$  in blood-brain barrier could be mainly related to endothelial-glia interactions [61].

**5.1.3. Interleukin-6.** Sleep onset is associated with an increase in circulating levels of IL-6 [62]; nevertheless, the potential

role of IL-6 in sleep regulation is controversial, and it may take a secondary role as compared to its primary role in the acute-phase response [63]. Some studies indicate an increase of IL-6 circulating levels in sleep-deprived subjects [64–66] and also in gene expression in immune cells [35, 50, 67], whereas others report a delay in the sleep-related peak of plasma IL-6 in sleep-restricted subjects [62]. Even some authors report that plasma levels of IL-6 are maintained without change despite sleep loss [30, 68]. Some studies also show that sleep recovery after total sleep deprivation increases plasma levels of IL-6 [69]; however, others found that in immune cells IL-6 levels remain unchanged during sleep recovery [50]. IL-6 is a pleiotropic cytokine key for immune regulation and if secreted during sleep loss and recovery may have neuroprotective effects; indeed, it has been reported that IL-6 appears to be neuroprotective and is involved in endothelial survival after shear stress [70]. However, given the high variability of IL-6 after sleep loss and recovery, the role of IL-6 as a possible modulator of blood-brain barrier during sleep is unclear. It is necessary to elucidate the precise changes in IL-6 levels both centrally and peripherally to clarify the role of IL-6 in blood-brain barrier modulation during sleep.

IL-6 has pyrogenic effects when endogenously released during systemic inflammation; it achieves this function by its binding to IL-6 receptor  $\alpha$  (IL-6R $\alpha$ ) on brain endothelial cells and the subsequent induction of PGE synthesis. However, those effects require high levels of IL-6 (>1 ng/mL). In humans, IL-6 serum levels were less than 100 pg/mL and the normal levels for IL-6 in CSF are around 10 pg/mL, significantly lesser than those measured in several *in vitro* and *in vivo* experiments [70]. For instance, treatment with 50 or 500 ng of IL-6 reduced the infarct volumes and symptoms of neurological deficit in a rat model of cerebral ischemia [71]. In addition, the administration of IL-6 decreased the blood-brain barrier permeability to Evans blue by suppressing the expression of matrix metalloproteinase-9 (MMP-9) [71]. The role of IL-6 as well as TNF- $\alpha$  and IL-1 $\beta$  may depend on the brain region, for example, the stimulation with lipopolysaccharide (LPS) induces in the brain the expression of the IL-6 receptor (IL-6R) in the cortex and hippocampus but not in the cerebellum [72]. Therefore, considering IL-6 a proinflammatory cytokine it is possible to suggest that its role in blood-brain barrier physiology during sleep loss may be related to the modulation of the expression of other proinflammatory cytokines.

**5.1.4. Interleukin-17A.** Th17 cells have been identified as a subset of T helper lymphocytes characterized by the production of a number of cytokines including IL-17A, IL-17F, and IL-22. Th17 cells have emerged as a key factor in the pathogenesis of autoimmune disorders. For instance, high expression of IL-17A is associated with autoimmune inflammatory diseases including multiple sclerosis [73], rheumatoid arthritis [74], inflammatory bowel disease [75], and systemic lupus erythematosus [76]. During sleep loss a subtle increase of IL-17A is reported (from 0.5 to 3 ng/mL in rat) [29]. IL-17A high levels were found in plasma even after 24 hours of sleep recovery in sleep-restricted rats [29]. Sleep loss also

increases the mRNA and protein expression of IL-17A on PHA activated PBMC in humans [50].

Particularly, the receptor for IL-17A is expressed in epithelial and endothelial cells and promotes the expression of inflammatory mediators such as IL-6 and chemokines [77]. IL-17A induces epithelial and endothelial dysfunction; it decreases the TEER and concomitantly increases tracer permeability; the mechanism is mediated through tight junction disruption [77]. Finally, from *in vitro* experiments it is known that IL-17A increases endothelial cell permeability at 10 or 100 ng/mL doses [78, 79]. These data suggest that IL-17A might be involved in blood-brain barrier disruption during sleep loss.

## 5.2. Other Inflammatory Molecules Altered during Sleep Loss and Their Role in Blood-Brain Barrier Regulation

### 5.2.1. C-Reactive Protein.

C-reactive protein (CRP) is the major acute-phase protein involved in the resistance to microbes and autoimmune diseases and is an important risk marker of cardiovascular and cerebrovascular disorders. The plasma levels of CRP increase faster and at higher magnitude than other acute-phase proteins [80]. Sleep loss increases the circulating levels of CRP (0.5  $\mu\text{g/mL}$ ), which is associated with increased risk of cardiovascular disease and stroke [4, 8, 50, 67, 81, 81–83].

The synthesis of CRP in the liver is controlled by proinflammatory cytokines, including TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and IL-17A [82, 84]. CRP (10–20  $\mu\text{g/mL}$ ) induces blood-brain barrier disruption [85] because brain endothelial cells express high levels of CRP receptors (CD16 and CD32) and also because brain endothelial cells express high levels of the p22phox subunit of the NAD(P)H-oxidase. The high expression of both exacerbates the generation of reactive oxygen species (ROS) with the resultant oxidation of tight junction proteins [86].

### 5.2.2. Intercellular Adhesion Molecule-1 (ICAM-1).

The expression of ICAM-1 in endothelial cells is pivotal in supporting lymphocyte migration across the vascular endothelium [87]. ICAM-1 associates with an endothelial cytoskeleton fraction, suggesting that ICAM-1 redistribution is an early event in the signalling cascade during inflammatory events, particularly in lymphocyte transmigration [87]. The expression of endothelial cell adhesion molecules increases in the central nervous system during inflammation secondary to pathogen intracerebral administration (e.g., *Corynebacterium parvum*). Brain vessels located in the centre of the cellular infiltrate began to express markers of fenestrate endothelium such as the endothelial-specific expression of MECA32 suggesting an altered functional status of the endothelial cell [88]. Abundant ICAM-1 expression has been observed after IL-1 or TNF- $\alpha$  stimulation of cultured heart endothelial cells [89].

Elevated levels of ICAM-1 may contribute to cardiovascular disease and are associated with obstructive sleep apnoea (OSA) and obesity, in which sleep deficiency is present [90]. In the same way, it has been shown that patients with diabetes mellitus type 2 and poor sleep present higher morbidity of cardiovascular diseases than diabetes mellitus patients

sleeping normally; those patients also present higher plasma levels of ICAM-1 [91]. ICAM-1 higher serum levels were also found during the sleep recovery period after 40 hours of total sleep deprivation in healthy men [69]. Therefore it seems that the mediator between poor sleep (with bad quality and poor sleep recovery) and higher risk for cardiovascular diseases is ICAM-1.

### 5.2.3. Vascular Endothelial Growth Factor.

Inflammation is characterized by upregulation of vascular endothelial growth factor (VEGF). In *in vivo* experiments, increases in VEGF during neuroinflammation (e.g., in experimental autoimmune encephalomyelitis (EAE)) are accompanied with increased blood-brain barrier permeability and decreased expression of tight junction proteins (e.g., claudin-5 and occludin). Likely, VEGF administration to human brain endothelial cells increases permeability of the monolayer and downregulates claudin-5 and occludin, but not junctional adhesion molecule-1 (JAM-1), cingulin, peripheral plasma membrane protein (CASK), or ZO-1 [92].

Given the role of VEGF in regulating blood-brain barrier during neuroinflammation, it may participate in generating the vascular changes associated with sleep loss. Indeed, it has been shown that VEGF is overexpressed in OSA patients and it is generally considered that VEGF increases are associated with hypoxia events [93]. However, OSA patients also have severe sleep fragmentation; therefore, in addition to chronic intermittent hypoxia, VEGF changes may be related to sleep loss [94]. In fact, in a study with major depressive disorder patients, sleep deprivation increased VEGF plasma levels [95].

### 5.2.4. Insulin-Like Growth Factor-1.

Sleep deprivation decreases IGF-1 levels in rats and humans and one night of sleep recovery is sufficient to restore its basal levels [96]. The neuroprotective effects of IGF-1 are unclear but it is known that IGF-1 receptors are present in brain endothelial cells, microglia, and astroglia and even in neurons [97]. Indeed, it has been suggested that IGF-1 may promote neuroprotection by acting on the blood-brain barrier; in an experimental model of ischemic stroke IGF-1 reduced the inflammatory infiltrate in the brain [97]. In an *in vitro* experiment with brain endothelial cells IGF-1 reverted the hyperpermeability to bovine serum albumin induced by oxygen-glucose deprivation (an *in vitro* model of ischemic stroke) [97].

Changes on inflammatory molecules during sleep loss are well described but we do not know what the source of those alterations is. In this way the role of microbiota could appear a good candidate to induce the low-grade proinflammatory status during sleep loss.

## 6. A Brief View of the Microbiota and Barriers Dysfunction as a Possible Source of Inflammatory Mediators in Sleep-Deficient Subjects

The source of inflammatory mediators during sleep loss remains unclear; however, microbiota may play a key role

in this event. In other conditions that exhibit low-grade systemic inflammation, such as chronic depression, obesity, and diabetes, evidence from murine models initially suggested a role for the gut microbiota in the generation of low-grade inflammation, with the consequent increased risk of endothelial and epithelial dysfunction [98, 99]. For instance, changes in gut microbiota composition increase intestinal permeability [100]. In the same way, during sleep deprivation gut microbiota has been detected in blood, suggesting the induction of systemic inflammation and deficits in gut epithelial permeability [26]. In addition, preclinical evidence from germ-free mice suggests that the microbiota can also modulate the blood-brain barrier; exposure of germ-free adult mice to the faecal microbiota from pathogen-free donors decreased the blood-brain barrier permeability and increased the expression of tight junction proteins in brain endothelial cells [101], therefore strengthening the hypothesis that the blood-brain barrier may also be sensible to changes in the gut microbiota composition [100]. The candidate pathways to induce barriers dysfunction under altered gut microbiota composition include serotonin, cytokines, toll-like receptor activation, and short chain fatty acids [100]. Moreover, the inflammatory response subsequent to microbiota-induced barriers disruption may underlie the sleep loss-related cognitive deficits and the exacerbation of neurological disorders such as depression [100].

These data might support the theory of a coevolution between sleep and blood-brain barrier proposed by Korth in 1995 [102]. Because the brain and blood-brain barrier react sensitively to the exposure to bacterial cell wall constituents and sleep is regulated by gut microbiota products, Korth proposed that low amounts of bacterial cell wall constituents that induce sleep under sleep loss conditions, by themselves or by cytokine production, increase the blood-brain barrier permeability ensuing their passage into the brain [102].

## 7. Molecular Mechanisms by Which Inflammatory Mediators Might Induce Blood-Brain Barrier Disruption during Sleep Loss

Cytokines and other inflammatory mediators induce blood-brain barrier disruption through mechanisms involving signalling pathways that converge in the disorganization of tight junctions (Figure 1). For instance, it has been reported that proinflammatory cytokines, including TNF- $\alpha$  and IL-1 $\beta$ , decreased ZO-1 expression and ZO-1-occludin coassociation, concomitant to increased ZO-1 phosphorylation in tyrosine and threonine residues [103]. Those effects are presumably mediated by ROS [103]. ZO-1 phosphorylation in tyrosine residues is also observed after VEGF administration [104]. In this way, VEGF-A also promotes disruption of blood-brain barrier by downregulating the expression of claudin-5 and occludin [92]. Low cytokine concentrations (>1 ng/mL) led to activation of effector caspases via c-Jun N-terminal kinases (JNK) and protein kinase C (PKC) signalling pathways, increased paracellular flux, and redistribution of ZO-1 and VE-cadherin but failed to induce apoptosis [105]. In addition

to caspase-3, TNF- $\alpha$  activates the production of MMP-9 [106], which is also associated with high levels of IL-1 $\beta$  in brain parenchyma [107].

TNF- $\alpha$  activates the NF $\kappa$ B signalling pathway, leading to increased PGE levels via COX-2 [108]. COX-2 plays a crucial role in the inflammatory response of the blood-brain barrier (for review see [109]); particularly COX-2 derived PGE<sub>2</sub> increases blood-brain barrier permeability [110]. Other cytokines, such as IL-1, use other signalling pathways that finally converge in COX-2 induction; particularly, the IL-1 receptor-1 (IL-1R1) signals via the p38 mitogen-activated protein kinase (MAPK) and the c-Jun pathway to induce COX-2 synthesis, whereas activation of the IL-6 receptor leads to COX-2 expression through activation of signal transducer and activator of transcription-3 (STAT-3) [111]. The activation of NF $\kappa$ B by TNF- $\alpha$  and IL-1 $\beta$  is also correlated with COX-2 expression in microvascular endothelial cells. Indeed, both I $\kappa$ B $\alpha$  and COX-2 are expressed within the same endothelial cells, suggesting a potential interaction between the transcription factor and COX-2 expression in the cerebral endothelium of animals with systemic inflammation [112].

TNF- $\alpha$  and IL-1 $\beta$  promote the release of CRP. The putative mechanism by which CRP increases blood-brain barrier permeability is by its action on CD16/CD32 receptors present in the cell membrane of brain endothelial cells [85]. This association activates the Myosin Light Chain (MLC) phosphorylation by MLC-kinase (MLCK) and the activation of p38-MAPK, with the subsequent formation of actin stress fibers [85]. Brain endothelial cells express the p22phox subunit located in the cell membrane; this enzyme uses NADH or NADPH as the electron donor for the single electron reduction of oxygen to produce ROS during CRP stimulation [86]. The assembly of active NADPH oxidase requires translocation of cytosolic subunits, p47phox, p67phox, and Rac1 (a cytosolic GTPase), to the plasma membrane, where they interact with gp91phox and p22phox and associate with other membrane cofactors to form a functional enzyme complex [113]. In addition, CRP stimulation also disorganizes ZO-1 via MLCK and ROS production [85]. In this way, IL-17A also induces NADPH oxidase- or xanthine oxidase-dependent ROS production and downregulates the expression of occludin by activation of MLCK [79].

The signalling of inflammatory mediators and particularly NADPH oxidase may promote the upregulation of adhesion molecules such as ICAM-1 via JAK/epidermal growth factor receptor (EGFR) signalling [113] contributing to a possible leukocyte infiltration. Therefore, these changes may be deemed as the mechanisms involved in brain endothelial cell dysfunction during sleep loss.

## 8. Conclusion and Future Directions

We propose that inflammatory mediators increased during chronic sleep loss might promote blood-brain barrier disruption (Figures 1 and 2). For aims of clarity the hypothesis does not explicitly distinguish between REM and non-REM sleep and we know that other molecules altered during sleep loss also should be studied because they may have a potent role in the blood-brain barrier disruption such as adenosine

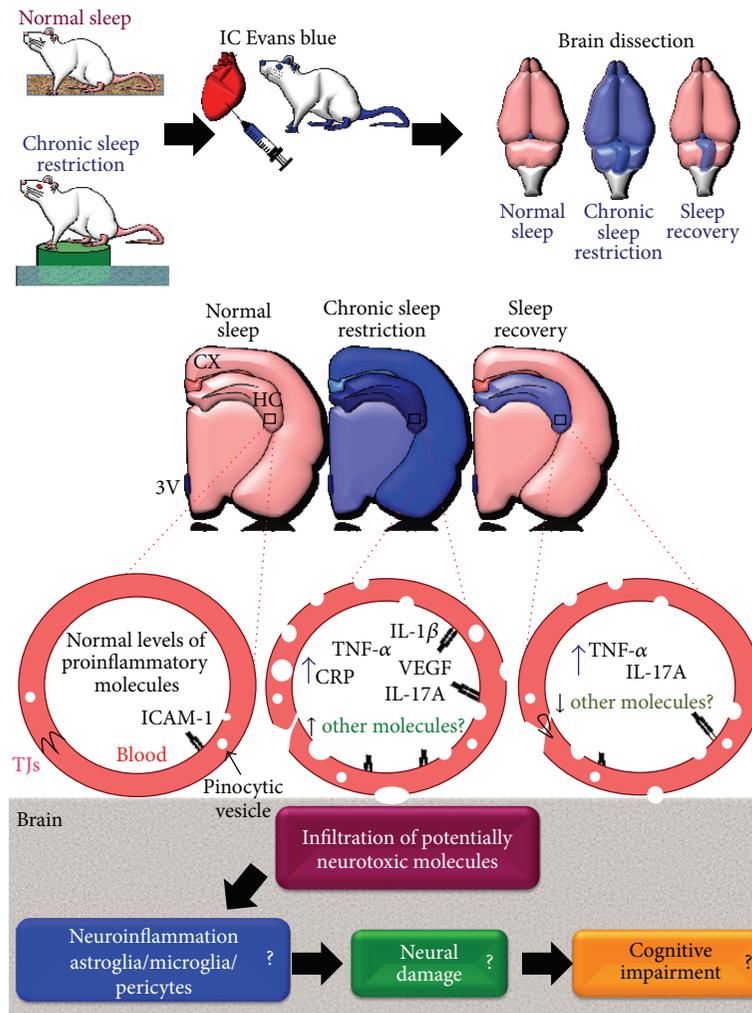


FIGURE 1: Potential inflammatory mediators participating in the regulation of blood-brain barrier permeability during sleep loss. The figure shows the platform method to induce sleep loss in the rat. Chronic sleep restriction increases blood-brain barrier permeability to circulating molecules (e.g., Evans blue) and sleep recovery promotes restoration of normal blood-brain barrier permeability. Inflammatory mediators with barrier regulation properties, such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), vascular endothelial growth factor (VEGF), interleukin-1 $\beta$  (IL-1 $\beta$ ), and IL-17A, are released during sleep loss conditions and some of them return to basal levels during sleep recovery; others, like IL-17A and TNF- $\alpha$ , are maintained at high levels despite sleep recovery. The barrier changes induced by inflammatory mediators may lead to neuroinflammation and potentially may underlie the cognitive impairments induced by sleep loss.

[114] and hormones [115]. In interpreting these data, a number of factors need to be considered. For instance, the cellular components of the blood-brain barrier that promote inflammation in the brain, such as microglia and astroglia, in addition to regulating blood-brain barrier may also be affecting several brain functions during sleep and sleep loss. On the other hand, pericytes have a unique synergistic relationship with brain endothelial cells in the regulation of capillary permeability through secretion of inflammatory mediators including cytokines, chemokines, nitric oxide, and matrix metalloproteinases. Those inflammatory mediators released during sleep restriction may directly induce pericyte detachment from the vessel wall ensuing blood-brain barrier disruption (for review see Hurtado-Alvarado, 2014 [116]).

Summarizing, chronic sleep loss induces systemic low-grade inflammation that may be related to epithelial and

endothelial disturbances both at the systemic and at the central level. Particularly, the role of inflammatory mediators in the blood-brain barrier disruption induced by sleep loss might explain the cognitive impairment associated with sleep loss. The systemic and local effect of inflammatory molecules accumulated during chronic sleep loss should be taken into account for the study of general consequences of sleep deficiency including the risk of developing neurologic and neurodegenerative diseases.

## Abbreviations

CSF:	Cerebrospinal fluid
COX:	Cyclooxygenase
CRP:	C-reactive protein
CXCL-1:	Chemokine (C-X-C motif) ligand 1

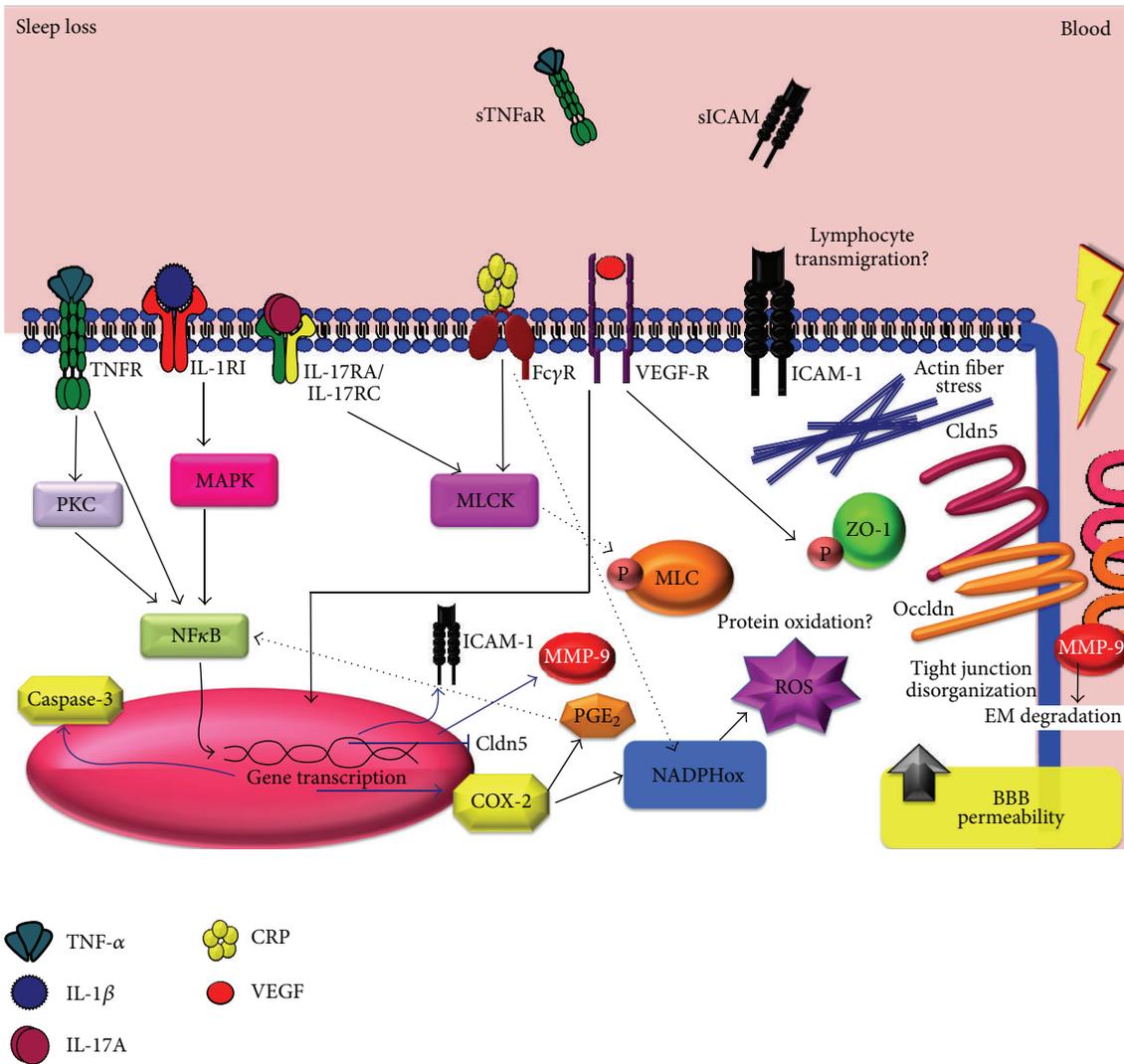


FIGURE 2: Hypothetical molecular mechanisms mediating sleep loss effect on blood-brain barrier permeability. During sleep loss the increase of soluble inflammatory mediators such as TNF- $\alpha$ , IL-1 $\beta$ , IL-17A, CRP, and VEGF activates several membrane receptors that converge in cellular pathways hallmark of inflammation, for example, the NF $\kappa$ B pathway. The final outcome involves the phosphorylation of tight junction proteins and the generation of actin fiber stress. But also other pathways are potentially activated, such as the NADPH oxidase pathway, leading to ROS generation and the subsequent lipoxidation and protein oxidation. The activation of transcription factors (eg., NF $\kappa$ B) and their translocation to the nuclei may promote the transcription of inflammatory-related genes (eg., ICAM-1, prostaglandins, and matrix metalloproteinases (MMP)) as well as death-related genes (eg., caspase 3) and the repression of genes involved in the maintenance of the barrier properties (eg., claudin-5). Conjointly, all those pathways could lead to increased blood-brain barrier permeability during chronic sleep loss. Cldn5: claudin-5, COX: cyclooxygenase, CRP: C-reactive protein, Fc $\gamma$  receptor: fragment crystallizable region, ICAM-1: intracellular adhesion molecule-1, IL: interleukin, NADPHox: nicotinamide adenine dinucleotide phosphate oxidase, NF $\kappa$ B: nuclear factor kappa-light-chain-enhancer, MMP: matrix metalloproteinase, MLC: myosin light chain, MLCK: myosin light chain kinase, PGE: prostaglandin, PKC: protein kinase C, sICAM: soluble ICAM, sTNF $\alpha$ : soluble TNF- $\alpha$  receptor, VEGF: vascular endothelial growth factor, TNF: tumor necrosis factor, and ZO: zonula occludens.

EGFR: Epidermal growth factor receptor  
 ET-1: Endothelin-1  
 ICAM-1: Intracellular adhesion molecule-1  
 IGF-1: Insulin-like growth factor-1  
 I $\kappa$ B $\alpha$ : Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha  
 IL: Interleukin  
 JNK: c-Jun N-terminal kinase

NADPH: Nicotinamide adenine dinucleotide phosphate  
 NF $\kappa$ B: Nuclear factor kappa-light-chain-enhancer  
 NOS: Nitric oxide synthase  
 MMP: Matrix metalloproteinase  
 MLC: Myosin light chain  
 MLCK: Myosin light chain kinase  
 PBMC: Peripheral blood mononuclear cells  
 PGE: Prostaglandin

PHA: Phytohaemagglutinin  
 PKC: Protein kinase C  
 REM: Rapid eye movement  
 STAT: Signal transducer and activator of transcription  
 TEER: Transendothelial resistance  
 TNF: Tumor necrosis factor  
 VEGF: Vascular endothelial growth factor  
 ZO: Zonula occludens.

## Competing Interests

The authors declare that there are no competing interests.

## Authors' Contributions

G. Hurtado-Alvarado took part in the conception and design of the review, critically revised the manuscript, and took primary responsibility of writing the manuscript. B. Gómez-González took part in the conception of the review and drafted and critically revised the manuscript. L. Pavon, E. Domínguez-Salazar, and J. Velázquez-Moctezuma drafted and critically revised the paper. All authors read and approved the final manuscript.

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## Research Article

# Immunomodulatory Effects of 1,25-Dihydroxyvitamin D<sub>3</sub> on Dendritic Cells Promote Induction of T Cell Hyporesponsiveness to Myelin-Derived Antigens

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While emerging evidence indicates that dendritic cells (DC) play a central role in the pathogenesis of multiple sclerosis (MS), their modulation with immunoregulatory agents provides prospect as disease-modifying therapy. Our observations reveal that 1,25-dihydroxyvitamin D<sub>3</sub> (1,25(OH)<sub>2</sub>D<sub>3</sub>) treatment of monocyte-derived DC results in a semimature phenotype and anti-inflammatory cytokine profile as compared to conventional DC, in both healthy controls and MS patients. Importantly, 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated DC induce T cell hyporesponsiveness, as demonstrated in an allogeneic mixed leukocyte reaction. Next, following a freeze-thaw cycle, 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated immature DC could be recovered with a 78% yield and 75% viability. Cryopreservation did not affect the expression of membrane markers by 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated DC nor their capacity to induce T cell hyporesponsiveness. In addition, the T cell hyporesponsiveness induced by 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated DC is antigen-specific and robust since T cells retain their capacity to respond to an unrelated antigen and do not reactivate upon rechallenge with fully mature conventional DC, respectively. These observations underline the clinical potential of tolerogenic DC (tolDC) to correct the immunological imbalance in MS. Furthermore, the feasibility to cryopreserve highly potent tolDC will, ultimately, contribute to the large-scale production and the widely applicable use of tolDC.

## 1. Introduction

Multiple sclerosis (MS) is a chronic inflammatory and neurodegenerative disease of the central nervous system (CNS) characterized by disseminated patches of demyelination and axonal loss in the brain and spinal cord. Although both genetic [1] and environmental [2] factors have been demonstrated to contribute to the onset of disease, it is currently

generally accepted that MS is a T helper type 1 (Th1) and Th17-driven immune-mediated disease. This was demonstrated by immune cell infiltration and accompanying inflammatory processes leading to damage of myelin [3, 4]. Moreover, Th1 and Th17 lineage-specific cytokines, interferon- $\gamma$  (IFN- $\gamma$ ), and interleukin-17 (IL-17) play a pivotal role in the pathogenesis of MS. Production of IFN- $\gamma$  and IL-17 by T cells has been associated with disease activity in MS patients [4]

and these cytokines are also expressed in brain lesions [5, 6]. Several clinical trials have been performed to determine if targeting effector T cells may be beneficial for MS patients. In particular, anti-IFN- $\gamma$  therapy showed promising results in a small clinical trial in MS [7] but was not beneficial in experimental autoimmune encephalomyelitis (EAE), an animal model of MS. Hence, since IFN- $\gamma$  and IL-17 are probably not the critical determinants of whether an effector T cell is capable of trafficking to the CNS and inducing inflammatory demyelination, the focus of research on effector T cells in MS should be on upstream pathways driving Th1 and Th17 cells. In this perspective, dendritic cells (DC), professional antigen-presenting cells, play an important role in polarizing the T cell response, thereby regulating the balance between immunity and tolerance. The possibility of modulating the function of DC using various biological or pharmacological agents makes DC interesting not only from an immunopathogenic point of view but also from a therapeutic perspective [8].

The identification of so-called tolerogenic, that is, tolerance-inducing, DC (tolDC) has paved the way for novel forms of cell-based tolerance-inducing therapies (CTT). TolDC can be characterized by low expression levels of costimulatory molecules, low production of proinflammatory cytokines, high secretion of anti-inflammatory cytokines, and a maturation-resistant phenotype [9, 10]. Importantly, tolDC can inhibit or suppress T cell responses via a multitude of mechanisms, including T cell deletion, T cell anergy, cytokine deviation, and/or the induction of regulatory T cells (Treg) [11]. In doing so, tolDC can reprogramme or modulate the immune system in order to reestablish self-tolerance in autoimmunity.

Various immunomodulatory strategies have been used to generate tolDC *in vitro*. In this respect, an exponentially increasing amount of studies is currently investigating the capacity of 1,25-dihydroxyvitamin D<sub>3</sub> (1,25(OH)<sub>2</sub>D<sub>3</sub>), the active form of vitamin D<sub>3</sub> [12–15]. Increasing evidence has highlighted the potential immunoregulatory functions of 1,25(OH)<sub>2</sub>D<sub>3</sub> including the capability of 1,25(OH)<sub>2</sub>D<sub>3</sub> to modulate both innate and adaptive immune responses [16, 17]. In particular, it was demonstrated that treatment of DC with 1,25(OH)<sub>2</sub>D<sub>3</sub> renders DC in a semimature state, as evidenced by low expression levels of costimulatory molecules, such as CD40, CD80, and CD86, increased IL-10 production, and impaired IL-12 secretion. Consequently, 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated DC display a reduced capacity to activate T cells [12–14, 18] and promising results were obtained following their administration in preclinical models of autoimmunity [15, 19–21].

So far, the first clinical trials evaluating the use of tolDC have been recently completed for type 1 diabetes, rheumatoid arthritis, and Crohn's disease [22–25]. The results were promising and the use of tolDC was safe and well tolerated. Nevertheless, several challenges still remain. First, it can be envisaged that, following migration to the inflamed tissues *in vivo*, clinically administered tolDC may acquire an immunostimulatory state upon encounter of inflammatory mediators. Hence, a stable maturation-resistant phenotype of tolDC should be aimed for. Similarly, tolDC-mediated T cell hyporesponsiveness should be persistent and robust

following *in vivo* rechallenge with proinflammatory stimuli. Moreover, T cell hyporesponsiveness should be directed to disease-specific antigens, while preserving T cell capacity to respond to unrelated antigens. Other remaining issues are dose, timing, route, and frequency of administration of tolDC. Regarding the latter, it was recently demonstrated that although murine tolDC were able to reduce disease activity in EAE, the clinical effect was transient but could be restored following a subsequent injection with tolDC [26] suggesting that repeated administration is necessary. For this, large numbers of DC manufactured in accordance with current good manufacturing practice (cGMP) guidelines are required. Since the manufacturing of a large number of DC is time-consuming and cost-intensive, cryopreservation of tolDC in ready-to-use aliquots for clinical application would significantly improve the feasibility of consecutive injections. Moreover, production of sufficient numbers of DC at one time point would not only facilitate the use of DC in clinical trials but also reduce batch-to-batch variations. Whereas an efficient cryopreservation method for tolDC would greatly contribute to their use in clinical trials, studies demonstrating the influence of cryopreservation on the properties of tolDC are scarce.

In the present study, the effects of the active form of vitamin D<sub>3</sub> on the differentiation, maturation, and function of monocyte-derived DC (mo-DC) from healthy controls as well as from MS patients were investigated. Given the risk of concomitant DC activation in a proinflammatory microenvironment *in vivo*, the *in vitro* stability of the maturation-resistant phenotype was also analyzed. Finally and importantly, we addressed the feasibility to cryopreserve tolDC by assessing the effects of cryopreservation on the phenotype and allogeneic T cell-stimulatory capacity of 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated DC.

## 2. Material and Methods

**2.1. Study Population.** Peripheral blood from healthy volunteers was obtained from buffy coats provided by the Antwerp Blood Transfusion Center (Red Cross-Flanders, Edegem, Belgium). MS patients, diagnosed according to the revised McDonald criteria [27], were recruited by the Department of Neurology from the Antwerp University Hospital (Edegem, Belgium). Ten patients (6 males and 4 females) with an average age of 38 years (range: 25–52 years) and a median expanded disability status scale (EDSS) score of 3 (range: 0–5) were included (Table 1). All subjects gave written consent after they were informed of the nature and possible risks of the study. The study was approved by the Ethics Committee of the Antwerp University Hospital and followed the tenets of the Declaration of Helsinki. Approximately 100 mL of heparinized blood was collected by venous puncture. Samples were processed within 24 hours after collection.

**2.2. Dendritic Cell Culture.** Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation (Ficoll-Paque™ PLUS, GE Healthcare, Chalfont St. Giles, UK). Next, CD14<sup>+</sup> monocytes were purified by CD14<sup>+</sup> immunomagnetic selection (CD14 Reagent, Miltenyi Biotec, Bergisch Gladbach, Germany), according to manufacturer's

TABLE 1: Clinical details of the patients recruited into the study.

UPN	Gender	Age	MS-type	EDSS score	Disease duration (years)	Medication
MS-DC 001	F	51	RR-MS	3	14.5	Glatiramer acetate
MS-DC 002	M	52	SP-MS	5	19	None
MS-DC 003	F	27	CIS	0	1	None
MS-DC 004	M	35	RR-MS	3	2	Natalizumab
MS-DC 005	F	35	RR-MS	3	6	Natalizumab
MS-DC 006	M	33	RR-MS	3.5	14	Natalizumab
MS-DC 007	F	42	RR-MS	2	18	IFN- $\beta$
MS-DC 008	M	45	RR-MS	2	19	None
MS-DC 009	M	32	RR-MS	2.5	1	IFN- $\beta$
MS-DC 010	M	25	RR-MS	1.5	2	IFN- $\beta$
M/F: 6/4		Median: 35 Range: 25–52	RR/CP: 8/1	Median: 3 Range: 0–5	Median: 10 Range: 1–19	

UPN, unique patient number; M, male; F, female; EDSS, expanded disability status scale; RR-MS, relapsing-remitting multiple sclerosis; SP-MS, secondary-progressive multiple sclerosis; and CIS, clinically isolated syndrome.

instructions, and were directly used for *in vitro* DC differentiation (Figure 1). The CD14-depleted cell fraction (i.e., peripheral blood lymphocytes (PBL)) was cryopreserved in freezing solution containing 90% fetal bovine serum (Life Technologies, Paisley, UK) supplemented with 10% dimethyl sulfoxide (DMSO, Sigma-Aldrich, Bornem, Belgium) and stored at  $-80^{\circ}\text{C}$  for later use in DC/T cell cocultures. In order to generate immature conventional DC, CD14+ monocytes were cultured at a density of  $1\text{--}1.2 \times 10^6/\text{mL}$  for 7 days in Iscove's modified Dulbecco's Medium (IMDM with L-glutamine, Life Technologies) supplemented with  $10 \mu\text{g}/\text{mL}$  gentamicin (Life Technologies),  $1 \mu\text{g}/\text{mL}$  amphotericin B (Life Technologies), 2.5% heat-inactivated human (h) AB serum (Life Technologies),  $25 \text{ ng}/\text{mL}$  IL-4 (Gentaur, Brussels, Belgium), and  $17.5 \text{ ng}/\text{mL}$  granulocyte macrophage colony-stimulating factor (GM-CSF, Gentaur). Simultaneously, tolDC were differentiated under the same conditions, but with the addition of  $10^{-5} \text{ M}$   $1,25(\text{OH})_2\text{D}_3$  (Sigma-Aldrich). Cells were replenished on day 3 with fresh medium and cytokines. On day 6, DC were (i) stimulated for 24 hours by adding a cocktail of proinflammatory cytokines consisting of  $100 \text{ U}/\text{mL}$  IL-1 $\beta$  (Biosource Europe, Nivelles, Belgium),  $500 \text{ U}/\text{mL}$  IL-6 (Life Technologies),  $2.5 \text{ ng}/\text{mL}$  tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) (Gentaur), and  $10^{-7} \text{ M}$  prostaglandin  $\text{E}_2$  (PGE $_2$ , Prostin E $_2$ , Pfizer, Elsenne, Belgium) (i.e., cytokine cocktail-matured DC (cc-mDC)), or (ii) stimulated for 24 hours by adding  $1 \mu\text{g}/\text{mL}$  lipopolysaccharide (LPS) (Invivogen, San Diego, CA, USA) and  $1000 \text{ IU}/\text{mL}$  IFN- $\gamma$  (ImmunoTools, Friesoythe, Germany) (i.e., LPS-matured DC (LPS-mDC)), or (iii) left untreated (i.e., immature DC (iDC)). Cells were cultured in a humidified atmosphere with 5%  $\text{CO}_2$  at  $37^{\circ}\text{C}$ . On day 7, conventional and  $1,25(\text{OH})_2\text{D}_3$ -treated DC were harvested and used in further experiments.

**2.3. Cryopreservation and Thawing Conditions.** On day 7, immature conventional and  $1,25(\text{OH})_2\text{D}_3$ -treated DC were resuspended in freezing medium containing 86% hAB serum, 10% DMSO, and 4% glucose and frozen in 2 mL cryotubes

(Sarstedt, Numbrecht, Germany) at a concentration of  $10^7$  cells/mL. Cell suspensions were slowly frozen at a cooling rate of  $-1^{\circ}\text{C}/\text{min}$  to  $-80^{\circ}\text{C}$  by using a Mr. Frosty freezing container (Nalgene, Rochester, USA). Within 4 days, cell suspensions were transferred to liquid nitrogen for long-term storage. Frozen samples were quickly thawed at  $37^{\circ}\text{C}$  in a warm water bath and subsequently transferred into preheated ( $37^{\circ}\text{C}$ ) CellGro medium (CellGenix, Freiburg, Germany) supplemented with 1% hAB serum. Next, cells were washed and resuspended in preheated CellGro medium supplemented with 1% hAB serum,  $25 \text{ ng}/\text{mL}$  IL-4, and  $17.5 \text{ ng}/\text{mL}$  GM-CSF. Following a 2 h resting phase at  $37^{\circ}\text{C}$  in an ultralow adherent 6-well plate, conventional and  $1,25(\text{OH})_2\text{D}_3$ -treated iDC were stimulated with a proinflammatory cytokine cocktail or left untreated. After 24 hours, cells were harvested and used in further experiments.

**2.4. Flow Cytometric Immunophenotyping.** For phenotypic characterization of DC, direct immunofluorescence staining was performed using the following fluorochrome-labeled mouse anti-human monoclonal antibodies: anti-CD86-fluorescein isothiocyanate (FITC) (BD Pharmingen, Erembodegem, Belgium), anti-CD80-phycoerythrin (PE) (BD Biosciences, Erembodegem, Belgium), anti-human leukocyte antigen-(HLA-) DR-peridinin chlorophyll (PerCP) (BD Biosciences), anti-CD83-FITC (Life Technologies), anti-dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin-(DC-SIGN-) PE (BD Pharmingen), anti-CD14-PerCP (BD Biosciences), anti-programmed death-ligand 1-(PD-L1-) FITC (BD Pharmingen), anti-CCR7-PE (R&D Systems, Abingdon, UK), and anti-immunoglobulin-like transcript 3-(ILT3-) PE-Cy5 (Immunotech, Marseille, France). Isotype-matched control monoclonal antibodies were used to determine non-specific background staining. Propidium iodide staining was done for analysis of cell viability. For analytical flow cytometry, at least  $10^4$  events were analyzed using a BD FACScan flow cytometer (BD Biosciences). All results were analyzed using FlowJo software (Tree Star, Ashland, USA).

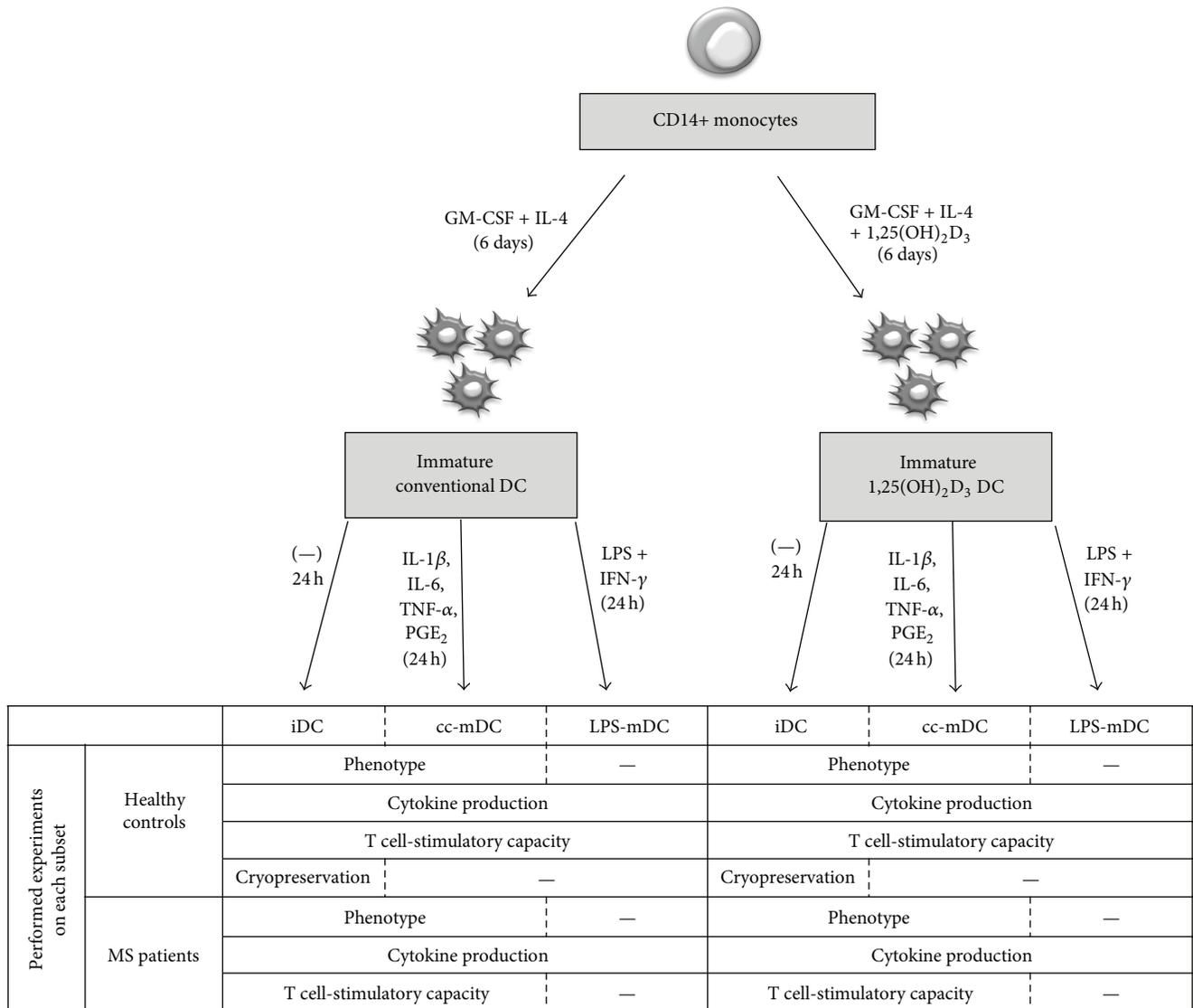


FIGURE 1: Experimental design. CD14+ monocytes were cultured for 6 days in the presence of IL-4 and GM-CSF or in the presence of IL-4, GM-CSF, and 1,25(OH)<sub>2</sub>D<sub>3</sub> to obtain immature conventional DC (iDC) or 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated iDC, respectively. On day 6, iDC were stimulated with a cocktail of proinflammatory cytokines (i.e., cc-matured DC (cc-mDC)) or with LPS and IFN-γ (i.e., LPS-matured DC (LPS-mDC)) or left untreated (i.e., iDC).

**2.5. Cytokine Release Assays.** For quantitative detection of the cytokine secretion profile of the different DC populations, a multiplex fluorescent bead immunoassay (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, TNF-α, TNF-β, and IFN-γ) (Bender MedSystems, Vienna, Austria) and a transforming growth factor-β (TGF-β) ELISA (eBioscience, San Diego, United States of America) were used according to the manufacturer's instructions. For this, iDC and mDC were harvested, washed, and resuspended in IMDM supplemented with 5% hAB serum at a concentration of  $5 \times 10^5$  cells/mL. After 24 hours, supernatant was collected for analysis of cytokine production.

**2.6. Allogeneic Mixed Lymphocyte Reaction (Allo-MLR).** In order to assess the allogeneic T cell-stimulatory capacity

of DC, DC were cocultured with allogeneic responder PBL at a 1:10 ratio. Nonstimulated responder PBL served as negative control, while allogeneic responder cells stimulated with mitomycin C-treated (Sigma-Aldrich) PBL were used as positive control. Cocultures were performed in IMDM supplemented with 5% hAB serum at 37°C. After 6 days, the secreted level of IFN-γ in the cell culture supernatant was determined as a measure for allostimulatory capacity using a commercially available ELISA kit (PeproTech, New Jersey, USA), where each condition is measured in triplicate.

**2.7. Antigen-Specific T Cell-Stimulatory Capacity of DC.** In order to determine the antigen-specific T cell-stimulatory capacity of DC,  $5 \times 10^6$  PBL were stimulated with a pool of myelin-derived peptides (5 μg/mL myelin oligodendrocyte

glycoprotein (MOG) (aa 1–22), 5  $\mu\text{g}/\text{mL}$  MOG (aa 34–56), 5  $\mu\text{g}/\text{mL}$  MOG (aa 64–86), and 5  $\mu\text{g}/\text{mL}$  MOG (aa 74–96) and 5  $\mu\text{g}/\text{mL}$  myelin basic protein (MBP) (aa 84–102) and 5  $\mu\text{g}/\text{mL}$  MBP (aa 143–168), all purchased from Severn Biotech Ltd. (Kidderminster, UK) in the presence or absence of  $5 \times 10^5$  autologous DC. After 7 days of coculture, PBL were analyzed for antigen-specific responsiveness by determining IFN- $\gamma$  production following antigenic restimulation by means of IFN- $\gamma$  ELISPOT (Mabtech, Nacka Strand, Sweden), according to the manufacturer's instructions. In brief,  $2 \times 10^5$  stimulated PBL were rechallenged with 5  $\mu\text{g}/\text{mL}$  of MOG- and MBP-derived peptides in anti-IFN- $\gamma$  antibody-coated 96-well polyvinylidene fluoride (PVDF) plates (Millipore, Bedford, MA, USA). Nonstimulated PBL were used as a control and each condition was measured in quadruple. In some experiments, PBL were harvested on day 7 of coculture and restimulated either with 0.5  $\mu\text{g}/\text{mL}$  cytomegalovirus (CMV) pp65-derived peptide pool or with 5  $\mu\text{g}/\text{mL}$  of MOG- and MBP-derived peptides combined with cryopreserved fully mature conventional DC of the same donor. Frequencies of antigen-specific IFN- $\gamma$ -secreting cells were calculated based on the number of spots counted using an automated AID ELISPOT Reader system (AID GmbH, Strassberg, Germany) and analyzed using AID ELISPOT software version 5.0. A positive responder was defined according to the guidelines of the ELISPOT proficiency panel from the Cancer Vaccine Consortium [28]: per  $10^6$  PBL, the mean antigen-specific spot count for a donor and condition must be greater than or equal to 15 spots per well and at least 2.5 times as high as the background reactivity.

**2.8. DC-Mediated Induction of Suppressive T Cell Populations.** The induction of different populations of Treg was determined following coculture of autologous PBL, stimulated with MOG- and MBP-derived peptides in the presence or absence of DC, as described above. At day 6, 10  $\mu\text{g}/\text{mL}$  brefeldin A (GolgiStop, BD Pharmingen) was added to the DC/T cell coculture and incubated overnight at 37°C. Next, cells were harvested and membrane markers were stained with the following mouse anti-human monoclonal antibodies: anti-CD3-PerCP-Cy5.5 (BD Biosciences), anti-CD4-allophycocyanin-H7 (anti-CD4-APC-H7) (BD Biosciences), anti-CD8-Pacific Blue (Life Technologies), and anti-CD25-PE-Cy7 (BD Biosciences). Subsequently, cells were fixed and permeabilized using a FOXP3 Staining Buffer Kit (eBioscience, Hatfield, UK), according to manufacturer's instructions, and intracellular markers were stained with anti-FOXP3-alexa488 (BD Pharmingen), anti-TGF- $\beta$ -PE (IQ Products, Groningen, Netherlands), and anti-IL-10-APC (BD Pharmingen). Labeled cells were analyzed on a Cyflow ML flow cytometer (Partec, Münster, Germany). For analytical flow cytometry, at least  $5 \times 10^4$  CD3+ CD4+ CD8- lymphocytes were acquired. All results were analyzed using FlowJo software.

**2.9. Statistical Analysis.** Results are expressed as mean  $\pm$  standard error of mean (SEM), unless stated otherwise. Comparisons were validated using one-way or two-way analysis of variance (ANOVA) with a Bonferroni *post hoc* test for pairwise group comparisons, when appropriate using

GraphPad version 5 software (Prism, La Jolla, CA, USA). A *p* value of  $\leq 0.05$  was considered as statistically significant.

### 3. Results

**3.1. 1,25(OH) $_2$ D $_3$ -Treated Immature DC Express Lower Levels of CD86 and HLA-DR and Display an Anti-Inflammatory Cytokine Profile as Compared to Conventional DC.** Previously, we reported no major differences in the phenotype of *in vitro* generated immature DC of MS patients as compared to those of healthy controls, except for the expression of the migration marker CCR7 [29]. Here, we demonstrate that 1,25(OH) $_2$ D $_3$  treatment of immature mo-DC from healthy controls results in significantly lower expression levels of CD86 and of HLA-DR as compared to conventional DC, while 1,25(OH) $_2$ D $_3$ -treated mo-DC from MS patients only show lower expression levels of HLA-DR as compared to conventional mo-DC (Figures 2(a) and 2(c)). However, it needs to be noted that immature conventional DC of healthy controls show a significantly higher expression level of CD86 as compared to those of MS patients. MS-derived mo-DC show lower expression levels of DC-SIGN following 1,25(OH) $_2$ D $_3$  treatment, despite the fact that both conventional and 1,25(OH) $_2$ D $_3$ -treated mo-DC from MS patients display significantly higher expression levels of DC-SIGN as compared to those of healthy controls (Supplementary Figure 1, in Supplementary Material available online at <http://dx.doi.org/10.1155/2016/5392623>). No differences for the expression of CD80 and CD83 were observed following 1,25(OH) $_2$ D $_3$  treatment of mo-DC from both healthy controls and MS patients (Figures 2(b) and 2(d)). Furthermore, 1,25(OH) $_2$ D $_3$  treatment did not affect the expression of CD14, of the chemokine receptor CCR7, and of the inhibitory molecules PD-L1 and ILT-3 by mo-DC from both healthy controls and MS patients (Supplementary Figure 1).

Subsequently, the cytokine secretion profile of both conventional and 1,25(OH) $_2$ D $_3$ -treated immature mo-DC from healthy controls and MS patients was assessed using a multiplex immunoassay and ELISA. No major differences regarding the cytokine secretion profile of mo-DC from MS patients as compared to mo-DC from healthy controls could be detected (Figures 2(e)–2(j)). Remarkably, immature DC from healthy controls as well as from MS patients produced more TGF- $\beta$  following 1,25(OH) $_2$ D $_3$  treatment as compared to conventional DC (Figure 2(j)).

**3.2. 1,25(OH) $_2$ D $_3$ -Treated DC Display a Semimature Phenotype.** Next, immature mo-DC were stimulated with a cocktail of proinflammatory cytokines (i.e., cc-mDC) for 24 hours. Conventional DC of both healthy controls (Table 2(a)) and MS patients (Table 2(b)) acquire a mature phenotype following activation with proinflammatory stimuli, as evidenced by upregulation of the expression of CD80, CD86, CD83, and HLA-DR (Figure 3(a)). Importantly, 1,25(OH) $_2$ D $_3$ -treated DC from both healthy controls and MS patients displayed a significantly lower expression of CD86, CD83, and HLA-DR upon stimulation with a proinflammatory cytokine cocktail in comparison with conventional DC. However, also 1,25(OH) $_2$ D $_3$ -treated DC underwent a maturation process

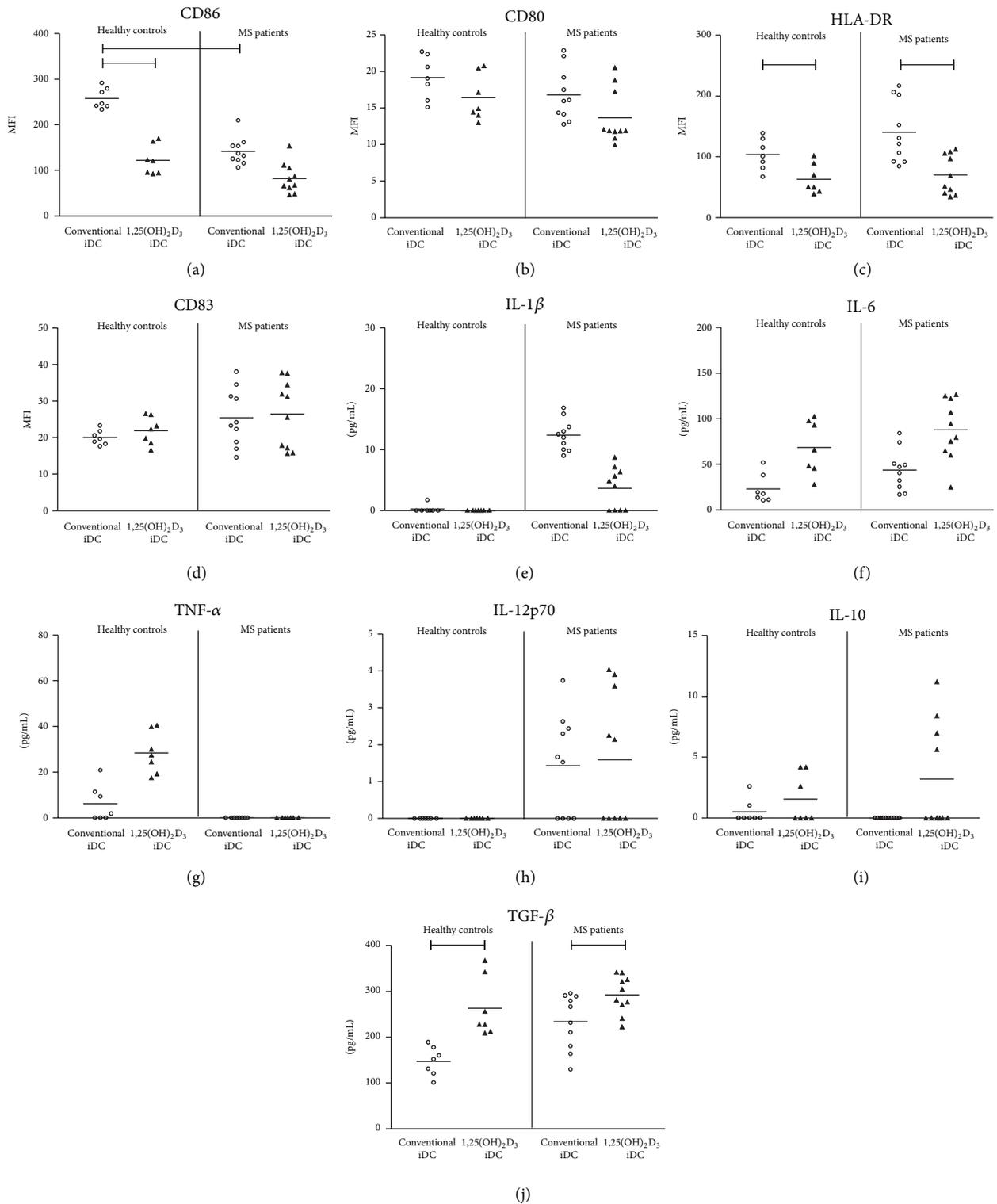


FIGURE 2: Characterization of *in vitro* differentiated iDC from healthy controls and MS patients. CD14<sup>+</sup> monocytes were cultured for 7 days in the presence of IL-4 and GM-CSF or in the presence of IL-4, GM-CSF, and 1,25(OH)<sub>2</sub>D<sub>3</sub> to obtain conventional iDC (open dots) or 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated iDC (filled triangles), respectively. The expression of (a) CD86, (b) CD80, (c) HLA-DR, and (d) CD83 by DC of healthy controls ( $n = 7$ ) and MS patients ( $n = 10$ ) is determined by flow cytometry. Cytokine secretion of (e) IL-1 $\beta$ , (f) IL-6, (g) TNF- $\alpha$ , (h) IL-12p70, (i) IL-10, and (j) TGF- $\beta$  by conventional iDC and 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated iDC is determined by a multiplex immunoassay or ELISA. Horizontal lines show the mean. MFI, mean fluorescence intensity, and iDC, immature DC.

TABLE 2: Immunophenotypic analysis of *in vitro* differentiated DC from healthy controls and MS patients upon stimulation with proinflammatory molecules.

(a) Healthy controls ( $n = 7$ ) cc-mDC					
Marker	Type	Fold change (cc-mDC/iDC)	MFI $\pm$ SD	$p$ value*	$p$ value**
CD86	Conventional DC	1.95	504 $\pm$ 99	$p < 0.05$	$p < 0.05$
	1,25(OH) <sub>2</sub> D <sub>3</sub> DC	1.89	232 $\pm$ 96	$p < 0.05$	
CD83	Conventional DC	4.44	89 $\pm$ 16	$p < 0.05$	$p < 0.05$
	1,25(OH) <sub>2</sub> D <sub>3</sub> DC	1.64	36 $\pm$ 18	n.s.	
CD80	Conventional DC	3.21	61 $\pm$ 14	$p < 0.05$	n.s.
	1,25(OH) <sub>2</sub> D <sub>3</sub> DC	2.03	33 $\pm$ 11	n.s.	
HLA-DR	Conventional DC	1.36	153 $\pm$ 46	$p < 0.05$	$p < 0.05$
	1,25(OH) <sub>2</sub> D <sub>3</sub> DC	1.43	86 $\pm$ 33	n.s.	
(b) MS patients ( $n = 10$ ) cc-mDC					
Marker	Type	Fold change (cc-mDC/iDC)	MFI $\pm$ SD	$p$ value*	$p$ value**
CD86	Conventional DC	2.63	372 $\pm$ 90	$p < 0.05$	$p < 0.05$
	1,25(OH) <sub>2</sub> D <sub>3</sub> DC	2.35	194 $\pm$ 77	$p < 0.05$	
CD83	Conventional DC	2.30	59 $\pm$ 16	$p < 0.05$	$p < 0.05$
	1,25(OH) <sub>2</sub> D <sub>3</sub> DC	1.17	31 $\pm$ 9	n.s.	
CD80	Conventional DC	2.42	41 $\pm$ 14	n.s.	n.s.
	1,25(OH) <sub>2</sub> D <sub>3</sub> DC	1.76	24 $\pm$ 8	n.s.	
HLA-DR	Conventional DC	1.81	258 $\pm$ 61	$p < 0.05$	$p < 0.05$
	1,25(OH) <sub>2</sub> D <sub>3</sub> DC	1.56	117 $\pm$ 59	n.s.	

CD14<sup>+</sup> monocytes were cultured for 6 days in the presence of IL-4 and GM-CSF or in the presence of IL-4, GM-CSF, and 1,25(OH)<sub>2</sub>D<sub>3</sub> to obtain conventional iDC or 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated iDC, respectively. On day 6, DC were stimulated with a cocktail of proinflammatory cytokines (i.e., cc-matured DC (cc-mDC)) or left untreated (i.e., iDC). The mean fluorescent intensity (MFI) of costimulatory molecules, CD80 and CD86, of maturation marker, CD83, and of HLA-DR by various DC subsets of healthy controls (a) ( $n = 7$ ) and MS patients (b) ( $n = 10$ ) was evaluated. Results are expressed as fold change, calculated as the ratio between the MFI value of cc-mDC to the MFI value of iDC.

\*The  $p$  values indicated are calculated for cc-mDC versus iDC.

\*\*The  $p$  values indicated are calculated for conventional cc-mDC versus cytokine cocktail-matured 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated DC.

MFI, mean fluorescence intensity; cc-mDC, cytokine cocktail-matured DC; iDC, immature DC; and n.s., nonsignificant.

as demonstrated by upregulated expression of CD80, CD83, CD86, and HLA-DR, albeit less pronounced as in conventional DC. No significant differences could be observed regarding the expression of DC-SIGN, CD14, and the inhibitory molecules ILT-3 and PD-L1 between 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated mo-DC and conventional mo-DC following stimulation with proinflammatory molecules (Supplementary Figure 1).

Additionally, we investigated the cytokine secretion profile of conventional and 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated mo-DC following stimulation with proinflammatory molecules. Our findings indicate significantly higher levels of secreted IL-1 $\beta$ , IL-6, IL-12p70, and TNF- $\alpha$  by conventional DC of both healthy controls and MS patients following stimulation with LPS and IFN- $\gamma$  (Figure 3(b)). Noteworthy, LPS and IFN- $\gamma$ -stimulated conventional mo-DC from MS patients secrete significantly higher, in particular 20-fold more, amounts of IL-12p70 as compared to conventional mo-DC from healthy controls. Similarly, also the secretion of IL-1 $\beta$  and IL-6 by mo-DC from MS patients was increased as compared to mo-DC from healthy controls. Importantly, 1,25(OH)<sub>2</sub>D<sub>3</sub> treatment of mo-DC drastically abrogated the secretion of IL-12p70 and TNF- $\alpha$  by mo-DC from both healthy controls and MS patients. Production of IL-1 $\beta$  and IL-6 following stimulation with LPS and IFN- $\gamma$  was only reduced in 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated mo-DC

from healthy controls as compared to conventional mo-DC. Even following 1,25(OH)<sub>2</sub>D<sub>3</sub> treatment, mo-DC from MS patients display a significantly higher secretion of IL-1 $\beta$ , IL-6, and TNF- $\alpha$  following stimulation with LPS and IFN- $\gamma$  as compared to 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated mo-DC of healthy controls. In our hands, we could not observe IL-10 secretion by 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated mo-DC from either healthy controls or MS patients. However, following stimulation with LPS and IFN- $\gamma$ , the secretion of IL-10 by conventional mo-DC of MS patients was significantly higher as compared to 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated mo-DC.

In summary, our findings demonstrate that 1,25(OH)<sub>2</sub>D<sub>3</sub> treatment of mo-DC renders DC of both healthy controls and MS patients in a semimature state as indicated by a significantly impaired upregulation of the expression of costimulatory molecules and activation markers as well as by a significantly reduced secretion of proinflammatory cytokines.

**3.3. Cryopreservation Did Not Affect the Expression of Membrane Markers by 1,25(OH)<sub>2</sub>D<sub>3</sub>-Treated DC.** In order to facilitate multiple injections with tolDC for clinical applications, we evaluated the feasibility to cryopreserve tolDC. For this, viability, recovery, and phenotype of cryopreserved iDC were assessed upon thawing. We demonstrate a yield

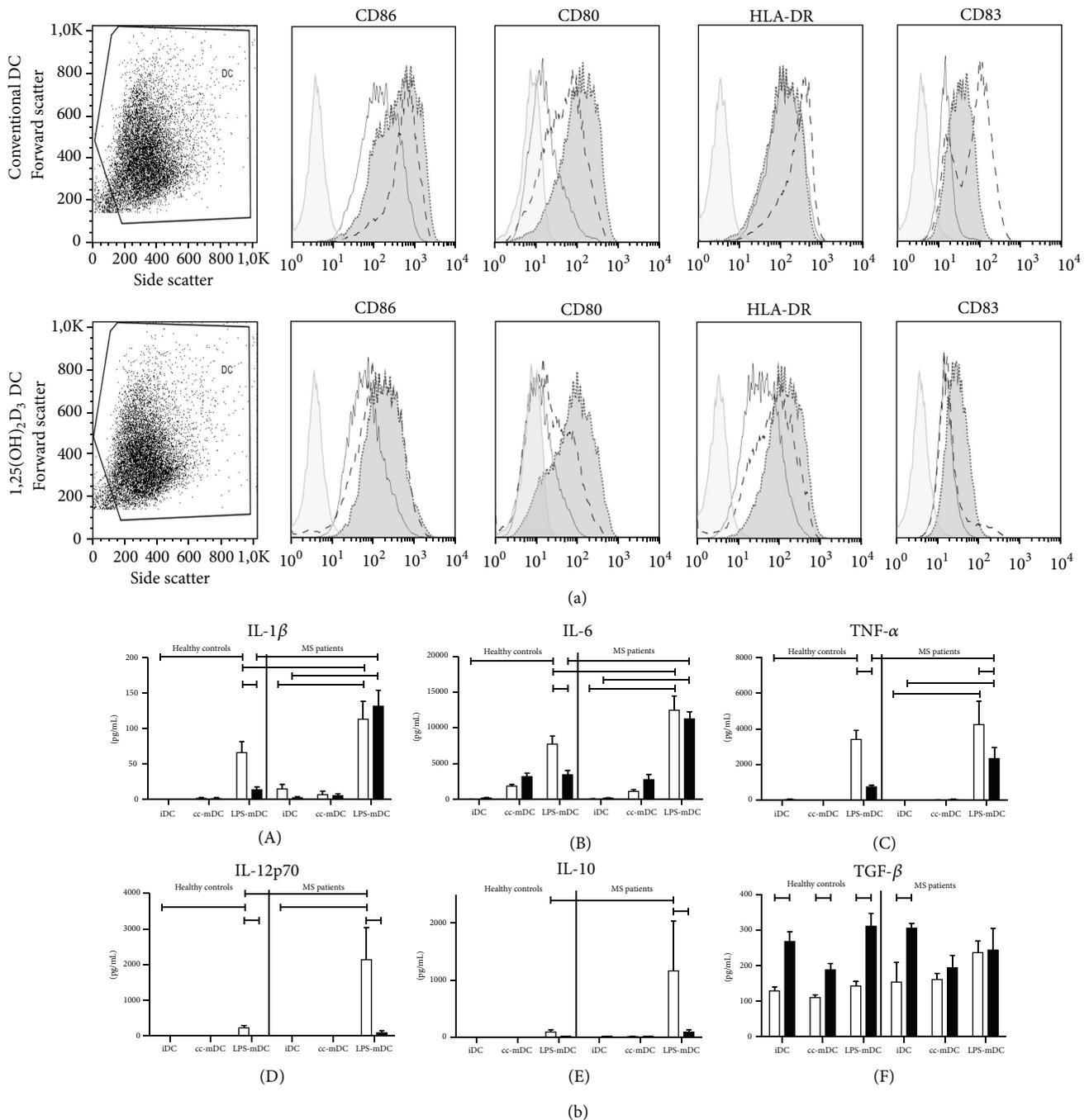


FIGURE 3: Immunophenotypic analysis and cytokine secretion profile of *in vitro* differentiated DC from healthy controls and MS patients upon maturation with proinflammatory stimuli. CD14<sup>+</sup> monocytes were cultured for 6 days in the presence of IL-4 and GM-CSF or in the presence of IL-4, GM-CSF, and 1,25(OH)<sub>2</sub>D<sub>3</sub> to obtain immature conventional DC (iDC) or 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated iDC, respectively. On day 6, DC were stimulated with a cocktail of proinflammatory cytokines (i.e., cc-mDC) or with LPS and IFN- $\gamma$  (i.e., LPS-mDC) or left untreated (i.e., iDC). (a) Representative example showing immunophenotypic analysis of DC. The expression of CD86, CD80, HLA-DR, and CD83 by conventional DC and 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated DC is determined by flow cytometry. Immature DC are represented by a solid line, cc-mDC are represented by a dashed line, and LPS-mDC are represented by a dark grey filled histogram. Isotype-matched controls are represented by the light grey filled histograms. For analysis, DC were gated on light scatter properties as depicted in the forward scatter (FSC) versus side scatter (SSC) dot plot. (b) Cytokine secretion of (A) IL-1 $\beta$ , (B) IL-6, (C) TNF- $\alpha$ , (D) IL-12p70, (E) IL-10, and (F) TGF- $\beta$  by conventional DC (open bars) and 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated DC (black bars) is determined by a multiplex immunoassay or ELISA. Results are expressed as mean  $\pm$  SEM (healthy controls:  $n = 7$ ; MS patients:  $n = 10$ ).

TABLE 3: Immunophenotypic analysis of *in vitro* differentiated mo-DC from healthy controls before and after cryopreservation.

Marker	Type	Healthy controls ( $n = 5$ ) cc-mDC			
		Fold change (cc-mDC/iDC)	MFI $\pm$ SD	$p$ value*	$p$ value**
CD86	Conventional DC	1.03	637 $\pm$ 98	n.s.	$p < 0.05$
	1,25(OH) <sub>2</sub> D <sub>3</sub> DC	1.19	209 $\pm$ 155	n.s.	
CD83	Conventional DC	1.43	45 $\pm$ 10	$p < 0.05$	$p < 0.05$
	1,25(OH) <sub>2</sub> D <sub>3</sub> DC	0.90	22 $\pm$ 6	n.s.	
CD80	Conventional DC	1.24	23 $\pm$ 7	n.s.	$p < 0.05$
	1,25(OH) <sub>2</sub> D <sub>3</sub> DC	1.00	15 $\pm$ 4	n.s.	
HLA-DR	Conventional DC	0.89	103 $\pm$ 21	n.s.	n.s.
	1,25(OH) <sub>2</sub> D <sub>3</sub> DC	0.92	61 $\pm$ 36	n.s.	

CD14<sup>+</sup> monocytes were cultured for 7 days in the presence of IL-4 and GM-CSF or in the presence of IL-4, GM-CSF, and 1,25(OH)<sub>2</sub>D<sub>3</sub> to obtain immature conventional DC or 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated DC, respectively. On day 7, iDC were frozen and stored at  $-80^{\circ}\text{C}$ . Next, cryopreserved iDC were thawed, rested for 2 h at  $37^{\circ}\text{C}$ , and stimulated with a proinflammatory cytokine cocktail for 24 h (i.e., cc-mDC). The mean fluorescent intensity (MFI) of costimulatory molecules, CD80 and CD86, of maturation marker, CD83, and of HLA-DR by various DC subsets of healthy controls ( $n = 5$ ) was determined. Results are expressed as fold change, calculated as the ratio between the MFI value after maturation following a freeze-thaw cycle and the MFI value obtained at immature stage following a freeze-thaw cycle.

\*The  $p$  values indicated are calculated for cc-mDC DC after cryopreservation versus iDC after cryopreservation.

\*\*The  $p$  values indicated are calculated for conventional cc-mDC after cryopreservation versus 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated cc-mDC after cryopreservation. MFI, mean fluorescence intensity; cc-mDC, cytokine cocktail-matured DC; iDC, immature DC; and n.s., nonsignificant.

of 78% and a viability of 75% of immature 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated DC following a freeze-thaw cycle (Figure 4(a)). No significant differences for the yield and viability were found between conventional DC and 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated DC. Furthermore, while conventional iDC display a significantly increased expression of CD86 and decreased expression of HLA-DR following cryopreservation, underscoring the plasticity of the phenotypic characteristics of conventional DC, no differences regarding the expression levels of HLA-DR, CD80, CD86, and CD83 were observed for 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated mo-DC following cryopreservation (Figure 4(b)).

In order to determine the ability of 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated iDC to maintain their semimature phenotype after cryopreservation, iDC were stimulated with a proinflammatory cytokine cocktail for 24 hours following a 2 h resting phase after thawing. Conventional DC display upregulated expression of CD83 (Table 3). No significant differences were detected for the expression of HLA-DR, CD80, and CD86, despite the fact that marked upregulation of CD86 expression by conventional DC was already observed following cryopreservation (Figure 4(b)). In addition, cryopreservation did not affect the expression profile of membrane markers by 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated mo-DC, not even upon stimulation with proinflammatory signals, indicative of a robust semimature phenotype of 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated DC (Table 3).

**3.4. Allogeneic T Cell-Stimulatory Capacity of 1,25(OH)<sub>2</sub>D<sub>3</sub>-Treated DC before and after Cryopreservation.** The immunostimulatory capacity of conventional and 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated DC was determined in an allogeneic mixed leukocyte reaction (allo-MLR). For this, responder PBL were stimulated with allogeneic iDC or mDC of healthy controls at a 10:1 ratio. The level of IFN- $\gamma$  secreted in the coculture supernatant was used as a measure for allogeneic T cell-stimulatory capacity. As demonstrated in Figure 5, conventional mo-DC have profound capacity to stimulate IFN- $\gamma$ -production

by responder PBL in an allo-MLR, which is not affected by cryopreservation of mo-DC, as compared to the negative control. In contrast, no allogeneic IFN- $\gamma$  production is induced by responder PBL following stimulation with 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated DC, irrespective of the maturation state of DC. Importantly, this T cell hyporesponsiveness was retained following stimulation with cryopreserved allogeneic 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated iDC or mDC.

**3.5. 1,25(OH)<sub>2</sub>D<sub>3</sub>-Treated DC Induce Antigen-Specific T Cell Hyporesponsiveness to Myelin-Derived Antigens.** In order to determine the antigen-specific T cell-stimulatory capacity of conventional and 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated DC of healthy controls ( $n = 7$ ) and MS patients ( $n = 4$ ), PBL were stimulated with myelin-derived peptides in the presence or absence of autologous iDC or mDC at a 10:1 ratio for 7 days. Subsequently, the number of antigen-specific IFN- $\gamma$ -secreting T cells was determined using IFN- $\gamma$  ELISPOT. Following rechallenge of *in vitro* stimulated PBL with myelin-derived peptides, antigen-specific IFN- $\gamma$  production by PBL stimulated with conventional mDC was significantly higher as compared to PBL stimulated with conventional iDC (Figures 6(a) and 6(b)). Hence, stimulation with fully mature conventional DC is mandatory to detect myelin-specific IFN- $\gamma$ -secreting T cells in both healthy controls and MS patients. Of interest, there was no significant difference in the number of MOG/MBP responders between healthy controls and MS patients. Seven out of 16 healthy controls and 4 out of 7 MS patients displayed a positive myelin-specific response following stimulation with conventional mDC, as defined in the Material and Methods. In contrast, PBL stimulated with 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated mDC fail to respond to a rechallenge with myelin-derived peptides, as evidenced by the significantly reduced number of IFN- $\gamma$ -secreting spot-forming cells as compared to PBL stimulated with conventional mDC (Figures 6(a) and 6(b)). Based on these observations, we

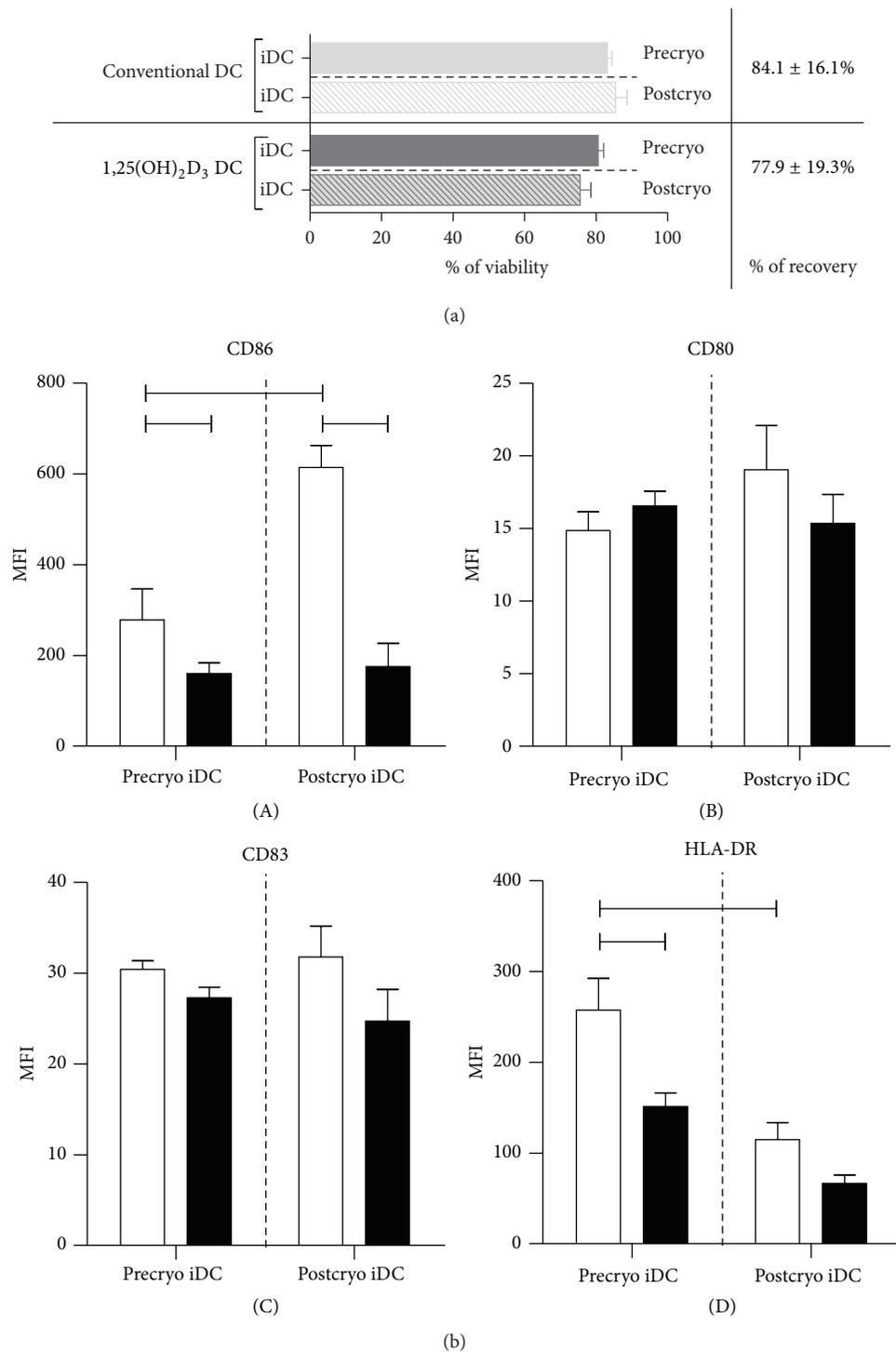


FIGURE 4: Viability, recovery, and phenotypic characteristics of conventional and 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated iDC from healthy controls before and after cryopreservation. CD14<sup>+</sup> monocytes were cultured for 7 days in the presence of IL-4 and GM-CSF or in the presence of IL-4, GM-CSF, and 1,25(OH)<sub>2</sub>D<sub>3</sub> to obtain immature conventional DC or 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated DC, respectively. On day 7, iDC were frozen (i.e., precryo iDC). Following a 2 h resting phase after thawing, iDC were left untreated for 24 h (i.e., postcryo iDC). (a) Viability of conventional and 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated iDC of healthy controls ( $n = 5$ ) was determined on day 7 of DC culture (i.e., precryo) and 26 h after thawing (i.e., postcryo). Recovery is expressed as the ratio of cells harvested before and after cryopreservation. (b) The MFI of (A) CD86, (B) CD80, (C) CD83, and (D) HLA-DR by immature conventional DC (open bars) or 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated DC (black bars) of healthy controls ( $n = 5$ ) was determined. Results are expressed as mean  $\pm$  SEM. MFI, mean fluorescence intensity, and iDC, immature DC.

TABLE 4: Flow cytometric analysis of CD4+ CD25hi FOXP3+ Treg and immunosuppressive cytokine-expressing Treg in cultures of PBL stimulated with MOG- and MBP-derived peptides in the presence or absence of conventional and 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated DC.

	% CD4+ CD25hi FOXP3+ within CD3+ CD4+ T cells	% TGF- $\beta$ + within CD3+ CD4+ CD25- T cells	% IL-10+ within CD3+ CD4+ CD25- T cells	% IL-10+ TGF- $\beta$ + within CD3+ CD4+ CD25- T cells	<i>p</i> value*
MOG/MBP peptide stimulated PBL + conventional iDC	2.29 $\pm$ 0.84	0.21 $\pm$ 0.10	0.15 $\pm$ 0.04	0.02 $\pm$ 0.01	n.s.
MOG/MBP peptide stimulated PBL + 1,25(OH) <sub>2</sub> D <sub>3</sub> iDC	1.62 $\pm$ 0.66	0.17 $\pm$ 0.08	0.12 $\pm$ 0.02	0.02 $\pm$ 0.01	n.s.

\*The *p* values indicated are calculated for MOG- and MBP-derived peptides-stimulated PBL versus PBL cocultured with iDC in the presence of MOG- and MBP-derived peptides.

MOG, myelin oligodendrocyte glycoprotein; MBP, myelin basic protein; PBL, peripheral blood lymphocytes; iDC, immature DC; and n.s., nonsignificant.

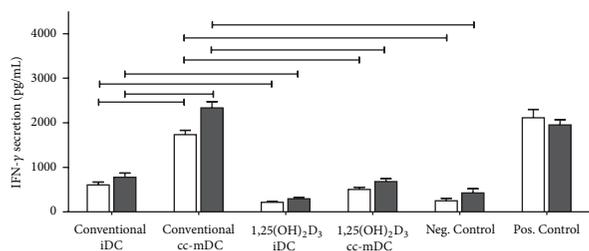


FIGURE 5: Allogeneic T cell-stimulatory capacity of 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated DC before and after cryopreservation. Fresh and frozen iDC and mDC were cocultured with allogeneic responder PBL at a 1:10 ratio. Nonstimulated PBL served as negative control, while allogeneic responder cells stimulated by mitomycin C-treated PBL (10:1 responder/stimulator ratio) were used as positive control. After 6 days, cell culture supernatant was collected and the secreted level of IFN- $\gamma$  was used as a measure for allostimulatory capacity by means of IFN- $\gamma$  ELISA. Each condition was measured in triplicate. Results of healthy controls (*n* = 5) are expressed as mean  $\pm$  SEM. The open bars and black bars represent the measured IFN- $\gamma$  secretion before cryopreservation and after cryopreservation, respectively. iDC, immature DC; cc-mDC, cytokine cocktail-matured DC; and PBL, peripheral blood lymphocytes.

demonstrate that 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated DC from both healthy volunteers and MS patients induce T cell hyporesponsiveness, irrespective of the maturation state of DC.

In order to investigate if the T cell hyporesponsiveness mediated by 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated DC is antigen-specific, we investigated the capacity of T cells stimulated with 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated DC and myelin-derived peptides to respond to an unrelated antigen, that is, cytomegalovirus (CMV) pp65-derived peptides. For this, PBL stimulated with a pool of myelin-derived peptides in the presence or absence of iDC or mDC were rechallenged with myelin-derived peptides or with a CMV pp65-derived peptide pool after 7 days of initial coculture. While a low frequency of myelin-specific IFN- $\gamma$ -secreting spot-forming cells was detected when PBL from healthy controls (Figure 6(c)) and from MS patients (Figure 6(d)) were rechallenged with myelin-derived peptides, PBL were still able to secrete IFN- $\gamma$  production following rechallenge with a CMV pp65-derived peptide pool in all conditions tested.

**3.6. Mode of Action of T Cell Hyporesponsiveness Mediated by 1,25(OH)<sub>2</sub>D<sub>3</sub>-Treated DC.** In order to evaluate whether the T cell hyporesponsiveness mediated by 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated DC could be reversed, PBL were stimulated with myelin-derived peptides in the presence or absence of autologous conventional and 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated iDC or mDC for 7 days. Next, PBL were rechallenged with myelin-derived peptides alone or with myelin-derived peptides and conventional cc-mDC. In both healthy controls (Figure 6(e)) and MS patients (Figure 6(f)), inclusion of a strong stimulus, such as fully mature conventional DC, together with antigen rechallenge is associated with a significantly higher number of antigen-specific IFN- $\gamma$ -secreting T cells as compared to PBL rechallenged with myelin-derived peptides alone. In contrast, rechallenge of PBL tolerized to myelin-derived peptides in the presence of 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated mDC using conventional cc-mDC did not affect the myelin-specific T cell response. For this, we conclude that PBL stimulated with 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated DC were rendered in a robust hyporesponsive state in both healthy volunteers and MS patients, irrespective of the maturation state of DC.

Since others described that CD4+ T cells primed by iDC acquire a Treg phenotype [30, 31], we assessed the presence of Treg populations in autologous DC/T cell cocultures. Hereto, PBL were stimulated with myelin-derived peptides in the presence or absence of autologous conventional or 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated iDC at a 10:1 ratio. After 7 days of coculture, multiparametric flow cytometry was performed to characterize the presence of CD4+ CD25+ FOXP3+ Treg as well as the expression of intracellular immunosuppressive cytokines. No differences in the number of Treg following stimulation with both conventional and 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated DC could be detected. In addition, we could not observe IL-10 and/or TGF- $\beta$ -expressing cells (Table 4).

## 4. Discussion

Current disease-modifying therapies to prevent or slow progressive disability in MS include IFN- $\beta$ , glatiramer acetate, natalizumab, and fingolimod. Recently, a number of new treatment strategies have been approved for clinical use by the regulatory authorities including teriflunomide [32], dimethyl fumarate (BG-12) [33], and alemtuzumab [34]. All

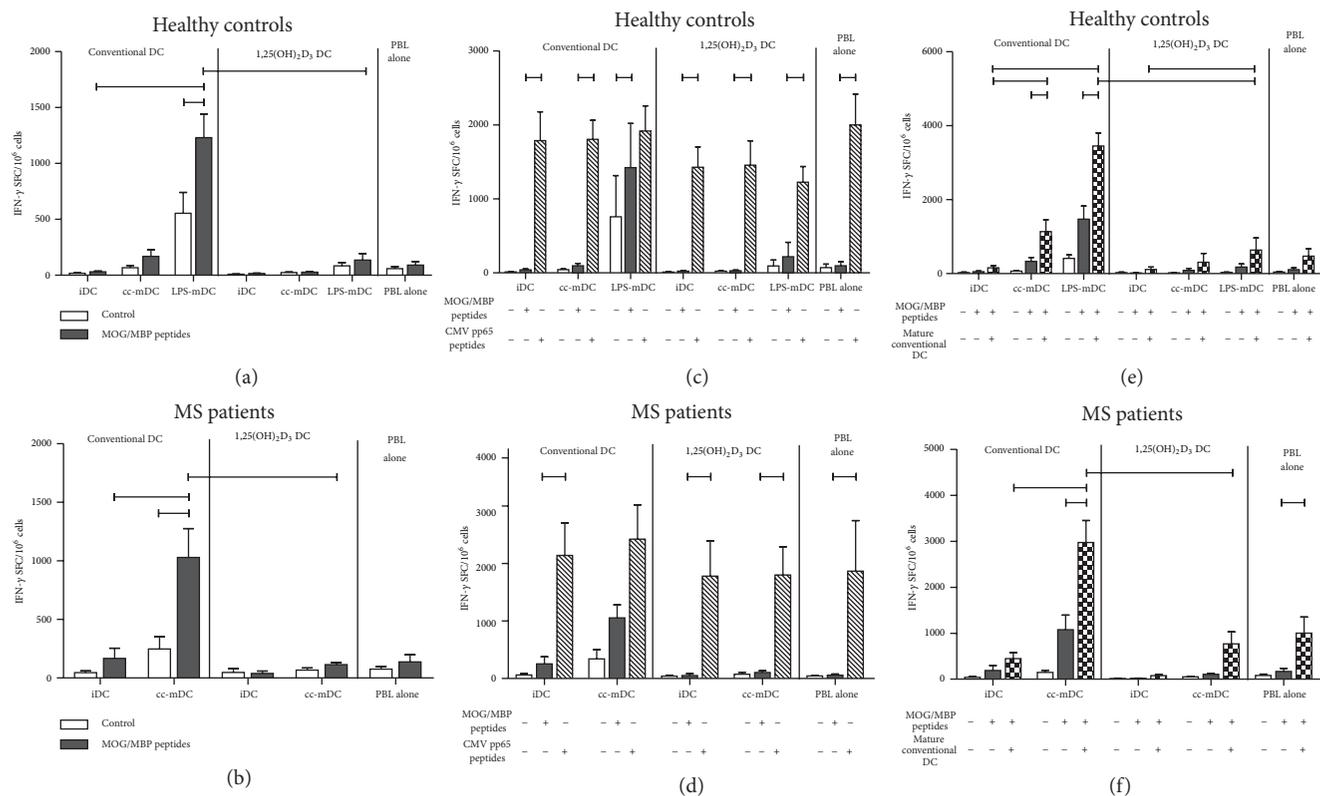


FIGURE 6: 1,25(OH)<sub>2</sub>D<sub>3</sub>-Treated DC induce stable antigen-specific T cell hyporesponsiveness to myelin-derived antigens (MOG/MBP peptides) in both healthy controls and MS patients. PBL stimulated with MOG/MBP peptides with or without autologous iDC or mDC were restimulated with MOG/MBP peptides (black bars) after 7 days of initial coculture. Controls represent nonrestimulated PBL (open bars). The secretion of IFN- $\gamma$  was used as a measure for autologous T cell-stimulatory capacity. Each condition was measured in quadruple. Results are expressed as mean  $\pm$  SEM. T cell hyporesponsiveness induced by 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated DC of healthy controls ( $n = 7$ ) and MS patients ( $n = 4$ ) is shown, respectively, in (a) and (b). The antigen specificity of T cell hyporesponsiveness induced by 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated DC was determined for healthy controls (c) and MS patients (d). PBL stimulated with MOG/MBP peptides with or without autologous DC were restimulated with either MOG/MBP peptides (black bars) or CMV pp65 peptides (dashed bars) after 7 days of initial coculture. ((e) and (f)) Stability of T cell hyporesponsiveness induced by 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated DC of healthy individuals (e) and MS patients (f). PBL stimulated with MOG/MBP peptides with or without autologous DC were restimulated either with MOG/MBP peptides (black bars) or with MOG/MBP peptides combined with fully mature conventional DC (blocked bars) after 7 days of initial coculture. iDC, immature DC; cc-mDC, cytokine cocktail-matured DC; LPS-mDC, lipopolysaccharide-matured DC; PBL, peripheral blood lymphocytes; MOG, myelin oligodendrocyte glycoprotein; MBP, myelin basic protein; CMV, cytomegalovirus; and SFC, spot forming cells.

are primarily aimed at reducing the number of relapses and slowing the disease progression; however, none induces a long-lasting, drug-free remission of MS, whereas several are accompanied by severe side effects such as secondary autoimmunity and infections. Therefore, continuous efforts are aimed at the development of new therapeutic approaches that specifically target the pathologic autoinflammatory processes in MS without generalized immune suppression. In this perspective, the identification of tolDC as cellular mediators to downmodulate unwanted autoimmune responses may provide new prospects. Indeed, preclinical evidence from animal models supports the therapeutic potential of tolDC as demonstrated by prevention of transplant rejection in skin and heart graft models [35, 36] or by attenuation of pathogenic T cells and reestablishment of self-tolerance following administration of *ex vivo* generated tolDC in collagen-induced arthritis (CIA), nonobese diabetic (NOD), and EAE

models [15, 19–21]. These promising outcomes resulted in a number of recently completed phase I clinical trials using tolDC in patients with type 1 diabetes [22], rheumatoid arthritis [23, 25], and Crohn's disease [24]. Treatment with autologous tolDC was well tolerated and safe without any discernible adverse events or toxicities. While these studies highlight the emergence of tolDC therapy as a new approach to treat autoimmune diseases, numerous questions still remain in view of the translation of bench findings to the bedside. Indeed, although different strategies using a variety of tolerogenic agents for the generation of tolDC *in vitro* are showing promising results, not all tolerogenic agents seem to have the ability to maintain a stable tolerogenic profile, once administered *in vivo* [37].

Here, we demonstrated that 1,25(OH)<sub>2</sub>D<sub>3</sub> treatment renders mo-DC in a semimature state as evidenced by impaired upregulation of the expression of CD86, CD80,

CD83, and HLA-DR upon stimulation with proinflammatory molecules as compared to the expression of these markers by conventional DC. Furthermore, no phenotypic differences were found between mo-DC from healthy controls and MS patients following  $1,25(\text{OH})_2\text{D}_3$  treatment. Similar results were previously demonstrated by others [14, 38]. Additionally, while  $1,25(\text{OH})_2\text{D}_3$ -treated immature DC secrete higher levels of  $\text{TGF-}\beta$  as well as of IL-6 and  $\text{TNF-}\alpha$ , as compared to conventional DC,  $1,25(\text{OH})_2\text{D}_3$ -treated DC show impaired secretion of proinflammatory cytokines following stimulation with LPS and  $\text{IFN-}\gamma$  as compared to conventional DC, except for IL-1 $\beta$  and IL-6 secretion by mature  $1,25(\text{OH})_2\text{D}_3$ -treated mo-DC from MS patients. Whereas concomitant DC activation following administration in an inflammatory microenvironment *in vivo* can be envisaged, our findings support a semimature phenotype of  $1,25(\text{OH})_2\text{D}_3$ -treated DC from both healthy controls and MS patients, as evidenced by impaired upregulation of the expression of costimulatory markers and of the secretion of proinflammatory cytokines following rechallenge with LPS, in agreement with previous observations by others [14, 38]. Nevertheless, the levels of IL-1 $\beta$ , IL-6, and  $\text{TNF-}\alpha$  secreted by mature DC of MS patients are significantly higher as compared to those from healthy controls, even following treatment with  $1,25(\text{OH})_2\text{D}_3$ . Hence, careful safety monitoring will be required when administering  $1,25(\text{OH})_2\text{D}_3$ -treated DC in a clinical setting, for example, for the induction of tolerance in MS patients.

Recently, it was demonstrated that one injection with murine tolDC in EAE resulted in a profound clinical effect [26]. However, the clinical improvements were transient underscoring the possible need for multiple injections with tolerance-inducing cell products if long-lasting regulation of the autoimmune response is aimed for. Therefore, cryopreservation of DC—allowing the generation of ready-to-use aliquots—may facilitate the clinical use of tolDC. In addition, this approach can minimize batch-to-batch variations. Hence, in order to guarantee the comparability of the cell product before and after cryopreservation, the function and phenotype of the DC must be preserved after a freeze-thaw cycle. Previously, others have described and standardized a number of approaches to generate immunogenic DC from cryopreserved monocytes or PBMC [39, 40]. However, the reported effects of cryopreservation on mo-DC differentiation, function, and allogeneic T cell-stimulatory capacity are conflicting [41–43]. In addition, frozen PBMC or monocytes require additional manipulations before a ready-to-use product is achieved which is cost-intensive and labor-intensive and introduces a higher degree of variation in DC characteristics. For this, efforts have been made to cryopreserve DC. Various reports using DC for cancer immunotherapy have demonstrated no differences regarding the morphology, phenotype, and function between cryopreserved and freshly generated DC [44–47]. However, to date, studies addressing the influence of cryopreservation on the characteristics of tolDC are limited.

Here, we report the development and optimization of a cryopreservation protocol which yielded a recovery of 78% and a viability of 75% of immature  $1,25(\text{OH})_2\text{D}_3$ -treated DC

following a freeze-thaw cycle. Previously, other studies investigating the effects of cryopreservation on immunostimulatory DC demonstrated a recovery of 86% on average [44, 47, 48]. In this study, the recovery of tolDC appears to be lower as compared to immunostimulatory DC, albeit not statistically significant. However, since it has been demonstrated that  $1,25(\text{OH})_2\text{D}_3$  can promote spontaneous apoptosis of mature DC *in vitro* [12], this can be attributed to a direct effect of  $1,25(\text{OH})_2\text{D}_3$  and not of the cryopreservation procedure. Overall, our observations underscore the feasibility to cryopreserve tolDC without affecting the viability. Furthermore, no differences regarding the expression of activation markers, including costimulatory molecules, by  $1,25(\text{OH})_2\text{D}_3$ -treated DC could be observed following cryopreservation, indicative of a robust semimature phenotype of  $1,25(\text{OH})_2\text{D}_3$ -treated DC. In contrast, conventional DC display increased levels of CD86 expression and decreased levels of HLA-DR expression as compared to freshly generated mo-DC. Similarly, John et al. have shown that cryopreservation of immature mo-DC resulted in enhanced cell maturation but decreased endocytic activity and efficiency of adenoviral transduction [48]. Importantly, we have demonstrated that  $1,25(\text{OH})_2\text{D}_3$ -treated mo-DC are unable to activate allogeneic T cells as compared to conventional DC, irrespective of their maturation state or cryopreservation. Our study confirms previous findings by Raich-Regué and coworkers [14] demonstrating that  $1,25(\text{OH})_2\text{D}_3$ -treated DC of both healthy individuals and MS patients were able to induce T cell hyporesponsiveness following antigen-specific T cell stimulation. Indeed, following stimulation with  $1,25(\text{OH})_2\text{D}_3$ -treated DC myelin-reactive T cells were unable to respond to myelin-derived antigen rechallenge whereas their ability to respond to an unrelated antigen was maintained, underlining the potential of tolDC to induce hyporesponsiveness in an antigen-specific manner. As clinical translation is aimed for and the need for repetitive injections of tolDC for a prolonged clinical effect in EAE is reported [26], the use of cryopreserved tolDC would highly contribute to the large-scale production and the widely applicable use of tolDC. Recently, the same group reported the *in vivo* clinical efficacy of frozen tolDC in EAE as administration of frozen tolDC was able to abrogate EAE disease progression, mediated by an inhibition of antigen-specific reactivity, the induction of Treg and regulatory B cells (Breg), and the activation of immunoregulatory natural killer T (NKT) cells. Importantly, long-term treatment was well tolerated and exhibited a prolonged clinical beneficial effect [49].

Although the exact mechanism by which tolDC induce T cell hyporesponsiveness remains unclear, several mechanisms by which tolDC can induce tolerance and orchestrate T cell fate have been identified. First, it has been described that tolDC induce tolerance in a “DC-specific” manner through the induction of T cell anergy or apoptosis or deletion of autoreactive T cells. For this, the expression of so-called negative regulatory molecules has been identified to contribute to T cell tolerance. Indeed, Unger et al. observed an increased expression of these inhibitory molecules by  $1,25(\text{OH})_2\text{D}_3$ -treated DC contributing to the induction of T cell anergy [18]. However, in our hands, no pronounced expression of PD-L1 and ILT-3 by  $1,25(\text{OH})_2\text{D}_3$ -treated DC was observed

as compared to conventional DC (Supplementary Figures 1A and B). Furthermore, we demonstrated that 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated DC rendered PBL in a robust hyporesponsive state, even following rechallenge with fully mature conventional DC, thereby excluding tolDC-mediated induction of T cell anergy in agreement with previous reports [14]. Van Halteren and coworkers demonstrated that 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated DC selectively can induce apoptosis in T cells stimulated via the HLA-peptide complex on the DC surface. Importantly, bystander T cells, either resting or activated by peptide-pulsed untreated DC, were unaffected [50]. In contrast, Raïch-Regué et al. ruled out specific apoptosis of autoreactive T cells [14]. In addition, tolDC can also initiate immune tolerance via the induction or expansion of Treg (i.e., “infectious tolerance”). Indeed, several groups have demonstrated that 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated DC are able to induce antigen-specific IL-10-secreting Tr1 cells, capable of suppressing proliferation of responder T cells *in vitro* [18, 51]. Furthermore, it was reported that the induction of Treg required repetitive boosting with 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated DC and was mediated by PD-L1 [18] and/or membrane-bound TNF- $\alpha$  expressed on the tolDC surface [51]. However, we and others [14, 52] could not observe any differences in the frequency of Treg induced by 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated DC. Altogether, further investigation is warranted in order to understand the complex cross talk between 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated DC and T cells.

In conclusion, we deliver proof-of-principle that 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated DC display a semimature phenotype and anti-inflammatory cytokine profile. Importantly, we demonstrate that 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated DC induce antigen-specific T cell hyporesponsiveness to myelin-derived antigens. Furthermore, we report the feasibility of cryopreservation of 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated DC. Since cryopreservation did not affect the viability, phenotype, and the allogeneic T cell-stimulatory capacity of 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated DC, our results contribute to the large-scale production and the widely applicable use of 1,25(OH)<sub>2</sub>D<sub>3</sub>-treated DC. Recent efforts by the European COST (European Cooperation in Science and Technology) network A FACTT (action to focus and accelerate cell-based tolerance-inducing therapies) have resulted in initiatives to harmonize tolDC therapy in a cost-effective and efficient way [53]. We expect that the demonstrated feasibility of the cryopreservation of tolDC in this study is an important step forward in the field of tolDC vaccination and may lay the groundwork for the development of a new form of cellular immunotherapy for MS and other autoimmune diseases.

## Competing Interests

The authors declare no conflict of interests.

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## Research Article

# The Relationship of Cytokines IL-13 and IL-17 with Autoantibodies Profile in Early Rheumatoid Arthritis

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**Aims.** In the present study, we aimed to assess the concentrations of IL-13 and IL-17 in serum of patients with early rheumatoid arthritis (eRA), the investigation of correlation between the concentrations of these cytokines and disease activity score, and the concentration of some autoantibodies and the evaluation of the utility of IL-13 and -17 concentration measurements as markers of disease activity. **Materials and Methods.** Serum samples were collected from 30 patients and from 28 controls and analysed parameters. **Results.** The serum concentrations of IL-13, IL-17, anti-CCP, and IgM-RF were statistically significantly higher in patients with eRA, compared to the controls. IL-13 concentrations in the severe and moderate groups with eRA were statistically higher than in the mild and control groups. Also, in the case of IL-17, serum concentrations increased proportionally with the disease activity of eRA. We observe that concentrations of IL-13 and -17 did not correlate with autoantibodies. IL-17 concentration significantly positively correlated with CRP, while IL-13 concentration significantly negatively correlated with CRP. Disease activity score, DAS28, was strongly positively correlated with levels of ESR and weakly positively correlated with concentrations of anti-RA33 autoantibodies. IL-13 has a higher diagnostic utility than IL-17, CRP, ESR, IgM-RF, and anti-CCP as markers of disease activity. **Conclusions.** The presence of higher IL-13 and IL-17 serum levels in patients, compared with those of controls, confirms that these markers, found with high specificity, might be involved in the pathogenesis of eRA. IL-13 and IL-17 might be of better usefulness in the prediction of eRA activity status than IgM-RF and anti-CCP.

## 1. Background and Aims

Rheumatoid arthritis (RA) is a chronic, progressive, inflammatory autoimmune disease in which the body's immune system mistakenly attacks the joint. The disease produces an inflammatory infiltrate of immune cells as well as a series of destructive events such as synovial hyperplasia, pannus setting, bone and cartilage erosion, and joint destruction. It

results in swelling and pain in the joints and around them [1, 2].

In RA, activation of innate immunity in early disease, followed by the appearance of adaptive immune responses ultimately leads to a destructive phase. The pathophysiology of RA implies the existence of T and B cells, various immune modulators (cytokines and effector cells), and signalling pathways. The complex interaction of immune modulators

causes joint damage starting at the synovial membrane and covering most structures [3].

This disequilibrium between pro- and anti-inflammatory cytokine activities facilitates the induction of autoimmunity, chronic inflammation, and joint damage. It is less known, though, how cytokines are organized within a hierarchical regulatory network and which cytokines may qualify as best targets for clinical intervention a priori [3–5].

RA pathogenesis is caused by B cells not only through antigen presentation, but also through the production of antibodies, autoantibodies, and cytokines [2, 5].

There are no disease-specific diagnostic features in RA and patients can have a wide range of manifestations. The diagnosis of RA is given by a combination of symptoms, signs, serologic tests, and radiologic findings, as established by the American College of Rheumatology [6].

Since early inflammatory arthritis is a clinically heterogeneous disease, cytokine networks are known to play a critical role in the pathogenesis of rheumatoid arthritis; a panel of pro- and anti-inflammatory cytokines and associated autoantibodies were measured to identify the biologically based subsets of early rheumatoid arthritis (eRA) [7].

Emery and Symmons [8] discuss in their article the difficulties of early diagnosis. They said that diagnosis of early rheumatoid arthritis (RA) has inherent difficulties. It requires assessment, not only of the current clinical picture but also of the potential for change.

Therefore, identifying the early rheumatoid arthritis (RA) is a crucial step in controlling the progress of the disease. A major outcome will be achieved through early diagnosis and treatment [9].

Early diagnosis of rheumatoid arthritis (RA) is essential, because there is strong evidence that early treatment with one or more disease-modifying antirheumatic drugs improves the evolution [10, 11]. When DMARDs therapy is introduced early in patients, function and radiological outcome in the long term are better than cases when it is delayed [12].

Our research objectives were to assess the concentrations of IL-13 and IL-17 in serum of patients with eRA, the investigation of correlation between the concentrations of these cytokines and disease activity score and the concentration of some autoantibodies in relation to the control group, and the evaluation of the utility of IL-13 and IL-17 concentration measurements as markers of disease activity.

## 2. Materials and Methods

**2.1. Subjects and Clinical Assessment.** We accomplished a study which included 30 patients diagnosed with early rheumatoid arthritis, gender ratio 6 M/24 F, and mean age 56.22 years; in parallel, we investigated a control group that included 28 persons unaffected by early rheumatoid arthritis or other diseases. Controls were matched for sex, age at the time point of blood sampling, and area of residence (rural or urban).

Early RA patients fulfilled the American College of Rheumatology (ACR) 1987 revised criteria for the classification of RA [13]. They were all investigated, diagnosed, and included

in the studied group, following the revised classification criteria of the American College of Rheumatology, in 2010 [6]. All patients accomplished the inclusion criteria for early rheumatoid arthritis (two or more swollen joints dating more than 2 weeks, but less than 12 months from onset).

We excluded, from the start of the study, patients with other autoimmune diseases, those who received treatment with DMARDs, glucocorticoids, or/and vitamins, the women during pregnancy, and persons with diabetes mellitus or metabolic syndrome. The study cohort comprised patients firstly evaluated for early arthritis because we aimed to investigate eRA patients to find a better and faster way of discrimination between affected and unaffected cases.

Based on the DAS28 results, the 30 eRA patients were subdivided into three groups: mild ( $2.6 < \text{DAS28} \leq 3.2$ ), moderate ( $3.2 < \text{DAS28} \leq 5.1$ ), and severe ( $5.1 < \text{DAS28}$ ).

Serum samples were collected from 30 patients and from 28 controls (healthy persons) and analyzed for concentrations of IL-13 and IL-17, anticyclic citrullinated peptide antibodies (anti-CCP), rheumatoid factor IgM isotype (IgM-RF), anti-cardiolipin IgG isotype (IgG-aCL), anti-RA33, erythrocytes sedimentation rate (ESR), and C-reactive protein (CRP).

**2.2. Samples Collection.** Blood samples were obtained from all subjects into tubes without additives by venous puncture in a fasting state in the morning. Peripheral venous blood was collected into separator vacutainers and allowed to clot for 30 minutes at room temperature. The test tubes were centrifuged at  $3.000 \times g$  for 10 minutes, and serum samples were further divided into aliquots and stored at  $-80^\circ\text{C}$ , until assessment. Before testing, frozen probes were brought to room temperature, avoiding freezing-unfreezing cycles.

**2.3. Immunological Investigations.** The analysis of serum parameters was based on a quantitative sandwich ELISA, according to the manufacturer's instructions. IgG anti-CCP 3.1 and IgG-aCL autoantibodies were determined by ELISA, using Quanta Lite™-INOVA Diagnostics kits, USA (autoantibodies seropositivity was defined as a titer  $>20 \text{ U/L}$ ). The investigation of serum IgM-RF concentrations was achieved using ELISA-AESCU Germany kits (positive  $>15 \text{ U/L}$ ) and of anti-RA33 antibodies using ELISA-kit Human, Wiesbaden, Germany (positive results  $>25 \text{ U/mL}$ ). For hsCRP dosage, DRG ELISA International Inc. USA kit was used (the positive values were  $>10 \text{ mg/L}$ ).

Serum concentrations of IL-13 and IL-17 were measured in patients with early, untreated inflammatory arthritis and in control persons, using ELISA techniques with Invitrogen Corporation kits (Camarillo, CA, USA). The values obtained were expressed in  $\text{pg/mL}$ . In looking for a method of measuring serum cytokine, concentrations were taken into account studies that show good stability for samples stored at  $-70^\circ\text{C}$  until dosage [14]. Most manufacturers recommended avoiding repeated freeze-thaw cycles for serum samples.

All the procedures were followed in accordance with the ethical standards of the institutional responsible committees for human studies and with the Helsinki Declaration of 1975, as revised in 2008.

For realisation of this study, we obtained the approval of the Committee of Ethics and Academic and Scientific Deontology of the University of Medicine and Pharmacy from Craiova number 76/2014.

**2.4. Statistical Analysis.** Patients' data, management system, and data processing were performed using Microsoft Excel and Data Analysis module; statistical analysis was done using *GraphPad Prism 5*. All tests were two-sided and  $p$  values  $\leq 0.05$  were considered significant.

The significance of differences between groups was examined with a Mann-Whitney  $U$  test or Kruskal-Wallis, when multiple comparisons were made. Correlation analysis between the concentration of IL-13 and IL-17 and the degree of disease activity (DAS28), as well as the concentration of some autoantibodies, CRP and ESR, were conducted with a Pearson's test. All tests were two-sided and  $p$  values  $\leq 0.05$  were considered significant.

The diagnostic values of studied markers were evaluated using receiver operating characteristic (ROC) curves analysis. The performance was expressed as the area under the ROC curve (AUC, area under ROC curve) together with 95% confidence interval (95% CI) and  $p$  statistics for the difference between calculated AUC and AUC = 0.5 (weak discriminative marker). Cut-off values corresponding to the highest accuracy were determined and for various threshold values investigated at each marker, we calculated the sensitivity (Sn), specificity (Sp), and Youden index (sensitivity + specificity - 1).

### 3. Results

**3.1. Clinical Characteristics of the Study Subjects.** Among the 30 patients, initially diagnosed with eRA, 80% were female (sex ratio: 24 female/6 male), with age, mean  $\pm$  stdev  $55.77 \pm 10.87$  years. In controls group, incidence for women was 78% and age was  $52.36 \pm 13.38$ . There was no significant difference in age between the two groups (Table 1).

**3.2. Cytokines Concentrations and Disease Activity Stage.** In our study, we found that both IL-13 (18.20 pg/mL, 95% CI: 16.47–19.92) and IL-17 (17.87 pg/mL, 95% CI: 12.99–22.75) concentrations in the serum of patients suffering from eRA were higher than those in the control group (4.80 pg/mL, 95% CI: 3.89–5.71,  $p < 0.0001$ , and 4.20 pg/mL, 95% CI: 3.36–5.05,  $p < 0.0001$ , resp.).

We also found differences in serum concentrations of IL-13 and IL-17 in subgroups of eRA disease patients with different clinical activity stages.

IL-13 concentrations were increasing along with the disease activity (Figure 1). The concentrations of IL-13 in the severe and moderate groups were statistically higher than in the mild and control groups ( $p < 0.05$ ). There were no statistical differences between severe and moderate groups.

Also, in the case of IL-17 serum concentrations increased proportionally with the disease activity of eRA, the highest concentrations were in patients with severe activity disease (Figure 2). Statistically significant differences were observed between both the moderate and the severe groups and a mild

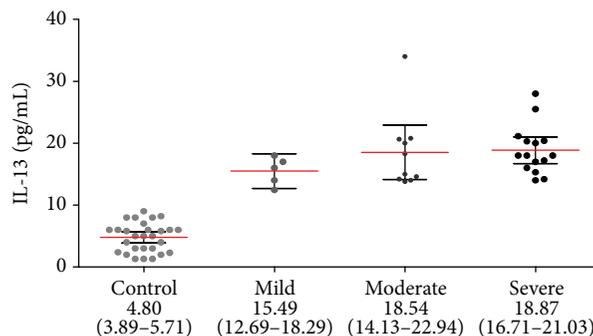


FIGURE 1: IL-13 concentration in serum of eRA disease patients with different clinical activity stages (black circles represent IL-13 concentration, pg/mL in individual serum samples; red lines represent mean values accompanied by 95% confidence interval, represented as black horizontal bars).

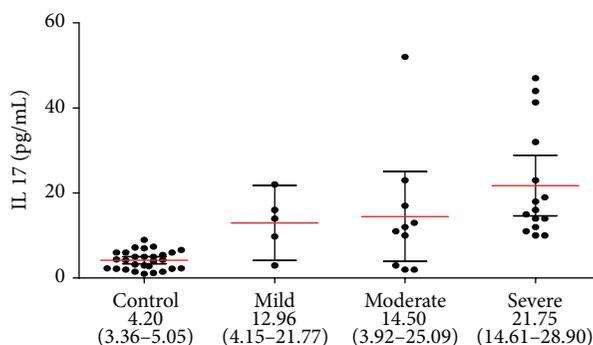


FIGURE 2: IL-17 concentration in serum of eRA disease patient with different clinical activity stages (black circles represent IL-17 concentration, pg/mL in individual serum samples; red lines represent mean values accompanied by 95% confidence interval, represented as black horizontal bars).

group ( $p < 0.05$ ), as well as between the group with moderate disease activity and the control group ( $p < 0.001$ ).

In the studied cohort of patients, we observe statistically significant differences in the concentrations of CRP and the levels of ESR between patients with eRA and the control group (CRP/control group,  $p < 0.0001$ ; ESR/control group,  $p < 0.0001$ ) (Table 1). Analyzing the relationship between serum levels of CRP and ESR and different disease activity stages, we observed only statistically significant differences between severe and moderate group ( $p < 0.0379$ ) (Table 2).

**3.3. Autoantibodies Concentrations and Disease Activity Stage.** Another objective of this study was to investigate autoantibodies profile in eRA. We reproduced in Table 2 concentrations of these autoantibodies investigated.

Following the analysis, our study showed different profiles of IgG anti-CCP and IgM-RF concentrations in serum of patients suffering from eRA in different clinical activity stages. IgG anti-CCP and IgM-RF concentrations were increasing along with the disease activity. In both cases, there were statistically significant differences between severe groups and moderate and mild groups (IgG anti-CCP: severe versus moderate group,  $p = 0.0011$ , severe versus mild group,

TABLE 1: Clinical characteristics of the study subjects.

Character	Controls ( <i>n</i> = 28)	eRA patients ( <i>n</i> = 30)	<i>p</i> value
Age (yrs) (mean ± stdev)	52.36 ± 13.38	55.77 ± 10.87	NS ( <i>p</i> = 0.391)
Gender (male/female)	6/22	6/24	—
DAS28	—	4.80 ± 0.84	—
Mild (2.6 < DAS28 ≤ 3.2) ( <i>n</i> )	—	5	
Moderate (3.2 < DAS28 ≤ 5.1) ( <i>n</i> )	—	10	
Severe (5.1 < DAS28) ( <i>n</i> )	—	15	
CRP (mg/L)	5.01 ± 2.22	16.97 ± 5.14	<i>p</i> < 0.0001
ESR (mm/h)	11.89 ± 6.24	33.60 ± 12.35	<i>p</i> < 0.0001

TABLE 2: CRP, ESR, and autoantibodies (IgG anti-CCP, IgG anti-cL, anti-RA33, and IgM-RF) concentrations in serum of patients with eRA and in the control group.

	Disease activity eRA					
	Severe		Moderate		Mild	
	Mean	95% CI	Mean	95% CI	Mean	95% CI
CRP (mg/L)	19.33	17.34–21.33	14.99	10.81–19.17	13.86	7.50–20.22
ESR (mm/h)	34.53	29.96–39.11	37.30	25.55–49.05	23.40	11.54–35.26
<i>Autoantibodies</i>						
IgG anti-CCP (U/L)	162.20	124.60–199.70	49.76	19.57–79.95	16.20	6.93–25.47
IgG anti-cL (U/L)	13.27	9.61–16.92	16.30	9.90–22.70	11.40	3.18–19.62
Anti-RA33 (U/mL)	15.80	11.15–20.45	19.40	10.91–27.89	12.20	9.96–26.37
IgM-RF (U/L)	99.27	72.28–126.30	33.50	11.33–55.67	26.80	6.27–59.87

TABLE 3: Correlations between IL-13 and IL-17 and eRA indices.

	DAS28	IL-13	IL-17	IgM-RF	Anti-cL	Anti-RA33	Anti-CCP	CRP	ESR
DAS28		<i>r</i> = 0.060 <i>p</i> = 0.753	<i>r</i> = 0.168 <i>p</i> = 0.376	<i>r</i> = 0.140 <i>p</i> = 0.459	<i>r</i> = 0.341 <i>p</i> = 0.065	<i>r</i> = 0.404 <i>p</i> = 0.027*	<i>r</i> = 0.075 <i>p</i> = 0.694	<i>r</i> = 0.051 <i>p</i> = 0.788	<b><i>r</i> = 0.967</b> <b><i>p</i> ≤ 0.0001*</b>
IL-13			<i>r</i> = -0.054 <i>p</i> = 0.775	<i>r</i> = 0.206 <i>p</i> = 0.274	<i>r</i> = -0.082 <i>p</i> = 0.668	<i>r</i> = 0.071 <i>p</i> = 0.711	<i>r</i> = -0.033 <i>p</i> = 0.864	<b><i>r</i> = -0.334</b> <b><i>p</i> = 0.041*</b>	<i>r</i> = 0.019 <i>p</i> = 0.919
IL-17				<i>r</i> = 0.320 <i>p</i> = 0.085	<i>r</i> = -0.049 <i>p</i> = 0.795	<i>r</i> = -0.249 <i>p</i> = 0.184	<i>r</i> = 0.231 <i>p</i> = 0.219	<b><i>r</i> = 0.366</b> <b><i>p</i> = 0.047*</b>	<i>r</i> = 0.197 <i>p</i> = 0.298
IgM-RF					<i>r</i> = -0.320 <i>p</i> = 0.085	<i>r</i> = 0.162 <i>p</i> = 0.391	<i>r</i> = 0.418 <i>p</i> = 0.022*	<i>r</i> = 0.294 <i>p</i> = 0.115	<i>r</i> = 0.071 <i>p</i> = 0.709
Anti-cL						<i>r</i> = -0.173 <i>p</i> = 0.362	<i>r</i> = 0.052 <i>p</i> = 0.784	<i>r</i> = 0.129 <i>p</i> = 0.496	<i>r</i> = 0.274 <i>p</i> = 0.142
Anti-RA33							<i>r</i> = -0.140 <i>p</i> = 0.460	<i>r</i> = -0.066 <i>p</i> = 0.731	<b><i>r</i> = 0.385</b> <b><i>p</i> = 0.035*</b>
Anti-CCP								<b><i>r</i> = 0.371</b> <b><i>p</i> = 0.044*</b>	<i>r</i> = -0.005 <i>p</i> = 0.979
CRP									<i>r</i> = -0.020 <i>p</i> = 0.916

*r*: Pearson correlation coefficient; \* statistically significant correlations.

*p* = 0.0030; IgM-RF: severe versus moderate group, *p* = 0.0014, severe versus mild group, *p* = 0.0039).

3.4. Correlations between IL-13, IL-17, and Indices of eRA. Concentrations of both interleukins are not correlated with each other (Table 3). Also we observe that concentrations of IL-13 and IL-17 are not correlated with autoantibodies. There

was a weak, statistically not significant correlation between IL-17 and IgM-RF (*r* = 0.320, *p* = 0.085).

There was a significant positive correlation between the concentrations of IL-17 and CRP (*r* = 0.366, *p* = 0.047) and a significant negative correlation between the concentrations of IL-13 and CRP (*r* = -0.334, *p* = 0.041).

Disease activity score, DAS28, was strongly positively correlated with levels of ESR (*r* = 0.967, *p* ≤ 0.001)

TABLE 4: Diagnostic performance of the investigated parameters.

Parameter	AUC accuracy	Cut-off value	<i>p</i> value	Sensitivity (%)	Specificity (%)	Youden index
IL-13	1.000	10.73	<0.0001	<b>100.00</b>	<b>100.00</b>	1.000
IL-17	0.902	9.40	<0.0001	<b>86.67</b>	<b>100.00</b>	0.866
<i>Autoantibodies</i>						
IgM-RF	0.981	9.50	<0.0001	<b>94.12</b>	<b>97.06</b>	0.910
Anti-CCP	0.947	12.50	<0.0001	<b>94.44</b>	<b>91.18</b>	0.856
Anti-cL	0.847	7.50	<0.0001	<b>83.33</b>	<b>76.47</b>	0.598
Anti-RA33	0.762	8.50	0.00062	<b>80.00</b>	<b>64.29</b>	0.422
<i>Markers of inflammation</i>						
CRP	0.969	8.70	<0.0001	<b>96.67</b>	<b>100.00</b>	0.966
ESR	0.948	20.50	<0.0001	<b>83.33</b>	<b>92.86</b>	0.762

and weakly positively correlated with concentrations of anti-RA33 autoantibodies ( $r = 0.404$ ,  $p = 0.027$ ).

Concentrations of anti-CCP autoantibodies positively correlated fairly well with CRP and IgM-RF, and concentrations of anti-RA33 positive correlated with levels of ESR.

**3.5. Diagnostic Performance of IL-13 and IL-17 as Disease Markers.** Comparing the ROC curves for the studied parameters in the patients with eRA indicated that IL-13 has a higher diagnostic utility than IL-17, CRP, ESR, IgM-RF, and anti-CCP as markers of disease activity (Table 4).

ROC analysis revealed that IL-13 concentration indicated eRA presence with 100% accuracy using the concentration of 10.73 pg/mL as an optimal cut-off value for discrimination between patients with eRA and controls (95% CI: 0.962–1.000,  $p < 0.0001$ ). The likelihood ratios of positive and negative results obtained on the basis of optimal threshold values specific for eRA were as follows: LR(+) = 28.00 and LR(–) = 1.12 with sensitivity and specificity equal to 100 and 100%, respectively; Youden index was 1.00 (Figure 3).

In case of IL-17, the calculated cut-off value for discrimination between patients with eRA and controls was 9.40 pg/mL and using this value the diagnostic accuracy of IL-17 was 90.2% (95% CI: 0.809–0.995,  $p < 0.0001$ ). The likelihood ratios of positive and negative results obtained on the basis of optimal threshold values specific for eRA were as follows: LR(+) = 24.27 and LR(–) = 1.14 with sensitivity and specificity equal to 86.67 and 100%, respectively; Youden index was 0.866.

In the studied cohort of patients, CRP and ESR have discriminative power towards diagnosis of eRA (sensitivities for both CRP and ESR were found to be lower in comparison to the IL-13; diagnostic accuracy of CRP was 96.9 and 94.8%, resp., for ESR).

We also noticed that although they have a specificity less than IL-13 and CRP, autoantibodies IgM-RF and anti-CCP have a good tendency to discriminate patients with eRA from healthy ones (diagnostic accuracy 98.1 and 94.7%, resp.).

#### 4. Discussions

RA is an inflammatory autoimmune disease characterized by systemic and articular effects. Chronic inflammatory and

autoimmune diseases are the result of an interplay between genetic factors and environmental ones that culminate in the phenotypes of the established disease. Owing to the prevalence and accessibility of joint samples for laboratory investigation, RA has been a suitable model for the study of numerous inflammatory and immune-mediated conditions [15].

The formulation of a definition for early RA was difficult, but the majority of the rheumatologists use the term “early” for symptoms shorter than three months. There was a tendency to accept the involvement of fewer affected joints [9, 16].

Changing from health to established disease in RA is generally clearly understood. Early rheumatoid arthritis (RA) and very early RA are major targets of research and clinical practice [15, 17].

We found a predominance (80%) of women in patients affected by eRA, finding which are congruent with the results of other studies having identified a female predominance in RA [9, 15, 18].

Both pro- and anti-inflammatory cytokines were found elevated in RA patients over controls claiming opinion that cytokine networks play critical rolls in the pathogenesis of rheumatoid arthritis [4, 7, 19–28].

The present study reveals that levels of serum IL-13 and IL-17 cytokines were significantly higher in eRA patients than in age- and sex-matched healthy persons. Our results are related to the cell-mediated immune response intervention in disease onset.

A broad range of inflammatory processes that are involved in the pathophysiology of rheumatoid arthritis are regulated by cytokines. The imbalance between pro- and anti-inflammatory cytokine activities favours the induction of autoimmunity, chronic inflammation, and thereby joint damage [5, 27].

Synovial inflammation is regulated by cytokines. Some cytokines, such as tumour necrosis factor- (TNF-) alpha, IL-17, and (IL)-1, function by promoting inflammatory responses and by inducing cartilage degradation. Others such as IL-4, IL-10, and IL-13 are mainly anti-inflammatory molecules [18, 20, 25].

Even if present in rheumatoid joints, in progressive RA anti-inflammatory cytokine levels are too low to neutralize

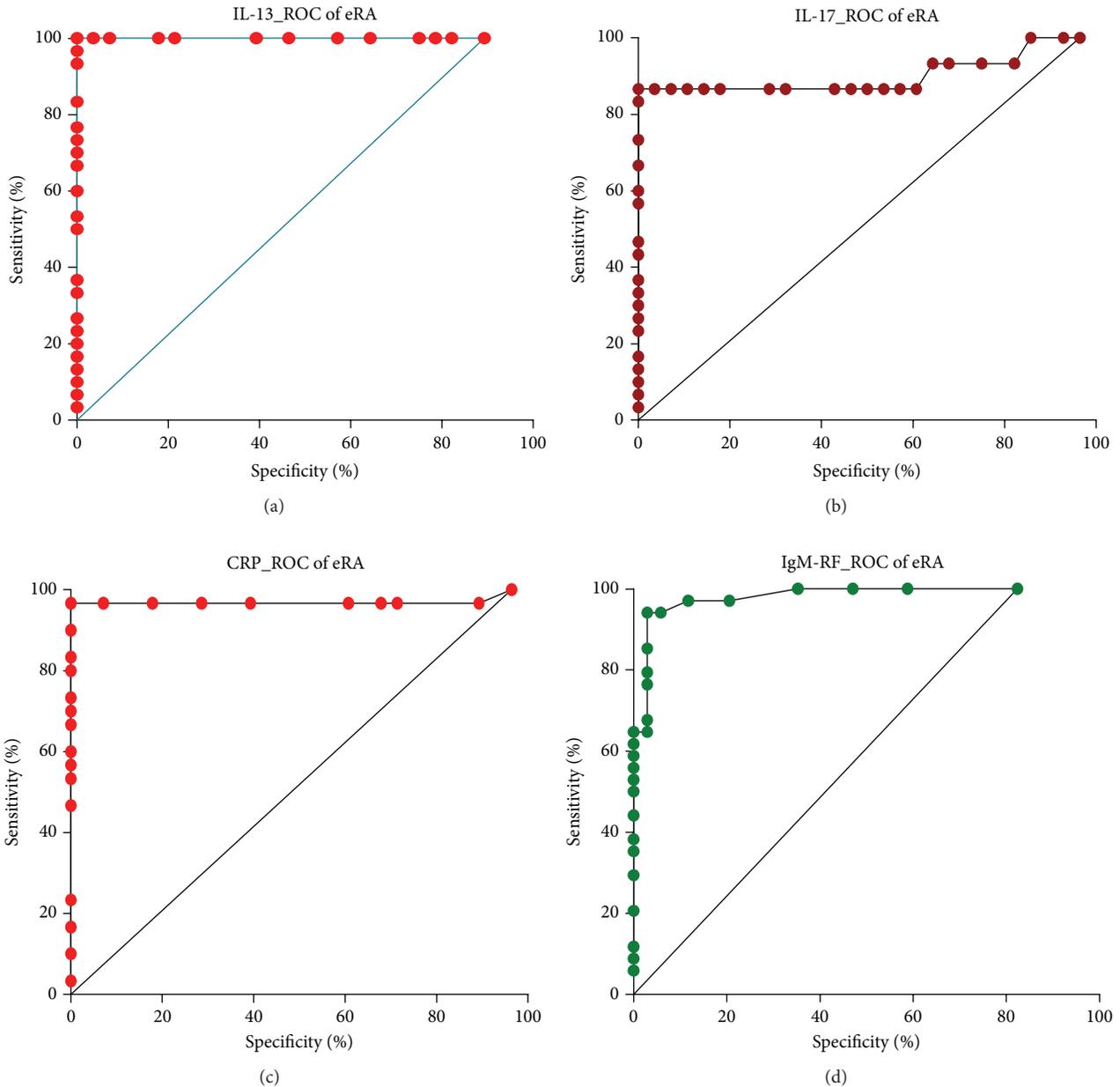


FIGURE 3: Comparison of ROC curves for IL-13 (a), IL-17 (b), CRP (c), and IgM-RF (d).

the deleterious effects of proinflammatory cytokines. The suppression of both the secretion and action of IL-17 by IL-13 is of potential clinical importance [19–22].

IL-13 is a protein, secreted by activated T cells, that modulates B cell function *in vitro* and plays an important part in their proliferation and differentiation; the high local IL-13 levels were observed in patients with RA, correlated with B lymphocyte proliferation [23]. Interleukin 13 induces interleukin-4-independent IgG4 and IgE synthesis and CD23 expression by human B cells [24].

Some researchers support a role for IL-13 as an *in vivo* antiangiogenic factor and provide a rationale for its use in RA to control pathologic neovascularization [25].

Treatment with Th2 cytokines (IL-4, IL-10, and IL-13) was tested in many animal models of arthritis based on the Th1 bias of T cells, showing considerable promise [26].

In patients suffering from RA, disruptions in self-tolerance lead to abnormalities such as recognition of citrullinated antigens by T and B cells. The proportion of lymphocyte differentiation in RA is skewed towards the Th1 phenotype, to the detriment of the Th2, Th17, and T regulator (Treg) ones. Imbalances appear in the main cytokine systems including IL-1, TNF, IL-6, IL-18, IL-15, IL-33, IL-22, and IL-13. However, the destruction of the joint in RA is caused not only by these cytokine imbalances but also by matrix production dysregulation responsible for cartilage damage.

IL-17 levels fall after administration of anti-inflammatory cytokines such as IL-4 or IL-13 [27].

IL-13 levels were significantly higher in patients with early RA ( $p < 0.001$ ) than in reference group, suggesting the different pathogenic mechanisms involved in joint inflammation. Serum IL-13 values increased in RA have been reported in many works [20, 22, 28].

Lower interleukin 13 levels were communicated in patients with arthritis by some investigators as Barra and contributors [29] and Woods et al. [30].

Spadaro et al. [28] assume that the production of RF and antinuclear antibodies by B cells could depend on different cytokines action. In their study, IL-13 serum levels correlated with those of RF in RA patients and they suggested that IL-13 may be involved in the pathogenesis of autoimmune rheumatic diseases, with a relevant role on RF production. McKenzie et al. [31] emphasize the involvement of IL-13 in regulating human monocyte and B cell function.

We found that detection of IL-13 in eRA patients was not affected by rheumatoid factor IgM ( $r = 0.206$ ,  $p = 0.274$ ), a fact revealed by other researchers too [32].

Interleukin 13 inhibits the production of proinflammatory cytokines, chemokines, and hematopoietic growth factors by activated human monocytes [30]. The increase of biologically active IL-13 in RA supports the hypothesis that IL-13 regulates immune cell (including dendritic cell) activity and indicates how the varied anatomical distribution of cytokines may play a role in the RA disease process. The differential regulation of circulating IL-13 and M-CSF levels by TNF antagonists further implies discrete roles in the TNF-cytokine network in RA [33, 34].

Isomäki et al. [34] showed that IL-13 was present in 27 out of 28 serum samples from patients with RA, indicating that this cytokine is constantly present in rheumatoid joints.

Raza et al. [35] detected increased levels of the Th2 cytokines IL-4 and IL-13 in synovial samples from early RA patients.

IL-13 causes B cell proliferation and differentiation, including IgE production, and the expression of certain adhesion molecules on endothelial cells. All these biological properties of IL-13 are shared with IL-4, but in contrast to IL-4, IL-13 does not act on T cells [36].

Besides the increased values of IL-13, we detected circulating IL-17 levels significantly higher in patients with eRA ( $p < 0.001$ ) when compared to those in the reference group.

IL-17 concentrations were associated in ten patients with seropositivity for IgM-RF. Strong correlations of serum IL-17A levels with anti-CCP were found by Roşu et al. [18].

Th17 with decreased circulating levels in eRA seems to be a marker of anti-CCP seropositivity [37]. Considering the complexity and heterogeneous nature of RA, it is unlikely that only cytokines investigation may provide sufficient discrimination; predicting the eRA is better with a combination of biomarkers [38].

IL-17 has several sources: Th17 cells, which are a subset of CD4+helper T cells, mast cells, NK cells, and  $\gamma\delta$  T cells; all of them contributing to the pathogenesis of inflammatory arthritis [39, 40].

In a recent study comparing individuals before the onset of symptoms (defined as prepatients) and after the onset of RA with matched control subjects, Kokkonen et al. [41] showed that IL-17 was present at its highest concentrations in prepatients, and the level had decreased within 7.7 months following the onset of disease.

IL-17A was detected at higher levels in early disease compared with late, established disease [42]. Roşu et al. [18] reported IL-17A levels significantly higher in synovial fluid (SF) and serum from eRA patients compared to osteoarthritis (OA). In other previous studies, there were higher serum and SF IL-17A levels in RA patients as compared to healthy controls, which suggests that the cytokine is mainly produced locally in the inflamed joint [43].

Other results sustain that despite the significant increases in Th17 and IL-17 CD4+ T cells in the blood of RA patients, these did not correlate with ESR, CRP, or DAS28, suspecting that the presence of IL-17 producing CD4+ T cells in the blood from patients with established RA is of limited use as a biomarker to indicate disease activity [44]. In our study, there were significant correlations ( $r = 0.366$ ,  $p = 0.047$ ) found between IL-17 and CRP serum levels, but not with ESR or disease activity score, supporting partially arguments of these investigators.

In another work, Leipe et al. [45] demonstrated that Th17 cells play an important role in inflammation in human autoimmune arthritides, both at the onset and in established disease. They claim that the levels of IL-17 are connected to the systemic disease activity at both the onset and the progression of RA.

Taken together, these data suggest that IL-17 may be a key activator of T cell-driven inflammation and thus may contribute to the pathogenesis of RA [46, 47].

It can be asserted that IL-17 represents a member of the proinflammatory cytokine family produced by RA synovium and inhibited by some Th2 cytokines. In this way, IL-17 contributes to the active, proinflammatory pattern characteristic to RA, whose production and function are regulated by IL-4 and IL-13.

Reduction of synovial inflammation may be protective through a direct effect on IL-17, inducing proinflammatory effects. Thus, IL-17 appears to represent a target for treatments of RA [48, 49].

Overexpression of IL-17 has been shown to be associated with a number of pathological conditions. Since IL-17 was found at high levels in the synovial fluid around the affected cartilage in patients with inflammatory arthritis, it is assumed that this determines the direct effect on articular cartilage. IL-17 functions as a direct and potent inducer of matrix breakdown and an inhibitor of matrix synthesis in articular cartilage explants. Such findings have important implications for the treatment of degenerative joint diseases from arthritis [50].

It was observed that individuals in whom RA later developed had significantly increased concentrations of several cytokines, closer to the onset of symptoms, and that there exists a relationship between Th1, Th2, Treg, and Th17-cytokines and the presence of anti-CCP antibodies. Sensitivity, though, was not increased by the combination

of anti-CCP antibodies and these cytokines [18, 41, 51, 52].

Because we know the statistical limits of the present study, the relatively small number of patients, we propose in the future longitudinal studies with regular serum analysis to determine more precise roles of IL-17, IL-13, and autoantibodies in RA pathogenesis.

## 5. Conclusions

With IL-13 and IL-17 serum concentrations increasing proportionally with the disease activity of eRA, the highest concentrations were in patients with severe activity disease. Based on the results of this study, we can conclude that the presence of higher IL-13, IL-17, IgM-RF, and anti-CCP serum levels in patients, compared to those of controls, confirms that these markers, found with high specificity, might be involved in the pathogenesis of eRA. IL-13 and IL-17 might be of better usefulness in the prediction of eRA activity status than IgM-RF and anti-CCP. Investigation of the association between cytokine profile and autoantibodies status may lead to prognostic and treatment decisions in eRA patients. The basis for the new therapies in patients with RA is represented by inhibiting the action of proinflammatory cytokines by using specific cytokine inhibitors or anti-inflammatory cytokines. The combination of IL-17 and anti-CCP autoantibodies may have the potential as biomarkers in early RA, especially for their clinical utility.

## Competing Interests

The authors report no conflict of interests regarding this study.

## Authors' Contributions

All authors participated equally in this work.

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## Research Article

# PD-1/PD-L1 Interaction Maintains Allogeneic Immune Tolerance Induced by Administration of Ultraviolet B-Irradiated Immature Dendritic Cells

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Our previous study demonstrated that transfusion of ultraviolet B-irradiated immature dendritic cells (UVB-iDCs) induced alloantigen-specific tolerance between two different strains of mice. Programmed death-1 (PD-1) and programmed death ligand-1 (PD-L1) have been suggested to play an important role in maintaining immune tolerance. In the present study, we seek to address whether PD-1/PD-L1 plays a role in the maintenance of UVB-iDC-induced tolerance. We first observe that the UVB-iDC-induced alloantigen-specific tolerance can be maintained for over 6 weeks. Supporting this, at 6 weeks after tolerance induction completion, alloantigen-specific tolerance is still able to be transferred to syngeneic naïve mice through adoptive transfer of CD4<sup>+</sup> T cells. Furthermore, skin transplantation study shows that the survival of allogeneic grafts is prolonged in those tolerant recipients. Further studies show that PD-1/PD-L1 interaction is essential for maintaining the induced tolerance as blockade of PD-1/PD-L1 by anti-PD-L1 antibodies largely breaks the tolerance at both cellular and humoral immunological levels. Importantly, we show that PD-1/PD-L1 interaction in tolerant mice is also essential for controlling alloantigen-responding T cells, which have never experienced alloantigens. The above findings suggest that PD-1/PD-L1 plays a crucial role in maintaining immune tolerance induced by UVB-iDCs, as well as in actively controlling effector T cells specific to alloantigens.

## 1. Introduction

The major obstacle of allogeneic transplantation is the allograft rejection due to mismatched major histocompatibility complex (MHC) antigens [1, 2]. Induction of immune tolerance across MHC barrier is an ideal approach for preventing allograft rejection. It has been demonstrated that steady-state cell apoptosis during self-renewal plays an important role in maintaining immune tolerance to self-antigens [3, 4]. In line with this, we have successfully induced immune tolerance to alloantigens between two different mouse strains through injection of ultraviolet B- (UVB-) irradiated immature dendritic cells (UVB-iDCs) and infusion of iDCs without UVB irradiation mounts potent immune response to alloantigens [5, 6]. Using this approach, we were able to significantly prevent graft-versus-host disease in a mouse model of allogeneic

hematopoietic stem cell transplantation [5]. However, how this UVB-iDC-induced tolerance is maintained remains to be determined.

The interaction of programmed death-1 (PD-1) and its ligand (PD-L1) has been proposed to be involved in the modulation of both central and peripheral tolerance [7]. Studies showed that PD-1/PD-L1 interaction was required for both induction and maintenance of T cell tolerance [8–10]. In an alloantigen tolerance induction model, it was shown that PD-1/PD-L1 plays an important role in maintaining long-term allogeneic tolerance induced by infusion of ethylene carbodiimide-fixed allogeneic splenocytes [11]. In our previous study, we demonstrated a significantly prolonged survival in the recipients receiving bone marrow and spleen cells from donor mice tolerant to alloantigens induced by infusion of UVB-iDCs in an allogeneic hematopoietic stem cell

transplantation mouse model [5], suggesting that UVB-iDC-induced immune tolerance to allogeneic MHC antigens could be long lasting. In this study, we firstly addressed whether UVB-iDCs treatment-induced alloantigen tolerance could be maintained after induction. Secondly, we addressed whether PD-1/PD-L1 played a role in maintaining this tolerance. The results are reported herein.

## 2. Materials and Method

**2.1. Mice.** 8–10-week-old Balb/c (H-2d) and C3H (H-2k) were purchased from Charles River Animal facility (Beijing, China) and housed in the Animal Care facility at Xuanwu Hospital, Capital Medical University, Beijing. All mice were used following the Chinese governmental and Capital Medical University guidelines for animal welfare. This study was approved by the Capital Medical University Animal Ethics Committee. All mice used in this study were euthanized in a CO<sub>2</sub> chamber with a CO<sub>2</sub> meter connected to it to control CO<sub>2</sub> flow as 1.5 L/min.

**2.2. Dendritic Cell Culture and Preparation.** Balb/c bone marrow derived immature dendritic cells (BM-iDCs) were cultured and irradiated by ultraviolet B (UVB) (1200 mJ/cm<sup>2</sup>) following the protocol we reported previously [5, 6]. After being irradiated, iDCs would initiate the process of apoptosis because of the DNA crosslinking induced by UVB irradiation. In our observation, 60–70% of UVB-irradiated iDCs underwent apoptosis 8 h after irradiation and almost all cells died 24 h after irradiation. Therefore, to avoid infusion of apoptotic cells at different stages of apoptosis, the irradiated iDCs were either injected immediately after irradiation or put on ice to stop biological activities of the cells and injected within 2 h.

**2.3. Alloantigen Tolerance Induction and Adoptive CD4<sup>+</sup> T Cells Transfer.** As previously reported [5, 6], C3H male mice received intravenous injection of UVB-irradiated Balb/c iDCs ( $2 \times 10^5$  cells/mouse) or PBS once a week for 4 weeks and then plasma anti-donor antibodies were measured by flow cytometry (FCM, Canto, BD) following the protocol described previously [6] to confirm that the tolerance was successfully induced. Six weeks later, we challenged the mice with intravenous injection of  $2 \times 10^5$ /mouse Balb/c spleen cells once a week for two weeks. Thereafter, we measured the anti-donor antibody levels using the method we reported previously [5, 6] with mild modifications. In brief, the anti-donor antibody assay was a flow cytometric analysis of plasma levels of antibodies against donor cells. Donor spleen cells ( $1 \times 10^6$ ) in 60  $\mu$ L of PBS were incubated with 40  $\mu$ L of plasma collected from mice in different experimental groups as indicated and anti-CD4-PerCP antibodies at room temperature for 30 min. Then, the cells were washed twice with PBS and resuspended in 100  $\mu$ L PBS and incubated with secondary antibody, anti-mouse IgG-FITC at room temperature for 30 min. The data were analyzed by flow cytometry. Samples with anti-mouse IgG-FITC staining positive were considered positive for anti-donor antibodies. Using CD4 T cell staining to represent spleen cells was for the purpose of eliminating

the false positivity caused by Fc binding of mouse plasma IgG in antigen-presenting cells, mainly B cells.

For CD4<sup>+</sup> T cell transfer studies, CD4<sup>+</sup> T cells from C3H mice from two different groups used for the above experiments were prepared by negative selection with CD4<sup>+</sup> T cell isolation kit (EasySep kit) according to the protocol from the manufacturer (StemCell Biotech). The purity of CD4<sup>+</sup> T cells was around 95%.  $5 \times 10^6$  purified CD4<sup>+</sup> T cells were adoptively transferred to naïve C3H mice via tail vein injection. After the injection, all recipient C3H mice were challenged with two weekly transfusions of  $2 \times 10^5$  Balb/c spleen cells. One week after the second challenge, plasma samples were prepared and assayed for antibodies against Balb/c WBCs. In the above experiments, the mice were monitored twice a week until the endpoint of the experiments and then euthanized as described in Section 2.1.

**2.4. In Vivo Alloantigen-Specific Immune Response Assay and Anti-PD-L1 Antibody Treatment.** Tolerant C3H mice received Balb/c spleen cells ( $1.5 \times 10^7$ /mouse) prelabeled with carboxyfluorescein diacetate succinimidyl ester (CFSE, from Invitrogen). In the meantime, 100  $\mu$ g of purified anti-mouse PD-L1 mAb (Biolegend, Clone 10F.9G2) or control IgG (Rat IgG, Sigma) was injected intraperitoneally (i.p.) into these mice, respectively. Nontolerant naïve C3H mice receiving injection of CFSE-labeled Balb/c spleen cells served as an additional control group. Twenty-four hours later, peripheral blood mononuclear cells (PBMNCs) of all recipient mice were prepared and stained by anti-CD4-PerCP antibody (BD BioScience, clone RM4-5). CFSE+CD4<sup>+</sup> T cells were measured by flow cytometry. The percentage of CFSE+CD4<sup>+</sup> T cells in total CD4<sup>+</sup> T cells served as a readout to demonstrate the acceptance of donor cells.

For examining the maintenance of humoral immune tolerance, the tolerant recipient C3H mice 6 weeks after tolerance induction received intravenous injection of Balb/c spleen cells ( $2 \times 10^5$ /mouse) along with injection of anti-mouse PD-L1 mAb (100  $\mu$ g) or control IgG (100  $\mu$ g), once a week for two weeks. Thereafter, plasma samples were prepared and assayed for antibodies against Balb/c WBCs as described above.

In assessing how PD-1/PD-L1 interaction in tolerant C3H mice affected alloantigen-responding T cells from naïve C3H mice, Balb/c spleen cells ( $1 \times 10^7$ /mouse) (as alloantigens) and CFSE-labeled naïve C3H spleen cells ( $1 \times 10^7$ /mouse) were injected intravenously (i.v.) into tolerant mice with anti-mouse PD-L1 mAb (100  $\mu$ g/mouse) or control IgG (100  $\mu$ g/mouse) at day 1 and day 3. Naïve C3H mice receiving injection of both types of cells served as an additional control. On day 4, the recipient mice were sacrificed and inguinal lymph node cells were prepared and stained with anti-CD4 and anti-CD8 fluorescent antibodies, and CFSE-labeled CD4<sup>+</sup> and CD8<sup>+</sup> T cell proliferation was examined by flow cytometry and analyzed using FCS express software (De Novo software, Vancouver, Canada).

**2.5. Mouse Skin Transplantation.** Recipient C3H mice were prepared with four weekly infusions of UVB-iDCs from Balb/c mice or PBS as described above. To assess the

maintenance of tolerance, we performed skin transplantation 6 weeks after finishing tolerance induction. Syngeneic skin transplantation in Balb/c mice was also performed to ensure the success of our surgical procedure. The surgical procedure is as follows: donor skin grafts were from the ears of Balb/c mice. The donor ears were surgically removed and placed in PBS on ice and then split and the dorsal flap was retained for transplant. C3H allogeneic and Balb/c syngeneic recipient mice were anesthetized and placed in lateral position. The transplant area was wetted with 75% alcohol; then the hairs were shaved and the operation area was cleaned with alcohol swab. In the area of skin preparation, 1 cm diameter circular flap was surgically removed and placed on the preprepared graft bed. Donor skin graft was carefully trimmed to fit the graft bed. The recipient mice were wrapped by a sterile bandage around the body to completely cover the surgical area and placed in a clean cage. All recipient animals were monitored on a daily basis. At day 6 after transplant, the bandage was removed and the graft was examined for the success of surgery and graft survival. The recipients were continued to be monitored daily for signs of rejection. When a graft showed signs of scabbing or contraction, skin graft rejection was considered. All mice in these experiments were euthanized using CO<sub>2</sub> as described in Section 2.1 at the endpoint of the experiment.

### 3. Result

**3.1. Infusion of UVB-iDCs to Allogeneic Recipients Induces Long-Lasting Alloantigen-Specific Tolerance, Which Is Transferable through Adoptive Transfer of CD4<sup>+</sup> T Cells.** Our previous study showed that four weekly transfusions of UVB-iDCs to allogeneic recipient mice induced tolerance to alloantigens [6]. In our allogeneic hematopoietic stem cell transplantation model, it appeared that this tolerance was maintained in the recipient mice 3 months after transplantation [5]. In these experiments, we sought to determine whether this tolerance could be maintained for a period of time after tolerance induction. We followed the same protocol reported previously [6], but, instead of performing assessment 1 week after the last treatment, we evaluated tolerance 6 weeks after finishing tolerance induction through alloantigen challenge by intravenous injection of allogeneic Balb/c spleen cells. We found that C3H mice tolerized by Balb/c UVB-iDC treatment remained tolerant to alloantigen challenge (Figures 1(a), 1(b), and 1(c)), whereas nontolerant C3H mice pretreated by injection of PBS developed high-level anti-Balb/c antibodies in response to Balb/c alloantigen challenge (Figures 1(a), 1(b), and 1(c)). Similar to the results reported previously that CD4<sup>+</sup> T cells from the tolerant mice could transfer tolerance [5], we found that this long-lasting immune tolerance could also be transferred to syngeneic naïve C3H mice through adoptive transfer of CD4<sup>+</sup> T cells from the tolerant mice, demonstrating that CD4<sup>+</sup> T cells from tolerant mice markedly suppressed alloantigen-induced anti-donor antibody development compared to CD4<sup>+</sup> T cells transferred from immunized mice (Figures 2(a), 2(b), and 2(c)). The above findings suggest that UVB-iDCs infusion-induced alloantigen immune tolerance can be maintained for

long term, and CD4<sup>+</sup> T cells play an important role for the maintenance of alloantigen tolerance.

**3.2. Skin Graft Survival in Tolerant Mice Is Significantly Prolonged.** To further address the strength and maintenance of alloantigen-specific tolerance induced by UVB-iDC treatment, we performed allogeneic skin transplantation in tolerant and PBS-treated C3H mice 6 weeks after tolerance induction. The results shown in Figures 3(a) and 3(b) demonstrated that skin graft survival was significantly prolonged in tolerant mice compared to nontolerant mice. The skin grafts were eventually rejected from the tolerant recipients (Figure 3(b)), suggesting that the tolerance was not sufficient in sustaining allogeneic skin graft survival in this most difficult organ transplantation model. As expected, syngeneic skin grafts were all survived (Figure 3), indicating no technical difficulty concurring in our skin transplantation experiment.

**3.3. PD-1/PD-L1 Blockade Breaks the Allogeneic Immune Tolerance Induced by Infusion of UVB-iDCs.** To determine whether PD-1/PD-L1 pathway plays a role in UVB-iDC-induced allogeneic tolerance, we employed anti-mouse PD-L1 antibodies to block the PD-1 signaling in the mice with long-lasting tolerance during alloantigen challenge. We intravenously injected fluorescent dye, CFSE-labeled Balb/c spleen cells into C3H mice tolerant to Balb/c alloantigens, with treatment of anti-PD-L1 antibody, or control IgG. Nontolerant naïve C3H mice receiving injection of CFSE-labeled Balb/c spleen cells served as an additional control. In nontolerant control mice, all CFSE-labeled cells were rejected within 24 h after injection (Figures 4(a) and 4(b)). Fair numbers of CFSE-labeled cells remained in the tolerant recipient mice receiving control IgG treatment, whereas these numbers were significantly reduced upon PD-1/PD-L1 blockade by the treatment of anti-PD-L1 antibodies with a complete rejection in one of three mice (Figures 4(a) and 4(b)). Further, we assessed how PD-1/PD-L1 blockade affected anti-alloantigen antibody development upon alloantigen challenge. We showed that tolerant mice receiving control IgG treatment failed to develop any levels of anti-alloantigen antibodies upon alloantigen challenge (Figures 5(a) and 5(b)). However, tolerant mice receiving anti-PD-L1 treatment developed relatively high levels of anti-alloantigen antibodies, which nonetheless were still lower than nontolerant mice challenged with alloantigens based on mean fluorescent intensity (MFI) (Figure 5(c)). This result is consistent with the result shown above that there were still certain numbers of CFSE-labeled cells remaining in some of tolerant mice receiving anti-PD-L1 treatment (Figures 4(a) and 4(b)). These findings suggest that PD-1/PD-L1 plays an important role in maintaining alloantigen tolerance in the tolerant mice.

**3.4. PD-1/PD-L1 Interaction Is Required to Prevent Naïve Alloantigen-Responding T Cells from Being Activated in the Tolerant Mice.** The results shown above suggest that tolerant T or B cells can be reinvigorated by alloantigen stimulation under PD-1/PD-L1 blockade. It is of interest to learn whether PD-1/PD-L1 interaction is essential in tolerant mice for

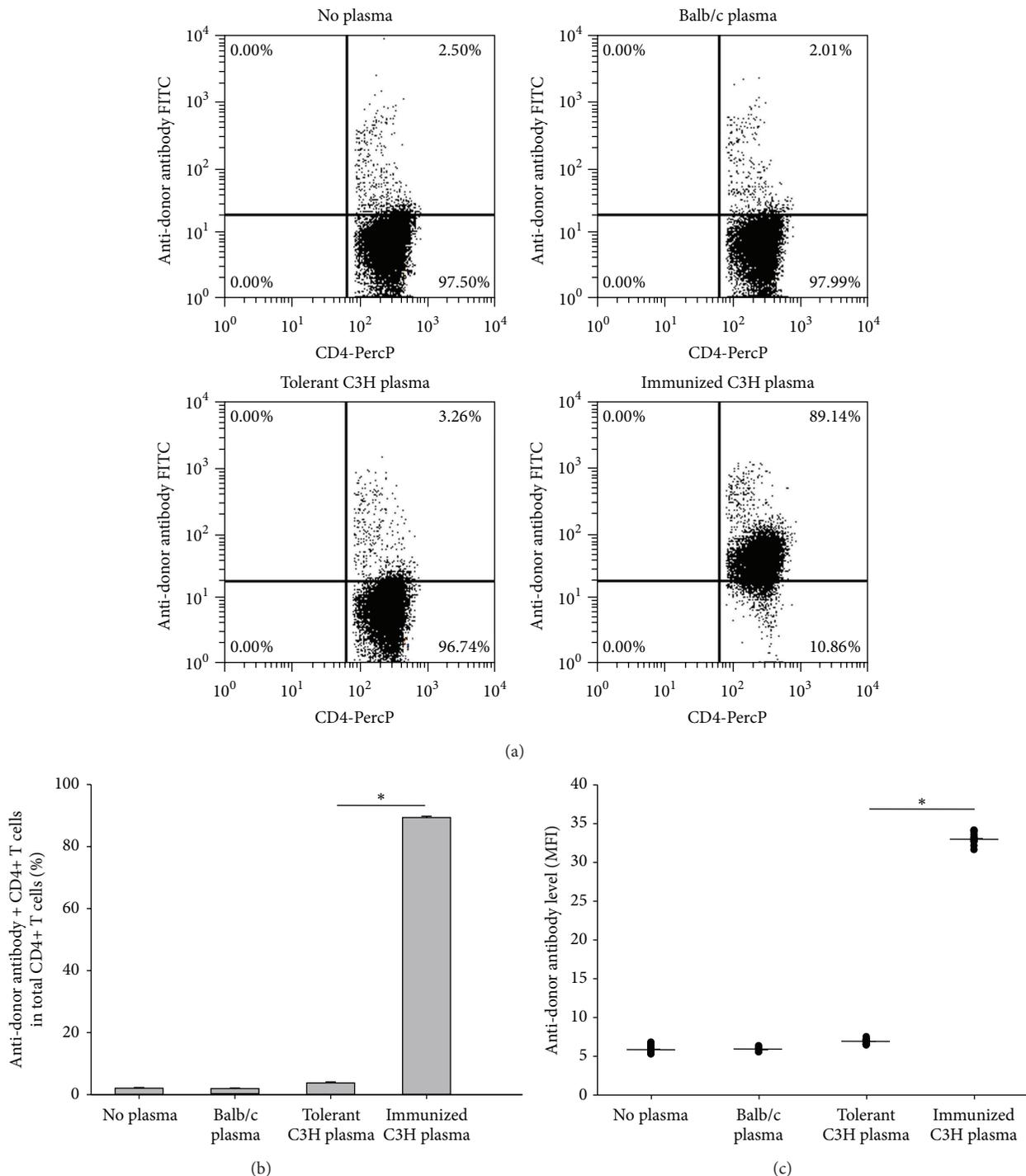


FIGURE 1: Alloantigen-specific tolerance induced by infusion of UVB-iDCs can be maintained for long term. (a) Recipient C3H mice (H2k) were treated with 4 weekly intravenous injections of donor Balb/c (H2d) UVB-iDCs or PBS (10 mice/group). Six weeks later, all mice were challenged by infusion of Balb/c spleen cells ( $2 \times 10^5$ /mouse) once a week for two weeks. One week after the second challenge, plasma levels of anti-donor antibodies were examined. Naïve Balb/c mouse plasma samples were utilized for testing the background binding. Balb/c spleen cells with plasma added were blank controls. ((b) and (c)) The summary of anti-donor antibodies levels in both percentage and mean fluorescent intensity (MFI) is depicted, respectively. Data represent mean  $\pm$  standard error. \*  $P < 0.05$ . The  $P$  values were calculated using Student's  $t$ -test.

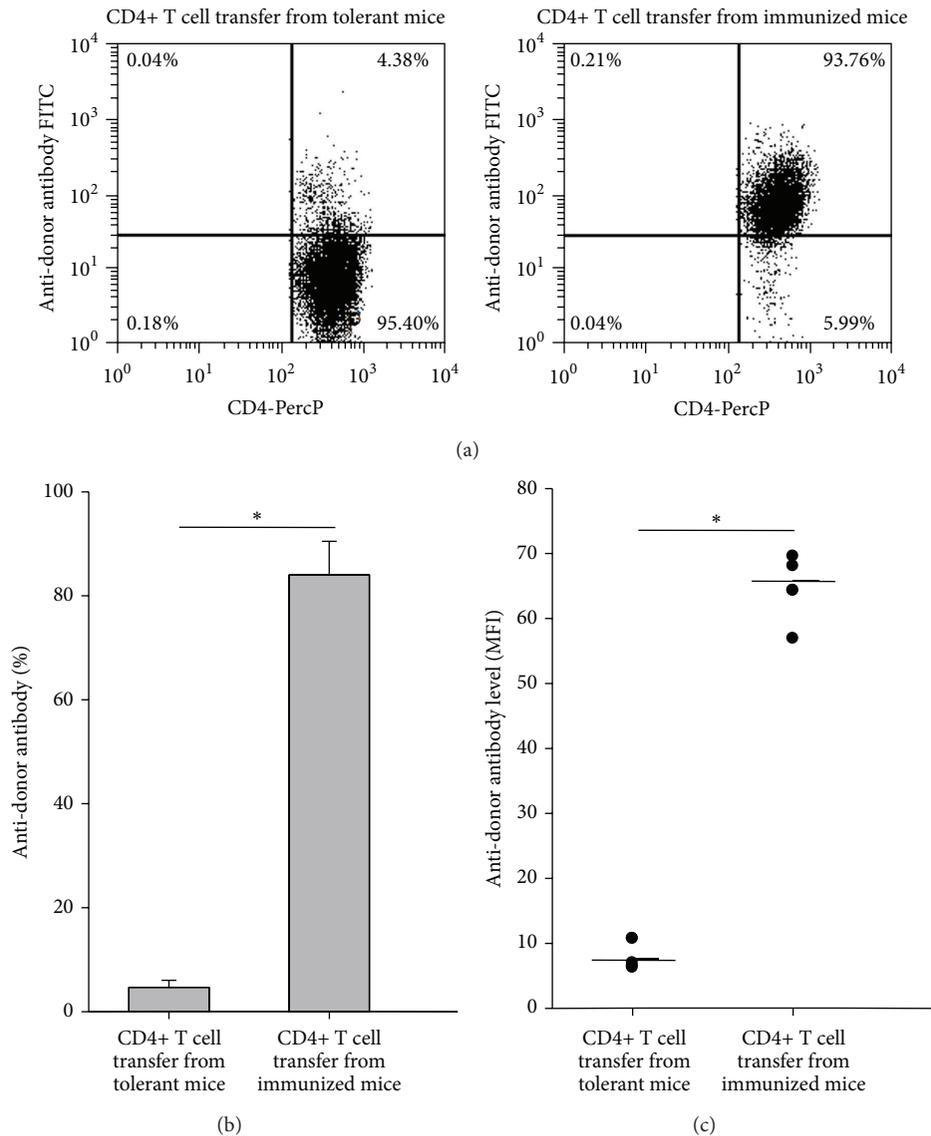
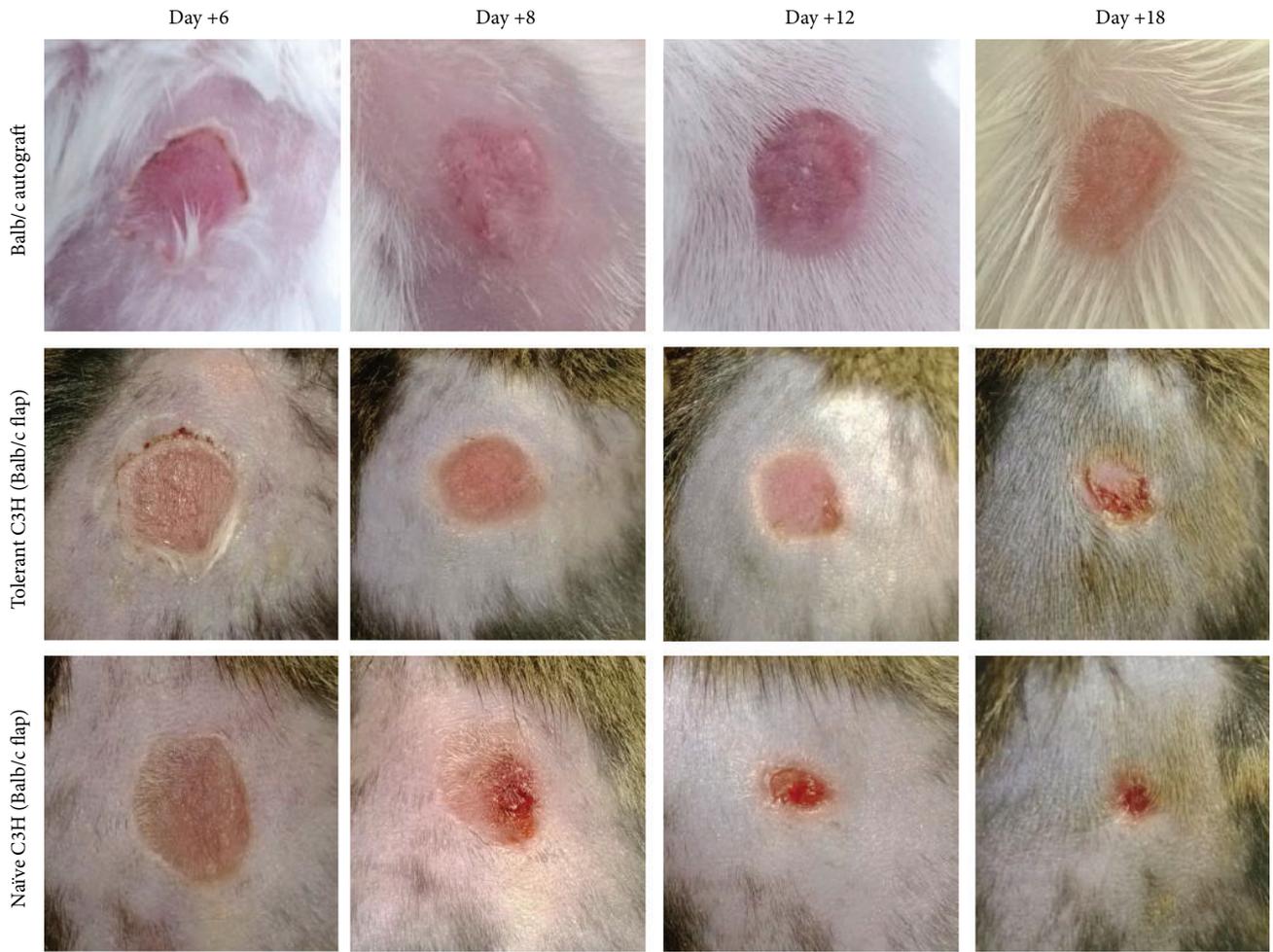


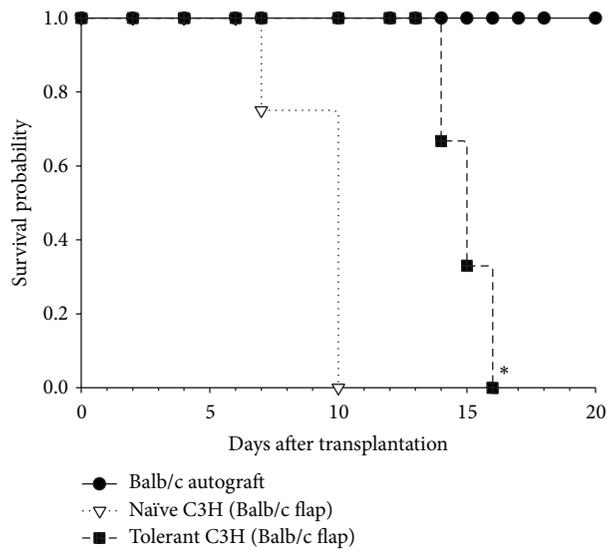
FIGURE 2: Alloantigen immune tolerance is transferable by adoptive transfer of CD4+ T cells from long-lasting tolerant mice. Splenic CD4+ T cells from C3H mice used for the experiments in Figure 1 were prepared by negative selection using Easysep kits from StemCell Biotechnology Company. Given that all mice had been challenged by injection of Balb/c spleen cells, the PBS-treated C3H mice were all immunized as shown in Figure 1, and the two groups were designated as tolerant group and immunized group, respectively. CD4+ T cells were adoptively transferred into naïve C3H mice ( $5 \times 10^6$  CD4+ T cells/mouse) (5 mice/group). One day after CD4+ T cell transfer, all mice were challenged with two weekly transfusions of  $2 \times 10^5$  Balb/c spleen cells. One week after the second antigen challenge, the development of antibodies against donor Balb/c WBCs was examined as described in Section 2. (a) A representative of each group was shown. ((b) and (c)) The summary of anti-donor antibody levels in percentage and MFI of each group was depicted, respectively. Data represent mean  $\pm$  standard error. \* $P < 0.05$ . The  $P$  values were calculated using Student's  $t$ -test.

controlling alloantigen-responding T cells that potentially arise later and never experience alloantigens. Those T cells could lead to allograft rejection or graft-versus-host disease if left unchecked in allogeneic transplantation. To assess whether PD-1/PD-L1 in those alloantigen tolerant mice keeps the alloantigen-responding T cells in check, we adoptively transferred CFSE-labeled naïve C3H spleen cells (syngeneic) along with Balb/c spleen cells (alloantigen) to the tolerant C3H mice or naïve C3H mice. Thereafter, anti-PD-L1 or control IgG was administered to two groups of tolerant

mice at day 1 and day 3, respectively. Four days later, we sacrificed the mice and prepared cells from inguinal lymph nodes and assessed the proliferation of injected CFSE-labeled C3H syngeneic CD4+ and CD8+ T cells not preexperiencing alloantigen, in response to injected Balb/c spleen cells (alloantigen) *in vivo*. In comparison to control IgG-treated mice, CD4+ and CD8+ T cell proliferation in response to *in vivo* alloantigen stimulation was significantly enhanced in anti-PD-L1 treated mice, which was similar to the T cell proliferation levels in naïve recipient mice (Figure 6).



(a)



(b)

FIGURE 3: Tolerance induced by injection of UVB-iDCs prolongs the skin allograft survival. Six weeks after the last treatment for tolerance induction as described elsewhere, the tolerant and naïve C3H mice were transplanted with Balb/c skin grafts as described in Section 2. The graft survival was monitored daily for 30 days. (a) The condition of a representative transplanted flap from each group is exhibited. (b) The graft survival of each group was shown. Six mice were included in each group. The survival data were analyzed by Log Rank test. \*  $P < 0.05$  (tolerant group versus naïve group).

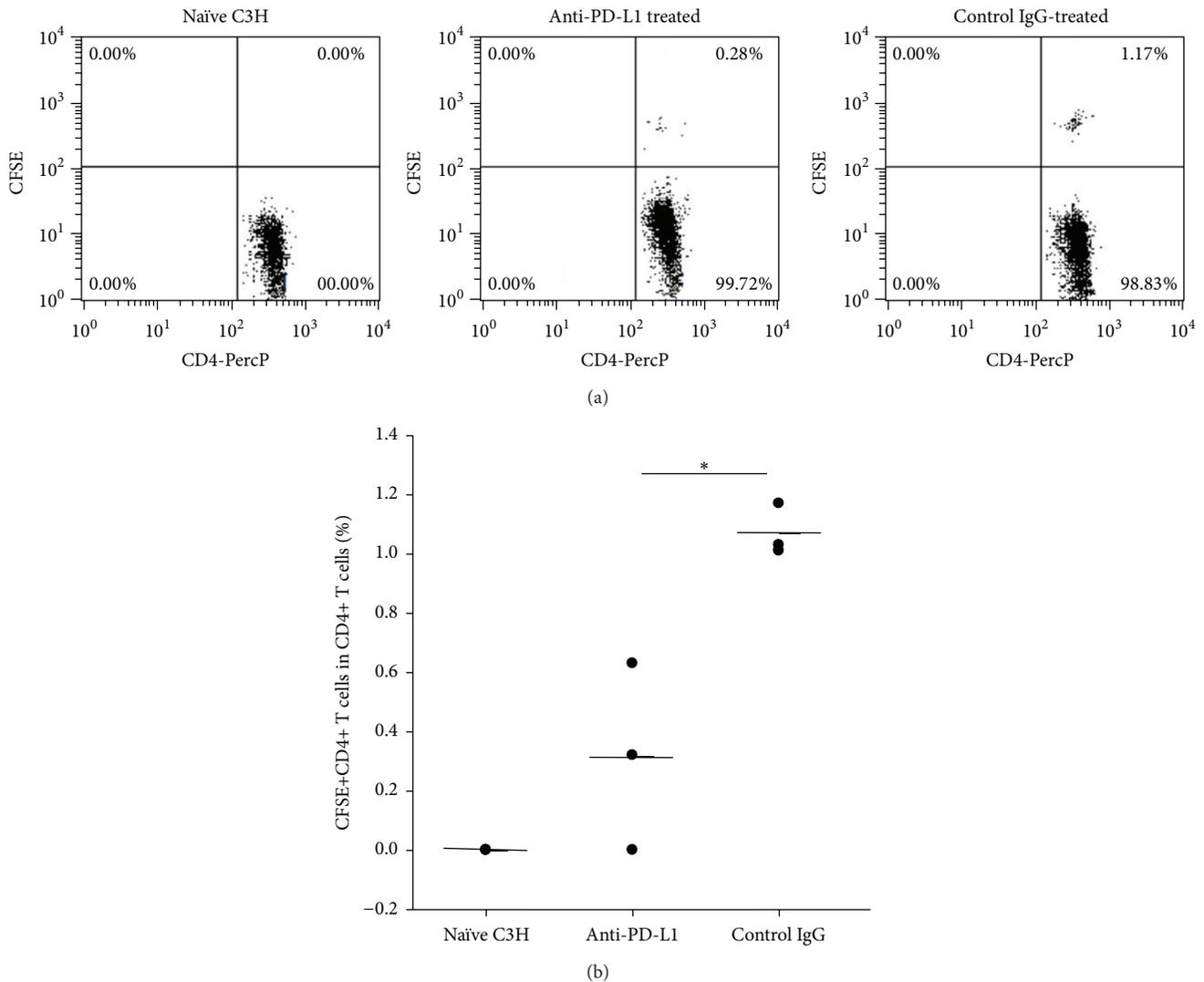


FIGURE 4: Anti-mouse PD-L1 antibody treatment enhances the rejection of intravenously injected Balb/c spleen cells in C3H mice with long-lasting tolerance. The tolerant C3H mice received purified anti-mouse PD-L1 mAb ( $100 \mu\text{g}/\text{mouse}$ ) or control IgG ( $100 \mu\text{g}/\text{mouse}$ ) along with administration of CFSE-labeled Balb/c spleen cells ( $1.5 \times 10^7/\text{mouse}$ ). Nontolerant C3H mice received the same numbers of CFSE-labeled Balb/c spleen cells as control. Three mice were included in each group. 24 hours after injection, peripheral blood samples were collected from all mice and stained with anti-CD4-PercP antibody. The CFSE+CD4+ cells to total CD4+ T cells were examined by flow cytometry. (a) Flow cytometric data are exhibited from a representative mouse of each group. (b) The data summary of all mice in each group is depicted. Data represent mean  $\pm$  standard error. \*  $P < 0.05$ . The  $P$  values were calculated using one-way ANOVA.

The above findings indicate that, in tolerant mice, PD-1/PD-L1 interaction plays an important role in keeping potentially arising alloantigen-responding T cells in check and preventing them from being activated.

#### 4. Discussion

We previously reported that transfusion of UVB-iDCs was able to induce immune tolerance across MHC barriers and this tolerance was associated with regulatory CD4+ T cells [5, 6]. However, it is unknown whether this tolerance state can be maintained for long term, which is highly desirable for allogeneic transplantation. In the present study, we assessed the tolerance maintenance 6 weeks after finishing tolerance

induction. We found that tolerant mice failed to produce any levels of antibodies against alloantigens upon two weekly alloantigen challenges (Figure 1). These results are the same as those observed in the tolerant mice undergoing alloantigen challenge immediately after tolerance induction [6]. These findings suggest that UVB-iDC infusion-induced alloantigen tolerance could be maintained for at least 6 weeks after tolerance induction without any loss. Consistent with these results, we also observed that the tolerant mice failed to reject the intravenously injected CFSE-labeled traceable Balb/c allogeneic spleen cells, which were completely rejected in nontolerant naïve mice (Figure 4) through flow cytometric assessment of peripheral blood. In addition, we found those injected CFSE-labeled allogeneic spleen cells homed to host

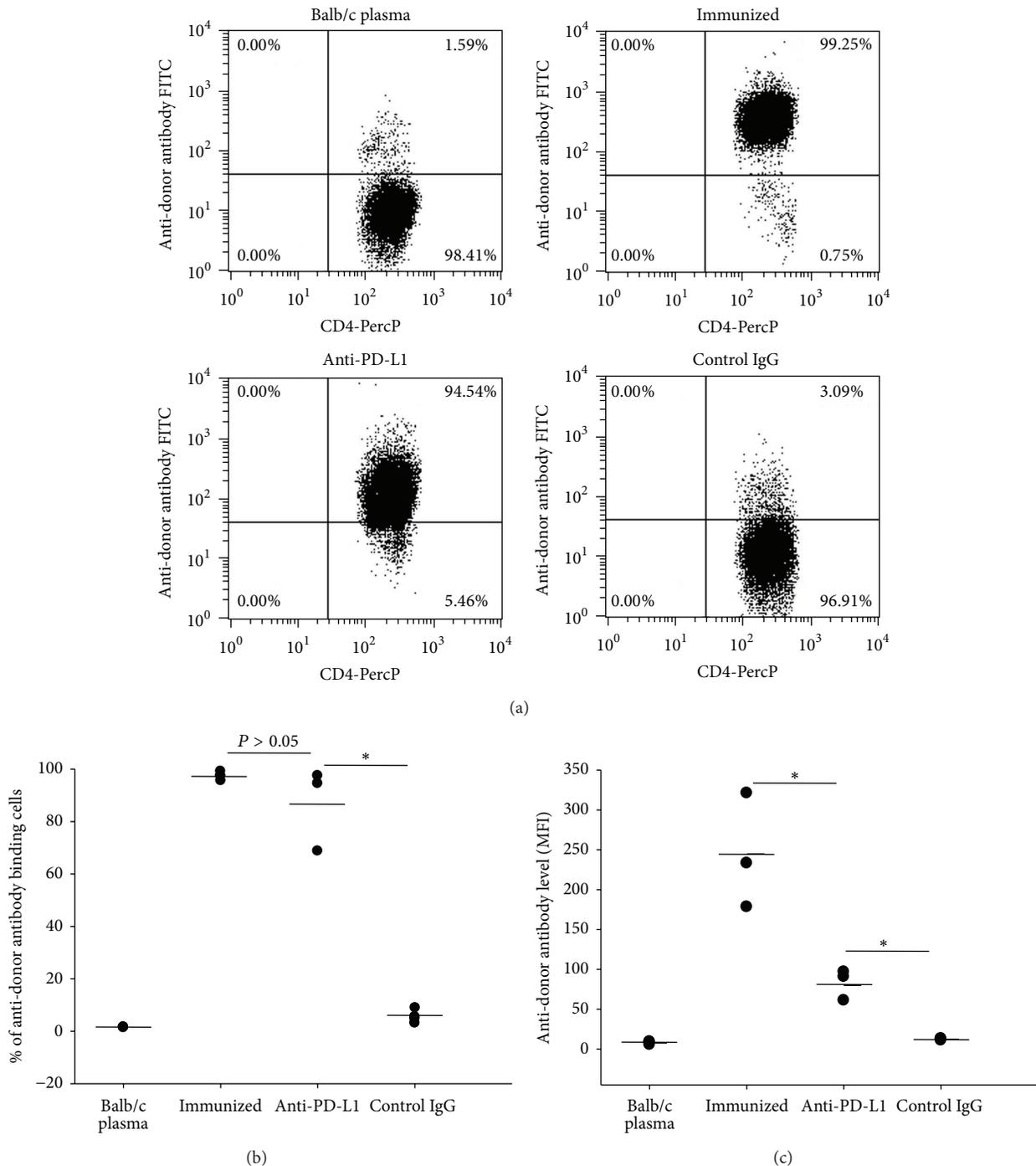


FIGURE 5: Anti-mouse PD-L1 antibody treatment partially recovers the development of antibodies against alloantigens in C3H mice with long-lasting tolerance to Balb/c alloantigens. Tolerant C3H mice 6 weeks after tolerance induction were given alloantigen challenge by injection of Balb/c spleen cells ( $2 \times 10^5$ /mouse) along with anti-mouse PD-L1 mAb ( $100 \mu\text{g}/\text{mouse}$ ) or control IgG ( $100 \mu\text{g}/\text{mouse}$ ) once a week for two weeks. The following week, plasma samples were prepared from all mice, and the levels of anti-donor (Balb/c) antibodies were examined as described elsewhere. The plasma samples from the Balb/c and naïve C3H mice immunized by Balb/c spleen cells served as controls. We only gated CD4+ T cells to analyze anti-donor antibody levels to eliminate false positivity caused by B cell IgG Fc binding. (a) A representative mouse of each group was exhibited. (b) The summary of 3 mice in each group was depicted. Data represent mean  $\pm$  standard error.  $*P < 0.05$ .  $P$  values were calculated using one-way ANOVA.

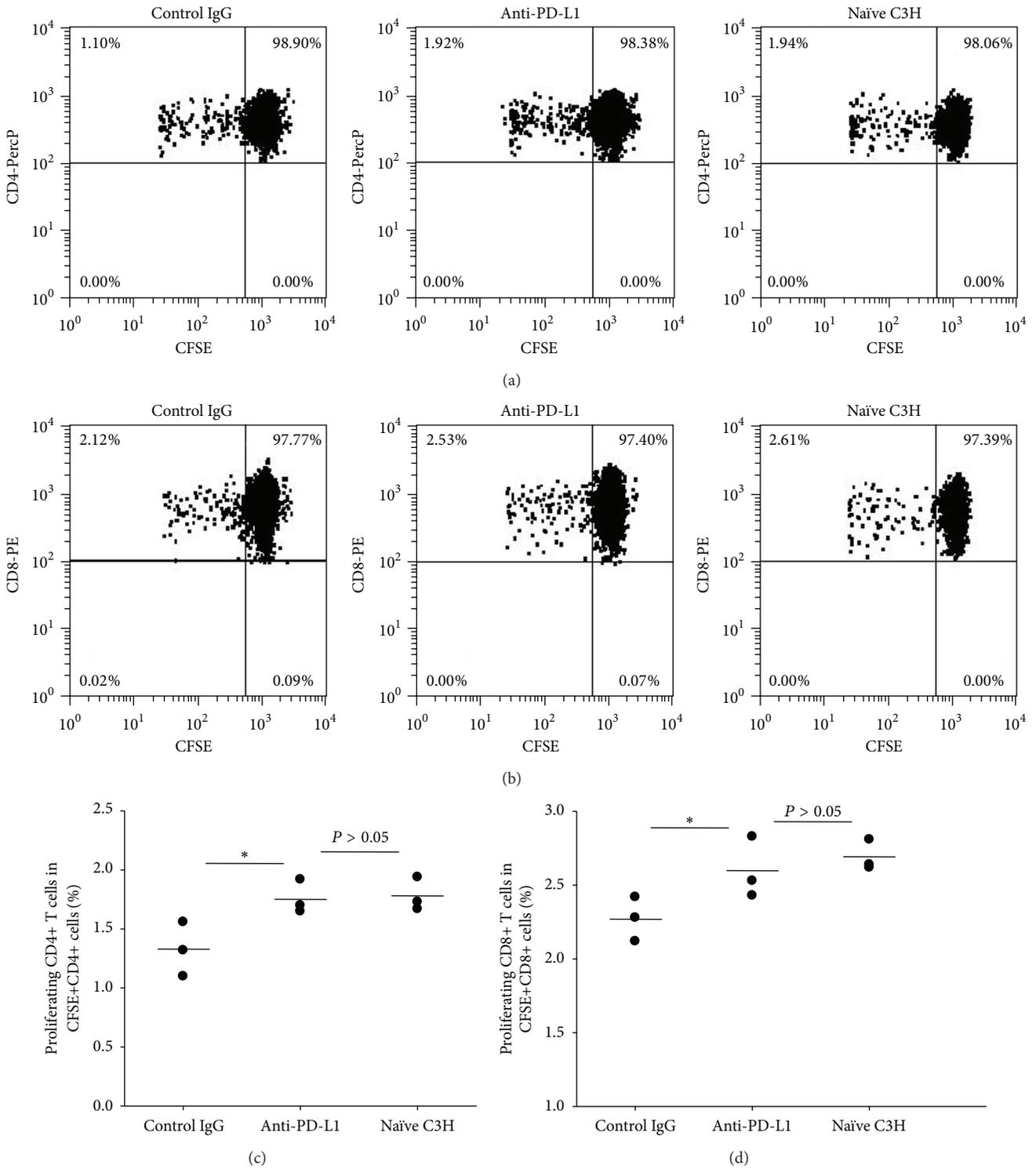


FIGURE 6: PD-1/PD-L1 interaction controls alloantigen-responding effector T cells in tolerant mice. C3H mice were treated with Balb/c UVB-iDCs to induce alloantigen tolerance as described elsewhere. Six weeks later, CFSE-labeled naïve C3H spleen cells ( $1 \times 10^7$ /mouse) together with Balb/c spleen cells ( $1 \times 10^7$ /mouse) were intravenously injected into the tolerant mice. One group received intraperitoneal injection of anti-mouse PD-L1 mAb ( $100 \mu\text{g}/\text{mouse}$ ); the other group received control IgG ( $100 \mu\text{g}/\text{mouse}$ ) at the same day when cells were injected. On day 3, the above treatments were administered again. One group of naïve C3H mice was set up only receiving injection of both types of cells. On day 4 after cell injection, all mice were sacrificed and inguinal lymph nodes were collected and prepared for cell suspension. The proliferating CD4+ T cells and CD8+ T cells in CFSE+ lymph node cells were analyzed by flow cytometry. (a) Representative flow cytometric data on CFSE-labeled CD4+ T cell proliferation in each group were shown; (b) representative flow cytometric data on CFSE-labeled CD8+ T cell proliferation in each group were shown; ((c) and (d)) the summary of CD4+ and CD8+ T cell proliferation was shown, respectively. Data represent mean  $\pm$  standard error. \*  $P < 0.05$ . The  $P$  values were calculated using one-way ANOVA.

lymph nodes and spleen in tolerant mice, which did not take place in nontolerant mice (data not shown). This tolerance assessment is a sensitive assay for evaluating allogeneic tolerance *in vivo* by looking at CFSE-labeled cells in the peripheral blood and could be a feasible method for future clinical evaluation of immune tolerance in allogeneic transplantation patients. Allogeneic skin transplantation is usually employed to test allogeneic tolerance. To assess whether tolerant mice 6 weeks after tolerance induction were able to accept allogeneic skin grafts, we performed allogeneic skin transplantation in tolerant and nontolerant mice. It is noted that, without any immunosuppression, the survival of allogeneic skin grafts was significantly prolonged in tolerant mice in contrast to that in nontolerant mice (Figure 3). These data further support that UVB-iDC-induced alloantigen tolerance is quite strong and durable.

We demonstrated previously that, among CD4<sup>+</sup> T cells in tolerant mice, there were significantly increased IL-10-producing and Foxp3<sup>+</sup> regulatory T cells [5], which may play a crucial role in maintaining tolerance. In the current study, we assessed whether CD4<sup>+</sup> T cells in the mice with long-lasting tolerance remained functioning to maintain immune tolerance to alloantigens. We found that the tolerance was transferrable with adoptive CD4<sup>+</sup> T cell transfer to syngeneic naïve recipient mice, showing that mice receiving CD4<sup>+</sup> T cells from tolerant mice failed to produce antibodies against alloantigens upon alloantigen challenge (Figure 2). Interestingly, the results shown in Figures 1 and 2 are quite similar, indicating that CD4<sup>+</sup> T cells exert critical effect on tolerance maintenance. Those CD4<sup>+</sup> T cells with regulatory function are likely to be Foxp3<sup>+</sup> Tregs and IL-10-producing Tr1 cells [5, 12].

PD-1/PD-L1 interaction has been considered playing an essential role in maintaining peripheral tolerance [7, 8, 13, 14]. Blocking PD-1/PD-L1 interaction can drastically accelerate allograft rejection [15–17]. It was also reported that PD-1/PD-L1 interaction is crucial for maintaining anti-CD3 treatment-induced immune tolerance to islet  $\beta$  cell antigens in nonobese diabetic mouse model [18]. Administration of ethylene carbodiimide- (ECDI-) fixed allogeneic splenocytes failed to induce alloantigen tolerance in PD-L1 deficient mice, suggesting that PD-1/PD-L1 is required for alloantigen tolerance induced by ECDI-treated allogeneic splenocytes [11]. In the current study, we were interested in learning whether PD-1/PD-L1 was required for maintaining alloantigen tolerance induced by UVB-iDC treatment. We employed our reliable and reproducible methods and tested the influence of PD-1/PD-L1 blockade by anti-PD-L1 antibodies on immune tolerance to alloantigens upon alloantigen challenge. We showed that PD-1/PD-L1 blockade by anti-PD-L1 antibodies significantly promoted the rejection of infused CFSE-labeled allogeneic spleen cells (Figure 4) compared to control IgG antibody-treated tolerant mice. As expected, the nontolerant naïve mice completely rejected the infused CFSE-labeled allogeneic spleen cells. In line with this finding, the levels of antibodies against alloantigens in response to alloantigen challenge in tolerant mice were largely recovered by the treatment of anti-PD-L1 antibodies but failed to reach the levels of nontolerant naïve mice challenged with alloantigens

(Figure 5). The above findings indicate that PD-1/PD-L1 interaction actively participates in maintaining allogeneic tolerance induced by UVB-iDC treatment. Incomplete recovery of immune response to alloantigen challenge might be attributed to incomplete blockade of PD-1/PD-L1 interaction because we only utilized one dose (100  $\mu$ g/mouse), which might not be the optimal dose to completely block PD-1/PD-L1 interaction. Another possibility is that other tolerance maintenance mechanisms may also be involved. A dose study for anti-PD-L1 antibodies or PD-L1 deficient mouse model may be needed to address whether PD-1/PD-L1 is the only factor to be involved in maintaining the tolerance induced by UVB-iDC treatment.

Finally, we investigated how the tolerance state in tolerant mice affected alloantigen-responding T cells that never experienced alloantigens and whether PD-1/PD-L1 interaction was also involved in this process. We injected CFSE-labeled syngeneic spleen cells from naïve C3H mice into tolerant C3H recipients to serve as responder cells, with simultaneous injection of allogeneic Balb/c spleen cells as allogeneic stimulators. One group was treated with anti-PD-L1 antibodies and the other group received control IgG treatment. As shown in Figure 6, blockade of PD-1/PD-L1 interaction significantly increased the proliferation of injected CFSE-labeled CD4<sup>+</sup> and CD8<sup>+</sup> T cells, suggesting that PD-1/PD-L1 plays an important role in keeping alloantigen-responding T cells in check. This effect may be through induced alloantigen-specific regulatory T cells as described in our previous study [5]. PD-1 has been reported to be expressed on regulatory T cells [19]. Our data show that CD4<sup>+</sup> T cells from UVB-iDC-treated mice express higher levels of PD-1 (Supplemental Figure 1, in Supplementary Material available online at <http://dx.doi.org/10.1155/2016/2419621>), and those CD4<sup>+</sup> T cells might gain regulatory function. Thus, UVB-iDC treatment-induced alloantigen-specific regulatory T cells may control the potential alloantigen-specific effector T cells through PD-1/PD-L1 interaction [20, 21].

Collectively, the present study demonstrates that UVB-iDC infusion-induced allogeneic immune tolerance is long lasting, and PD-1/PD-L1 interaction plays an important role in the maintenance of immune tolerance.

## Competing Interests

The authors have declared that there are no competing interests.

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## Research Article

# Effect of N-Feruloylserotonin and Methotrexate on Severity of Experimental Arthritis and on Messenger RNA Expression of Key Proinflammatory Markers in Liver

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Rheumatoid arthritis (RA) is a chronic inflammatory disease, leading to progressive destruction of joints and extra-articular tissues, including organs such as liver and spleen. The purpose of this study was to compare the effects of a potential immunomodulator, natural polyphenol N-feruloylserotonin (N-f-5HT), with methotrexate (MTX), the standard in RA therapy, in the chronic phase of adjuvant-induced arthritis (AA) in male Lewis rats. The experiment included healthy controls (CO), arthritic animals (AA), AA given N-f-5HT (AA-N-f-5HT), and AA given MTX (AA-MTX). N-f-5HT did not affect the body weight change and clinical parameters until the 14th experimental day. Its positive effect was rising during the 28-day experiment, indicating a delayed onset of N-f-5HT action. Administration of either N-f-5HT or MTX caused reduction of inflammation measured as the level of CRP in plasma and the activity of LOX in the liver. mRNA transcription of TNF- $\alpha$  and iNOS in the liver was significantly attenuated in both MTX and N-f-5HT treated groups of arthritic rats. Interestingly, in contrast to MTX, N-f-5HT significantly lowered the level of IL-1 $\beta$  in plasma and IL-1 $\beta$  mRNA expression in the liver and spleen of arthritic rats. This speaks for future investigations of N-f-5HT as an agent in the treatment of RA in combination therapy with MTX.

## 1. Introduction

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease affecting predominantly joints, synovial membranes, articular cartilages, and subchondral bones [1]. Disease progression is attributed to increases in reactive oxygen species (ROS) and oxidative stress (OS) in the lesion sites [2]. Proinflammatory cytokines, such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-1 $\beta$  (IL-1 $\beta$ ), and IL-6, regulate the inflammatory and immune responses and play a pivotal role in the disease [3]. Overproduction of nitric oxide (NO), as a result of induction of inducible nitric oxide synthase (iNOS)

due to enhanced production of these cytokines, is associated with persistent inflammation and tissue destruction in experimental arthritis models, including rheumatoid arthritis [4, 5]. A number of inflammation stimuli, including TNF- $\alpha$ , IL-1 $\beta$ , IL-6, or ROS, can activate proinflammatory pathways involved in RA pathogenesis, concerning predominantly nuclear factor- $\kappa$ B (NF- $\kappa$ B), mitogen activated protein kinases (MAPKs), or Janus kinases/signal transducers and activators of transcription (JAK/STAT1/3) [6–8]. This results in translocation of relevant downstream transcription factors from the cytoplasm to the nucleus, where they activate messenger RNA (mRNA) expression of target genes, including IL-1 $\beta$ , TNF- $\alpha$ ,

iNOS, and 12/15-lipoxygenase (LOX), leading to overproduction of corresponding proteins. Cytokines released into the synovium reach also the systemic circulation and act in other tissues and organs such as lungs, vascular tissue, liver, and heart [9]. Several recent investigations reported damage of vital organs with various degrees of impairment, considered to be secondary complications of RA and a major predictor of mortality in RA patients [10]. Increasing evidence is pointing to the critical role of the liver in modulating the immune response in autoimmune and chronic inflammatory diseases including RA [5, 11, 12]. The hepatic biochemical and immunological alterations are associated with and influenced by changes in the oxidative state of liver cells [5].

Adjuvant-induced arthritis (AA) in rats not only is an experimental model of polyarthritis but also induces pathological changes in a variety of other tissues, including the liver and spleen [13]. It is a useful tool to study immunopathologic processes, autoimmune chronic inflammation, and inflammatory cachexia in rodents. In addition, at the molecular level, mRNA profiling suggests that this model is also similar to human RA, particularly in tissue gene expression and in the activation of regulatory pathways [11, 14].

Numerous studies reported natural polyphenols as potential therapeutic agents of diseases caused by OS and inflammation [15–17]. N-feruloylserotonin (N-f-5HT, N-feruloyl-5-hydroxy-tryptamine) is a conjugated serotonin, a member of the indole hydroxycinnamic acid amides, with serotonin (5-HT) and ferulic acid (fa) as representative components of its structure. Hydroxycinnamic acid amides of serotonin, synthesized by serotonin N-hydroxycinnamoyltransferase, are present in several vegetables [18] and wild-growing plants whose seeds are used in herbal medicine in Eastern countries [18–20]. In cell-based studies, under short-term high-glucose conditions, N-f-5HT exerted an inhibitory effect on overproduction of mitochondrial superoxide by acting as scavenger of superoxide [21]. N-f-5HT attenuated the upregulation of mRNA and proteins of ROS-dependent adhesion (vascular cell adhesion protein-1 (VCAM-1)) and migration factors (monocyte chemoattractant protein-1 (MCP-1)), crucial in early atherosclerosis lesions in human aortic endothelial cells, and inhibited the activation of transcription factor NF- $\kappa$ B [21]. Furthermore, N-f-5HT showed a protective effect on ROS-related neuronal damage by decreasing the activity of proapoptotic caspase-3 [22]. N-f-5HT isomers isolated from seeds of *Leuzea carthamoides* were shown to inhibit protein kinase C  $\alpha/\beta$  II activation and decrease the oxidative burst of human whole blood and isolated neutrophils *in vitro* [23]. N-f-5HT was also found to have a protective effect against LDL oxidation and atherogenesis in experimental animals and in human studies [24–26].

Methotrexate (MTX), used as a standard drug in our study, represents the most frequently used pharmacotherapy of RA in clinical practice. Its administration is, however, limited due to its toxic side effects [27, 28]. Yet application of a combination therapy of MTX with other potential immunomodulators, synthetic drugs [29] or natural substances [30–32], might elevate the therapeutic efficacy: decrease the dose of MTX and thus its side effects. In our previous study, we showed that administration of N-f-5HT to MTX-treated

arthritic rats lowered the dose of MTX for the required sustained antirheumatic impact [33]. In this study, we focused on the therapeutic impact of N-f-5HT and MTX administered in monotherapy and on details of the inflammatory state in the arthritic rat liver with the aim to elucidate the molecular mechanisms of their effect. One of the possible clarifying approaches is to study the mRNA expression of key proinflammatory markers (IL-1 $\beta$ , TNF- $\alpha$ , and iNOS) in the liver of treated and untreated arthritic rats. Further, it is of particular interest to expand our knowledge on the effect of N-f-5HT and MTX in the AA model, which in turn should allow extrapolations of these results to RA patients. To this aim we evaluated also conventional arthritic parameters (HPV, arthritic score, body weight change, and weight of the liver) along with changes in plasmatic levels of IL-1 $\beta$  and CRP and the activity of 12/15-LOX in the liver.

## 2. Materials and Methods

**2.1. Animals.** Adult male Lewis rats weighing 160–180 g were obtained from Charles River Wiga, Germany. The rats had free access to standard pelleted diet and tap water. The experimental protocol was approved by the Ethics Committee of the Institute of Experimental Pharmacology and Toxicology and by the Slovak State Veterinary and Food Administration in accordance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes and was in line with Slovak legislation.

**2.2. Induction of Adjuvant Arthritis.** To induce a rat model of adjuvant arthritis (AA), rats were intradermally injected with a suspension of heat-inactivated *Mycobacterium butyricum* in incomplete Freund's adjuvant (Difco Laboratories, Detroit, MI, USA). The injection was performed near the tail base on the first experimental day.

**2.3. Experimental Design and Animal Treatments.** The experiments included 4 groups of animals.

**Group 1.** The first group comprised healthy control rats (CO).

**Group 2.** The second group comprised untreated adjuvant arthritis rats (AA).

**Group 3.** The third group comprised adjuvant arthritis rats treated with methotrexate (Methotrexat® EBEWE *sol inj* 20 mg/2.0 mL) in oral dose of 0.4 mg/kg twice a week (AA-MTX).

**Group 4.** The fourth group comprised adjuvant arthritis rats treated with N-feruloylserotonin dissolved in suspension of methylcellulose Tween 80 at a dose of 3 mg/kg/day orally (AA-N-f-5HT).

Drugs were administered orally by gastric gavage from day 0 (the day of treatment) to day 28 of the study. Blood for plasma preparation was taken by retroorbital puncture on day 14 and by cardiac puncture on day 28 under deep ketamine/xylazine anesthesia. After the animals had been sacrificed under deep ketamine/xylazine anesthesia, tissues for liver and

TABLE 1: Composition of the crystalline N-feruloylserotonin complex fraction, where the content of N-feruloyl- and N-isoferuloyl- (E = trans- and Z = cis-) serotonin isomers was determined by HPLC analysis.

Compound	Content [%]
N-(E)-Feruloylserotonin	18.3
N-(E)-Isoferuloylserotonin	67.4
N-(Z)-Feruloylserotonin	6.1
N-(Z) Isoferuloylserotonin	8.2

spleen homogenate preparation were taken at the end of the experiment (day 28). Blood in heparinized tubes for plasma preparation was centrifuged at 3000 rpm for 15 minutes at 4°C. Samples were stored at -80°C until biochemical and immunological analysis.

Fraction of four isomers of N-f-5HT (Table 1) was isolated from the seeds of *Leuzea carthamoides* (Wild) DC by solvent extraction. This was then followed by column chromatography on silica gel and HPLC separations under conditions previously reported [35, 36].

**2.4. Change of Hind Paw Volume (HPV).** The hind paw volume (HPV) was recorded on days 14, 21, and 28 with the use of an electronic water plethysmometer (UGO BASILE, Comerio, Varese, Italy). Calculation of the increase in hind paw volume in mL assessed the intensity of the edema.

**2.5. Arthritic Score.** The arthritic score was measured as the total score of HPV (mL, max. points 8) + paw diameter of forelimb (mm, max. points 5) + diameter of scab in the site of MB application, measured in parallel to the spinal column (mm, max. points 5) for each animal on all experimental days monitored [33].

**2.6. Body Weight Change.** Body weight change (BWC; g) was measured on days 1, 14, 21, and 28. BWC was calculated as the difference of the body mass measured on days 14, 21, and 28 to the body weight measured at the beginning of the experiment (day 1).

**2.7. Measurement of C-Reactive Protein (CRP) in Plasma.** For the determination of rat CRP concentration in plasma ( $\mu\text{g/mL}$ ), the ELISA kit from Immunology Consultant Laboratories, Inc. was used. The reaction of secondary biotin-conjugated anti-rat CRP antibody was evaluated by streptavidin-HRP. The tetramethylbenzidine reaction with HRP bound to immune complex was measured at 450 nm (microplate reader, Labsystems Multiskan RC). The results were calculated using the standard calibration curve on internal standards.

**2.8. Measurement of Interleukin-1 $\beta$  (IL-1 $\beta$ ) in Plasma.** For the determination of IL-1 $\beta$  concentration in plasma, the ELISA kit from R&D Systems Quantikine<sup>®</sup> was used. The assay procedures followed the description in the product manual. Rat cytokine present in the samples binds to anti-rat cytokine antibodies absorbed in the microwells. The reaction of

secondary biotin-conjugated anti-rat cytokine antibody is evaluated by HRP. The tetramethylbenzidine reaction with HRP bound to immune complex was measured at 490 nm in comparison with the reference wavelength of 620 nm (microplate reader MRX II). The results were calculated using the standard calibration curve on internal standards.

**2.9. Tissue Activity of 12/15-Lipoxygenase (LOX) in Liver.** Concentration of proteins in liver homogenates was determined by using the Bradford method [37] and expressed in mg/mL of enzyme preparation (cytosolic fraction from rat lung and liver tissues). Linoleic acid (99%, Sigma-Aldrich, USA) was used as a substrate prepared in solubilized state as described [38] in the concentration of  $0.2143 \times 10^{-5}$ – $0.7143 \times 10^{-5}$  M. The assay of LOX was monitored for 60 seconds as an increase in the absorbance at 234 nm, reflecting the formation of hydroperoxylinoleic acid. For the LOX activity assay, an UV/VIS Spectrometer Perkin-Elmer Lambda 35 (USA) was used. The reaction medium contained a 50 mM Tris-HCl buffer (pH 7.0), 2.5  $\mu\text{L}$  of the enzyme, and solubilized linoleic acid.

**2.10. Total RNA Isolation and Quantitative RT-PCR.** Total RNA was isolated from the rat liver and spleen using RNAzol RT (Sigma-Aldrich) and converted into complementary DNA (cDNA) using the PrimeScript RT Reagent Kit (Takara) following the protocols of the manufacturers. Amplification and detection of cDNA of reference and target genes were performed on a 7300 Real-Time PCR System (Applied Biosystems) using HOT FIREPol EvaGreen<sup>®</sup> qPCR Mix Plus (ROX) (Solis BioDyne). Relative mRNA expressions of IL-1 $\beta$ , TNF- $\alpha$ , and iNOS were analyzed using the  $\Delta\Delta\text{Ct}$  value method [39]. PCR products were evaluated by melting curve analysis to confirm the specific amplification.  $\beta$ -actin was used as a reference gene. The sequences of the primers were designed and checked using Primer3 and Oligo Analyzer 1.0.3 (Table 2).

**2.11. Statistical Analyses.** Mean and SEM values were calculated for each parameter in each group (8–10 animals in each experimental group). All measurements were done in duplicate or triplicate. Statistically significant differences among treated, untreated, and control groups were tested using parametric Analysis of Variance (ANOVA). *Post hoc* tests (Tukey-Kramer (ANOVA)) were applied in situations where differences among groups were significant at the level of significance  $\alpha = 0.05$ . After *post hoc* testing, the following significance levels were specified: extremely significant ( $***p < 0.001$ ), highly significant ( $**p < 0.01$ ), significant ( $*p < 0.05$ ), and not significant ( $p > 0.05$ ).

### 3. Results and Discussion

**3.1. Effect of N-f-5HT on Clinical Parameters: Arthritic Score and Change of Hind Paw Volume (HPV) and Parameters of Cachexia.** Antioxidant properties of polyphenols including N-f-5HT have been reported [21, 40, 41]. Nevertheless, the N-f-5HT impact on chronic inflammatory and OS-inducing arthritis, which could widen the possibilities of the RA

TABLE 2: Primer sequences.

Product	Sense primer (5'-3')	Antisense primer (5'-3')
IL-1 $\beta$	CCTCTGTGACTCGTGGGATG	GGGTGTGCCGTCTTTCATCA
TNF- $\alpha$	CTTCTGTCTACTGAACTTCG	GAACCTGGGAGTAGATAAGG
iNOS	AAAACCCCAGGTGCTATTC	GTGGTGAAGGGTGTCTGTGAA
$\beta$ -actin [34]	CCGCGAGTACAACCTTCTTG	GCAGCGATATCGTCATCCA

TABLE 3: Parameters of cachexia, liver weight, and severity of arthritis (hind paw volume and arthritic score) in rats with adjuvant-induced arthritis on experimental days 14 and 28 treated with N-f-5HT and MTX.

Cachexia	CO	AA	AA-MTX	AA-N-f-5HT
<i>Parameter</i>				
<i>Severity of arthritis</i>				
Hind paw volume				
Day 14	1.67 $\pm$ 0.01	1.82 $\pm$ 0.04	1.69 $\pm$ 0.03	1.87 $\pm$ 0.06
Day 21	1.78 $\pm$ 0.02	2.07 $\pm$ 0.08**	1.72 $\pm$ 0.04+++	2.07 $\pm$ 0.05
Day 28	1.81 $\pm$ 0.02	1.98 $\pm$ 0.07*	1.72 $\pm$ 0.03+++	1.95 $\pm$ 0.04
Arthritic score				
Day 14	10 $\pm$ 0	15.1 $\pm$ 0.79**	12.4 $\pm$ 0.72	16.7 $\pm$ 1.36
Day 21	11.25 $\pm$ 0.32	19.71 $\pm$ 1.11***	14.4 $\pm$ 1.68 <sup>+</sup>	19.6 $\pm$ 1.37
Day 28	11.37 $\pm$ 0.26	22.29 $\pm$ 0.71***	16.3 $\pm$ 1.11 <sup>++</sup>	19.5 $\pm$ 1.23
<i>Cachexia</i>				
Body weight change (g)				
Day 14	59.37 $\pm$ 1.80	33.25 $\pm$ 4.80***	42.82 $\pm$ 3.74	39.92 $\pm$ 5.33
Day 21	75.28 $\pm$ 2.68	14.14 $\pm$ 4.97***	31.15 $\pm$ 6.17	29.6 $\pm$ 4.52
Day 28	102.69 $\pm$ 4.32	28.1 $\pm$ 4.61***	38.9 $\pm$ 8.03	55.32 $\pm$ 4.99 <sup>+</sup>
Liver weight (g)				
Day 28	7.94 $\pm$ 0.17	7.99 $\pm$ 0.21	7.05 $\pm$ 0.23 <sup>+</sup>	8.32 $\pm$ 0.23

CO, control group; AA, adjuvant arthritis group; AA-N-f-5HT, adjuvant arthritis group given N-feruloylserotonin; AA-MTX, adjuvant arthritis group given methotrexate. The data represent the mean  $\pm$  SEM;  $n = 9-10$ . The symbols \* and + show significant difference: \* $p < 0.05$  versus CO; \*\* $p < 0.01$  versus CO; \*\*\* $p < 0.001$  versus CO; <sup>+</sup> $p < 0.05$  versus AA; <sup>++</sup> $p < 0.01$  versus AA; <sup>+++</sup> $p < 0.001$  versus AA.

therapy, remains to be elucidated. In our previous study in the model of AA, N-f-5HT in the dosage of 15 mg/kg markedly potentiated the therapeutic effect of low-dose (nontherapeutic dose) MTX (0.3 mg/kg) on arthritic (hind paw volume and arthritic score) and inflammatory parameters (IL-17, MCP-1, and CRP), yet it resulted in insignificant effect in monotherapy [33]. As data about the optimal N-f-5HT dose in the rat model are scarce, we decided to study two doses of N-f-5HT: (i) when 15 mg/kg exceeded the physiologically acceptable concentration, we used 3 mg/kg, and (ii) when 15 mg/kg was too low to reach the maximal effect, we used 30 mg/kg. Unexpectedly, contrary to the lower dose of N-f-5HT, the higher dose exhibited minor effect on the parameters examined and/or these varied strongly among the animals. For this reason, this report shows only the data evaluating the lower dose of N-f-5HT. In this study, we used the therapeutic dose of MTX (0.4 mg/kg) with the intention to compare each mechanism of action of MTX and N-f-5HT, both evaluated in monotherapy.

The significant rise in arthritic parameters, arthritic score, and HPV confirmed the arthritis in our model in rats. The arthritic score showed an increase in the untreated arthritic group compared to the control group on all days monitored

(AA versus CO, day 14, \*\* $p < 0.01$ ; day 21 and day 28, \*\*\* $p < 0.001$ ; Table 3). At the end of the experiment, the arthritic score was almost doubled in the AA group compared to controls. A trend toward reduction was observed after administration of N-f-5HT to AA animals on day 28, but the effect was not statistically significant. The treatment with MTX significantly reduced the arthritic score on observation days 21 and 28, compared to the untreated arthritic group, proving the therapeutic potential of the applied dose of MTX (AA-MTX versus AA, day 21, <sup>+</sup> $p < 0.05$ ; day 28, <sup>++</sup> $p < 0.01$ ; Table 3).

Similarly, the change in HPV showed an increase in the untreated arthritic group compared to the control group on days 21 and 28 (AA versus CO, day 21, \*\* $p < 0.01$ ; day 28, \* $p < 0.05$ ; Table 3). The administration of N-f-5HT induced no modification of HPV of the arthritic animals on any day monitored. MTX therapy significantly reduced the observed swelling on days 21 and 28 compared to the untreated arthritic group (AA-MTX versus AA, day 21 and day 28, <sup>+++</sup> $p < 0.001$ ; Table 3).

The muscle wasting condition due to high catabolic activity, known as rheumatoid cachexia, occurring in approximately two-thirds of all patients with RA, is mediated by TNF- $\alpha$  and IL-1 $\beta$  in RA [42]. Papers published over the past

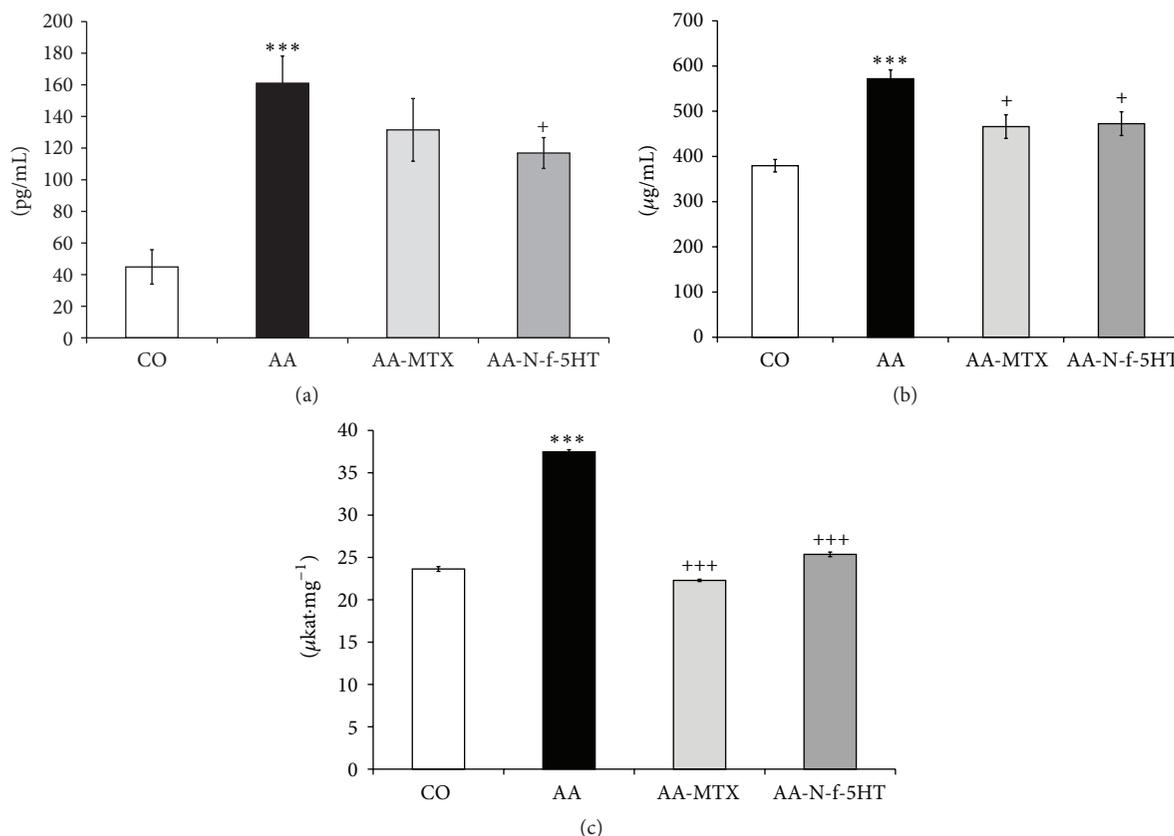


FIGURE 1: (a) Level of IL-1 $\beta$  in plasma in pg/mL measured on day 14. (b) Level of CRP in plasma in  $\mu$ g/mL measured on day 28. (c) 12/15-LOX activity in  $\mu$ kat $\cdot$ mg $^{-1}$  in liver. CO, control group; AA, adjuvant arthritis group; AA-N-f-5HT, adjuvant arthritis group given N-feruloylserotonin; AA-MTX, adjuvant arthritis group given methotrexate. Results are mean  $\pm$  SEM;  $n = 8-10$ . The symbols \* and + show significant difference: \*\*\*  $p < 0.001$  versus CO, +  $p < 0.05$  versus AA, and +++  $p < 0.001$  versus AA.

years confirmed that oxidative metabolism was considerably enhanced in the liver of adjuvant-induced arthritis in rats [43–46]. Rats used in this study revealed signs of cachexia (Table 3). A significant decrease in body weight change (BWC) was observed on all experimental days in the AA group. The BWC of the arthritic rats was 56% on day 14, 19% on day 21, and 27% on day 28 (AA versus CO, days 14, 21, and 28, \*\*\*  $p < 0.001$ ; Table 3) of the BWC of healthy controls. N-f-5HT treatment led to a significant increase of BWC on day 28 (AA-N-f-5HT versus AA, +  $p < 0.05$ ; Table 3). Administration of MTX elevated the BWC, yet the effect was not significant.

No difference was noted in the fresh weight of the liver. These results are comparable with reported manifestations in this experimental arthritis model [5]. The administration of N-f-5HT in arthritic animals did not change these parameters on any of the days observed. The liver weights were significantly lower (AA-MTX versus AA, +  $p < 0.05$ ; Table 3) only in the group of rats treated with MTX. The reduced weight of the liver in MTX-treated rats was assumed to be the result of inhibition of the pathway of *de novo* DNA synthesis by MTX [47, 48].

In summary, the statistical significance of 3 mg/kg of N-f-5HT treatment was determined only for BWC. The arthritic

score revealed a trend toward the positive effect increasing with time, indicating a late onset of N-f-5HT action (Table 3). As expected, significant differences were found in the arthritic score and HPV in the arthritic animals treated with the therapeutic dose of 0.4 mg/kg MTX compared to those treated with the low dose of 0.3 mg/kg MTX [33].

**3.2. Effect of N-f-5HT and MTX on IL-1 $\beta$  Plasmatic Level Measured on Day 14.** IL-1 $\beta$ , a prototypic proinflammatory cytokine, is a major mediator of the inflammatory cascade in RA, which is involved in the mechanisms leading to progressive joint destruction [3]. In the model of AA, the early phases of the disease seem to be characterized by a systemic increase of IL-1 $\beta$  [49]. The inflammatory process in AA is then self-remitting with time [50]. The plasmatic level of IL-1 $\beta$ , a protein of multiorgan origin, was significantly increased in arthritic animals compared to the control group in the early phase of AA, on day 14 (AA versus CO, \*\*\*  $p < 0.001$ ; Figure 1(a)), ascertaining the presence of inflammation. Administration of MTX did not lead to a significant change of plasmatic IL-1 $\beta$  concentration; only a trend toward reduction was observed on day 14. It is noteworthy that N-f-5HT treatment resulted in a significant decrease of IL-1 $\beta$  level in plasma (AA-N-f-5HT versus AA, +  $p < 0.05$ ; Figure 1(a)). This result

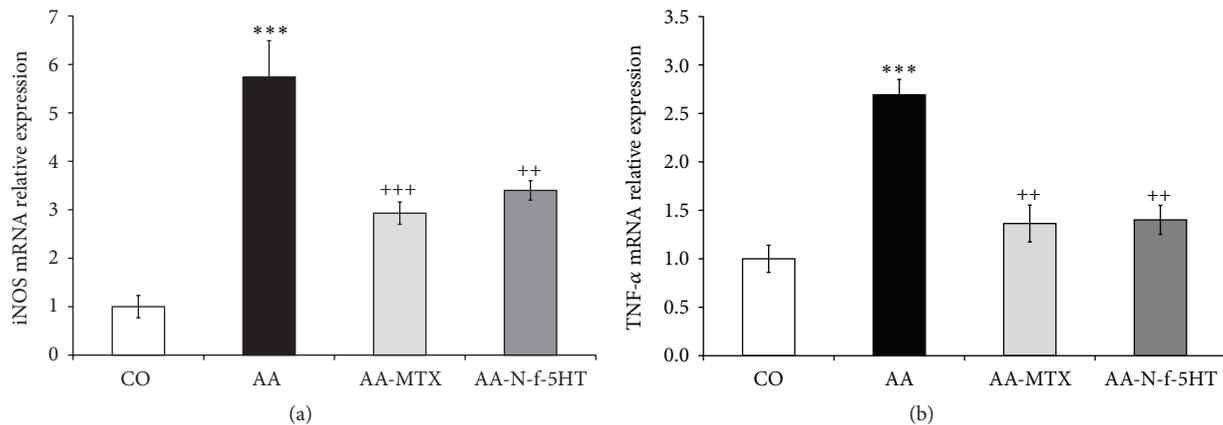


FIGURE 2: Relative changes of iNOS (a) and TNF- $\alpha$  (b) mRNA expressions normalized to  $\beta$ -actin mRNA in the rat liver. Control was present at 1. CO, control group; AA, adjuvant arthritis group; AA-N-f-5HT, adjuvant arthritis group given N-feruloylserotonin; AA-MTX, adjuvant arthritis group given methotrexate. The results are given as average  $\pm$  SEM;  $n = 8-10$ . The symbols \* and + show significant difference: \*\*\* $p < 0.001$  versus CO; ++ $p < 0.01$  versus AA; +++ $p < 0.001$  versus AA.

is interesting, as this molecule was reported to be relevant in driving the transition from the acute phase to the chronic irreversible phase of the disease and it has been suggested that it could be the target of early intervention to stop the course toward the chronic form of the disease [49]. The blocking IL-1 $\beta$  protects bone and cartilage from progressive destruction in RA and its inhibition could be effective in the treatment of this disease [7].

**3.3. Effect of N-f-5HT and MTX on C-Reactive Protein (CRP) Level in Plasma on Day 28.** The AA model represents a model of polyarthritis, which expands to systemic inflammation [13]. Activation of T and B cells, macrophages, and inflammatory mediators TNF- $\alpha$ , IL-1 $\beta$ , and IL-6 aggravates the oxidative damage of the vital organs in rheumatoid arthritis, such as the liver. The liver, in turn, influences the systemic inflammation via producing inflammatory cytokines and mediators such as TNF- $\alpha$ , IL-1 $\beta$ , IL-6, NO, CRP, and LOX. IL-6, IL-1 $\beta$ , and TNF- $\alpha$  promote the synthesis of CRP in hepatocytes via STAT3 [51, 52] and NF- $\kappa$ B [53] pathways. The level of the systemic inflammatory parameter CRP in plasma, resulting from liver synthesis, was increased significantly in the group of arthritic animals compared with control animals in the chronic phase of the disease on experimental day 28 (AA versus CO, \*\*\* $p < 0.001$ ; Figure 1(b)). Administration of N-f-5HT and MTX significantly reduced the plasmatic levels of CRP on day 28 compared to the untreated group of arthritic animals (AA-N-f-5HT versus AA, + $p < 0.05$ ; AA-MTX versus AA, + $p < 0.05$ ; Figure 1(b)). Interaction of CRP with Fc-gamma receptors (Fc $\gamma$ R) Fc $\gamma$ RI and Fc $\gamma$ RIIA is known to promote the production of proinflammatory cytokines, resulting in the amplification loop of inflammatory reaction [54]. These processes are initiated through the induction of the receptor activator of nuclear factor- $\kappa$ B ligand (RANKL) protein and direct stimulation of osteoclastogenesis, causing a loop between inflammation and bone destruction in RA. CRP enhances both the proinflammatory response and bone destruction. In the treatment of RA, a lowered CRP level thus

not only is a significant parameter in terms of disease progression elimination but also has a direct impact on decreasing the degree of bone destruction [55].

**3.4. Effect of N-f-5HT and MTX on 12/15-LOX Activity in the Liver.** Alterations in the oxidative state lead to the activation of NF- $\kappa$ B and NF- $\kappa$ B-dependent genes, such as LOX. The enzyme 5-LOX catalyzes the conversion of arachidonic acid to leukotrienes, whose production has been associated with inflammation in arthritis. Suppression of 5-LOX expression ameliorates clinical parameters in RA and AA [56, 57]. A similar role can be attributed to 15-LOX [58]. Increased levels of NF- $\kappa$ B in the lung and liver as well as increased activity of LOX in the lung highlight the importance of extra-articular manifestations of AA [38]. In our experiment, liver 12/15 LOX activity increased in arthritic animals in comparison to healthy animals (AA versus CO, \*\*\* $p < 0.001$ ; Figure 1(c)). The effect of N-f-5HT on the activity of 12/15-LOX in liver homogenate was comparable with that of MTX. After administration of MTX or N-f-5HT, a significant decrease to control levels was assessed in the liver of the AA group (AA-N-f-5HT versus AA, +++ $p < 0.001$ ; AA-MTX versus AA, +++ $p < 0.001$ ; Figure 1(c)). Thus the anti-inflammatory effect of N-f-5HT in AA was supported by the ability of the molecule to inhibit 12/15-LOX activity. Similar to this result, recent observations also reported that several other flavonoids may act as LOX inhibitors [59].

**3.5. Effect of N-f-5HT and MTX on mRNA Expression of iNOS and TNF- $\alpha$  in the Liver.** In AA, the gene expression levels of TNF- $\alpha$  and iNOS produced in the liver were reported to increase [60, 61]. Also, in our study, the levels of TNF- $\alpha$  and iNOS mRNA expressions were significantly increased in arthritic animals (both \*\*\* $p < 0.001$ , AA versus CO; Figures 2(a) and 2(b)). It was proposed that these modifications in the liver of arthritic rats not only were a consequence of the metabolic alterations caused by the disease, especially the increased oxidative metabolism [17], but also depended on

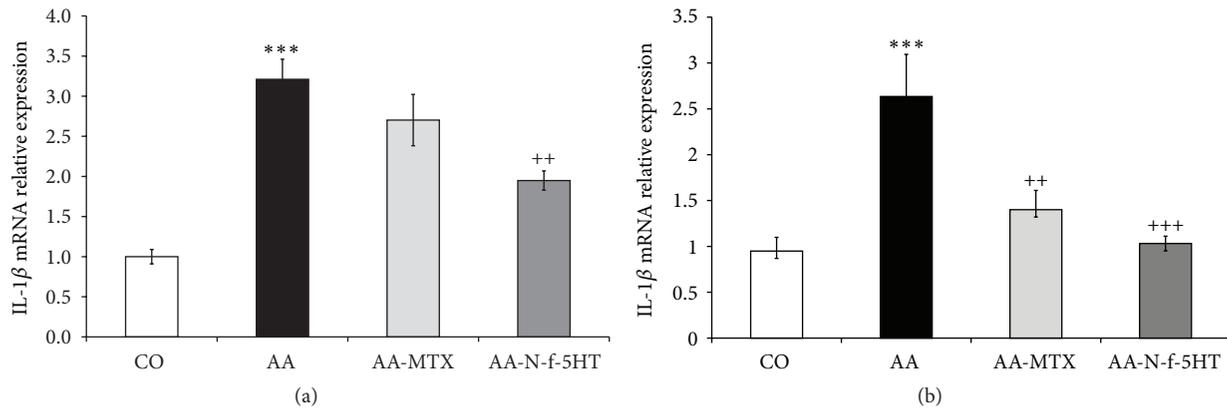


FIGURE 3: Relative changes of IL-1 $\beta$  mRNA expression normalized to  $\beta$ -actin mRNA in the liver (a) and in the spleen (b). Control was preset at 1. CO, control group; AA, adjuvant arthritis group; AA-N-f-5HT, adjuvant arthritis group given N-feruloylserotonin; AA-MTX, adjuvant arthritis group given methotrexate. The results are given as average  $\pm$  SEM;  $n = 8-10$ . The symbols \* and + show significant difference: \*\*\* $p < 0.001$  versus CO; ++ $p < 0.01$  versus AA; +++ $p < 0.001$  versus AA.

increased inflammatory parameters in the liver. The same agents that increase oxidative metabolism, TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and others [62], are responsible for increasing the activity of iNOS in several tissues. An increase of iNOS activity as a consequence of elevated iNOS mRNA expression was considered to play a dominant role in the pathogenesis of RA [4]. NO generation by iNOS induced in chondrocytes in the initial stage of AA may play a key role in triggering the subsequent events in arthritis [4]. In general, the use of NOS inhibitors has been shown to exert beneficial effects in experimentally induced arthritis [63]. However, which types of cells expressing iNOS are associated with the induction or progression of adjuvant-induced arthritis via NO generation remains uncertain. mRNA expression of iNOS in rat liver was reduced following MTX (AA-MTX versus AA, +++ $p < 0.001$ ; Figure 2(a)) and N-f-5HT treatment (AA-N-f-5HT versus AA, ++ $p < 0.01$ ; Figure 2(a)).

The effect of MTX treatment on TNF- $\alpha$  protein and mRNA expression differs among studies, depending on the conditions of the given study, concerning gender of patients, type of cell line, duration of treatment, MTX dose, and so forth [64]. In our study in the rat AA model, administration of MTX attenuated significantly the mRNA expression of TNF- $\alpha$  (AA-MTX versus AA, ++ $p < 0.01$ ; Figure 2(b)). In many patients, however, MTX treatment does not result in lower TNF- $\alpha$  plasma concentration. When MTX fails to produce an adequate response, newer therapies are used in combination with MTX. Blocking TNF- $\alpha$  with anti-TNF- $\alpha$  monoclonal antibodies significantly decreased the signs and symptoms of RA compared to placebo in RA patients with active disease receiving MTX [65, 66]. Thus, the N-f-5HT-driven significant reduction of TNF- $\alpha$  mRNA expression (A-N-f-5HT versus AA, ++ $p < 0.01$ ; Figure 2(b)) suggests an intriguing effect on RA treatment, calling for deeper investigation.

**3.6. Effect of N-f-5HT and MTX on mRNA Expression of IL-1 $\beta$  in Liver and Spleen.** Increase of mRNA expression was observed for IL-1 $\beta$  in the liver of arthritic animals (AA versus

CO, \*\*\* $p < 0.001$ ; Figure 3(a)) as expected [60]. Administration of MTX did not lead to significant attenuation of IL-1 $\beta$  transcription in the liver. This is in concert with previous studies of MTX function in different types of cells (e.g., human peripheral blood mononuclear cells and murine peritoneal and splenic cells) [67, 68]. On the other hand, MTX exhibits another mechanism of IL-1 $\beta$  function inhibition, which involves blocking the binding of IL-1 $\beta$  to IL-1 $\beta$  receptor in the membrane of peripheral blood cells (monocytes, lymphocytes, and granulocytes) [69]. Contrary to MTX, treatment with N-f-5HT led to a substantial inhibition of IL-1 $\beta$  gene expression (AA-N-f-5HT versus AA, ++ $p < 0.01$ ; Figure 3(a)).

Further, we examined IL-1 $\beta$  mRNA expression in the main immunocompetent organ, in the rat arthritic spleen, which has not been studied previously in terms of the AA model, related to IL-1 $\beta$  expression. We observed IL-1 $\beta$  mRNA expression activation comparable to that in the liver (AA versus CO, \*\*\* $p < 0.001$ ; Figure 3(b)). Interestingly, both MTX and N-f-5HT exhibited a significant and remarkably stronger inhibition of IL-1 $\beta$  mRNA expression in comparison to that in the liver (AA-MTX versus AA, ++ $p < 0.01$ ; AA-N-f-5HT versus AA, +++ $p < 0.001$ ; Figure 3(b)). In the spleen of N-f-5HT treated rats, the relative mRNA expression decreased even to control level.

Besides other events, MTX treatment leads to suppression of NF- $\kappa$ B, a heterodimer consisting of two subunits p65 and p50, one of the most prominent inflammatory transcription factors activated in RA [64]. This was confirmed in our previous work, along with the finding that also N-f-5HT (15 mg/kg) suppressed the activation of NF- $\kappa$ B (p65) in the arthritic rat liver [21, 33]. Interestingly, combination therapy (MTX + N-f-5HT) potentiated the effect of a single drug [33], suggesting different mechanisms leading to NF- $\kappa$ B inhibition. MTX driven reduction of cytokine transcription was attributed to abrogation of I $\kappa$ B $\alpha$  kinase activation and thereby suppression of I $\kappa$ B $\alpha$  (NF- $\kappa$ B inhibitor) phosphorylation and degradation, resulting in retaining the inactive NF- $\kappa$ B form

in cytoplasm [70]. However, the contribution of N-f-5HT to NF- $\kappa$ B pathway suppression needs to be further investigated.

Studies of the proposed pathways involved in the transcription of TNF- $\alpha$ , IL-1 $\beta$ , and iNOS in RA could help evaluate the mechanism of action of these drugs [6–8, 71, 72]. The gene expression of iNOS is mostly under the control of synergistically activating NF- $\kappa$ B (IL-1 $\beta$  and TNF- $\alpha$  stimulated) and STAT1 (IFN- $\gamma$  stimulated) key proinflammatory signals in the liver [6]. In contrast to iNOS, TNF- $\alpha$  does not contain the STAT binding element in its promoter region. Hence, its expression is under the control of NF- $\kappa$ B and AP-1 [7]. The inhibition of TNF- $\alpha$  and iNOS transcription observed in our study might be mostly attributed to the suppressed NF- $\kappa$ B pathway for both MTX and N-f-5HT [33, 64, 70]. However, the contribution of AP-1 to TNF- $\alpha$  and STAT1 for iNOS cannot be excluded.

MTX-dependent suppression of NF- $\kappa$ B was reported [33, 70, 73, 74], but in other cases MTX was not found to be effective in the attenuation of arthritic-increased mRNA expression of IL-1 $\beta$  [68, 75]. Taking into account our results, where MTX treatment did not lead to inhibition of IL-1 $\beta$  mRNA expression in the arthritic liver in contrast to the significant N-f-5HT impact, yet treatment of both MTX and N-f-5HT decreased the presumably NF- $\kappa$ B-dependent LOX activity and iNOS and TNF- $\alpha$  transcription to a similar extent, the involvement of N-f-5HT in another pathway for transcription regulation of this cytokine in the arthritic liver should be considered. After analysis of the reported pathways involved in the regulation of IL-1 $\beta$  mRNA expression, we hypothesized that TNF- $\alpha$ -driven AP-1 transcription factor activation or JAK/STAT3 pathway activated via IL-6 or IFN- $\gamma$  might play a role ([7, 8, 71, 72, 76, 77], Figure S1 in Supplementary Material available online at <http://dx.doi.org/10.1155/2016/7509653>). Papers reporting involvement of other polyphenols in anti-inflammatory regulation, for example, resveratrol, claim that these compounds exhibit their anti-inflammatory effect through suppression of NF- $\kappa$ B and JAK/STAT signaling pathways [78, 79].

The enhanced influence of MTX and N-f-5HT on IL-1 $\beta$  transcription in the spleen in comparison to the liver may be the consequence of different predominance of inflammatory pathways in this organ, presumably with a stronger NF- $\kappa$ B contribution. Details about the relevance of these pathways and the role of N-f-5HT in the transcription regulation of IL-1 $\beta$ , iNOS, and TNF- $\alpha$  in the liver and other organs in RA are to be further elucidated.

#### 4. Conclusions

The present study contributed additional evidence about the beneficial effect and mechanism of action of N-f-5HT and of MTX on a systemic inflammatory process in the liver and its association with the pathogenesis of adjuvant arthritis. N-f-5HT treatment led to amelioration of inflammatory parameters tested (plasmatic CRP and IL-1 $\beta$  protein levels, liver LOX activity, and liver and spleen cytokine expression). However, this did not result in a significant change of HPV, although a trend of improvement of the arthritic score was observed after 28 days. Chronic inflammation is an important mediator

of weight loss in the model of AA [80]. A synergistic effect of TNF- $\alpha$  and IL-1 $\beta$  was shown to influence the balance between protein degradation and protein synthesis causing among others an increase in resting energy expenditure and net efflux of amino acids from muscle to liver [81]. The significant increase of BWC in N-f-5HT treated rats, probably sign of the partial improvement of rheumatoid cachexia, might be the result of lowered mRNA expression of TNF- $\alpha$  and IL-1 $\beta$  determined in the arthritic liver. Moreover, taking into account the reported association of weight loss with the IL-1 $\beta$  production by splenic cells [80], the N-f-5HT mediated attenuation of increased IL-1 $\beta$  mRNA expression in the arthritic spleen might contribute to this complex process. The contribution of the affected expression of TNF- $\alpha$  and IL-1 $\beta$  originating from other organs cannot be excluded and is to be further elucidated.

Unexpectedly, chronic daily treatment with a high concentration of N-f-5HT (30 mg/kg) exhibited either a minor effect on the parameters examined and/or a strong variation among the animals (not shown) and that in contrast to a much lower concentration (3 mg/kg). Since N-f-5HT possesses a serotonin (5-hydroxytryptamine, 5-HT) moiety, the question if there might be some interplay between effects of these two molecules on RA pathogenesis is to be raised. Since N-f-5HT inhibited the increase of cytosolic free Ca<sup>2+</sup> concentration in rat vascular smooth muscle cells induced by serotonin mediated by 5-HT<sub>2</sub> receptors, it was hypothesized that at a sufficient concentration N-f-5HT may act as a competitive antagonist, which displaces serotonin from its binding site [82]. Intake of a high concentration of a 5-HT<sub>2</sub> receptor antagonist may lead to a variety of effects: it may influence the receptor density, even enhance the effect of serotonin, or lead to desensitization and with time to receptor resistance (through inhibitory feedback due to binding-induced enhanced production of serotonin) [83]. Interestingly, serotonin is known not only as a neurotransmitter. Increasing but contradictory reports associate serotonin with immunoinflammatory pathways in the periphery [84]. Serotonin, *via* its 5-HT<sub>2A</sub>, 5-HT<sub>2B</sub>, and 5-HT<sub>3</sub> receptors, has been implicated to have both proinflammatory and anti-inflammatory roles in a number of studies of rheumatoid arthritis [84–88]. The reported effects of 5-HT receptor antagonist on macrophage-like synovial cells encourage the interest to study the effect of N-f-5HT from this point of view [89]. To confirm this hypothesis, a precise characterization of interaction between N-f-5HT and 5-HT receptors is to be done.

On comparing the effects of the two drugs, administration of MTX (0.4 mg/kg) or N-f-5HT (3 mg/kg) was found to lead to a decrease of the main plasma marker of systemic inflammation CRP, the liver origin protein, and to inhibition of proinflammatory LOX in the liver. The impact of MTX and N-f-5HT on mRNA expression of TNF- $\alpha$ , IL-1 $\beta$ , and iNOS in the liver and on the level of CRP in plasma was mentioned at the conference [90]. MTX and N-f-5HT reduced the arthritis-increased transcription of TNF- $\alpha$  and iNOS in the liver to a comparable extent [90]. We suppose that the inhibition of TNF- $\alpha$  and iNOS transcription might be mostly attributed to the suppressed NF- $\kappa$ B pathway for the two drugs [21, 33, 70].

As previously reported [67, 68] and also proven by our study, MTX was not able to diminish the arthritic-induced IL-1 $\beta$  mRNA transcription in the liver [90]. This handicap might be compensated by coadministration of N-f-5HT, since this drug was shown to lower the level of proinflammatory cytokine IL-1 $\beta$  in plasma in the acute phase of AA and to attenuate significantly the elevation of IL-1 $\beta$  mRNA expression in the arthritic rat liver and spleen in the chronic phase. Detailed studies are required to confirm the hypothesis that N-f-5HT might function through potentially different mechanisms of inhibition of the inflammatory pathway NF- $\kappa$ B and not through MTX, as well as the possibility of an additional pathway influencing IL-1 $\beta$  transcription under control of N-f-5HT but not MTX. The confirmation would support N-f-5HT as a promising agent for the treatment of RA in combination therapy with MTX. The positive effect was shown in our previous study, where N-f-5HT markedly potentiated the therapeutic effect of low-dose MTX [33]. As the therapeutic dose of MTX was used in this study and the purpose of combination study is to lower the MTX dose to decrease the side effects of this drug, the effect of combination therapy was not included.

Oral daily intake of N-f-5HT could overcome the inconvenient administration and high costs of biological therapy using IL-1 $\beta$  monoclonal antibody, which was shown in clinical trials to be superior to placebo in combination with MTX in reducing signs, symptoms, and radiographic progression in patients with advanced RA [91, 92]. Future studies of N-f-5HT mechanisms of action should shed more light on the immunomodulatory function of this natural polyphenol. It is to be expected that N-f-5HT is able to positively affect the activity of other markers of inflammation and oxidative stress not only in the liver and spleen but also in other organs (lung, brain, etc.), a hypothesis to be tested by future work. However, to establish the optimal dosing in light of the effects achieved is of primary importance.

## Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

## Authors' Contributions

L'udmila Pašková and Viera Kuncírová contributed equally to the presented work.

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## Review Article

# Effects of Adoptive Transfer of Tolerogenic Dendritic Cells on Allograft Survival in Organ Transplantation Models: An Overview of Systematic Reviews

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**Objective.** To dissect the efficacy of Tol-DC therapy with or without IS in multiple animal models of transplantation. **Methods and Results.** PubMed, Medline, Embase, and the Cochrane Library were searched for reviews published up to April 2015. Six systematic reviews and a total of 61 articles were finally included. Data were grouped by organ transplantation models and applied to meta-analysis. Our meta-analysis shows that Tol-DC therapy successfully prolonged allograft survival to varying extents in all except the islet transplantation models and with IS drugs further prolonged the survival of heart, skin, and islet allografts in mice, but not of heart allografts in rats. Compared with IS drugs alone, Tol-DC therapy with IS extended islet allograft survival in rats but failed to influence the survival of skin, small intestine, and heart allografts in rats or of heart and skin allografts in mice. **Conclusion.** Tol-DC therapy significantly prolonged multiple allograft survival and further prolonged survival with IS. However, standardized protocols for modification of Tol-DC should be established before its application in clinic.

## 1. Introduction

Transplantation is one of the most effective methods of extending life for patients with end-stage organ failure. However, the immunosuppressive (IS) agents commonly used to prevent graft-versus-host disease and host-versus-graft disease compromise the recipient's immune system and are associated with side effects such as infection and recurrence of disease, thus decreasing the patient's quality of life. For this reason, induction of donor-specific tolerance without impairment of immune defense remains the holy grail of transplantation research.

Dendritic cells (DCs), first described in 1973 [1], are the most effective antigen-presenting cells and are key regulators of a balanced immune system by virtue of their dual immunogenic and tolerogenic functions. Immunogenic DCs have been developed as positive therapeutic vaccines to elicit antitumor responses. The first DC vaccine, sipuleucel-T

(PROVENGE®), was approved by the FDA in 2010 and has since been successfully used in prostate cancer treatment [2]. In contrast, tolerogenic DCs (Tol-DCs) lack essential costimulatory signals and/or express inhibitory signals and play a role in tolerance induction. Evidence indicates that Tol-DCs have great therapeutic potential in autoimmunity and allergy [3]. To date, several phase I trials assessing safety of Tol-DCs in rheumatoid arthritis and refractory Crohn's disease patients were conducted [4, 5]. Moreover, mounting evidence shows that Tol-DCs are able to induce donor-specific T cell hyporesponsiveness and prolong allograft survival. As such, negative vaccines based on Tol-DCs have great potential to prevent transplant rejection. The safety of autologous Tol-DCs has so far been demonstrated in type I diabetes patients [6] and is currently being tested by Moreau et al. in kidney transplant recipients [7]. However, whether Tol-DCs can effectively prolong allograft survival and show superiority to other forms of IS therapy remains controversial. Here,

we present the results of a meta-analysis of the efficacy of Tol-DCs in multiple animal models of transplantation. We evaluated allograft survival time after treatment with Tol-DCs alone, compared the relative superiority of single therapy with Tol-DCs or IS, and looked for evidence of synergy between Tol-DC and IS therapy in promoting allograft survival.

## 2. Methods

**2.1. Criteria for Considering Reviews for Inclusion and Exclusion.** We included systematic reviews that focused on the effects of Tol-DC injection on allograft survival compared with untreated groups in any kind of transplantation model. To be included, the reviews had to describe the outcome of interest.

**2.2. Search Methods for Identification of Reviews.** Comprehensive literature searches were conducted in PubMed, Medline, Embase, and the Cochrane Library from database inception until April 2015. We identified relevant systematic reviews using the following as MeSH or text words: “transplantation,” “dendritic cells,” “tolerance,” and “review.” To ensure comprehensive and up-to-date coverage of the evidence base and to make recommendations for future reviews, we also searched for and considered primary articles that were potentially eligible for, but not yet included in, published reviews.

**2.3. Selection of Reviews and Articles.** We screened reviews according to the inclusion criteria above and also included new primary studies, excluding duplicates and those already included in the reviews. For primary articles, we included only those that provided data applicable to meta-analysis on (i) Tol-DCs versus untreated and/or (ii) Tol-DCs in combination with IS agents (including immunosuppressive drugs and/or costimulatory blockers) versus Tol-DCs alone and (iii) Tol-DCs in combination with IS agents versus IS alone. We also excluded studies that were included in the reviews but did not provide data applicable to meta-analysis.

**2.4. Data Extraction.** For the eligible reviews, two reviewers independently extracted information on author name, publication year, transplantation model, outcomes measured, whether a meta-analysis was conducted, and quality assessment of the original articles. For primary articles, information was extracted on transplantation model, interventions, group comparisons, and outcomes measured. Disagreements were resolved by consensus.

**2.5. Quality Assessment of Systematic Reviews.** The methodological quality of the included systematic reviews was appraised by two independent reviewers using the Assessment of Multiple Systematic Reviews (AMSTAR) tool [9]. AMSTAR consists of 11 questions, each with “Yes,” “No,” “Can’t Answer,” or “Not Applicable” answers, and checks for the following items: (1) “a priori” study design; (2) duplicate reviewers for study selection and data extraction; (3) comprehensive literature search; (4) publication status as

an inclusion criterion (i.e., gray or unpublished literature); (5) list of studies included/excluded; (6) characteristics of the included studies; (7) scientific quality assessment and documentation; (8) appropriate formulation of conclusions (based on methodological rigor and scientific quality of the studies); (9) appropriate methods of combining studies (homogeneity test, effects model, and sensitivity analysis); (10) assessment of publication bias (graphic and/or statistical test); and (11) inclusion of conflict of interest statement. Disagreements were resolved by consensus.

**2.6. Data Synthesis.** Data were divided into six groups according to the transplantation model and then further divided into subgroups based on animal species. For each model, we grouped the data by intervention as follows: Tol-DCs versus untreated, Tol-DCs in combination with IS versus Tol-DCs, and Tol-DCs in combination with IS versus IS. The primary end point of our meta-analysis was allograft survival time. For each study, we calculated the summary mean difference and 95% confidence intervals (CI) for the end point. We pooled studies using a random effects model, making the assumption that individual studies estimated different treatment effects. We examined heterogeneity in the main analysis and subgroup analysis by  $Q$  statistic and  $I^2$  index. Three articles were excluded from our summary table (Table 3) and discussion because they contained only a single set of data and the evidence was too weak to be included [16–18]. However, data from those articles are mentioned individually in the Results.

**2.7. Ethics.** No ethical approval was required.

## 3. Results

**3.1. Results of Search and Selection.** Our research identified 1121 reports, of which 87 were excluded as duplicates. Screening by the titles and abstracts, we excluded 1027 articles for irrelevant themes or unwanted article types and 7 were selected to be read in their entirety. Of those, 1 systematic review was excluded for irrelevant theme and 6 systematic reviews assessing the efficacy of Tol-DC treatment in animal models of heart, liver, kidney, small intestine, skin, and islet transplantation satisfied our inclusion and exclusion criteria and were further evaluated (Figure 1) [10–15]. Of the 112 studies included in the six systematic reviews, 65 studies were excluded because of inadequate data for meta-analysis (heart 28, skin 16, kidney 9, islet 8, small intestine 3, and liver 2), and the remaining 47 studies were included in our overview [8, 16, 17, 19–63]. We also included 14 newly identified primary articles (heart 8 [64–71], skin 3 [8, 71, 72], and islet 3 [18, 73, 74]). Thus, we evaluated a total of 61 studies (Table 1).

**3.2. Description of Included Reviews.** Of the six included reviews, which were published between 2012 and 2014, only one conducted a meta-analysis [10–15]. The remaining five had incomplete information, such as omission of sample size or standard deviation, and applied semiquantitative methods to analyze the collected data. The kidney and islet

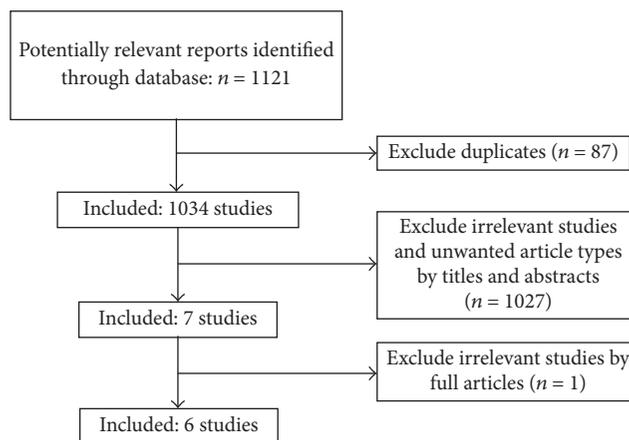


FIGURE 1: Flow diagram of searching and selection for included systematic reviews.

transplantation studies included both mouse and rat models, whereas the skin and heart studies included only mouse models and the small intestine and liver studies included only rat models (Table 1). Studies using either model were eligible for our overview.

**3.3. Methodological Quality of Included Reviews.** We assessed the methodological quality of the six included reviews using AMSTAR. The scores ranged from 5 to 8, with points deducted for Item 4 (status of the publication as an inclusion criterion), Item 5 (list of studies included/excluded), Item 9 (appropriate methods of combining studies), and Item 10 (assessment of publication bias) (Table 2). Although the systematic reviews are of only moderate to high quality, it should be borne in mind that there are no conventional criteria for quality assessment of animal studies and no clinical data are available for an equivalent analysis of humans.

### 3.4. Effects of Interventions on the Survival of Organ Allografts

**3.4.1. Liver Transplantation Models.** In rats, infusion of Tol-DCs promoted liver allograft survival for an additional 18 days compared with no treatment (mean and 95% CI; 18.17, 11.02 to 25.33) (Figure 3). One study (excluded from the overall evaluation) reported that Tol-DC + IS therapy was more effective in prolonging graft survival than either Tol-DCs or IS alone (mean  $\pm$  SD, 112 days  $\pm$  19.0 versus 58  $\pm$  3.7 versus 54  $\pm$  2.4, resp.) [16].

**3.4.2. Renal Transplantation Models.** Tol-DC therapy prolonged renal graft survival by 17 days in rats (17.72, 13.35 to 22.10) (Figure 3). Moreover, one study reported that Tol-DCs + IS extended graft survival significantly longer than Tol-DCs or IS alone (38.7 days  $\pm$  40.0 versus 5.0  $\pm$  2.2 versus 7.5  $\pm$  1.2, resp.) [17].

**3.4.3. Heart Transplantation Models.** Heart grafts survived 14 days longer in Tol-DC-infused rats than in untreated rats

(14.21, 6.11 to 22.31) (Figure 2). However, Tol-DC + IS therapy failed to further prolong allograft survival compared with Tol-DCs alone (60.21 days,  $-43.78$  to 164.20) (Figure 4) or IS alone (57.56 days,  $-59.15$  to 174.27) (Figure 5). In mice, infusion of Tol-DCs extended graft survival by 11 days (11.61, 7.73 to 15.49) (Figure 2). Tol-DC + IS therapy extended graft survival compared with Tol-DCs alone (5.05 days, 1.53 to 8.57) (Figure 4) but not with IS alone (1.72 days,  $-3.67$  to 7.10) (Figure 5).

**3.4.4. Small Intestine Transplantation Models.** In rats, Tol-DC therapy prolonged graft survival by 8 days (8.89, 6.16 to 11.61) (Figure 3); however, Tol-DC + IS therapy failed to promote graft survival longer than IS therapy alone (8.97 days,  $-3.75$  to 21.07) (Figure 5).

**3.4.5. Islet Transplantation Models.** Infusion of Tol-DCs failed to prolong allograft survival in rats (7.28 days,  $-2.91$  to 17.46) (Figure 3). However, Tol-DC + IS therapy was significantly better than Tol-DCs or IS alone in prolonging graft survival (137.49 days, 96.59 to 178.40 and 177.83 days, 160.05 to 195.62, resp.) (Figures 4 and 5). In mice, Tol-DC therapy prolonged allograft survival by 6 days (6.81, 2.97 to 10.64) (Figure 3). One included study reported that Tol-DCs + IS facilitated graft survival for significantly longer than Tol-DCs or IS alone (77.4 days  $\pm$  10.7 versus 24.9  $\pm$  4.5 versus 38.9  $\pm$  6.1, resp.) [18].

**3.4.6. Skin Transplantation Models.** The systematic reviews did not include studies of the effects of Tol-DC therapy alone on skin allograft survival in rats. Nevertheless, our analysis indicates that graft survival was no better in rats treated with Tol-DC + IS therapy than with IS alone (7.15 days,  $-3.84$  to 18.13) (Figure 5). In mice, Tol-DC therapy prolonged graft survival by 5 days (5.45, 2.31 to 8.59) (Figure 3), and Tol-DCs + IS had a significantly better outcome compared with Tol-DCs alone (3.84 days, 3.40 to 4.29) (Figure 4) but not with IS alone (0.45 days, 0.00 to 0.89) (Figure 5).

TABLE 1: Characteristics of included systematic reviews.

Included reviews	Tx models	Animal models		Data synthesis	Included studies	Excluded studies	Potential new studies	Final included studies
Wu et al. 2012 [10]	Heart	Mice	Included		44	28	2	
		Rats	Not included		0	0	6	
		<i>Total</i>		Description	44	28	8	24
Sun et al. 2012 [11]	Islet	Mice	Included		9	7	3	
		Rats	Included		4	1	0	
		<i>Total</i>		Description	13	8	3	8
Xia et al. 2014 [12]	Liver	Mice	Not reported		0	0	0	
		Rats	Included		7	2	0	
		<i>Total</i>		Description	7	2	0	5
Xia et al. 2013 [13]	Renal	Mice	Included		5	5	0	
		Rats	Included		11	4	0	
		<i>Total</i>		Description	16	9	0	7
Zhou et al. 2013 [14]	Skin	Mice	Included		21	15	3	
		Rats	Not included		0	0	0	
		<i>Total</i>		Description	21	15	3	9
Sun et al. 2013 [15]	Small intestine	Mice	Not reported		0	0	0	
		Rats	Included		11	3	0	
		<i>Total</i>		Description & meta-analysis	11	3	0	8
<i>Total</i>	5				112	65	14	61

TABLE 2: Methodological quality assessment of systematic review.

SR	Model	Methodological quality assessment of the included systematic reviews, AMSTAR items											Rating
		1	2	3	4	5	6	7	8	9	10	11	
Sun et al. [15]	Small intestine	Yes	Yes	Yes	No	No	Yes	Yes	Yes	No	Yes	Yes	8
Zhou et al. [14]	Skin	Yes	Yes	Yes	No	No	Yes	Yes	Yes	N/A	No	Yes	7
Sun et al. [11]	Islet	Yes	Yes	Yes	No	No	Yes	Yes	Yes	N/A	No	Yes	7
Wu et al. [10]	Heart	Yes	Yes	Yes	No	No	Yes	Yes	No	N/A	No	Yes	6
Xia et al. [13]	Renal	Yes	No	Yes	No	No	Yes	Yes	Yes	N/A	No	Yes	6
Xia et al. [12]	Liver	Yes	No	Yes	No	No	Yes	Yes	No	N/A	No	Yes	5
Total		6	4	6	0	0	6	6	4	0	1	6	
%		100%	67%	100%	0%	0%	100%	100%	67%	0%	17%	100%	

N/A: not applicable. There are 11 items in total, "Yes" making 1 score and "No" or "N/A" 0.

### 3.5. Effects of Different Interventions on Allograft Survival

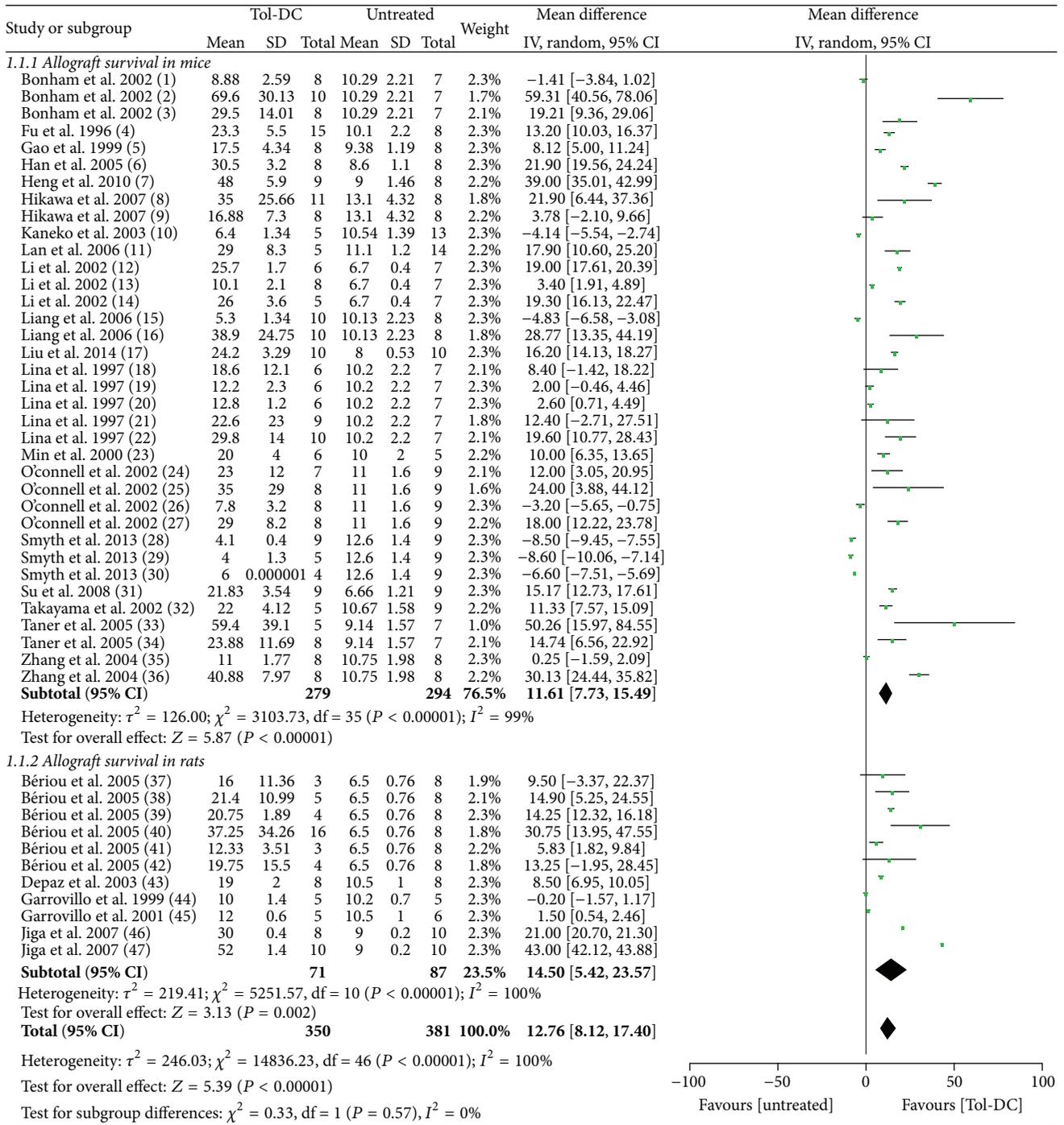
**3.5.1. Tol-DCs versus No Treatment.** Tol-DC therapy prolonged allograft survival in all transplantation models in rats and/or mice, with the exception of the islet transplantation model in rats. Ranked in order from longest to shortest allograft survival time, Tol-DC therapy was most efficacious for liver, kidney, heart, small intestine, and islet allografts in rats and heart, islet, and skin allografts in mice (Table 3, Figures 2 and 3).

**3.5.2. Tol-DCs + IS versus Tol-DCs Alone.** In rats, Tol-DCs + IS further prolonged the survival of islet allografts (137.49 days, 96.59 to 178.40), but not heart allografts (60.21 days, -43.78 to 164.20), compared with Tol-DCs alone. In mice, Tol-DCs + IS were superior to Tol-DCs alone in prolonging survival of both heart and skin allografts (5.05 days, 1.53 to 8.57 versus 3.84 days, 3.40 to 4.29, resp.) (Table 3 and Figure 4). Three studies of mouse islet [18], rat liver [16], and rat kidney [17] transplantation models reported better outcomes with Tol-DCs + IS than with Tol-DCs alone.

TABLE 3: Effects of Tol-DC alone and Tol-DC in combination with IS on allografts survival. Values are mean difference (95% confidence intervals).

Transplantation models	Animal models	Tol-DC versus untreated	Q statistic	I <sup>2</sup> index	Tol-DC + IS versus Tol-DC	Q statistic	I <sup>2</sup> index	Tol-DC + IS versus IS	Q statistic	I <sup>2</sup> index
Liver Tx	Rats	18.17 (11.02 to 25.33)	$P < 0.00001$	High (95%)	NA*	NA	NA	NA*	NA	NA
	Rats	17.72 (13.35 to 22.10)	$P < 0.00001$	High (99%)	NA*	NA	NA	NA*	NA	NA
Heart Tx	Rats	14.21 (6.11 to 22.31)	$P < 0.00001$	High (100%)	60.21 (-43.78 to 164.20)	$P < 0.00001$	High (100%)	57.56 (-59.15 to 174.27)	$P < 0.00001$	High (100%)
	Mice	11.61 (7.73 to 15.49)	$P < 0.00001$	High (99%)	5.05 (1.53 to 8.57)	$P < 0.00001$	High (91%)	1.72 (-3.67 to 7.10)	$P < 0.00001$	High (93%)
	Total	12.78 (8.30 to 17.26)	$P < 0.00001$	High (100%)	31.47 (-33.22 to 96.16)	$P < 0.00001$	High (100%)	34.67 (-24.09 to 93.42)	$P < 0.00001$	High (100%)
Small intestine Tx	Rats	8.89 (6.16 to 11.61)	$P < 0.00001$	High (87%)	—	—	—	8.97 (-3.75 to 21.07)	$P < 0.00001$	High (99%)
Islet Tx	Rats	7.28 (-2.91 to 17.46)	$P < 0.00001$	High (98%)	137.49 (96.59 to 178.40)	$P = 0.75$	Low (0%)	177.83 (160.05 to 195.62)	$P = 0.22$	Low (33%)
	Mice	6.81 (2.97 to 10.64)	$P = 0.01$	High (70%)	NA*	NA	NA	NA*	NA	NA
	Total	7.70 (1.49 to 13.92)	$P < 0.00001$	High (97%)	108.21 (46.57 to 169.86)	$P = 0.0009$	High (82%)	133.26 (34.64 to 231.88)	$P < 0.00001$	High (100%)
Skin Tx	Mice	5.45 (2.31 to 8.59)	$P < 0.00001$	High (94.7%)	3.84 (3.40 to 4.29)	$P = 0.22$	Low (34%)	0.45 (0.00 to 0.89)	$P = 0.22$	Low (35%)
	Rats	—	—	—	—	—	—	7.15 (-3.84 to 18.13)	$P < 0.00001$	High (93%)
	Total	—	—	—	—	—	—	4.03 (-1.60 to 9.65)	$P < 0.00001$	High (92%)

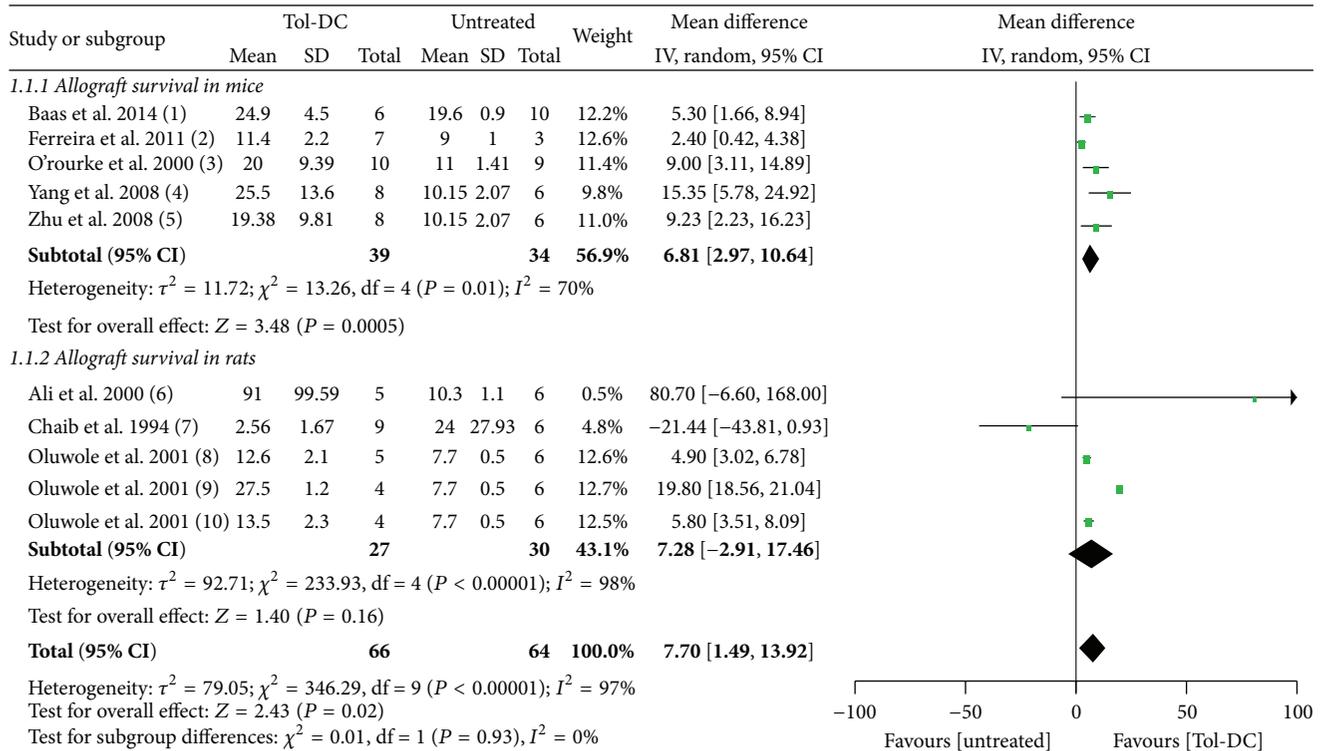
—: no data. NA: not applicable. \* Only one set of data in the subgroup.



Footnotes

- |   |   |   |   |
|---|---|---|---|
| (1) CTLA4lg-DC                                | (13) IL-3+GM-CSF+Mitomycin-C+DC                 | (25) GM-CSF+LPS+mCD8 $\alpha^+$ DC                    | (37) DC( $7 \times 10^6$ , injection on days -1 and 4)      |
| (2) (si-NF- $\kappa$ B decoy ODN+CTLA4lg)-DC  | (14) GM-CSF+Mitomycin-C+DC                      | (26) GM-CSF+LPS+mCD8 $\alpha^+$ DC                    | (38) DC( $15 \times 10^6$ )                                 |
| (3) si-NF- $\kappa$ B decoy ODN-DC            | (15) CD11c $^+$ DC                              | (27) GM-CSF+donor spleen iCD8 $\alpha^+$ DC           | (39) DC( $3 \times 10^6$ )                                  |
| (4) GM-CSF+DC                                 | (16) Donor liver B220 $^+$ DC                   | (28) Dex+D3+LPS+DC(B6D2F1)                            | (40) DC( $7 \times 10^6$ , injection on days -1)            |
| (5) Donor spleen DC coculture with leucocytes | (17) mPD-L1-Ig+DC                               | (29) Dex+D3+DC(BALB/c)                                | (41) DC( $7 \times 10^6$ , injection on days -1, 6, and 13) |
| (6) Mycophenolate mofetil+DC                  | (18) GM-CSF+TGF- $\beta$ +DC( $1 \times 10^6$ ) | (30) Dex+D3+DC(B6D2F1)                                | (42) DC( $7 \times 10^6$ , injection on day 0)              |
| (7) FITC20+DC                                 | (19) GM-CSF+TGF- $\beta$ +DC( $4 \times 10^6$ ) | (31) NBD peptide+DC                                   | (43) Low dose GM-CSF+DC                                     |
| (8) GM-CSF+DC                                 | (20) GM-CSF+IL-4+DC( $2 \times 10^6$ )          | (32) TGF- $\beta$ 1-DC                                | (44) Donor MHC1 peptide+ recipient BMDC                     |
| (9) GM-CSF+IL-4+DC                            | (21) GM-CSF+TGF- $\beta$ +DC( $8 \times 10^6$ ) | (33) AlloAg+Rapa+DC(injection on days -10, -3, and 0) | (45) Donor MHC1 peptide+ recipient thymus DC                |
| (10) IL-4-DC                                  | (22) GM-CSF+TGF- $\beta$ +DC( $2 \times 10^6$ ) | (34) AlloAg+Rapa+DC(injection on day -7)              | (46) Mitomycin+DC   |
| (11) GM-CSF+IL-10+TGF- $\beta$ +LPS+DC        | (23) FasL-DC                                    | (35) IL-10-DC(i.v.)                                   | (47) Anti-CD80/86/ICAM1+Mitomycin+DC                        |
| (12) IL-4+GM-CSF+Mitomycin-C+DC               | (24) GM-CSF+iCD8 $\alpha^+$ DC                  | (36) IL-10-DC(p.v.)                                   |   |

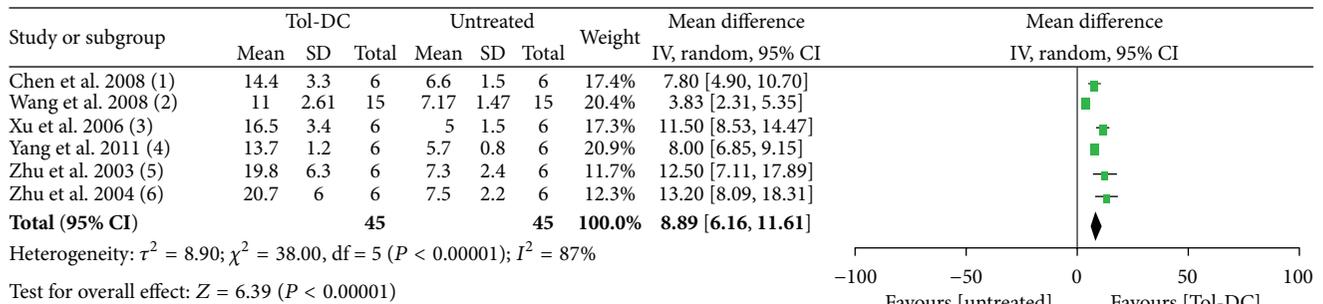
FIGURE 2: Mean difference (95% confidence intervals) for Tol-DC infusion versus untreated groups for allografts survival in heart. All studies involved used MHC complete mismatch models either in rats or in mice. In the footnotes, “-” before “DC” means genetic modification and “+” means substances added in the culture medium. If not specialized, DC refers to donor bone marrow DC; when injection time is involved, we take the transplantation day as day 0. i.v.: intravenous; p.v.: portal vein.



Footnotes

- (1) GM-CSF+recipient adherent BMDC
- (2) IFN $\gamma$ +LPS+1,25 D $_3$ +donor Ag+recipient BMDC
- (3) CTLA4lg-DC(donor-derived cell line)
- (4) Donor thymic CTLA4lg-DC
- (5) Thymic IL-10-DC
- (6) Donor MHC I peptide+recipient BMDC
- (7) Donor spleen DC
- (8) Donor MHC I peptide+recipient BMDC( $1 \times 10^6$ )
- (9) Donor MHC I peptide+recipient thymus DC( $5 \times 10^6$ )
- (10) Donor MHC I peptide+recipient BMDC( $2 \times 10^6$ )

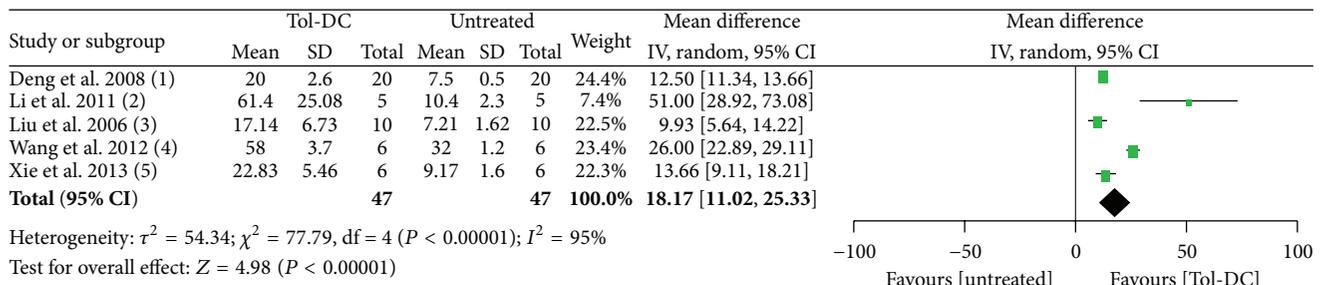
(a) Islet transplantation



Footnotes

- (1) Triptolide+DC
- (2) iBMDC
- (3) si-IL-12 p35-DC
- (4) LPS+si-M $\gamma$ D88-DC
- (5) Donor spleen IL10-DC
- (6) IL10-DC

(b) Small intestine transplantation in rats

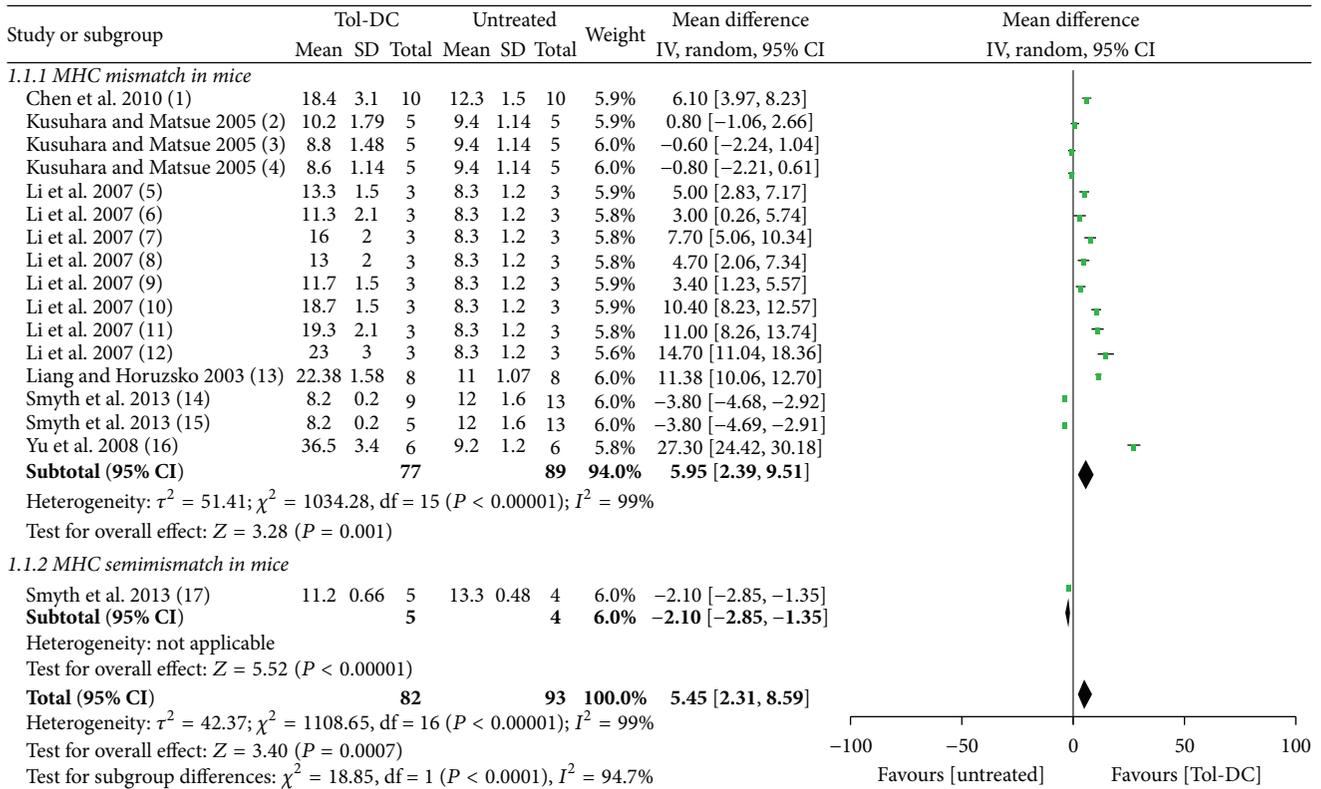


Footnotes

- (1) IL-10+DC
- (2) IL-10+recipient BMDC
- (3) TGF- $\beta$ 1-DC
- (4) iBMDC
- (5) si-RelB-DC

(c) Liver transplantation in rats

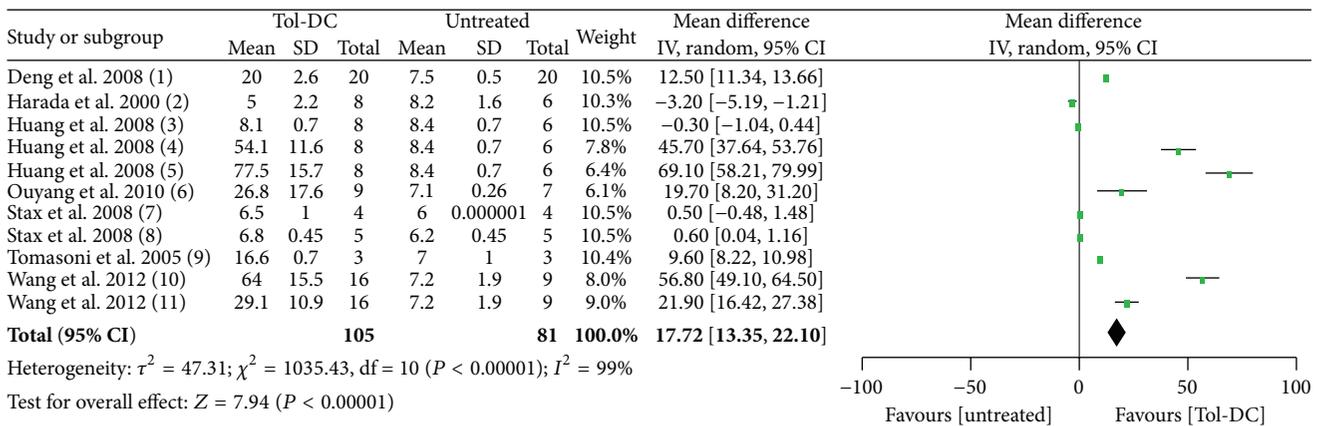
FIGURE 3: Continued.



Footnotes

- |   |  |                                       |
|---|--|---------------------------------------|
| (1) Tetrandrine+DC                                | (7) 10 $\mu$ g/L IL-10+donor spleen DC                 | (13) PIR-B+DC                         |
| (2) CD95L-DC(donor skin derived cell line) (i.v.) | (8) 30 $\mu$ g/L IL-10+20 mg/L Medron+donor spleen DC  | (14) Dex+D3+LPS+DC(B6K <sup>d</sup> ) |
| (3) CD95L-DC(donor skin derived cell line) (i.p.) | (9) 10 $\mu$ g/L IL-10+20 mg/L Medron+donor spleen DC  | (15) Dex+D3+DC(B6K <sup>d</sup> )     |
| (4) CD95L-DC(donor skin derived cell line) (s.c.) | (10) 10 $\mu$ g/L IL-10+10 mg/L Medron+donor spleen DC | (16) IDO-DC                           |
| (5) 10 mg/L Medron+donor spleen DC                | (11) 30 $\mu$ g/L IL-10+donor spleen DC                | (17) Dex+D3+DC(K <sup>bm1</sup> )     |
| (6) 20 mg/L Medron+donor spleen DC                | (12) 30 $\mu$ g/L IL-10+10 mg/L Medron+donor spleen DC |                                       |

(d) Skin transplantation

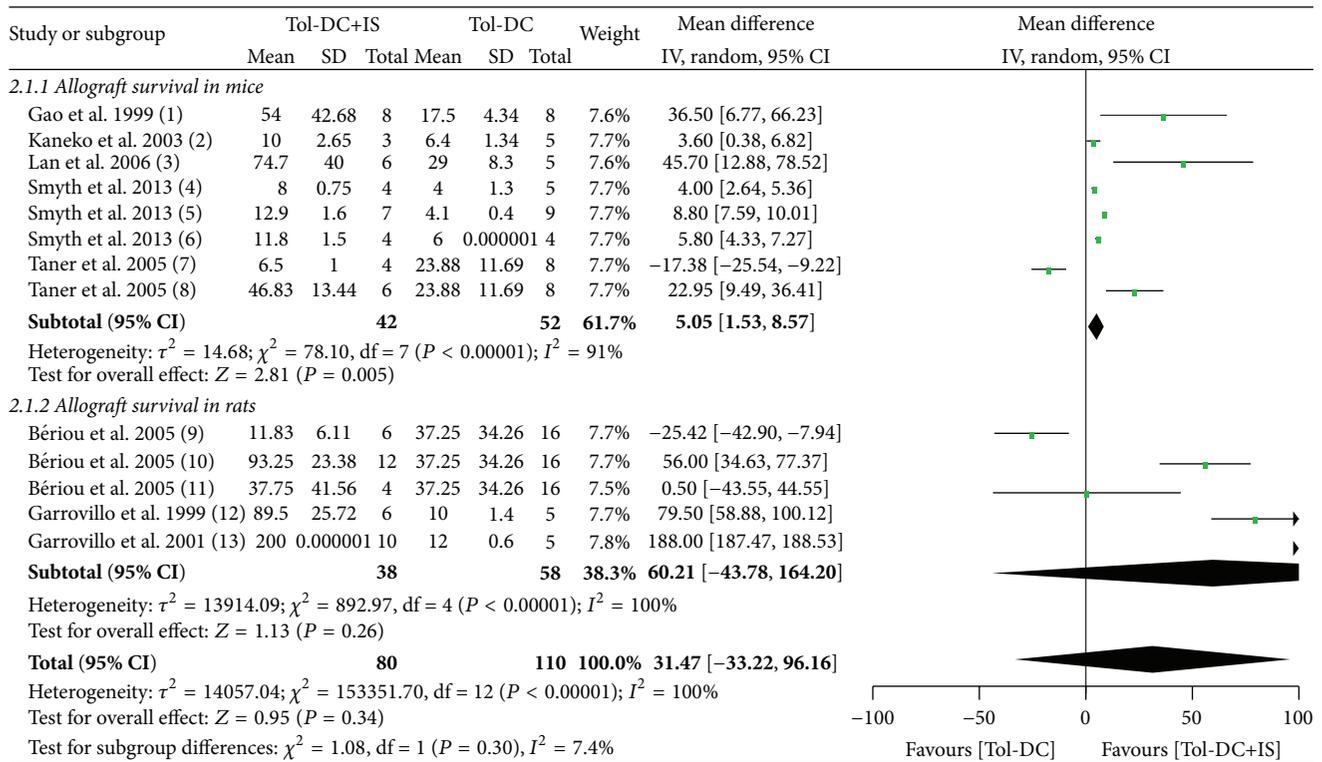


Footnotes

- |                            |                                      |                                       |
|----------------------------|--------------------------------------|---------------------------------------|
| (1) IL-10+DC               | (5) Donor and recipient CTLA4lg-BMDC | (9) dnIKK2-DC                         |
| (2) Donor spleen DC        | (6) IKK2dn-DC                        | (10) Donor antigen+CD4+recipient BMDC |
| (3) Recipient CTLA4lg-BMDC | (7) Dex + donor(Dark Agouti) DC      | (11) CD4+recipient BMDC               |
| (4) CTLA4lg-DC             | (8) Dex + donor(Brown Norway) DC     |                                       |

(e) Renal transplantation in rats

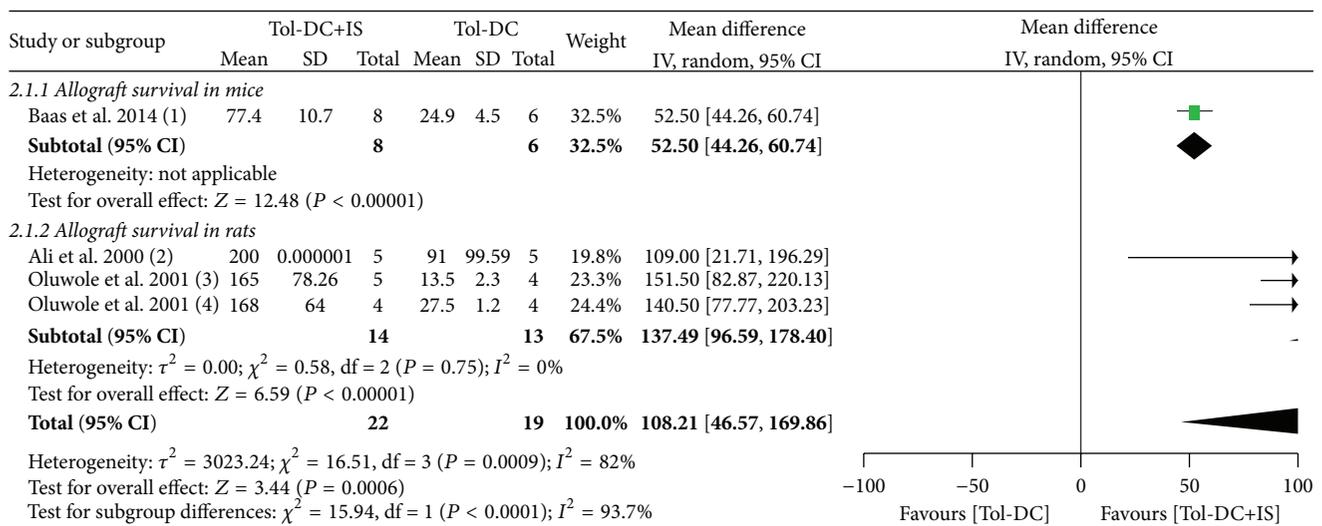
FIGURE 3: Mean difference (95% confidence intervals) for Tol-DC infusion versus untreated groups for allografts survival in islet (a), small intestine (b), liver (c), skin (d), and renal (e) transplantation models. Except for MHC semimismatch model in skin transplantation group, all other studies involved used MHC complete mismatch models either in rats or in mice. In the footnotes, “-” before “DC” means genetic modification and “+” means substances added in the culture medium. If not specialized, DC refers to donor bone marrow DC; when injection time is involved, we take the transplantation day as day 0. i.v.: intravenous; i.p.: intraperitoneal; s.c.: subcutaneous.



Footnotes

- |   |  |   |
|---|--|---|
| (1) (Donor spleen DC coculture with leucocytes) + CD4 mAb | (6) (Dex+D3+DC(B6D2F1))anti-CD8 antibody | (10) (DC) + LF 10-day treatment                       |
| (2) (IL-4-DC) + anti-CD40L mAb                            | (7) (alloAg+Rapa+DC) + IL-2              | (11) (DC) + LF 5-day treatment                        |
| (3) (GM-CSF+IL-10+TGF- $\beta$ +LPS+DC) + CTLA4-Ig        | (8) (alloAg+Rapa+DC) + FK506             | (12) (Donor MHC I peptide+ recipient BMDC) + ALS      |
| (4) (Dex+D3+DC(BALB/c)) + anti-CD8 antibody               | (9) (DC) + RAPA                          | (13) (Donor MHC I peptide+ recipient thymus DC) + ALS |
| (5) (Dex+D3+LPS+DC(B6D2F1))anti-CD8 antibody              |  |   |

(a) Heart transplantation



Footnotes

- |  |  |
|--|--|
| (1) (GM-CSF+recipient adherent BMDC) + anti-CD3 Ab | (3) (Donor MHC I peptide+recipient BMDC( $2 \times 10^6$ )) + ALS      |
| (2) (Donor MHC I peptide+recipient BMDC) + ALS     | (4) (Donor MHC I peptide+recipient thymus DC( $5 \times 10^5$ )) + ALS |

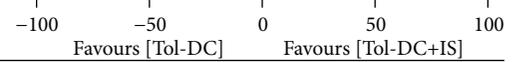
(b) Islet transplantation

FIGURE 4: Continued.

Study or subgroup	Tol-DC+IS			Tol-DC			Weight	Mean difference IV, random, 95% CI	Mean difference IV, random, 95% CI
	Mean	SD	Total	Mean	SD	Total			
Smyth et al. 2013 (1)	12.4	0.8	5	8.2	0.2	5	28.8%	4.20 [3.48, 4.92]	
Smyth et al. 2013 (2)	11.9	0.6	14	8.2	0.2	9	71.2%	3.70 [3.36, 4.04]	
<b>Total (95% CI)</b>			<b>19</b>			<b>14</b>	<b>100.0%</b>	<b>3.84 [3.40, 4.29]</b>	

Heterogeneity:  $\tau^2 = 0.04$ ;  $\chi^2 = 1.50$ ,  $df = 1$  ( $P = 0.22$ );  $I^2 = 34\%$   
 Test for overall effect:  $Z = 16.97$  ( $P < 0.00001$ )

Footnotes  
 (1) (Dex+D3+DC(B6K<sup>d</sup>)) + anti-CD8 antibody  
 (2) (Dex+D3+LPS+DC(B6K<sup>d</sup>)) + anti-CD8 antibody



(c) Skin transplantation in mice

FIGURE 4: Mean difference (95% confidence intervals) for Tol-DC in combination with IS versus Tol-DC alone for allografts survival in heart (a), islet (b), and skin (c) transplantation models. In the footnotes, “-” before “DC” means genetic modification and “+” inside the parentheses means substances added in the culture medium and “+” outside the parentheses means combined IS agents. If not specialized, DC refers to donor bone marrow DC.

**3.5.3. Tol-DCs + IS versus IS Alone.** In rats, Tol-DCs + IS led to better outcomes than IS alone in the survival of islet allografts, but not of heart, skin, or small intestine allografts, whereas in mice, Tol-DCs + IS were not significantly better than IS alone in prolonging the survival of heart or skin allografts (Table 3 and Figure 5). In three studies, the survival of mouse islets, rat liver, and rat kidneys was extended for significantly longer with Tol-DCs + IS than with IS alone [16–18].

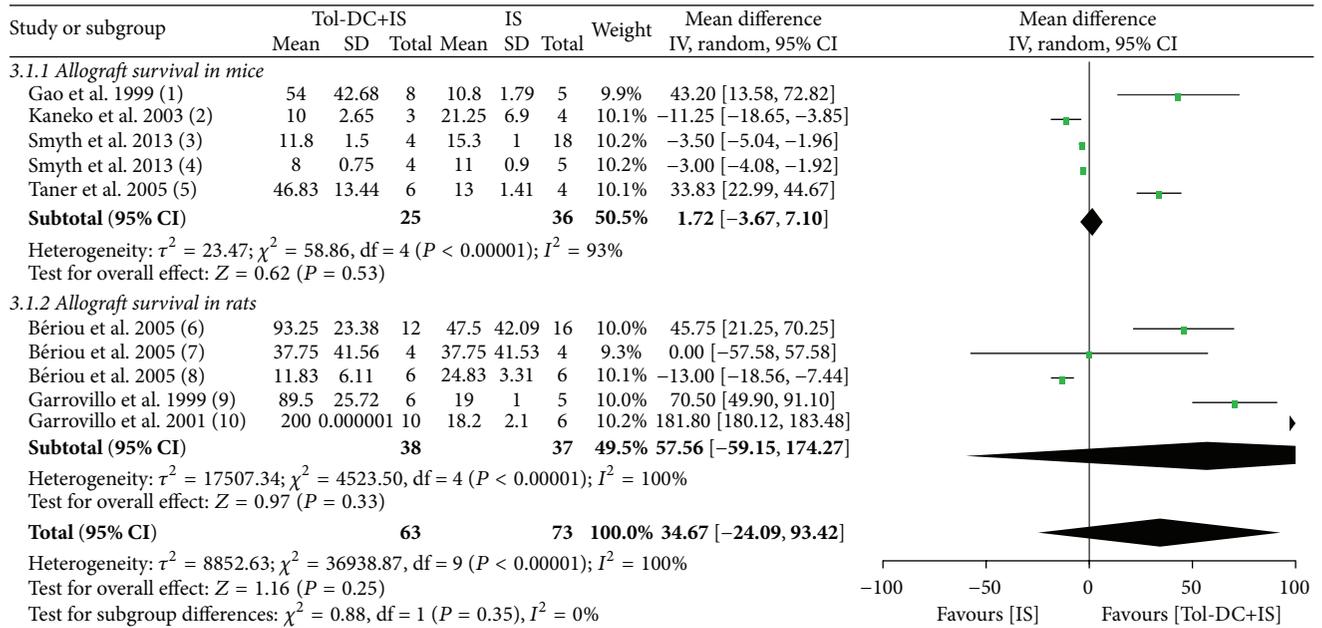
**3.5.4. Subgroup Analysis.** All of the mice used in the included studies were inbred strains, while the rats used in the liver, kidney, and small intestine studies included both inbred strains and closed colony randomly bred animals. A subgroup analysis of the inbred strains and closed colonies revealed similarly prolonged survival times in the kidney and small intestine transplantation models, and the merged results were identical. In the liver transplantation model, despite negative merged result (28.98 days, -11.16 to 69.12) in closed colony, we tended to take it as positive because of positive results of both included studies, then identical to inbred strains (Figure 6). Therefore, no heterogeneity in allograft survival was observed in the inbred strains and closed colony animals.

## 4. Discussion

The current overview included a total of 61 articles (47 studies from six systematic reviews and 14 primary studies) dissecting the efficacy of adoptive transfusion of Tol-DCs with or without IS drugs in promoting the survival of heart, liver, kidney, small intestine, skin, and islet allografts in animals. Tol-DC therapy prolonged allograft survival to varying extents in all except the islet transplantation models. Moreover, Tol-DC combined with IS drug therapy further prolonged the survival of heart, skin, and islet allografts in mice, but not of heart allografts in rats. Compared with IS drugs alone, Tol-DC + IS therapy extended islet allograft survival in rats but failed to influence the survival of skin, small intestine, and heart allografts in rats or of heart and skin allografts in mice (Table 3). Although three articles reported that Tol-DC + IS therapy had better outcomes than IS alone in the mouse islet,

rat liver, and kidney transplantation models, the evidence was based on single sets of data and we therefore did not include the results in our discussion. In addition, we did not directly compare Tol-DC and IS single therapies because most of the IS drugs have long histories of clinical use, whereas Tol-DC therapy has not yet been standardized and protocol differences undoubtedly affected the outcomes of the studies included here [10–15]. Comparisons of outcomes with single versus combination Tol-DC and IS therapy suggest that IS drugs have advantages over Tol-DCs. Our meta-analysis also shows that the efficacy of Tol-DC and Tol-DC + IS therapy varied with the transplantation model in both mice and rats, presumably reflecting the diversity of immune environments, organ-specific responses, and therapeutic protocols. Indeed, the severity and acuteness of rejection have been reported to vary for different grafts within a single organism, indicating organ-specific immune responses [75]. Moreover, in one transplantation model, the outcomes were different in rats and mice, suggesting species-specific responses to Tol-DC therapy.

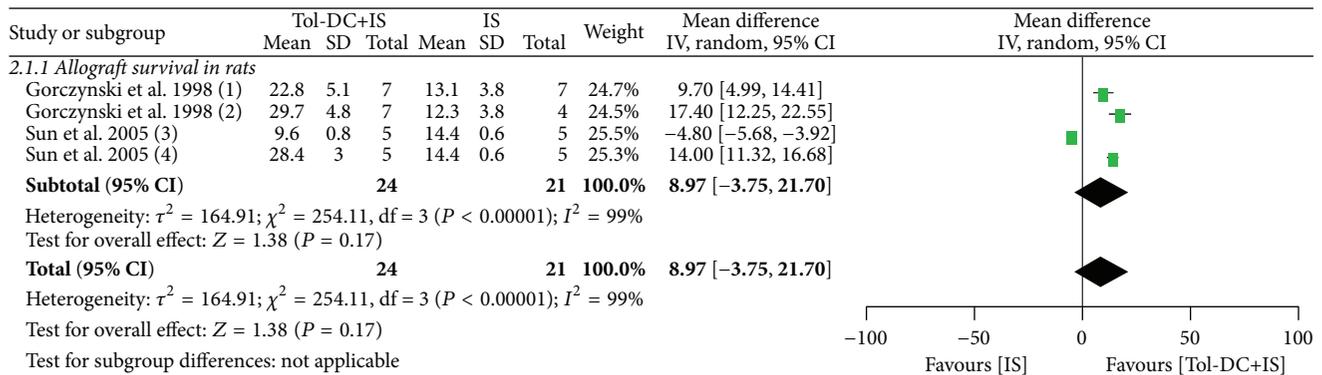
**4.1. Limitations.** The following limitations of this overview are noted. (1) The studies used different strains of rats and mice. Gene expression in primary immunocytes varies greatly across inbred mouse strains, suggesting that the same Tol-DC therapy may have variable efficacy in different strains [76]. (2) The Tol-DC modification protocols and organ donor/recipient strain derivation differed among the studies. Thus, Tol-DCs with the same modification gave different outcomes depending on the transplanted organ and the donor/recipient combinations [54, 74, 77]. Additionally, different gene modifications, drugs, cytokines, and culture media can induce Tol-DCs with immature, mature, or semimature phenotypes. Immature DCs are conventionally considered to be tolerogenic and mature DCs to be immunogenic. However, immature Tol-DCs are not always superior to mature Tol-DCs in terms of allograft survival [38, 78]. (3) The studies differed in Tol-DC injection time, route, dose, and frequency. These factors influence Tol-DC efficacy to variable extents [37, 40, 65], presumably by affecting the distribution, maintenance, and homing of Tol-DCs [79, 80].



Footnotes

- (1) (Donor spleen DC coculture with leucocytes) + CD4 mAb
- (2) (IL-4-DC) + anti-CD40L mAb
- (3) (Dex+D3+DC(B6D2F1)anti-CD8 antibody
- (4) (Dex+D3+DC(BALB/c) + anti-CD8 antibody
- (5) (alloAg+Rapa+DC) + FK506
- (6) (DC) + LF 10-day treatment
- (7) (DC) + LF 5-day treatment
- (8) (DC) + RAPA
- (9) (Donor MHC I peptide+ recipient BMDC) + ALS
- (10) (Donor MHC I peptide+ recipient thymus DC) + ALS

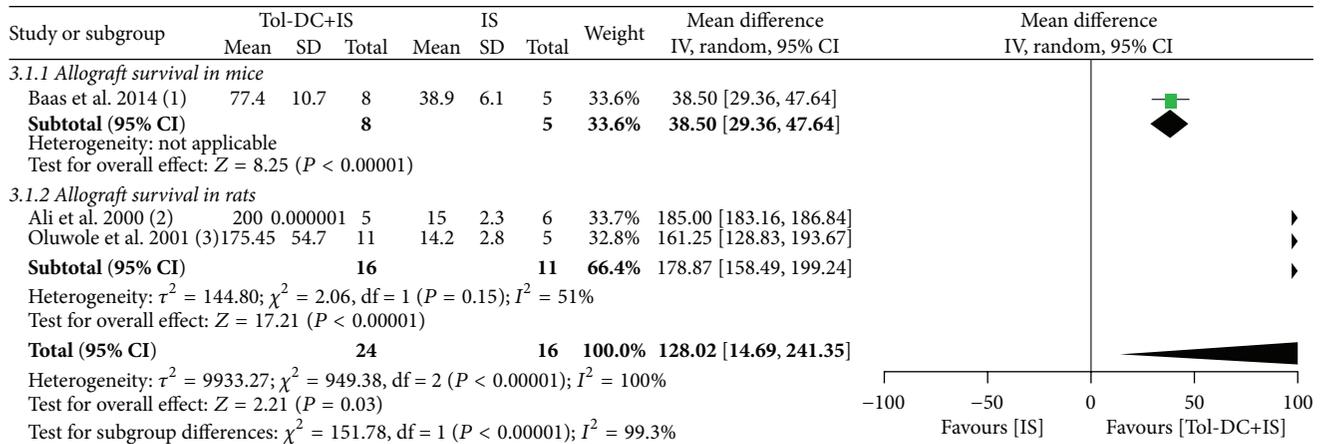
(a) Heart transplantation



Footnotes

- (1) (Donor BMDC) + CsA+anti-CD54+anti- $\alpha_1$
- (2) (Donor BMDC) + CsA+anti- $\alpha_4$
- (3) (Mature BMDC) + FK506
- (4) (iBMDC) + FK506

(b) Small intestine transplantation in rats

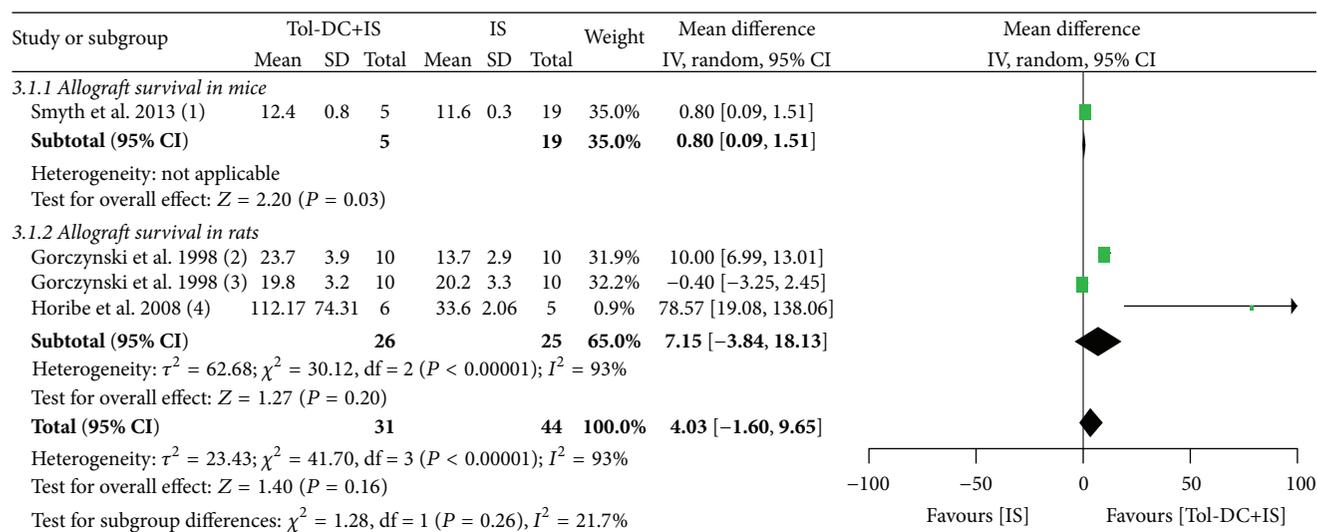


Footnotes

- (1) (GM-CSF+recipient adherent BMDC) + anti-CD3 Ab
- (2) (Donor MHC I peptide+recipient BMDC) + ALS
- (3) (Donor MHC I peptide+recipient BMDC( $1 \times 10^6$ )) + ALS

(c) Islet transplantation

FIGURE 5: Continued.



## Footnotes

- (1) (Dex+D3+DC(B6K<sup>d</sup>)) + anti-CD8 antibody  
 (2) (Donor BMDC) + CsA+anti- $\alpha 4$   
 (3) (Donor BMDC) + CsA+anti-CD54+anti- $\alpha 4$   
 (4) (Rapa+donor Ag+recipient BMDC) + ALS

## (d) Skin transplantation

FIGURE 5: Mean difference (95% confidence intervals) for Tol-DC in combination with IS versus IS alone for allografts survival in heart (a), small intestine (b), islet (c), and skin (d) transplantation models. Rats used in study Gorczyński 1998 [8] in small intestine and skin models were MHC semimismatch. In the footnotes, “-” before “DC” means genetic modification and “+” inside the parentheses means substances added in the culture medium and “+” outside the parentheses means combined IS agents. If not specialized, DC refers to donor bone marrow DC.

(4) The type of IS drug and the dose, time, and frequency of drug injection also differed considerably among the studies. (5) The statistical heterogeneity of the meta-analysis was very large for all transplantation models.

**4.2. Clinical and Preclinical Implications.** Our meta-analysis suggests that infusion of Tol-DCs alone is able to promote survival of allografts. However, there are currently no standardized protocols for the modification or application of Tol-DCs. To date, clinical experience with Tol-DCs is limited to a phase I trial of autologous Tol-DCs for type I diabetes, rheumatoid arthritis and refractory Crohn’s disease, and an ongoing safety trial of autologous Tol-DCs for kidney transplantation [4–7]. Nevertheless, our results indicate that a number of problems must be solved before Tol-DC therapy successfully moves from bench to bedside. For instance, standardized protocols must be established for the modification and dose of Tol-DCs; the time, frequency, and route of injection; and the type of IS drugs to be administered in combination. Considering that Tol-DC efficacy may be organ-specific, the therapeutic protocol may also need to be tailored to the transplanted organ. Although Tol-DC therapy did not give better outcomes than IS therapy or show synergy with IS drugs, Tol-DC therapy does have advantages over drug therapy. First, Tol-DCs are generally infused before transplantation, and since they exert their effect at the very earliest stages of the immune response, they are very likely to inhibit hyperacute rejection. Second, Tol-DCs

are likely to induce tolerance or prolong allograft survival without impairing the recipient’s immune defense against other antigens. Third, the studies included in this overview did not administer IS drugs continuously. Therefore, Tol-DC therapy may allow IS drug use to be reduced, thus decreasing their toxicity and improving the recipients’ quality of life, which is particularly important for IS-sensitive and IS-tolerant recipients. Notably, tacrolimus has been reported to inhibit the functions of Tol-DCs in mice [81], suggesting that further preclinical studies of Tol-DC and drug combinations are needed.

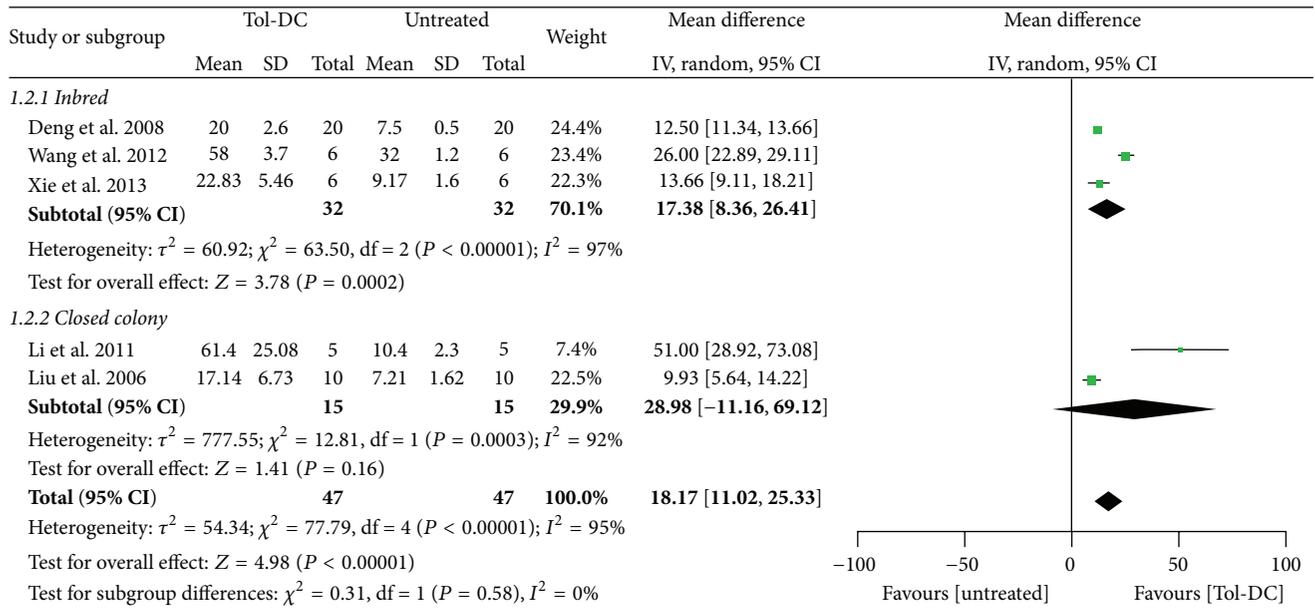
Our results indicate that Tol-DC efficacy may be species-specific, suggesting that studies in primates will be more clinically relevant. A kidney transplantation model has been successfully established in rhesus macaques and infusion of donor-derived Tol-DCs in combination with IS prolonged allograft survival [82]. This nonhuman primate model will help to translate research findings from animals to the clinic.

## Competing Interests

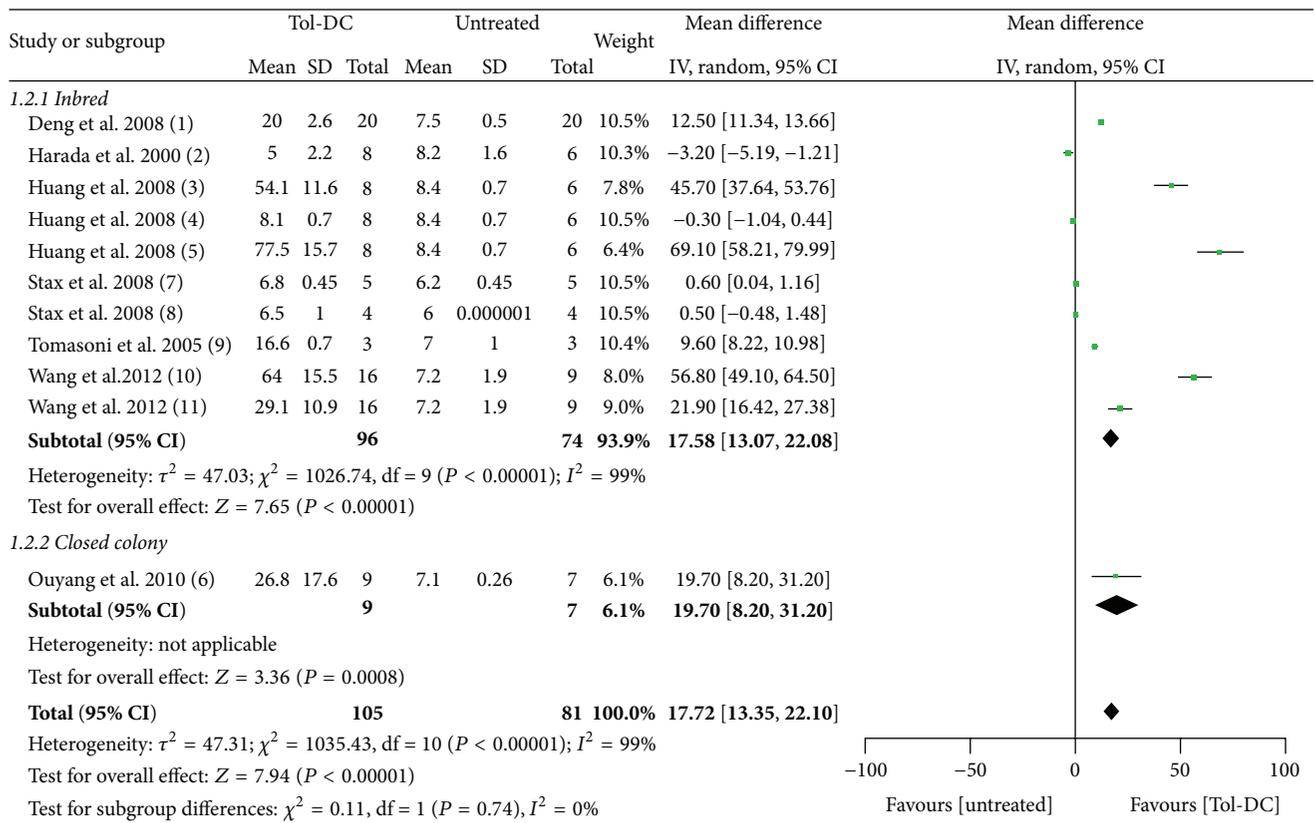
All authors declared no competing interests.

## Acknowledgments

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(a) Liver transplantation in rats

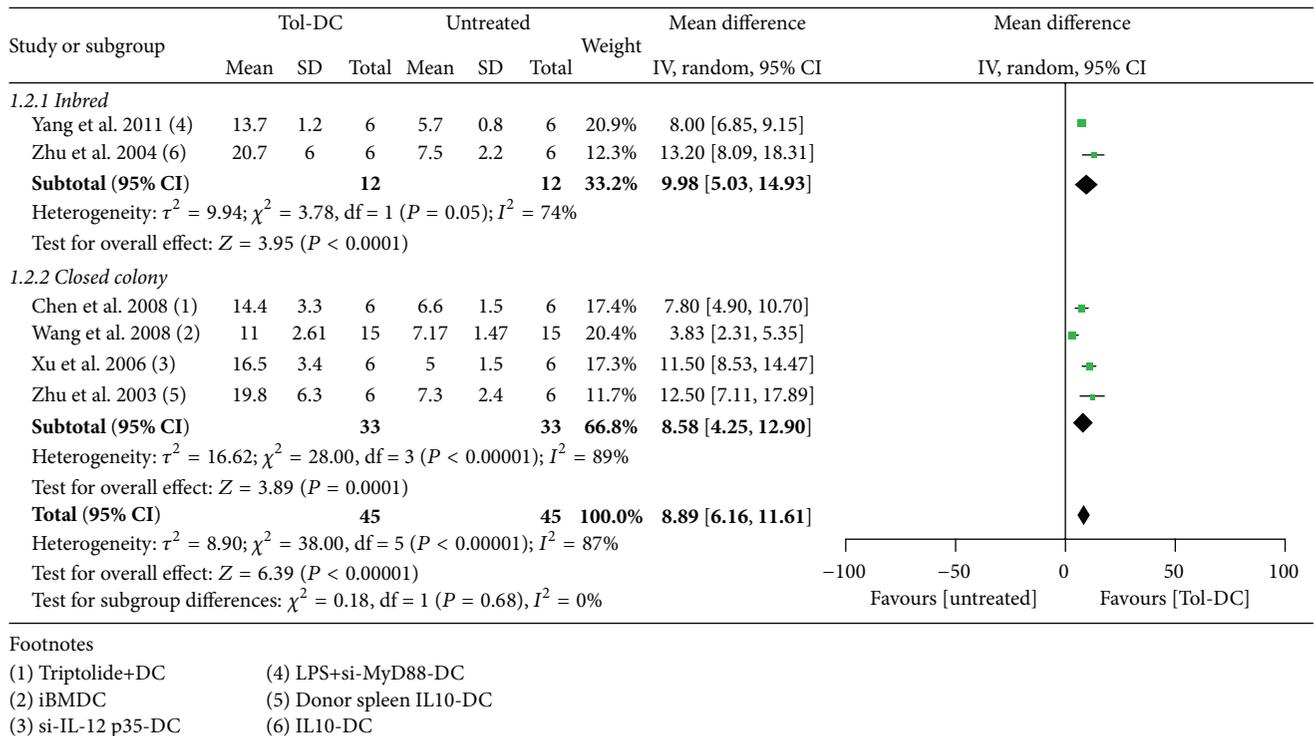


Footnotes

- |                                      |                                       |
|--------------------------------------|---------------------------------------|
| (1) IL-10+DC                         | (7) Dex+donor(Brown Norway) DC        |
| (2) Donor spleen DC                  | (8) Dex+donor(Dark Agouti) DC         |
| (3) Recipient CTLA4Ig-BMDC           | (9) dnIKK2-DC                         |
| (4) CTLA4Ig-DC                       | (10) Donor antigen+CD4+recipient BMDC |
| (5) Donor and recipient CTLA4Ig-BMDC | (11) CD4+recipient BMDC               |
| (6) IKK2dn-DC                        |                                       |

(b) Renal transplantation in rats

FIGURE 6: Continued.



## Footnotes

- (1) Triptolide+DC                      (4) LPS+si-MyD88-DC  
 (2) iBMDC                                (5) Donor spleen IL10-DC  
 (3) si-IL-12 p35-DC                    (6) IL10-DC

(c) Small intestine transplantation in rats

FIGURE 6: Mean difference (95% confidence intervals) for subgroups of inbred and closed colony rats for allografts survival in liver (a), renal (b), and small intestine (c) transplantation models. All mice and rats in other models are inbred.

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## Review Article

# Mesenchymal Stem Cells and Myeloid Derived Suppressor Cells: Common Traits in Immune Regulation

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To protect host against immune-mediated damage, immune responses are tightly regulated. The regulation of immune responses is mediated by various populations of mature immune cells, such as T regulatory cells and B regulatory cells, but also by immature cells of different origins. In this review, we discuss regulatory properties and mechanisms whereby two distinct populations of immature cells, mesenchymal stem cells, and myeloid derived suppressor cells mediate immune regulation, focusing on their similarities, discrepancies, and potential clinical applications.

## 1. Introduction

Immune response protects host against pathogen invasion and cancer. However, if uncontrolled, it may induce severe tissue damage and therefore under steady-state conditions is tightly regulated. Understanding cells and mechanisms that regulate immune response is critical to unravel pathogenesis of many diseases and develop new strategies for immune modulation during cancer, chronic infections, autoimmune disorders, allergies, and following organ transplantation.

Several populations of immune cells have been implicated in the control of immune response including natural and induced CD4<sup>+</sup> T regulatory cells (Treg), CD8<sup>+</sup> Treg, Breg, macrophages, and dendritic cells. To control immune response, these cells utilize a set of core suppressive mechanisms, the main of which are the secretion of inhibitory cytokines (e.g., IL-10, TGF- $\beta$ , and IL-35), the expression of inhibitory receptors (e.g., PD-L1), the inhibition of antigen-presenting cell maturation, and cytolysis [1–4].

Besides mature immunocompetent cells designated to control immune response, other populations may also contribute to immune regulation. In particular, two distinct populations of functionally immature cells, mesenchymal stem cells (MSCs), and a population of immature myeloid cells, myeloid derived suppressor cells (MDSCs), have

been implicated in immune suppression and regulation [5, 6].

MSCs and MDSCs belong to distinct differentiation lineages; however, their immunoregulatory properties have several common traits. Here, we review the underlying mechanisms and regulatory properties of MSCs and MDSCs focusing on their similarities and distinctions.

## 2. MSCs and MDSCs: General Characteristics

**2.1. MSCs.** MSCs are multipotent stromal self-renewing cells capable to differentiate into mesenchymal tissues like osteocytes, chondrocytes, and adipocytes [7]. MSCs exhibit paracrine effects and participate in immunomodulation and tissue repair. The cells are found in the bone marrow (BM) and other embryonic and adult tissues such as cord blood, placenta, adipose tissue, and perivascular sources. In the BM, MSCs fulfill a supportive function for hematopoietic cells and participate in the control of their renewal and differentiation [8–10]. Phenotypically, MSCs are characterized by the expression of CD105, CD90, and CD73 and lack of the expression of haemopoietic markers, such as CD45, CD34, CD14, CD11b, CD79 $\alpha$ , CD19, and HLA-DR [11–13].

The immunomodulatory properties of MSCs were first demonstrated by Di Nicola and coauthors, who showed that

BM-MSCs inhibited T cell proliferation in mixed lymphocyte reaction (MLR) [14]. Since then, the ability of MSCs to suppress immune responses has been extensively studied. Currently, it is understood that MSCs possess rather immunoregulatory than immunosuppressive properties: depending on the microenvironment they can inhibit, modulate or even enhance immune function of various immune cells [5, 15]. Proinflammatory conditions induce suppressive properties in MSCs. Due to their immunoregulatory properties and the feasibility of generating the large numbers of autologous MSCs, MSCs are considered as a potentially valuable tool for clinical immunomodulation.

**2.2. MDSCs.** MDSCs belong to the hematopoietic lineage and represent the heterogeneous population of early myeloid progenitors/precursors of granulocytes, macrophages, and dendritic cells (DCs) able to mediate immune suppression [6]. In steady-state conditions, MDSCs are rare and are primarily found in the BM. During different pathologies accompanied by inflammation, MDSCs accumulate abundantly in the BM, blood, spleen, lungs, and other organs [16–19].

In mice, MDSCs are defined as Gr-1<sup>+/dim</sup>CD11b<sup>+</sup> cells. In human, MDSCs are generally identified based on the expression of CD33 and CD11b and lack of the expression of HLA-DR. Two main subsets of MDSCs, monocytic and granulocytic, have been described according to their nuclear morphology and phenotype. In mice, monocytic and granulocytic MDSCs are identified as Ly-6G<sup>-/low</sup>Ly-6C<sup>hi</sup>CD11b<sup>+</sup> (F4/80<sup>+</sup>CD115<sup>+</sup>CD49d<sup>+</sup>) and Ly-6G<sup>+</sup>Ly-6C<sup>low</sup>CD11b<sup>+</sup> (F4-80<sup>-</sup>CD115<sup>-</sup>CD49d<sup>-</sup>) cells, respectively. In human, they are CD14<sup>+</sup>HLA-DR<sup>-</sup> and CD15<sup>+</sup>/CD66b<sup>+</sup>, respectively [6, 19–22].

MDSCs are the main negative regulators of immune response in cancer [6, 23] and are also involved in the immune suppression in many other pathological conditions [19, 24–26]. Similar to MSCs, the suppressive activity of MDSCs is inducible and dramatically increases under proinflammatory conditions [6]. Expressed immunosuppressive properties and abundant accumulation of MDSCs under proinflammatory conditions make them an attractive target for immunomodulation in cancer and other diseases.

### 3. Molecular Mediators of Immune Suppression

To regulate immune response, MSCs and MDSCs utilize a set of mediators and mechanisms, which they partially share with other immune regulatory cells. In this section, we summarize the main mechanisms whereby MSCs and MDSCs mediate immune regulation focusing on their cellular targets and activation.

#### 3.1. Indoleamine 2,3-dioxygenase (IDO) and Tryptophan Metabolism

**3.1.1. Effects.** IDO enzymes (expressed as two distinct enzymes, IDO1 and IDO2) catalyze the essential amino acid tryptophan into metabolites, that is, kynurenine, quinolinic

acid, and picolinic acid. Tryptophan consumption increases the level of uncharged tryptophan tRNA in immune cells. This activates general control nonderepressible 2 (GCN2) stress-response kinase, eukaryotic translation initiation factor 2 (eIF2), and GCN2-eIF2-mediated pathway, which leads to the reduction in protein synthesis, retards cellular proliferation, arrests T cells in G0/G1 cell cycle, and increases lymphocyte sensitivity to Fas-mediated apoptosis [27, 28]. The activation of GCN2-mediated pathway also downregulates IL-6 supporting the suppressive status of Tregs and restricting their conversion to Th17-like cells [29]. In a model of Th17-associated experimental autoimmune encephalomyelitis (EAE), halofuginone, a small molecule that induces amino acid starvation, selectively inhibited the differentiation of Th17, verifying a role of amino acid deficiency in the suppression of Th17 [30]. Tryptophan depletion decreases the expression of costimulatory molecules and increases the expression of the inhibitory receptors ILT3 and ILT4 by DCs; DCs differentiated under low-tryptophan conditions become tolerogenic [31] (Figure 1).

Tryptophan metabolites are directly toxic to CD8<sup>+</sup> and CD4<sup>+</sup> Th1 cells, whereas Th2 cells are more resistant to their toxicity. Therefore, IDO releases Th2 from Th1-mediated suppression and skews T helper response towards Th2 type [27, 32, 33]. Kynurenines are also natural ligands for the aryl hydrocarbon receptor (AhR); their interaction with AhR promotes the differentiation of CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells, interferes with the generation of Th17, and decreases the immunogenicity of DCs [34].

**3.1.2. IDO Expression by MSCs and MDSCs.** In the immune system, IDO are expressed primarily by professional antigen-presenting cells [28]. Both MSCs and MDSCs express IDO and utilize IDO mediated mechanisms for immune suppression [35–37].

**3.1.3. Regulation of IDO Expression.** The IDO gene is inducible in the presence of IFN- $\gamma$  and regulated by upstream IFN- $\gamma$ -responsive elements that bind activated STAT1, interferon regulatory factor-1 (IRF-1), and NF- $\kappa$ B [27, 35, 38, 39]. IRF-8 contributes to IFN- $\gamma$ -induced IDO expression by enhancing the expression of IDO and decreasing DAPI2 which basally opposes IDO expression [40]. It was suggested that MSCs utilize IDO mediated mechanism in the presence of IFN- $\gamma$  but not in basal state [41]. IDO expression is also increased by PGE2 [42], thus relating the two mechanisms of immune control utilized by MSCs and MDSCs.

#### 3.2. Cyclooxygenase-2 and Prostaglandin E2

**3.2.1. Effects.** PGE2 synthesizes from the arachidonic acid after the latter releases from membrane phospholipids and is metabolized by either the constitutive cyclooxygenase-1 (COX-1) or the inducible cyclooxygenase-2 (COX-2) [43]. PGE2 mediates pain, edema, and fever, the main features of inflammation. At the same time, it exerts anti-inflammatory effects. The interaction of PGE2 with EP2 and EP4 receptors expressed by immune cells leads to increase in cyclic AMP, activates protein kinase A and

Inducers	Mediators	Downstream mechanisms	Effects on DC and Mphs	Effects on T cell generation and function					Produced by
				Proliferation	Th1	Th17	Th2	Treg	
IFN- $\gamma$ [27, 38–41] PGE2 [42], TNF- $\alpha$ [42]	IDO	TRP consumption $\rightarrow$ GCN2 $\rightarrow$ eIF2 [27, 28, 31] TRP metabolites $\rightarrow$ toxicity, AhR [27, 28, 34]	$\downarrow$ costimulation [31] $\uparrow$ ILT3, ILT4 [31] $\uparrow$ tolerogenic DCs [31, 34]	$\downarrow$ [27, 28, 135] (G0/G1 arrest)	$\downarrow$ [27] (toxicity)	$\downarrow$ [29, 30] ( $\downarrow$ IL-6)	$\uparrow$ [32, 33] (release from Th1 control)	$\uparrow$ [31, 34] (AhR, tolerogenic DCs)	MSCs MDSCs
IFN- $\gamma$ , TNF- $\alpha$ , T cells [41, 59]	COX-2/PGE2	Cyclic AMP, Ca <sup>2+</sup> mobilization [43, 44]	$\downarrow$ maturation [44, 166] $\uparrow$ M2 [47] $\uparrow$ maturation [44]	$\downarrow$ [44, 134] $\uparrow$ [44]	$\downarrow$ [44–46] $\uparrow$ [44] (at low doses)	$\uparrow$ [43, 44, 48, 49] $\downarrow$ [50]	$\uparrow$ [44, 45]	$\uparrow$ [51]	MSCs MDSCs
IFN- $\gamma$ , TNF- $\alpha$ [35, 59, 91] IL-13 [92]	TGF- $\beta$	SMAD $\rightarrow$ histone acetyltransferase/deacetylase [85]	$\downarrow$ costimulation $\downarrow$ MHC II [85]	$\downarrow$ [85]	$\downarrow$ [87]	$\uparrow$ (in the presence of IL-6, IL-1 $\beta$ , IL-23) [88]	$\downarrow$ [86]	$\uparrow$ [5, 15, 88]	MSCs MDSCs
TLR4, TLR9 [94, 100] PGE2 [101]	IL-10	JAK1/TYK2/STAT3/SOCS3 [98]	$\downarrow$ costimulation [93] $\downarrow$ MHC II [93] $\uparrow$ M2 [97]	$\downarrow$ [93, 94]	$\downarrow$ [93, 94]	$\downarrow$ [95]	$\downarrow$ [94, 214]	$\uparrow$ [95, 96]	MSCs MDSCs
IFN- $\gamma$ [105]	HGF	ERK1/2 [104]	$\downarrow$ costimulation $\uparrow$ M2, IL-10 [102, 104]	$\downarrow$ [14]	$\downarrow$ [102]	$\downarrow$ [102, 103]	$\uparrow$ [102, 103]	$\uparrow$ [102]	MSCs MDSCs
IFN- $\gamma$ [79], IL-17 [80]	NO (iNOS)	$\downarrow$ [69, 70] Jak3/STAT5/ERK/AKT	$\downarrow$ MHC II [6, 71]	$\downarrow$ [69, 70]	$\downarrow$ [72, 73] (mice, human)		$\downarrow$ [73] (human)		MSCs MDSCs
IL-4, IL-13, TLR [64], PGE2 [81, 205]	ARG-1	L-arginine depletion, $\downarrow$ TCR- $\zeta$ [6, 61]	$\downarrow$ [230]	$\downarrow$ [6, 60, 61]	$\downarrow$ [62]	$\downarrow$ [63]	$\uparrow$ [64, 65] $\downarrow$ [66]	$\uparrow$ [63, 67, 68]	MDSCs
IL-10 [108], IFN- $\gamma$ TNF- $\alpha$ [114]	HLA-G5	ILT2, ILT4, KIR2DL4 [107–109]	$\downarrow$ [107–109]	$\downarrow$ [113, 114]	$\downarrow$ [111] $\uparrow$ [115] (at low doses)	$\downarrow$ [111]	$\downarrow$ [111] $\uparrow$ [115] (at low doses)	$\uparrow$ [112]	MSCs
IFN- $\gamma$ , TNF- $\alpha$ , IL-1 [105]	Galectins	Apoptosis [121]	$\downarrow$ [120]	$\downarrow$ [122]	$\downarrow$ [121]	$\downarrow$ [121]	No effect [121]	$\uparrow$ [122, 124]	MSCs

$\uparrow$ : enhances function and/or generation;  $\downarrow$ : inhibits function and/or generation.

The main effects, inhibition (pink), stimulation (green), and both inhibition and stimulation, have been described (checked). Not enough data or no effect (white).

AhR: aryl hydrocarbon receptor; DC: dendritic cells; eIF2: eukaryotic translation initiation factor 2; GCN2: general control nonderepressible 2 stress-response kinase; JAK1, Janus kinase 1; Mphs, macrophages; SOCS3: suppressors of cytokine signaling 3; STAT3: Signal transducer and activator of transcription 3; TRP: tryptophan.

FIGURE 1: Main mediators utilized by MSCs and MDSCs to suppress T cell responses and their effects.

phosphatidylinositol-3 kinase dependent signaling pathways, and inhibits Ca<sup>2+</sup> mobilization. Cyclic AMP interferes with IL-2-mediated pathways, inhibits the expression of proinflammatory cytokines and chemokines (i.e., IL-12p70, TNF- $\alpha$ , CCL3, and CCL4), and induces the expression of IL-10, IL-4, and IL-5 [43, 44]. This suppresses cell proliferation, induces alternative macrophages (M2), stimulates Th2, and weakens Th1 responses [44–47]. Besides inducing immunosuppression, PGE2 may play proinflammatory role in T cell function. In some studies, exogenous PGE2 enhanced DC maturation and T cell proliferation (reviewed in [44]). The above-mentioned inhibition of IL-12p70 induced by PGE2 is due to the inhibition of IL-12p35 chain. IL-12p40 chain is not affected by PGE2. IL-12p40 and p19 form IL-23, the cytokine involved in the generation of Th17. PGE2 increases the expression of p19 resulting in the increased production of IL-23 and Th17 polarization [43, 48]. In a model of EAE, EP4 antagonist decreased the accumulation of Th17 and Th1 cells and suppressed disease progression [49]. However, in some other studies, PGE2 inhibited Th17 differentiation [50]. Recent advances suggest that effects of PGE2 on Th17 and even Th1 depend on its concentration in such a way that micromolar concentrations suppress the responses while nanomolar concentrations promote the responses [44].

PGE2 enhances the differentiation of Foxp3<sup>+</sup> Treg cells [51], elevates TGF- $\beta$  secretion by monocytes, and induces the generation of MDSCs and their accumulation in tumor environment. The inhibition of COX-2 suppresses these processes [52–54].

**3.2.2. Regulation of COX-2/PGE2 and Their Expression by MSCs and MDSCs.** Both MSCs and MDSCs express COX-2 [41] and can produce PGE2 [41, 54–58]. PGE2 production increases in inflammatory conditions, that is, in the presence of IFN- $\gamma$  and TNF- $\alpha$  and after cell coculture with peripheral blood cells [41, 59].

### 3.3. Arginase-1, Inducible Nitric Oxide Synthase, and Arginine Metabolism

**3.3.1. Effects.** Arginase-1 (ARG1) hydrolyses L-arginine to ornithine and urea reducing local arginine concentration. The latter activates GCN2, which inhibits cell cycling [60]. ARG1 downregulates the  $\zeta$ -chain of the T cell receptor (TCR) complex, disturbing the process of T cell activation [6, 61]. There is only limited data on the subset-specific effects of ARG1. The studies have reported the inhibition of IFN- $\gamma$  [62] and Th17 [63], and both the stimulation [64, 65] and the suppression [66] of Th2 responses by ARG1 produced by various cells. Tregs are expanded by ARG1; the inhibitor of ARG1 N-hydroxy-L-arginine (NOHA) abrogates this effect [67, 68].

Besides ARG1, L-arginine is metabolized by inducible nitric oxide (NO) synthase (iNOS) that generates NO. NO suppresses T cell function through the inhibition of JAK3, STAT5, ERK, and AKT involved in IL-2 signaling and the control of T cell proliferation [69, 70]. NO also inhibits the expression of MHC class II and induces T cell apoptosis [6, 71]. In murine T cells, NO was shown to suppress

the secretion of Th1 cytokines [72]; in human T cells, it suppressed the secretion of both Th1 and Th2 cytokines [73].

**3.3.2. ARG1 and iNOS Expression by MSCs and MDSCs.** In the immune system, ARG1 and iNOS are generally expressed by polymorphonuclear cells (PMN) and monocyte/macrophages [74]; T helper cells are also able to produce NO [72]. In M1 and M2 macrophages, ARG1 and iNOS are expressed reciprocally: ARG1 is expressed by M2, whereas iNOS by M1 subset [75]. MDSCs express both ARG1 and iNOS [6, 70]; however, the levels of their expression in monocytic and granulocytic populations may differ so that ARG1 is expressed predominantly by granulocytic MDSCs [76] and iNOS by monocytic MDSCs [6]. MSCs express iNOS and can produce NO [77], but there is no evidence for their expression of ARG1. In spite of this, MSCs can contribute to the depletion of L-arginine by promoting the generation of MDSCs [78].

**3.3.3. The Regulation of ARG1 and iNOS.** Generally, ARG1 and iNOS undergo reciprocal induction: ARG1 is induced by Th2 cytokines, whereas iNOS by Th1 cytokines [79]. Recently, IL-17 was shown to contribute to iNOS expression by enhancing its mRNA stability [80]. PGE2 stimulates ARG1 [81].

### 3.4. Reactive Oxygen Species and Peroxynitrite

**3.4.1. Effects.** Reactive oxygen species (ROS) are generated by NADPH oxidase which produces superoxide anion ( $O_2^-$ ). Superoxide anion reacts with NO to form peroxynitrite. Peroxynitrate oxidates membrane molecules and nitrates amino acids. Nitration of TCRs alters antigen-recognition and inhibits the responses of  $CD4^+$  and  $CD8^+$  cells [82]. Nitration of the chemokine CCL2 was shown to block T cell migration to the inflammatory site [83].

**3.4.2. ROS Production by MSCs and MDSCs.** NADPH oxidase is generally expressed by leukocytes. In MDSCs, it is expressed predominantly by the granulocytic population [6]. MSCs do not generate ROS, but they are responsive to them: ROS promote MSCs' aging. In physiological levels, ROS improve MSCs' proliferation and differentiation [84].

**3.5. Cytokines and Growth Factors.** The main immunoregulatory cytokines produced by MSCs and MDSCs are TGF- $\beta$  and IL-10.

**3.5.1. TGF- $\beta$ .** TGF- $\beta$  binds to the heterodimeric TGF- $\beta$  receptor and initiates SMAD-dependent and SMAD-independent signal transduction pathways. SMAD-dependent pathway induces the recruitment of histone acetyltransferase and deacetylase to the promoters of target genes [85]. This leads to the blockade of IL-2 production, downregulates cell cycle promoting factors, upregulates cyclin-dependent kinase inhibitors, and inhibits the expression of MHC class II and costimulatory molecules in DCs and effector molecules (i.e., IFN- $\gamma$  and perforin) in T cells. Consequently, the proliferation, helper, and cytotoxic

activity of T and NK cells are suppressed. TGF- $\beta$  inhibits the differentiation of both Th1 and Th2 cells [86, 87]. In contrast, it promotes the generation of Treg and Breg cells. TGF- $\beta$  is a key regulator of Foxp3 expression [5, 15, 88]. In the presence of IL-6, IL-1 $\beta$  or IL-23 TGF- $\beta$  promotes the generation of Th17 [88]. Recently, TGF- $\beta$  was shown to inhibit the expression of iNOS in MSCs reversing their suppressive effect on T cell proliferation and manifesting immunostimulatory effect [89].

MSCs constitutively secrete TGF- $\beta$  [90] and upregulate its production in the inflammatory environment, that is, in the presence of IFN- $\gamma$  and TNF- $\alpha$  [35, 59, 91]. In MDSCs, the expression of TGF- $\beta$  was induced by IL-13 [92].

**3.5.2. IL-10.** IL-10 is produced by various immune cells, including DCs, macrophages, Th1, Th2, Th17, Treg,  $CD8^+$  T cells, and B lymphocytes, and also by MSCs and MDSCs. IL-10 directly acts on antigen-presenting cells (APC), decreasing their maturation, and the expression of MHC and costimulatory molecules [93]. IL-10 inhibits the production of proinflammatory cytokines and chemokines (i.e., IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, IL-12, TNF- $\alpha$ , CCL2, and CCL5, IL-8) and hampers DC migration to lymph nodes and the generation of effector T cells. Direct effects of IL-10 on T lymphocytes include the inhibition of proliferation, IL-2, IFN- $\gamma$ , TNF- $\alpha$ , IL-4, and IL-5 production and memory formation [93, 94]. IL-10 also inhibits the differentiation of Th17 [95] but enhances the differentiation of IL-10 producing Treg cells and M2 macrophages [96, 97]. The anti-inflammatory effects of IL-10 are mediated through the phosphorylation of JAK1, TYK2, the activation of STAT3, and the induction of SOCS3, which negatively regulates various cytokine genes [98]. Apart from the immunosuppressive activity, IL-10 may display immunostimulatory properties: it inhibited or stimulated  $CD8^+$  T cells depending on the type of pathogen and cell microenvironment [99]. Induction of IL-10 goes on as a result of toll-like receptors (TLR4 and others) activation [100]. PGE2 is a potent inducer of IL-10 [101].

**3.5.3. Hepatocyte Growth Factor (HGF).** HGF is primarily produced by MSCs. HGF displays pleiotropic immunosuppressive activity. It stimulates IL-10 production by monocytes, downregulates costimulatory molecules on DCs, inhibits Th1, and induces IL-10 producing Treg cells [102–105]. HGF produced by MSCs expanded human MDSCs [78]. One study reported the production of HGF by tumor-infiltrating MDSCs [106], which highlights the similarities between the two populations.

MSCs and MDSCs produce a number of other cytokines that contribute to cell biological activity, for example, IL-6, IL-8, and GM-CSF.

### 3.6. Other Mechanisms

**3.6.1. HLA-G.** HLA-G are nonclassical class I tolerogenic molecules expressed as four membrane-bound (HLA-G1 and HLA-G4) and three secreted (HLA-5 and HLA-G7) isoforms. In the immune system, HLA-G are largely expressed by DCs and macrophages. HLA-G act through the inhibitory

Cells	DCs and Mphs	T cell generation and function					B cells		PMN	NK cells	
		Proliferation & viability	Th1	Th17	Th2	Treg	B cells/plasma cells	Breg			
MSCs	↓ DC maturation, ↑ tolerogenic DCs [91, 163–177] ↑ M2 [178–186]	↓ [14, 131–137]	↓ [55, 136, 138, 139] ↑ (in Th2-conditions) [144]	↓ [50, 140–142] ↑ [145]	↓ (in Th2 conditions) [144]	↑ [55]	↑ [90, 91, 112]	↓ [131, 189–191, 193] ↑ [192, 193]	↑ [190, 194, 195]	↑ [207–211]	↓ [36, 112, 201]
MDSCs	↓ DC maturation [187, 188] ↑ M2 [153, 156]	↓ [19, 26, 146–152]	↓ [18, 19]	↑ [157] ↓ [158]	↑ [154–156], ↓ [231]	↑ [67, 159–162]	↓ [197–200]			↓ [212, 213]	↓ [203–206]

↑: enhances function and/or generation; ↓: inhibits function and/or generation.

The main effects, inhibition (pink), stimulation (green), and both inhibition and stimulation, have been described (checked). Not enough data (white).

DC: dendritic cells; Mphs: macrophages; PMN: polymorphonuclear cells.

FIGURE 2: Cellular targets and modulatory effects of MSCs and MDSCs.

receptors ILT2 and ILT4 expressed by myeloid DCs, CD4<sup>+</sup> and CD8<sup>+</sup> T cells, B cells, monocytes/macrophages, and NK cells [107–109]. HLA-G inhibit alloproliferative T cell response, Th1 cell migration, Th1, and Th17 cytokine production, induce Treg cells, and suppress the cytotoxic function of NK cells [110–114]. This general pattern has been reported by several groups. However, the effect of HLA-G may depend on their concentration: in the study by Kapasi and coauthors, high doses of HLA-G promoted Tregs, whereas low doses fostered the development of Th1 cells [115].

Human MSCs express and secrete HLA-G5. The secretion of HLA-G5 by MSCs is stimulated by IL-10, IFN- $\gamma$ , and TNF- $\alpha$  [112–114]. Myeloid APCs were shown to express HLA-G in pathological context (e.g., in cancer and viral infections); it was suggested that HLA-G-expressing myeloid APCs may be viewed as suppressor cells [108]. Yet, the role for HLA-G in the regulatory functions of MDSCs remains to be evaluated.

**3.6.2. CD39 and CD73.** MSCs express ectonucleotidase CD73 that catabolizes AMP to adenosine. AMP is generated from ATP under the action of ectonucleoside CD39 that is expressed at low levels by MSCs and at high levels by activated T cells. Extracellular ATP exhibits proinflammatory effects; adenosine triggers inhibitory pathways mediated by cAMP and protein kinase A. Thus, the concerted action of CD39 and CD73 cleaves ATP to adenosine resulting in the immune suppression [116, 117]. Our search for data on the expression of CD39 and/or CD73 by MDSCs resulted in two original studies. One study reported the expression of CD73 by granulocytic MDSCs and the involvement of the nucleotidase activity in MDSCs-mediated suppression [118]. In another study, the anticancerogenic drug  $\alpha$ -difluoromethylornithine hampered MDSC suppressive activity, in particular, by inhibiting the CD39/CD73-mediated pathway [119].

**3.6.3. Galectins.** Galectins (Gal), soluble glycan-binding proteins, bind to cell surface glycoproteins. MSCs express Gal-1 and Gal-9. Gal-1 inhibits tissue emigration of immunogenic DCs [120] and selectively binds to Th1 and Th17 cells inducing their apoptosis but does not affect Th2 cells [121, 122]. Gal-1 upregulates the expression of AhR in T cells and the production of IL-10 by Th1 and Th17 cells [122]. Gal-9 mediates antiproliferative effects on T and B cells. In B lymphocytes, it also reduces immunoglobulin release. Gal-9 is upregulated

by IFN- $\gamma$  [123]. We found no reports on the usage of galectins by MDSCs in the available literature. However, galectins were shown to participate in the induction and the accumulation of MDSCs at tumor site [124].

**3.6.4. CCL2.** The chemokine CCL2 interacts with CCR2 receptor expressed by myeloid cells and NK cells, activated Th1 and Th17 cells, and recruits them to the site of inflammation. MSCs produce CCL2 and express metalloproteinase that truncates CCL2, generating CCR2 antagonist that suppresses the migration of inflammatory cells. This mechanism seems to be critical for MSC-mediated suppression during autoimmune disorders. Defects in CCL2 processing have been associated with the pathogenesis of SLE [125]. In EAE, adoptively transferred wild-type MSCs induced immune suppression, whereas CCL2<sup>-/-</sup> MSCs did not [126]. We found no reports on the usage of CCL2-mediated mechanism by MDSCs. However, MDSCs express CCR2 and readily respond to CCL2 by accumulating at the corresponding inflammatory sites [127].

**3.6.5. B7-H1.** MSCs and MDSCs express negative costimulatory molecules, in particular, B7-H1. B7-H1 interacts with PD-1 [128]. The expression of B7-H1 by MSCs was induced by IFN- $\gamma$  [129], whereas on MDSCs it could be induced by IL-13 [37]. Whether these differences are due to different experimental settings or are characteristic for MSCs and MDSCs remains to be clarified.

## 4. Cellular Targets

This section reviews immunomodulatory effects of MSCs and MDSCs on different immune cells (Figure 2).

**4.1. T Lymphocytes.** Effector T lymphocytes generate after naïve T cells recognize antigen, activate, proliferate, and differentiate into effector subsets. MSCs and MDSCs interfere with T cells at different stages of their differentiation and function.

**4.1.1. MSCs.** MSCs hamper antigenic presentation by DCs and thus interfere with the antigenic stimulation of T cells, both *in vitro* and *in vivo* (see below). Activation of T cells,

measured by the expression of CD69 and CD25, was inhibited by MSCs in some [130] but not in all [131, 132] studies. MSCs readily suppress T cell proliferation induced by mitogens, anti-CD3/CD28 stimulation, or transplantation antigens in MLR [14, 131–133]. The inhibitory effect is due to cell arrest in G0/G1 phase of cell cycle [131] and can be mediated by iNOS, PGE2 [134], IDO [135], TGF- $\beta$  [14, 130], IL-10 [136], or PD-1 [134, 137]. The role for these molecular mediators in the suppression of T cell proliferation varies in different experimental settings. For example, the inhibition depended on IDO in some studies [135] but was IDO-independent in others [134, 137].

Functional activity of various T helper subsets is differentially affected by MSCs. Th1 and the production of IFN- $\gamma$  are inhibited by MSCs through the production of PGE2 [55], IL-10 [136], IDO [138], cell contacts, and other mechanisms [139]. MSCs also suppress the generation of Th17, the expression of ROR $\gamma$ c in differentiating cells, and the production of IL-17 and IL-22 by Th17. The effects are mediated by PGE2 [50, 140, 141], IDO [50], and IL-10 [142].

MSCs do not suppress Th2 proliferation [138], stimulate the production of IL-4, and may switch from Th1 to Th2 response augmenting the production of IL-4, IL-10, and IL-13, supposedly through the PGE2-dependent mechanism [55].

MSCs promote the generation of Treg and enhance their activity and IL-10 production. The effect is mediated by TGF- $\beta$  [90], by HLA-G5 [112], and indirectly through the generation of tolerogenic DCs (reviewed in detail in [91]).

This pattern is characteristic for MSCs derived from various sources and examined at different experimental settings. However, several exemptions have been reported. MSCs promoted the survival of quiescent T cells [143]. In Th2-predominating conditions, MSCs inhibited IL-4 and IL-5 and increased the production of IFN- $\gamma$  and IL-2 [144]. BM-MSCs derived from rheumatoid arthritis and osteoarthritis patients induced the activation and the expansion of Th17 [145]. Dysfunction of MSCs has been associated with several autoimmune disorders [125, 145].

**4.1.2. MDSCs.** In general, there are less data on the regulatory properties of MDSCs compared to MSCs. MDSCs inhibit the proliferation, IL-2, and IFN- $\gamma$  production by T cells stimulated *in vitro* with anti-CD3/CD28, specific antigens, or in MLR [146–151]. The suppression is mediated through the production IL-10 [150], NO, and peroxynitrite [19, 26, 148, 151], and ARG1 [151, 152] and indirectly through the formation of M2 [153].

Data on the effects of MDSCs on Th2 and Th17 cells are limited. Several studies have reported that endogenous or adoptively transferred MDSCs increase Th2 response and inhibit graft-versus-host disease (GVHD) [154–156]. Both, promotion and suppression of Th17 by MDSCs have been shown [157, 158].

MDSCs promote *de novo* development of Foxp3+ Tregs *in vivo*. Different studies have associated this effect with the production of ARG-1 [67], IDO [159], IL-10 [160], CD40, and direct MDSC-Treg contacts [161, 162].

Comparison of the effects, which MSCs and MDSCs exert on T cells, shows similarities in (i) the inhibition of T

cell proliferation and Th1 responses and (ii) the stimulation of Treg cells (Figure 2). This pattern corresponds to the mode of action of molecular mediators produced by MSCs and MDSCs (Figure 1). Th17 are generally suppressed by MSCs, although there are exemptions. Data on MDSCs-Th17 interactions are limited and contradictory. In line with this, different molecular mediators, utilized by MSCs and MDSCs, affect Th17 in different ways, suggesting that the final effect may depend on the combination of mediators that the cells produce in a given experimental setting. The same is likely true for Th2 cells.

As discussed above, most of the mediators produced by MSCs and MDSCs are induced by proinflammatory type 1 cytokines (e.g., IFN- $\gamma$ ). This suggests that the cells play immunoregulatory role and control Th1 responses through the negative feedback loop. On the other hand, several mediators (i.e., ARG-1, TGF- $\beta$ , and HLA-G5) can be induced by type 2 and regulatory cytokines (i.e., IL-13, IL-4, IL-10, and TGF- $\beta$ ). Whether in these “type 2 conditions” MSCs and MDSCs inhibit Th1 and support Th2 responses in a positive feedback manner, or switch their activity towards the suppression of Th2 (as it was demonstrated by Cho and coauthors [144]), is not completely clear. Further complication comes from the observations that the same mediator may play stimulatory or suppressive role depending on its concentration [44, 115] and that mediators produced by MSCs/MDSCs influence each other (see Figure 1). Evidently, studies are needed to create a quantitative model of cellular and molecular interactions that determine the final immunoregulatory properties of MSCs and MDSCs.

## 4.2. DCs and Macrophages

**4.2.1. MSCs.** MSCs suppress monocyte differentiation into DCs, decrease the expression of MHC class II, CD80, CD86, CD83, and CD40 by DCs, lower DC capacity for endocytosis, suppress the production of IL-12 and TNF- $\alpha$  by DC type 1, and stimulate the production of IL-10 by DC type 2. Overall, MSCs inhibit antigen presentation and T cell stimulation and promote the generation of tolerogenic DCs [163–170]. These effects have been attributed to the production of PGE2 [166], IL-6 [164, 167], IL-10 [168, 171], HGF [104, 165, 172], and TNF-stimulated gene 6 protein (TSG-6) [169]. Many of these factors operate by activating JAK/STAT pathway and suppressing the activation of mitogen-activated protein kinases (MAPKs) and NF- $\kappa$ B signaling pathways within DCs responding to TLR4 stimulation [168, 169, 173, 174]. Direct MSCs-DC contacts inhibit DC maturation and induce their tolerization by activating the Notch pathway [175] and altering actin cytoskeleton in the DCs [176]. *In vivo* administration of MSCs decreased DC migration to the draining lymph node and hampered local CD4 T cell priming. The effect was attributed to the inhibition of MyD88 and the impairment of MAPKs and NF- $\kappa$ B signaling pathways within DCs after TLR4 stimulation [177].

Two main and opposite types of macrophages have been defined, classically activated inflammatory (M1) and alternatively activated anti-inflammatory (M2) [178]. MSCs inhibit M1 and stimulate the generation of M2 macrophages:

coculture of MSCs with BM-derived macrophages decreased the expression of iNOS, TNF- $\alpha$ , IL-6, IL-12, and CCL2 (i.e., the markers of M1) and upregulated the expression of IL-10, ARG-1, CD206, and STAT3 (i.e., the markers of M2) [179, 180]. Similar effects were observed *in vivo* [181]. The underlying factors were PGE2 [181], TSG-6 [182], IDO [183], IL-6 [184], and direct cell contacts.

The activation of M2 likely plays a role in the therapeutic effects of MSCs. In experimental settings, systemically infused gingival MSCs homed to the wound site, promoted host macrophage differentiation into M2, and enhanced wound repair [184]. In the models of acute lung injury, MSCs shifted macrophage phenotype from M1 to M2 attenuating lung tissue inflammation [185, 186]. In one study, the effect was partially due to insulin-like growth factor I (IGF-I) secreted by MSCs [186].

**4.2.2. MDSCs.** MDSCs are cells that in the presence of appropriate cytokines differentiate into mature DCs and macrophages [6]. However, in pathology this differentiation is inhibited and MDSCs accumulate to affect different branches of immune response. Information on MDSCs-DCs and MDSCs-macrophages interactions is limited. In most studies, the effects were similar to those mediated by MSCs; that is, MDSCs inhibited DC maturation and polarized macrophages towards M2 phenotype, largely through the production of IL-10. In a clinical vaccine study, MDSCs copurified with monocytes suppressed DC maturation, antigen uptake, migration, and Th1 induction in a dose-dependent manner [187]. MDSCs isolated from hepatocellular carcinoma mice decreased the production of IL-12 by DCs [188].

When cocultured with macrophages, MDSCs reduced macrophage expression of MHC class II [17]. MDSCs from mice with spontaneous metastatic 4T1 mouse mammary carcinoma skewed macrophages towards the generation of M2 through IL-10 and cell contact dependent mechanisms [153]. In patients with esophageal cancer, increased ratio of MDSCs and augmented plasma levels of ARG1 were associated with high tissue expression of CD163, decreased IL-12 and IFN- $\gamma$ , and increased GATA3, IL-4, IL-13, and IL-6 expressions (evidences of M2 polarization) [156].

In tumor environment, MDSCs can directly differentiate into suppressive tumor-associated macrophages (TAM) [6]. Of note, suppressive macrophages were shown to stimulate MDSCs for the production of IL-10 [17]. Thus, MDSCs may form a positive feedback loop with TAM.

### 4.3. B Cells

**4.3.1. MSCs.** Data on MSCs-B lymphocyte interactions is not uniform. In some studies, MSCs inhibited proliferation, plasma-cell differentiation, IgM, IgG, and IgA production by B cells stimulated *in vitro* with CpG, recombinant CD40L, anti-Ig antibodies or IL-2, IL-4, and IL-10 cytokines. MSCs also suppressed B cell expression of CXCR4, CXCR5, and CCR7 and reduced B cell chemotaxis [131, 189–191].

In other studies, MSCs did not affect B cell proliferation [138] or even augmented it. In the study by Traggi and coauthors, MSCs enhanced the proliferation of purified B

cells obtained from healthy donors or pediatric systemic lupus erythematosus (SLE) patients and their differentiation into plasma cells [192]. One possible explanation for these discrepancies comes from the study by Rasmusson and coauthors. The study suggests that MSCs' activity depends on the level of basal B cell response: MSCs reduced high-level IgG response and enhanced low-level production of IgG induced by LPS, cytomegalovirus, or varicella zoster virus, that is, played a homeostatic role [193].

Breg cells are induced by MSCs. The induction has been registered both *in vitro* and *in vivo* and resulted in the amelioration of GVHD [190, 194, 195].

Mechanisms underlying MSCs-B-cell interactions are complex and not fully understood. Different authors reported the involvement of IDO [195], MSC-T-cell contacts [190, 191], IL-10, soluble factor other than IDO, TGF- $\beta$  or IL-10 [196], Galectin-9 [123], and CCL2 [125]. In SLE patients, BM-MSCs had reduced production of CCL2, which was associated with their defective capacity to suppress B cells. These findings suggest a potential role for MSCs in disease pathogenesis and demonstrate that MSCs generated in healthy and pathological conditions can exhibit different properties, uncovering another potential cause for conflicting data on MSCs-B cell interactions.

**4.3.2. MDSCs.** Data on MDSCs-B lymphocytes interactions are highly limited and only start to accumulate. Most of existing data report inhibitory effect of MDSCs on B lymphocytes. Following murine retroviral LP-BM5 infection, MDSCs expanded and suppressed *ex vivo* B cell responses, partially through iNOS/NO- and VISTA-mediated mechanisms [197, 198]. MDSCs generated in the presence of adipocyte-conditioned medium inhibited B lymphopoiesis largely through IL-1 [199]. MDSCs from mice with collagen-induced arthritis inhibited autologous B cell proliferation and antibody production in NO, PGE2, and cell-cell contact dependent manner [200]. Administration of monocytic MDSCs reduced autoantibody production and rescued CCR2<sup>-/-</sup> mice from the exacerbated collagen-induced arthritis [125, 200].

### 4.4. NK Cells

**4.4.1. MSCs.** MSCs inhibit NK cell proliferation, expression of activating receptors, and decrease NK cytotoxicity and IFN- $\gamma$  production [36]. In different settings, the effects were mediated by IDO, PGE2, TGF- $\beta$ , HLA-5, and cell contacts [36, 112, 201]. Following their coculture with MSCs, NK upregulate the expression of CD73 that has anti-inflammatory effect [202].

**4.4.2. MDSCs.** NK cultured with MDSCs produce less IFN- $\gamma$ . The suppression has been attributed to the production of ARG1 [203], COX2/PGE2 [204], cell-cell contacts involving NK cell activation receptor NKG2D, and membrane-bound TGF $\beta$  [205]. The role for MDSCs in the inhibition of NK *in vivo* was demonstrated in the study by Zhu and coauthors, who described the generation of granulocytic MDSCs following the administration of adenoviral vectors in mice;

depletion of MDSCs enhanced NK responses and accelerated virus clearance [206].

**4.5. Neutrophils.** Neutrophils are nonproliferating short-living cells that rapidly migrate to the site of infection/inflammation and eliminate pathogens or cellular debris.

**4.5.1. MSCs.** MSCs generally exhibit proneutrophilic action supporting neutrophil survival and inhibiting their apoptosis. The proneutrophilic effect was demonstrated for MSCs derived from various sources (i.e., BM, glandular, and adipose tissue) and was largely mediated by IL-6 [207]. It has been suggested that the proneutrophilic effect of MSCs plays a role in supporting this short-living population in the BM. MSCs activated by LPS stimulate the expression of CD11b by neutrophils [208] and are able to recruit neutrophils in IL-8 and MIF-dependent manner [209]. Data on the influence of MSCs on antibacterial properties of neutrophils are not uniform. In some studies, BM-MSCs dampened neutrophil respiratory burst [207], while in others enhanced it [208]. The stimulatory effect depended on IL-6, IFN- $\beta$ , and GM-CSF [208]. Hall and coauthors have demonstrated that MSCs may affect neutrophil function *in vivo*: the administration of BM-MSCs to septic mice stimulated bacteria clearance; neutrophil depletion abrogated the effect [210]. In one study, proneutrophilic effect of MSCs was mediated through the induction of Th17 [211].

**4.5.2. MDSCs.** The influence of MDSCs on neutrophils remains underinvestigated. Existing data are largely limited by the observation that the accumulation of MDSCs is accompanied by gradual disappearance of mature neutrophils [19]. These opposing relationships between MDSCs and neutrophils may be due to the incomplete differentiation of immature myeloid cells in inflammatory conditions leading to the accumulation of MDSCs and simultaneously to the reduction of mature neutrophils. Another suggested mechanism is efferocytosis of apoptotic neutrophils by MDSCs, which has been described in mice infected with *Klebsiella pneumoniae* or challenged with LPS [212, 213]. Thus, differentially from MSCs, MDSCs and neutrophils seem to be mutually exclusive. However, this speculation needs further investigation.

**4.6. Interactions between MSCs and MDSCs.** Only few studies directly addressed the interplay between MSCs and MDSCs. In the study by Yen and coauthors, human MSCs expanded CD14<sup>-</sup>CD11b<sup>+</sup>CD33<sup>+</sup> MDSCs that expressed ARG1 and NO, suppressed lymphocyte proliferation, and promoted Treg generation. The effect was mediated through the secretion of HGF and the induction of STAT3 [78]. In another study, growth-regulated oncogene GRO- $\gamma$  secreted by MSCs suppressed the generation of monocyte-derived DCs and stimulated the formation of MDSCs. The latter secreted IL-10 and IL-4 and expressed ARG1 and iNOS [214]. Galectins, known to be produced by MSCs, were reported to participate in the expansion of MDSCs at tumor sites [124]. MDSCs produce ROS. In physiological levels, ROS support MSCs' proliferation and differentiation, and, in higher amount, ROS

promote MSCs' aging [84]. Other mediators produced by MSCs and MDSCs (e.g., PGE2) can activate both populations of cells in a positive feedback manner.

## 5. Concluding Remarks

In this review, we have compared mechanisms and modes of immunoregulatory action of two immature cell populations: MSCs and MDSCs. The populations belong to two distinct differentiation lineages, but both are able to regulate immune response. MSCs and MDSCs share many immunomodulatory mechanisms and exert similar effects. In particular, they inhibit DC and macrophage maturation, antigen presentation, and suppress T cell proliferation, Th1 responses and NK activity. Both populations promote the generation of tolerogenic DCs, M2 macrophages, and regulatory T cells. Proinflammatory conditions activate suppressor capacities of both MSCs and MDSCs. This likeness is largely due to the usage of similar set of mediators, for example, IDO, PGE2, IL-10, and TGF- $\beta$  (Figure 1).

In spite of these similarities, comparative analysis reveals the discrepancies between the two populations. First, some mediators are produced by one but not by another subset. In particular, ARG1 and ROS seem to be restricted to MDSCs; the production of galectins and HLA-G has been attributed to MSCs but not to MDSCs. Second, MSCs and MDSCs are generally activated by proinflammatory type 1 cytokines. However, in some conditions, they can be stimulated by type 2 cytokines, and MDSCs seem to be more prone to this type of stimulation. Indeed, ARG1, which is expressed in MDSCs but not in MSCs, can be induced by IL-4, IL-13, and TGF- $\beta$ . In MSCs, the expression of B7-H1 and the production of TGF- $\beta$  were induced by IFN- $\gamma$ , whereas in MDSCs by IL-10 and IL-13 [37, 129]. Third, MSCs exert proneutrophilic effects, supporting neutrophil survival and function [207–210]. MDSCs, in contrast, seem to oppose neutrophilic inflammation [19, 212, 213]. Fourth, MSCs expand MDSCs. Whether and how MDSCs affect MSCs is largely unknown. Fifth, MSCs are generally considered as immunoregulatory cells that can inhibit or enhance immune function depending on cell microenvironment [5, 15]. MDSCs are currently regarded as immunosuppressive cells. Whether MDSCs may be considered as immunoregulatory cells that act by supporting immune homeostasis is not yet clear.

Several other questions remain unanswered and need further investigation.

In particular, the pattern of the interplay between MSCs/MDSCs and Th2, Th17, and B lymphocytes is not fully clear. Both stimulation and inhibition of these responses by MSCs and MDSCs have been documented [55, 144, 154–156, 189–193, 231], and exact factors that determine the ultimate result are yet to be determined. One group of factors is represented by TLR ligands. The contribution of different TLR-mediated pathways to pro- or anti-inflammatory functions of MSCs/MDSCs is one of the recently emerged areas of research [213, 215].

In proinflammatory conditions, MSCs and MDSCs are activated to inhibit type 1 response, that is, act in a negative feedback manner. Whether in “type 2 conditions” the cells

inhibit Th1 or Th2 responses, that is, participate in positive or negative feedback loop, remains unclear.

As noted above, MSCs and MDSCs share a set of core regulatory mediators and mechanisms. However, they differentially affect some immune cells. Molecular mechanisms underlying these discrepancies remain unknown.

MSCs and MDSC can simultaneously produce a wide range of immunoregulatory factors that have similar but not identical activity (Figure 1). Furthermore, the subsets of the produced factors and the amounts secreted may vary in different conditions. Overall, this creates the possibility for MSCs/MDSCs to fine-tune different branches of the immune response and simultaneously makes their final effect difficult to predict. Quantitative models of cellular and molecular interactions that determine the final immunoregulatory properties of MSCs and MDSCs would help to predict their effects in various microenvironments, both *in vitro* and *in vivo*.

Speaking about possible clinical applications, MSCs are widely considered for the purposes of clinical immunomodulation due to their homeostatic properties and the feasibility of generating the large numbers of autologous cells. MSCs have been suggested as a mean to treat severe life-threatening forms of autoimmune and autoinflammatory diseases (e.g., SLE, systemic sclerosis, and inflammatory bowel disease [216–219]), prevent and treat steroid-refractory graft-versus-host disease [220], improve the outcome after organ transplantation [221], and stimulate tissue repair, regeneration, and wound healing [222–227]. Detailed analysis of MSC therapeutic potential, risks, and limitations of their application is beyond the focus of the current review. In contrast to MSCs, MDSCs are usually regarded as the target for immunomodulation, particularly, in cancer where they accumulate abundantly and contribute to pathology [228, 229]. However, in autoimmune pathology, MDSC dysfunction may be a factor driving disease progression and can be limited by the administration of exogenous MDSCs [24]. Thus, the question whether MDSCs can be used for therapeutic immunomodulation in some pathological conditions remains to be explored. It is important to note that MDSCs can be grown *in vitro* and they are more differentiated compared to MSCs; thus, they have a lower risk of cell transformation.

In conclusion, this review does not imply to describe all effects and mechanisms mediated by MSCs/MDSCs, as they are multiple and vary in different conditions. Rather, it is an attempt to compare the main patterns of MSCs/MDSCs' activities in a way to detect cell similarities and discrepancies and identify new directions for their investigation.

## Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

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## Research Article

# *Lactobacillus rhamnosus* GG Activation of Dendritic Cells and Neutrophils Depends on the Dose and Time of Exposure

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This study evaluates the ability of *Lactobacillus rhamnosus* GG (LGG) to activate DC and neutrophils and modulate T cell activation and the impact of bacterial dose on these responses. Murine bone marrow derived DC or neutrophils were stimulated with LGG at ratios of 5:1, 10:1, and 100:1 (LGG: cells) and DC maturation (CD40, CD80, CD86, CD83, and MHC class II) and cytokine production (IL-10, TNF- $\alpha$ , and IL-12p70) were examined after 2 h and 18 h coculture and compared to the ability of BCG (the present immunotherapeutic agent for bladder cancer) to stimulate these cells. A 2 h exposure to 100:1 (high dose) or an 18 h exposure to 5:1 or 10:1 (low dose), LGG: cells, induced the highest production of IL-12 and upregulation of CD40, CD80, CD86, and MHC II on DC. In DCs stimulated with LGG activated neutrophils IL-12 production decreased with increasing dose. LGG induced 10-fold greater IL-12 production than BCG. T cell IFN $\gamma$  and IL-2 production was significantly greater when stimulated with DC activated with low dose LGG. In conclusion, DC or DC activated with neutrophils exposed to low dose LGG induced greater Th1 polarization in T cells and this could potentially exert stronger antitumor effects. Thus the dose of LGG used for immunotherapy could determine treatment efficacy.

## 1. Introduction

*Lactobacillus rhamnosus* GG (LGG) is a gram positive lactic acid bacterium that is part of the commensal microflora in humans. It is generally regarded as safe and has been used extensively in food products and health supplements. LGG has been reported to alleviate allergies and dermatitis [1, 2]. Meta-analysis of probiotic supplementation during pregnancy and early infancy indicates a reduced risk ratio of developing eczema in early infancy [3]. A meta-analysis of LGG supplementation showed increased treatment responders in subjects with abdominal pain related gastrointestinal disorders and Irritable Bowel Syndrome [4]. Ohashi et al. also found that long-term consumption of *Lactobacillus casei* was associated with the reduced risk of bladder cancer [5]. LGG was also shown to possess antitumor effects in animal models of bladder cancer [6, 7]. The antitumor effects were

comparable to that induced by *Mycobacterium bovis*, bacillus Calmette-Guérin [7]. Intravesical instillations of LGG resulted in an influx of dendritic cells (DCs) and neutrophils [7]. Despite BCG's efficacy it is associated with significant side-effects and less toxic therapies are needed [8]; thus this study further evaluates the immunotherapeutic potential of LGG.

DCs are antigen presenting cells that play an important role in cancer immunotherapy by stimulating cytotoxic T lymphocytes (CTL) and polarizing T helper cells towards a Th1 profile. DC maturation causes enhanced expression of surface costimulatory molecules and production of cytokines and chemokines. However, extensive stimulation of DC can result in DC exhaustion that is characterized by diminished production of IL-12 [9] which is necessary for CTL induction and interferon gamma (IFN $\gamma$ ) [10] production. DC exhaustion can be the result of prolonged exposure to a stimulus

or exposure to a very high dose of a stimulus either of which scenarios are possible when analyzing the interaction of microbes with immune cells.

Miyazaki et al. [11] showed that, upon inflammation, neutrophils migrate from the site of infection to neighboring lymph nodes where they undergo apoptosis and are taken up by DCs, thus ensuring that neutrophil derived antigens are presented to T cells. Neutrophils are also able to directly transfer antigens to DCs as was demonstrated by Morel et al. [12], studying BCG infected neutrophils.

This work evaluates the impact of the dose of LGG and time of exposure on DC activation in the absence and presence of neutrophils and the consequent stimulation of T cells. The mouse orthotopic tumor model used to assess the intravesical instillation of LGG into the bladder followed the clinical protocol of BCG immunotherapy and was performed over a 2-hour time frame [7]. Thus, this was the minimum time of interaction that was analyzed and 18 h was chosen as the maximum time of interaction as, beyond this time frame, DC viability was greatly reduced after exposure to a high dose of LGG. The death induced by LGG on longer exposure may be a consequence of lactic acid production as observed with cancer cells exposed to LGG [13].

## 2. Materials and Methods

**2.1. Bacterial Preparation.** *L. rhamnosus* GG (National Collections of Industrial and Marine Bacterial Ltd., UK) was streaked onto deMan Rogosa Sharpe (MRS) agar (Difco Laboratories, USA) and incubated at 37°C in 5% CO<sub>2</sub> [14]. Single colonies were used to produce seed cultures (9 h) which were used to start 50 mL cultures. Bacteria were harvested at the late log phase by centrifugation (1699 ×g for 10 minutes at room temperature) and washed twice with sterile saline (0.85% NaCl). The colony forming units (CFU) were determined by plating serial dilutions of the bacterial samples on MRS agar plates which were incubated at 37°C in 5% CO<sub>2</sub>. BCG Connaught was prepared in the lab as previously described [15].

**2.2. Preparation of Bone Marrow Derived Neutrophils, DC, and T Cells.** Protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the National University of Singapore. Bone marrow derived neutrophils and DC were generated as previously described [16]. In brief, neutrophils were derived by positive selection with anti-Ly6G microbead kit (Miltenyi Biotec, Germany) and were at least 95% positive for Ly6G, by flow cytometry. DCs were obtained from the bone marrow derived cells after 9 days of culture (with fresh media replacement every other day) in RPMI 1640 supplemented with 10% heat-inactivated FCS, 50 μM 2-mercaptoethanol, 1% penicillin, streptomycin, glutamine, MEM (minimum essential medium), and 0.1% sodium pyruvate with 40 ng/mL of GM-CSF (BD Bioscience, USA). The DCs were at least 95% positive for CD11c, by flow cytometry. The media used for both neutrophil and DC experiments were DMEM supplemented with 10% fetal bovine serum (Hyclone, USA), 2 mM L-glutamine (Gibco,

Japan), 50 μg/mL penicillin G (Sigma Aldrich, USA), and 50 μM of 2-mercaptoethanol (Merck, Germany) with the addition of 20 ng/mL of GM-CSF for the culture of dendritic cells.

T lymphocytes were isolated from spleens of naive C57BL/6 mice and enriched with the EasySep™ T cell isolation kit (STEMCELL Technologies, Vancouver, Canada). The desired fraction was about 95–98% CD3 positive.

**2.3. Neutrophil, Dendritic Cell LGG Coculture, and Blocking of IL-10 and COX-2.** The LGG to cell ratios of 5:1 and 10:1 were defined as exposure to low dose LGG, while exposure to a ratio of 100:1 was defined as exposure to high dose LGG. Neutrophils ( $5 \times 10^5$ ) and DCs ( $2.5 \times 10^5$ ) were cocultured with LGG at bacteria to mammalian cell ratios of 5:1, 10:1, and 100:1 for 2 h in 24-well plates, before 200 μg/mL of gentamicin was added for 2 h at 37°C to kill extracellular LGG. Cells were washed thrice with PBS to remove extracellular bacteria and then neutrophils were incubated with DCs ( $2.5 \times 10^5$ ) for 18 h and DCs were incubated in fresh media for 18 h. Untreated neutrophils and DCs were evaluated as controls. All controls were given the same treatment as above. For  $2.5 \times 10^5$  DCs the bacteria CFU that corresponded to 5:1, 10:1, and 100:1 were  $1.25 \times 10^6$ ,  $2.5 \times 10^6$ , and  $25 \times 10^6$  CFU. The neutrophils ( $5 \times 10^5$  cells) were treated with  $2.5 \times 10^6$ ,  $5 \times 10^6$ , and  $50 \times 10^6$  CFU of LGG that corresponded to 5:1, 10:1, and 100:1, LGG to cells. For the DC 18 h experiment the DCs were exposed to LGG for 18 h. Similarly cells were treated with BCG at a 5:1 ratio. The supernatants were assayed for TNF-α, IL-12p70, and IL-10 (eBioscience, San Diego, USA) and prostaglandin E2 (PGE2) (Cayman Chemical, USA) by ELISA using a GENios Pro™ microplate reader (Tecan, Switzerland). The cells were harvested in PBA (PBS with 1% bovine serum albumin and 0.01% sodium azide) for flow analysis of surface markers.

IL-10 and COX-2 were inhibited by pretreatment with 400 ng/mL of anti-IL-10 antibody (Biolegend, San Diego, USA) and 10 μM of NS398 (Sapphire Bioscience, Australia), respectively, for 30 mins at 37°C prior to addition of bacteria and then further incubated for another 18 hours. The respective isotype and solvent controls were included for comparison. The efficacy of the blocking was confirmed by ELISA.

**2.4. Flow Cytometry and Antibodies.** Fixed DCs were double stained with anti-CD11c antibody and antibodies of the following surface markers: CD40, CD80, CD83, CD86, and MHC II (Biolegend) or the respective isotype controls in PBA (1x PBS with 1% BSA, 0.05% sodium azide) for 20 mins at 4°C. After that the cells were washed once and resuspended with PBA before they were analyzed with BD FACS Canto using FACS Diva software (Becton Dickinson, USA).

**2.5. DC-T Cell Coculture.** DCs or T cells were resuspended in LDA medium (NCTC 109 and RPMI 1640 [1:1]), supplemented with 10% heat-inactivated FCS, 10 mM L-glutamine, 1 mM oxaloacetic acid, 0.2 U of bovine insulin per mL, and 50 μM 2-mercaptoethanol. Naive T cells ( $1.0 \times 10^7$  cells/mL)

TABLE 1: Surface marker expression on DC after direct and indirect exposure via neutrophils to BCG and LGG.

Marker	DC	Percentage of CD11c <sup>+</sup> cells				
		Control	5:1 (BCG)	5:1 (LGG)	10:1 (LGG)	100:1 (LGG)
CD40	2 h	6.0 ± 3.3	22.5 ± 3.3	32.1 ± 4.9	32.7 ± 0.3	50.7 ± 2.6 <sup>b</sup>
	18 h	2.2 ± 0.5	19.3 ± 5.4	20.5 ± 6.9	19.0 ± 6.8	7.0 ± 0.8 <sup>b</sup>
	+neutrophils (2 h)	2.2 ± 0.4	27.8 ± 3.5	22.9 ± 5.2	25.4 ± 8.7	10.4 ± 2.0 <sup>b</sup>
CD80	2 h	9.9 ± 1.4	39.3 ± 10.4	18.2 ± 0.8	18.1 ± 3.3	34.0 ± 4.2 <sup>b</sup>
	18 h	9.6 ± 1.6	30.7 ± 7.5	17.9 ± 0.5	19.7 ± 2.5	19.6 ± 0.8
	+neutrophils (2 h)	6.1 ± 1.6	33.8 ± 8.9	21.2 ± 5.0	16.6 ± 7.6	16.1 ± 7.8
CD83	2 h	6.5 ± 0.2	29.6 ± 5.7	38.4 ± 3.7	32.7 ± 1.3	36.2 ± 4.7
	18 h	5.1 ± 1.5	27.5 ± 10.5	8.4 ± 1.9	4.8 ± 0.8	6.5 ± 1.4
	+neutrophils (2 h)	3.6 ± 0.2	31.2 ± 4.8	9.8 ± 1.3	8.0 ± 0.8	11.1 ± 2.8
CD86	2 h	17.0 ± 0.3	72.0 ± 5.4	37.1 ± 8.1	41.6 ± 1.4	58.5 ± 6.1 <sup>b</sup>
	18 h	14.8 ± 1.9	71.0 ± 7.9	57.6 ± 0.1	56.5 ± 1.3	29.7 ± 6.0 <sup>b</sup>
	+neutrophils (2 h)	13.7 ± 0.2	68.0 ± 4.8	68.3 ± 6.4	76.5 ± 4.4	54.9 ± 3.8 <sup>b</sup>
MHC class II	2 h LGG	31.7 ± 1.8	61.9 ± 2.5	69.9 ± 8.8	70.7 ± 12.8	77.8 ± 11.1
	18 h LGG	32.6 ± 2.7	59.2 ± 6.1	58.4 ± 12.5	52.9 ± 7.2	57.4 ± 11.6
	+neutrophils (2 h)	34.7 ± 10.1	55.3 ± 10.2	58.5 ± 12.9	52.1 ± 6.3	64.0 ± 9.4

<sup>b</sup>  $p < 0.05$  compared to groups treated with 5:1 and 10:1 LGG, respectively. Data are presented as the mean ± SEM.

were cultured with untreated DCs ( $1.0 \times 10^5$  cells/mL) or DCs stimulated with lactobacilli for 2 h and 18 h (treated as described above), in 200  $\mu$ L of LDA medium in 96-well U-bottom plates at 37°C under 5% CO<sub>2</sub> for 5 days. After 5 days, supernatants were harvested and analyzed for IFN gamma or IL-2.

**2.6. Statistical Analysis.** One-way ANOVA with post hoc Bonferroni test was used to analyze all the data except for the comparison of cytokine profile of the treatment groups with anti-IL-10 antibody or NS398 and their respective controls, which were analyzed with Student's *t*-test. A significant difference was taken to exist when the *p* value was <0.05.

### 3. Results

**3.1. LGG Dose, Exposure Time, and Neutrophils Modulate DC and Neutrophil Maturation and Viability.** A short exposure (2 h) to low dose LGG (LGG to cell ratios of 10:1 and 5:1) reduced DC viability slightly ( $91.7 \pm 2.0\%$  and  $94.7 \pm 1.7\%$ , resp.) and there was little loss in viability even at 18 h. Exposure to activated neutrophils had a similar effect. However at a prolonged exposure of 18 h to high dose LGG (100:1 LGG to DC ratio), there was reduced DC viability ( $63.7 \pm 1.8\%$ ).

About 50% of neutrophils were dead (apoptotic and necrotic death) at 18 h after the initial 2 h exposure to LGG regardless of dose. But in contrast there was increased LGG internalized with exposure to increased LGG dose. At a 5:1 ratio of LGG:neutrophils there were  $228 \pm 51$  CFU internalized/ $5 \times 10^5$  neutrophils and this almost doubled after exposure to 10:1 and more than doubled again after exposure

to 100:1 LGG to neutrophils. The LGG in the neutrophils were still viable at 18 h after internalization.

Activation markers on naïve DC were examined and after exposure to LGG there was a significant increase in all markers with respect to naïve DC, Table 1. As a further control DCs were also exposed to BCG, Table 1. After high dose LGG exposure for 2 h, there was significantly ( $p < 0.05$ ) higher expression of CD80, CD86, and CD40 compared to low dose LGG. But at 18 h coincubation with LGG, expression of CD86 and CD40 was significantly reduced ( $p < 0.05$ ) after exposure to high dose LGG compared to low dose LGG. DCs cocultured with neutrophils, activated with low doses of LGG for 2 h, showed higher expression of CD86 and reduced CD83 compared to DCs exposed directly to low dose LGG for 2 h. DC exposed to BCG at a dose of 5:1, for 2 h or 18 h, or stimulated by neutrophils activated with BCG did not show a difference in surface marker expression. In contrast LGG at the same dose showed changes in the expression of CD83 and CD86.

Neutrophils cultured with LGG showed decreased MHC class I expression, no increase in MHC class II expression, and an increase in CD11b expression when placed directly in contact with LGG. CD11b is an activation marker for neutrophils and has been shown to activate DC maturation via interaction with DC-SIGN [17].

MHC class II mean fluorescence index (MFI) showed a doubling on exposure to low dose LGG or activated neutrophils, Table 2. The MFI for MHC class II, CD40, and CD80 was decreased after exposure to DC activated for 18 h, with either high dose LGG or neutrophils activated with high dose LGG ( $p < 0.05$ ). But the reverse was true for CD83 when DCs were exposed to high dose LGG for 18 h

TABLE 2: LGG dose and time of exposure modulated the MFI of DC surface markers.

Marker	Group	Control	5:1	10:1	100:1
MHC class II	DC 2 h	1191 ± 10	2522 ± 2 <sup>a</sup>	2545 ± 94 <sup>a</sup>	2978 ± 245 <sup>a</sup>
	DC 18 h	1303 ± 37	2066 ± 154 <sup>a</sup>	2124 ± 29 <sup>a</sup>	1530 ± 3 <sup>bc</sup>
	DC + neutrophils	1191 ± 28	2010 ± 107 <sup>a</sup>	2352 ± 78 <sup>a</sup>	1946 ± 80 <sup>ac</sup>
CD40	DC 2 h	539 ± 1	629 ± 1 <sup>a</sup>	654 ± 1 <sup>a</sup>	684 ± 6 <sup>abc</sup>
	DC 18 h	489 ± 42	735 ± 27 <sup>a</sup>	721 ± 1 <sup>a</sup>	370 ± 8 <sup>bc</sup>
	DC + neutrophils	437 ± 61	576 ± 15	642 ± 18 <sup>a</sup>	390 ± 21 <sup>c</sup>
CD83	DC 2 h	465 ± 20	417 ± 11	554 ± 79	452 ± 22
	DC 18 h	668 ± 20	506 ± 33 <sup>a</sup>	681 ± 7 <sup>b</sup>	702 ± 12 <sup>b</sup>
	DC + neutrophils	704 ± 34	609 ± 61 <sup>a</sup>	441 ± 54 <sup>a</sup>	351 ± 3 <sup>ab</sup>
CD80	DC 2 h	515 ± 4	537 ± 5	516 ± 14	568 ± 15 <sup>ac</sup>
	DC 18 h	513 ± 3	544 ± 12	535 ± 10	478 ± 28 <sup>b</sup>
	DC + neutrophils	476 ± 6	613 ± 16 <sup>a</sup>	553 ± 10 <sup>a</sup>	478 ± 21 <sup>bc</sup>

<sup>a</sup>  $p < 0.05$  compared to control with no bacteria.

<sup>b,c</sup>  $p < 0.05$  compared to groups treated with 5:1 and 10:1 LGG, respectively.

Data are presented as the mean ± SEM.

( $p < 0.05$ ), Table 2. The MFI of CD86 did not vary with treatment conditions.

**3.2. LGG Dose, Exposure Time, and Neutrophils Modulate DC Cytokine Production.** More IL-10 was produced after exposure to high dose LGG (Figure 1(a)) for 2 h and 18 h and via neutrophil mediated activation. TNF- $\alpha$  production was higher in DC exposed to high dose LGG (Figure 1(a)) for 2 h, but at 18 h, DC exposed to low dose LGG produced more TNF- $\alpha$ . Both indirect (via neutrophils) and direct DC activation for 18 h resulted in more IL-12p70 production after low dose LGG exposure (Figure 1(a)). With a short 2 h exposure to LGG, IL-12p70 production was independent of the bacterial dose.

Neutrophils stimulated with LGG produced IL-12p70 and TNF- $\alpha$  and very little IL-10. LGG activated neutrophils (2 h) induced more IL-10, TNF- $\alpha$ , and IL-12p70 production in DC compared to DC exposed directly to low dose LGG for 2 h. At high dose LGG there was no significant difference in IL-12p70 and TNF- $\alpha$  production, whether the DC was stimulated directly with LGG or with activated neutrophils.

In contrast, when DC and neutrophils were exposed to BCG at a 5:1 ratio [12], there was comparable production of IL-10 from all groups except DC exposed to BCG for 18 h. The amount of IL-12p70 produced after BCG stimulation was at least 10-fold lower than that produced by LGG. The TNF- $\alpha$  response was comparable to LGG (Figure 1(a)).

**3.3. IL-12p70 Production after Exposure to LGG Stimulated Neutrophils Is Dependent on IL-10.** Since, at high dose LGG, IL-10 production is significantly higher in DCs, as well as DC treated with activated neutrophils, we determined if the dose dependent effects on IL-12 production were due to IL-10 levels. PGE<sub>2</sub> is known to modulate IL-10 expression; induce indoleamine 2,3-dioxygenase (a potent suppressor of DC function); and modulate chemokine production and DC maturation and IL-12p70 production [18–20]. Therefore

PGE<sub>2</sub> and IL-10 production/function were inhibited individually and the impact on IL-12p70 production was monitored, Figure 1(b). There was a significant increase in PGE<sub>2</sub> levels on DC stimulation with high rather than low dose LGG. At the concentration of NS398 that completely blocked the production of PGE<sub>2</sub> (Figure 1(b)) there was no significant effect on either IL-10 or IL-12p70 levels, Figure 1(b). Blocking IL-10 with a neutralizing antibody caused a sharp increase in IL-12p70 production, Figure 1(b). This corresponded to 2.1- and 4.4-fold increases, respectively, in DC stimulated with neutrophils activated with low and high dose LGG. Expression of surface markers on dendritic cells was not significantly affected by the presence of either the anti-IL-10 antibody or NS398 (data not shown).

**3.4. T Cell Activation Is Dependent on LGG to DC Ratios and Time of Exposure.** Neutrophils stimulated with LGG did not induce IFN $\gamma$  production by T cells (Figure 2(a)) but induced IL-2 production (Figure 2(b)). DCs stimulated with LGG activated neutrophils induced a significant increase ( $p < 0.05$ ) in IFN $\gamma$  production (Figure 2(a)) and a slight increase in IL-2 production by T cells (Figure 2(b)) similar to DC directly activated with low dose LGG. The DC-neutrophil-T cell triple cell culture by itself induced IL-2 production. A dose dependent effect was clearly seen with IFN $\gamma$  production ( $p < 0.05$ ) (Figure 2(a)) which was consistent with the decrease in IL-12p70 production that was observed earlier. Direct or indirect DC activation with low dose LGG for 2 h induced more IFN $\gamma$  and IL-2 production by T cells ( $p < 0.05$  for IFN $\gamma$ ) compared to high dose LGG, Figures 2(a) and 2(b). At 18 h the differential effect of the dose was lost for IFN $\gamma$ .

## 4. Discussion

On activation by LGG, there was an increase in the percentage of DC expressing CD40, CD80, and CD86 with increasing dose. But only CD40 had a significant increase in MFI

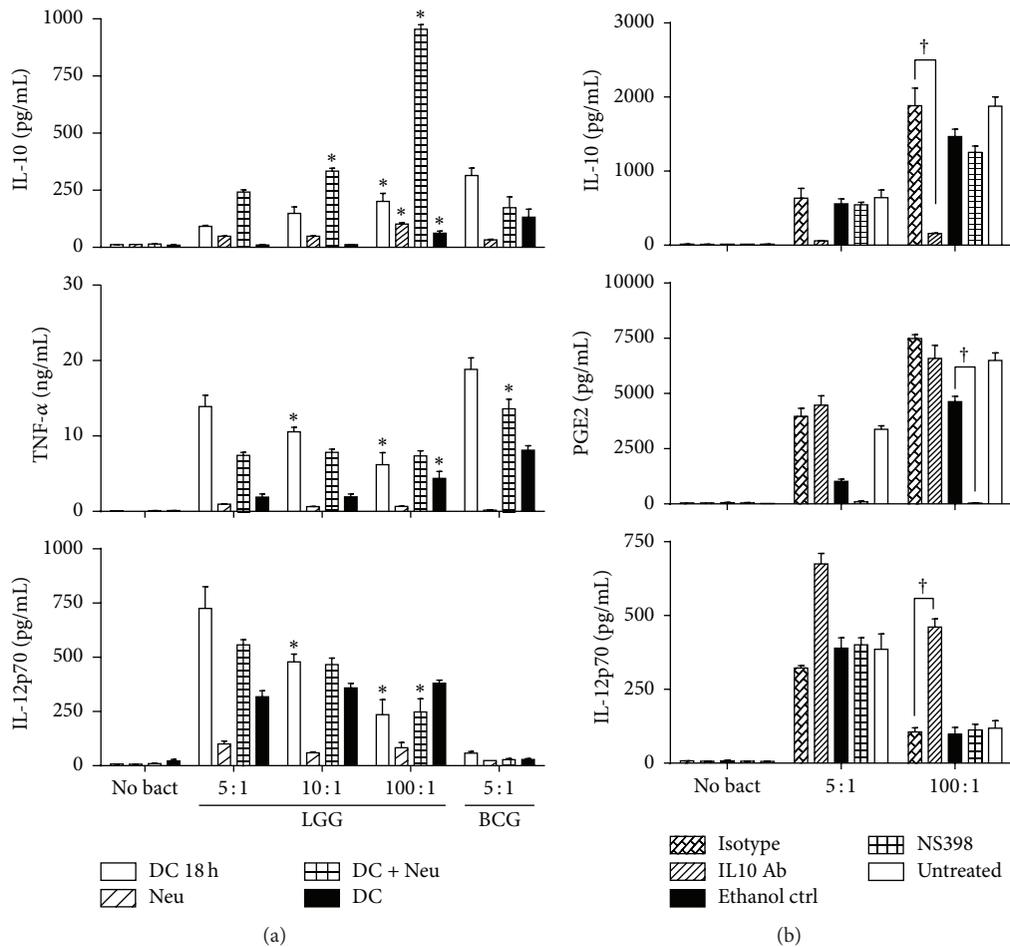


FIGURE 1: LGG and BCG induced dose and time dependent effects on DC cytokine production by direct or indirect stimulation via neutrophils. LGG was assessed at 5:1, 10:1, and 100:1 ratios and BCG at 5:1 ratios. (a) Production of IL-10, TNF- $\alpha$ , and IL-12p70 after 18 h of continuous coculture of DCs with LGG/BCG (white bar), 2 h of exposure of DCs (black bar) or neutrophil (striped bar) to LGG/BCG, followed by 18 h of bacteria free incubation and 18 h of DC coculture with neutrophils pretreated with LGG/BCG for 2 h (crisscross bar). “\*” indicates a significant difference ( $p < 0.05$ ) compared to low dose (5:1). For BCG “\*” indicates a significant difference from neutrophils and for TNF- $\alpha$  significance with respect to DC. Data are presented as the mean  $\pm$  SEM. (b) Neutrophils were prestimulated with low (5:1) and high (100:1) dose of LGG for 2 h before they were cocultured with DC for 18 h in the presence and absence of a COX-2 inhibitor, NS398 (crisscross bar); COX-2 inhibitor solvent control (black bar); IL-10 neutralizing antibody (IL-10 Ab) (striped bar); and the isotype control for the antibody (double striped bar). The impact on PGE<sub>2</sub>, IL-10, and IL-12p70 secretion is shown. “†” indicates a significant difference from the respective controls ( $p < 0.05$ ). Data are presented as the mean  $\pm$  SEM.

( $p < 0.05$ ), indicating increased protein expression. CD86 and CD80 interact with CD28 on T cells while CD40 binds to the CD40 Ligand on T cells to induce T cell activation. On prolonged exposure (18 h) to high dose LGG, the percentage of cells expressing CD86 and CD40 and the MFI of CD40, CD86, and CD80 were reduced. Thus, these DCs may not be as able to activate T cells as efficiently; that is, there is a point beyond which LGG dose can be inhibitory to DC activation. A similar effect was observed with *L. casei* [21] which also has antitumor effects [22] and other commensal lactobacilli such as *L. gasseri*, *L. johnsonii*, and *L. reuteri* [23].

Different *Lactobacillus* species induce variable levels of IL-10, IL-12, and TNF- $\alpha$  via Toll-like receptor (TLR) dependent activation of DC [24]. Indirect DC stimulation via LGG activated neutrophils showed no difference in TNF- $\alpha$  production with increasing dose. But primary interaction

between DC and LGG showed dose dependent effects. This could be due to TLR engagement and phagocytosis [25]. LGG is known to adhere to epithelial cells with greater ability than other *Lactobacillus* species. Such binding to DC may also increase the cellular signals triggered by direct interaction with DC. Tytgat et al. found that LGG pili S SpaCBA could interact with DC-SIGN and that blocking this interaction reduced DC cytokine production [26]. DC-SIGN also modulates TLR activation and it is possible that LGG pili interaction with DC-SIGN could have modulated TLR activated cytokine production. An 18 h exposure to low dose LGG produced more TNF- $\alpha$  and IL-12 than exposure to high dose LGG. It is likely that prolonged exposure led to increasing phagocytosis of LGG with time which resulted in triggering the downregulation of TNF- $\alpha$  and IL-12 production. IL-12 production is TLR2 independent [25] but

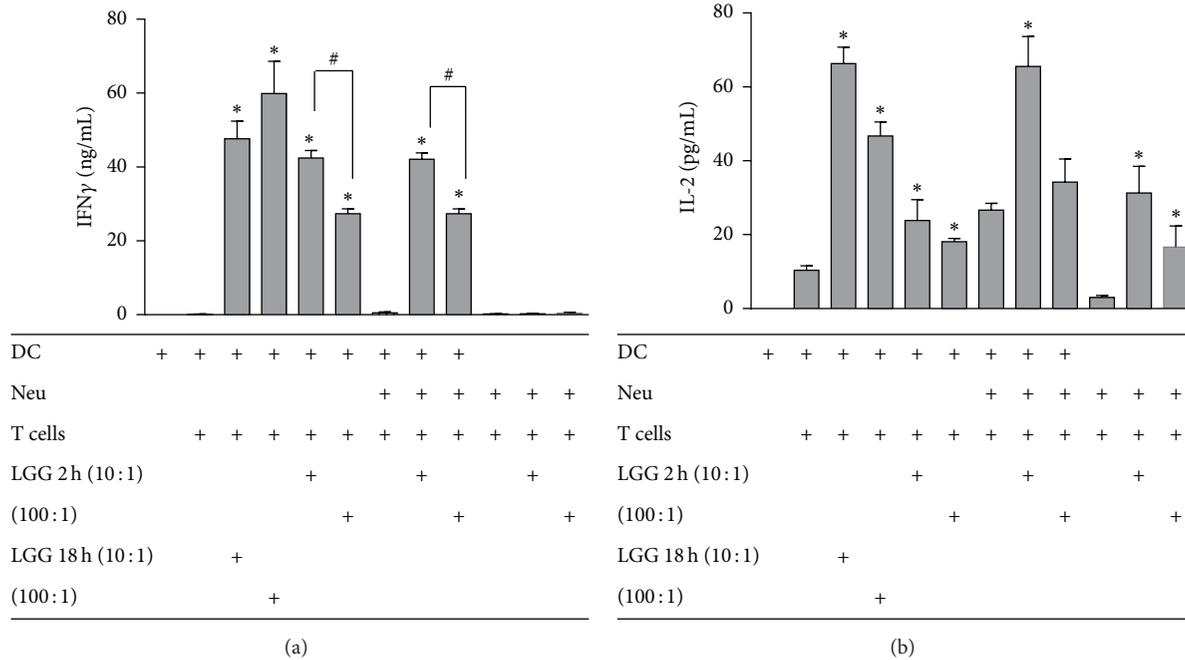


FIGURE 2: T cell activation is dependent on the dose of LGG used to stimulate DC or neutrophils. IFN $\gamma$  (a) and IL-2 (b) production by T cells after 5 days of coculture with DC, neutrophil, or DC-neutrophil (stimulated with low or high dose of LGG for 2 h and then DC for 18 h). Data are presented as the mean  $\pm$  SEM. “\*” indicates a significant difference compared to their respective no LGG controls ( $p < 0.05$ ). “#” indicates significant difference between high and low dose of LGG treatment ( $p < 0.05$ ).

TLR9 [27], MyD88, and ROS [28] dependent. Phagocytosis of LGG is important, as is the presence of undigested bacteria [29]. However, with a short exposure to LGG there was a dose dependent effect only on TNF- $\alpha$  production which might reflect TLR2 engagement [25].

As neutrophils are generally the first to encounter microbes and move to the lymph nodes to educate DC [30], we evaluated the dose effect of LGG on the ability of neutrophils to activate DC. Stimulating DC with LGG treated neutrophils exposed to low dose LGG induced higher CD86 than direct stimulation of DCs with LGG. Neutrophils cultured with high dose LGG induced a decrease in the MFI of CD40, CD80, and CD83 ( $p < 0.05$ ) on DCs. Though the number of neutrophils undergoing apoptosis was similar at all doses of LGG the number of internalized LGG increased with dose of LGG. The latter may have resulted in increased LGG or LGG components being transferred to DC [11, 12] causing strong stimulation of DC and consequently DC exhaustion. In line with this hypothesis, IL-12p70 and TNF- $\alpha$  production were much higher when the DCs were cultured with neutrophils activated with low dose LGG. DCs are known to internalize apoptotic cells [31] which like necrotic cells are able to stimulate DC [32]. Phagocytosis of apoptotic cells induces anti-inflammatory signals [33] such as the high levels of IL-10 which was found in this study.

IFN $\gamma$  production was higher in T cells cocultured with DC and neutrophils treated with low dose LGG for 2 h rather than high dose LGG. Similarly, when DCs were treated with LGG at 200:1 bacteria to cell ratio, phenotypic maturation and cytokine production but not Th1 polarization

were observed [21]. Instead, the CD4<sup>+</sup> cells were converted to hyporesponsive T cells that secrete low IFN $\gamma$ . Thus, for optimal T cell activation, low dose LGG is overall the better therapeutic option.

Prolonged stimulation of DCs (for 24 h or longer) can result in the loss of the ability to produce cytokines like IL-12, which is termed DC exhaustion [9, 34]. These “exhausted” DCs tend to induce Th2 cell differentiation. Langenkamp et al. reported that the optimal temporal window to induce DC maturation in order to have sustained IL-12 production for cancer immunotherapy is narrow, with a time frame of 10–18 h [9], but our results indicate that a 2 h exposure is sufficient for DC maturation. Further LGG was much better at inducing IL-12p70 production than BCG, the current standard therapy for bladder cancer.

IL-10 is widely reported to downregulate DC maturation [35, 36] and its ability to activate T cells [37] as well as induce DC apoptosis [38]. PGE<sub>2</sub>, a potent inducer of IL-10 [20], was also found to be produced in greater amounts when DCs were stimulated with neutrophils treated with high dose LGG. Neutralization of IL-10 substantially increased the IL-12p70 production. However, it was still lower than the levels produced by DC coculture with neutrophil stimulated with low dose LGG, suggesting that there are other inhibitory factors aside from IL-10.

## 5. Conclusion

Low dose LGG stimulates DC to induce greater Th1 polarization in T cells compared to high dose LGG. Thus, low dose

LGG would potentially be able to exert stronger antitumor effects. In mice LGG ( $1 \times 10^8$  CFU/100  $\mu$ L) was effective at reducing tumor growth with comparable efficacy to BCG Connaught ( $1 \times 10^7$  CFU/mL) [7]. The former is roughly in the range of a 100 : 1, LGG to cells for 2 h. Thus future analysis should consider the effect of a 10-fold lower dose of LGG as an immunotherapeutic agent. The dose response is an important consideration if LGG is to be used for human bladder cancer therapy.

## Abbreviations

LGG: *Lactobacillus rhamnosus* GG

BCG: Bacillus Calmette-Guérin

DCs: Dendritic cells.

## Competing Interests

None of the authors has competing interests.

## Authors' Contributions

Shirong Cai and Matheswaran Kandasamy performed the DC and neutrophil studies with LGG and Juwita N. Rahmat performed the studies with BCG and DC and Sin Mun Tham performed the PGE<sub>2</sub> analysis of LGG and DC. Boon Huat Bay, Yuan Kun Lee, and Ratha Mahendran conceived the experimental design. Shirong Cai, Juwita N. Rahmat, and Ratha Mahendran wrote the paper and the others reviewed it.

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## Review Article

# An Update on Inflamm-Aging: Mechanisms, Prevention, and Treatment

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Inflamm-aging is a challenging and promising new branch of aging-related research fields that includes areas such as immunosenescence. Increasing evidence indicates that inflamm-aging is intensively associated with many aging diseases, such as Alzheimer's disease, atherosclerosis, heart disease, type II diabetes, and cancer. Mounting studies have focused on the role of inflamm-aging in disease progression and many advances have been made in the last decade. However, the underlying mechanisms by which inflamm-aging affects pathological changes and disease development are still unclear. Here, we review studies of inflamm-aging that explore the concept, pathological features, mechanisms, intervention, and the therapeutic strategies of inflamm-aging in disease progression.

## 1. Introduction

Inflamm-aging [1] was first named by Franceschi et al. in 2000, and it is a new addition to the types of aging studies. Inflamm-aging plays an increasingly important role in the rate of aging and age-related diseases. Research in this area has attracted attention of scholars in many fields and significant progress has been made in the last decade. Here, we review the concept of inflamm-aging and describe various research strategies that have led to insights into its inflammatory characteristics and mechanisms of action. We also discuss the relationship of inflamm-aging with diseases and novel interventions to delay or prevent inflamm-aging-related diseases.

## 2. The Concept of Inflamm-Aging

A main feature of the aging process is a chronic progressive increase in the proinflammatory status, which was originally

called “inflamm-aging” [1]. Subsequently other similar terms were used such as “inflammaging” [2], “inflamm-ageing” [3], and “inflammageing” [4]. Inflamm-aging is the expansion of the network theory of aging [5] and the remodeling theory of aging [6, 7]. The network theory of aging posits that aging is indirectly controlled by the network of cellular and molecular defense mechanisms. The remodeling theory, which was put forward to explain immunosenescence, is the gradually adaptive net result of the process of the body fighting malignant damage and is a dynamic process of optimization of the trade-off in immunity [6, 7]. In the process of aging, some researchers pointed out that the phenomenon where adaptive immunity declines is called immunosenescence, while the phenomenon where innate immunity is activated, coupled with the rise of proinflammation, is called inflamm-aging [8]. Some regard the chronic inflammatory process with age as inflamm-aging [9], while others proposed the oxidation-inflammation theory of aging [10]. Despite the lack

of agreement on definitions and terminology, there is consensus that the primary feature of inflamm-aging is an increase in the body's proinflammatory status with advancing age. Furthermore, a new concept of "anti-inflammaging" was also proposed, which influences progressive pathophysiological changes, as well as lifespan, and acts along with inflamm-aging [11]. In the next section the characteristics of inflamm-aging are described in more detail.

### 3. The Inflammatory Characteristics of Inflamm-Aging

The five states of inflamm-aging are as follows [12]: low-grade, controlled, asymptomatic, chronic, and systemic. However, the inflammation during inflamm-aging is not in a controlled inflammatory state. We propose that inflammation in the process of inflamm-aging belongs to nonresolving inflammation [13]. Inflammation is a series of complex response events which are caused by the host system facing a pathogen infection or various types of tissue injury. These response events are characterized by interactions between the cells and factors in the microenvironment and by regulation of the balance between physiological and pathological signaling networks. In common conditions, inflammatory responses disappear when proinflammatory factors in infection and tissue injuries are eliminated and then change into a highly active and well regulated balanced state, which is called resolving inflammation [13]. However, in the presence of some as yet uncertain factors, such as persistent and low intensity stimulation and long-term and excessive response in target tissues, inflammation fails to move into a steady state of anti-infection and tissue injury repair; instead the inflammation continues and moves to a nonresolving inflammation state [13]. Given this background, inflammation in the process of inflamm-aging belongs to the state of nonresolving inflammation.

### 4. The Relationship between Inflamm-Aging and Diseases

Like the immune response, inflammation has a physiological function in the normal body. Moderate inflammatory response is beneficial to the body but when excessive, the response becomes harmful. Changes in the inflammatory cytokine network control the direction of the development of inflammation. The dynamic balance of the network of proinflammatory cytokines and anti-inflammatory cytokines maintains the physiologic function of inflammation in the normal body. Tipping the balance from anti-inflammation to proinflammation can lead to pathological changes. Persistent inflammation during the inflamm-aging process may cause inflammation-related diseases.

Inflamm-aging is a determinant of the speed of the aging process and of lifespan and is highly related to Alzheimer's disease [2], Parkinson's disease, acute lateral sclerosis, multiple sclerosis, atherosclerosis, heart disease, age-related macular degeneration [14], type II diabetes [15], osteoporosis and insulin resistance [16], cancer, and other diseases. Inflamm-aging also increases morbidity and mortality, significantly

harming the health of patients, and causes a decline in the quality of life of patients [16]. Chronic, subclinical inflammation and immune disorders coexist in the process of inflamm-aging. Epidemiological studies show that with age there is an imbalance in the loss of old bone and the formation of new bone. Inflamm-aging may be one of the contributing factors to the imbalance and to the subsequent excessive loss of bone. Inflammatory markers of inflamm-aging provide clinicians with the necessary data for risk assessment of osteoporosis. Inflammatory cytokines may be therapeutic targets for improving the formation of bone in the elderly after bone operations [16]. Excessive inflammation during inflamm-aging increases the morbidity and mortality of patients after bone operations, even though the mechanism for this remains unclear [17]. In the process of inflamm-aging, the pathophysiological changes in the colon are revealed at the cellular and molecular levels, and these culminate in the inflammation that leads to injury of the gastric mucosa and epithelium as well as a decrease in the epithelium's ability to regenerate [18] (Figure 1).

However, inflamm-aging seems to be a double-edged sword in that it decreases immune function but also increases the autoreactivity of the body [17]. Inflamm-aging is beneficial to the body by neutralizing the harmful cytokines in the early stage of the life but has a detrimental role in the later life [17].

Unfortunately, the strong correlation between inflamm-aging and disease development is complex and unclear. Because immunosenescence and inflamm-aging coexist, it is difficult to distinguish whether the inflammation-related diseases are caused by inflamm-aging or immunosenescence. Moreover, the crucial question is whether there is a causal relationship between inflamm-aging and diseases, which needs integrated biological and clinical research to resolve.

### 5. The Mechanisms of Inflamm-Aging

While the mechanism of inflamm-aging is not completely understood, the current theories in the field are summarized below.

*5.1. The Theory of Stress.* Generally, stress is either beneficial or harmful to the body. During inflamm-aging, the body is constantly in the stress environment, which is caused by different kinds of stressors that induce and maintain the chronic proinflammatory status in the body. Stress, as one of regulated factors of immunity, provokes the greatest immune response in the bodies of young persons, whereas it provokes that weakest response in elderly persons with signs of immunosenescence and inflamm-aging [19, 20]. According to a series of studies involving different species from invertebrates to humans, from an evolutionary perspective, inflammation is closely related to innate immunity and stress [20]. Based on evolutionary studies, immune response, stress response, and inflammation form a defensive network in the body. However, the compatibility between inflammatory status and longevity and the paradoxical proinflammatory character in healthy centenarians strongly suggest the existence of physiological inflammation [21]. Therefore, inflammation and the

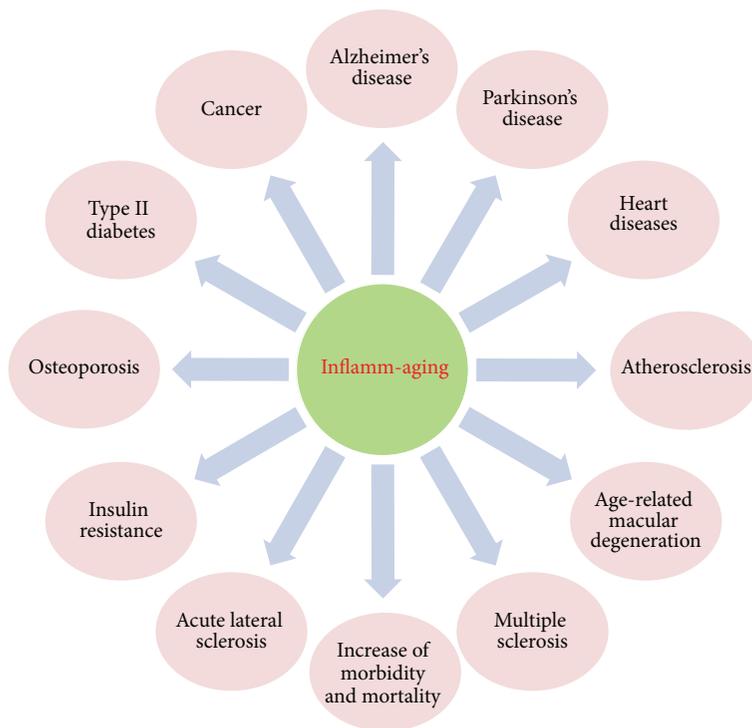


FIGURE 1: The relationship between inflamm-aging and diseases.

proinflammatory status in healthy persons and centenarians show a beneficial response that helps the elderly deal with the stimuli generated by chronic antigen stressors [19]. However, excessive stress response, as well as an accompanying increasingly high proinflammatory response, leads to human inflamm-aging.

**5.2. The Theory of Oxidation-Inflammation.** There are close relationships between oxidative stress and inflamm-aging [21]. Based on the close relationship between oxidative stress, inflammation, and aging, the oxidation-inflammatory theory of aging (oxi-inflamm-aging) was proposed [10]. In this theory, oxidative stress leads to inflamm-aging and influences the homeostasis and health of the body. The relationship between the redox state and the function of immune cells influences the speed of aging and lifespan [10]. According to this theory, sufficient antioxidants in food may improve immune function, decrease oxidative stress, and extend the lifespan [22].

**5.3. The Theory of Cytokines.** Proinflammatory cytokines play an important role in inflamm-aging caused by chronic inflammation [23]. Type I cytokines (such as IFN- $\gamma$  and TNF- $\alpha$ ) and type II cytokines (IL-4) in unactivated and memory CD4+ T lymphocytes participate in the proinflammatory process [24]. Further research shows that CD8+ and CD4+ T lymphocytes play a pivotal role in the developing cytokine network, and this can lead to the chronic proinflammatory state and inflamm-aging [25]. In animal experiments, increased expression of IL-1 $\beta$ , IL-15, IL-18, TNF- $\alpha$  mRNA,

and TNF- $\alpha$  protein in the peripheral blood of elderly horses appears to be a distinctive feature of inflamm-aging [26].

Elevated levels of IL-6 and TNF- $\alpha$  in the serum of the elderly are associated with disease, disability, and mortality [3]. Studies employing large patient cohorts provide evidence that the level of serum IL-6 is a reliable marker, or a predictive index, of inflamm-aging [3]. Experiments with healthy elderly people show that aging relates to increased proinflammatory status. The cause of the increased proinflammatory status is elevated levels of proinflammatory cytokines in the circulation including IL-1, IL-6, TNF- $\alpha$ , and PGE2 [27, 28]. Although the identity of cells that secrete proinflammatory mediators in elderly persons remains controversial, the prevailing view is that, during inflamm-aging, high levels of proinflammatory cytokines in the circulation create an inflammatory environment for tissues and organs [29]. However, differences in levels of IL-10 and TNF- $\alpha$  in individuals may play an important role in the final outcome of inflammation [29]. IL-6 and TNF- $\alpha$  are upregulated, while growth hormone and IGF-1 are downregulated in the process of aging. The overall balance of cytokines, such as IL-6 and TNF- $\alpha$ , appears to play a decisive role in aging. As well, genetic variations in the promoter regions of proinflammatory and regulated cytokine genes have effects on inflamm-aging and susceptibility to age-related diseases [29].

Pes et al. [30] found that the frequency of the -174C single nucleotide polymorphism (SNP) in the promoter region of IL-6 gene is increased in Italian male centenarians and the frequency of the -1082G SNP at the 5' flanking region of the IL-10 gene coding sequence is increased

among male centenarians. These data indicate that different alleles in different cytokine gene coding regions for pro- (IL-6) or anti-inflammatory (IL-10) cytokines may influence immune-inflammatory responses and individual lifespan expectancy, suggesting that inflammatory cytokine gene polymorphisms for immune system genes may regulate immune-inflammatory responses. Gene polymorphisms of proinflammatory cytokines associated with high levels of IL-6 have decreased capacity to reach extremely old age, whereas genotypes associated with high levels of IL-10 were increased in centenarians [30]. Genetic polymorphism in proinflammatory cytokine genes is necessary and has important consequences in the body. On the one hand, moderate levels of proinflammatory cytokines contribute to inducing a protective response, when the body is invaded by pathogens. On the other hand, excessive proinflammatory cytokines may cause immune-inflammatory diseases and even death. Indeed, the process of evolution has shaped the ability of the body to fight and control pathogens. Therefore, the proinflammatory response may be beneficial to the body in fighting potentially fatal infections. Thus, high levels of IL-6 and low levels of IL-10 are associated with enhanced ability against pathogens [30].

The vicious cycle of reciprocal causation between the proinflammatory cytokines and cellular senescence aggravates inflamm-aging. On the one hand, proinflammatory cytokines induce cellular senescence. Proinflammatory cytokines, such as TNF- $\alpha$ , IFN- $\gamma$ , and IFN- $\beta$ , induce cellular senescence in epithelial cells by producing reactive oxygen species and activating the ATM/P53/P21 (WAF1/Cip1) signaling pathway [31]. CXCR2, a chemokine receptor, induces cellular senescence of fibroblasts [32]. DNA damage produces proinflammatory cytokines (such as IL-1, IL-6, and IL-8) by activating the NF- $\kappa$ B signaling pathway, blocking the cellular cycle and inducing and maintaining the phenotype of cellular senescence [32]. On the other hand, senescent cells secrete growth factors, proteases, chemokines, and cytokines such as IL-6 and IL-8 [33].

Most phenotypes of aging can be explained by an imbalance between proinflammation and anti-inflammation, which results in inflamm-aging with a low chronic proinflammatory status. However, centenarians have high levels of inflammatory mediators and more anti-inflammatory cytokines, suggesting that inflamm-aging can coexist with longevity, even though the underlying mechanisms have not been uncovered [34].

**5.4. The Theory of DNA Damage.** Sustained telomere DNA and mitochondrial DNA damage, caused by exogenous and endogenous factors, can induce errors of DNA replication or translation, which leads to point mutations or chromosomal rearrangements and stress reactions via various signaling pathways, which eventually contributes to cellular senescence. Researchers found that, in human senescent primary cells, the shortest telomeres lack most of the telomere repeat sequence, which leads to DNA damage accumulation and terminal cell cycle arrest and further induces replicative senescence [35]. A persistent DNA damage response (DDR) caused by telomere shortening is a key mechanism involved

in replicative senescence and aging process [36]. New evidence indicates that DNA damage response (DDR) signaling is a major link between cell senescence and organism aging. DDR activation of senescent cells contributes to an increase in the proinflammatory secretory phenotype (PSP), which in turn triggers the activation of adjacent cell DDR and PSP. This local inflammatory environment eventually becomes systemic. The increasing number of cells with DDR activation may exacerbate inflamm-aging [37]. These results suggest that cells in an inflammatory environment induce aging at the systemic level. Stem cells and stromal fibroblasts differentiate into proinflammatory cytokine overexpressing cells and consequently the cytokine network breaks down, inducing inflamm-aging as a result of the accumulation of DNA damage [38]. Proinflammatory cytokines in the microenvironment of cells with DNA damage further induce inflamm-aging. Macrophages, which mediate the main effects of inflamm-aging, amplify inflamm-aging self-propagation via a cascade effect on the local and systemic proinflammatory response [38].

**5.5. The Theory of Autophagy.** Autophagy plays an important role in stress, removing harmful substances in cells to maintain homeostasis and normal metabolism [39, 40]. Autophagy transfers the abnormal substances of the cell to lysosomes for degradation and also plays a role in many pathophysiological processes [41]. For example, autophagy plays important roles in removing abnormal proteins, adapting to hunger, and cancer. More and more evidence shows that autophagy is important in increasing longevity. For example, knocking out the autophagy gene Atg7 leads to the accumulation of proteins and organelles in the cell, causing cellular senescence [42].

The process of aging accompanies disorder in homeostasis. However, autophagy plays an important role in maintaining homeostasis and delaying aging. In the process of aging, autophagic cleansing capacity declines gradually, which induces mitochondrion disordering and protein accumulation. This leads to increased reactive oxygen species (ROS) and consequently oxidative stress. Destabilized lysosomes release ROS, which activate Nod-like receptor 3 (NLRP3), and this initiates an inflammatory cascade reaction. During this process, inactive precursors of IL-1 $\beta$  and IL-18 are increased, and IL-1 $\beta$  and IL-18 release is stimulated, which causes an inflammatory reaction and accelerated aging [43].

**5.6. The Theory of Stem Cell Aging.** Stem cell aging is closely related to inflammation [44]. Stem cell aging is the cellular basis of aging and chronic inflammation is one of the main factors that induces stem cell aging. In the chronic inflammatory process, proinflammatory factors activate NF- $\kappa$ B/MAPKs, TOR, RIG-I, and JAK/STAT signaling pathways to induce cells to synthesize and to secrete large amounts of inflammatory cytokines, such as TNF $\alpha$  and IL-1 $\beta$  [45], which leads to a chronic low degree of inflammation in the environment of cells, thereby inhibiting the regenerative capacity of stem cells. This leads to dysfunctional differentiation of stem cells, damage of the stem cell microenvironment (i.e., that stem cell niche), homeostasis, and stem cell aging [44].

## 6. Regulatory Signaling Pathways of Inflamm-Aging

In principle, the pathways controlling inflammation are potential regulatory signaling pathways of inflamm-aging. The NF- $\kappa$ B and TOR signaling pathways, in particular, have been investigated.

**6.1. NF- $\kappa$ B Signaling Pathway.** NF- $\kappa$ B, a nuclear transcription factor, is regarded as the main molecular switch of inflammatory pathways. The NF- $\kappa$ B signaling pathway may also regulate inflamm-aging [8]. However, the longevity gene, SIRT1, can be combined with a subunit of NF- $\kappa$ B, Rel/p65, to deacetylate K310 and inhibit the transcriptional activity of NF- $\kappa$ B [46, 47]. NF- $\kappa$ B can regulate the occurrence of aging, whereas SIRT1 may regulate NF- $\kappa$ B to delay aging [48]. Thus, NF- $\kappa$ B can regulate both aging and inflammation [49]. NF- $\kappa$ B can also inhibit inflammatory reactions by regulating SIRT1 (Sir2 homolog) and FoxODAF-16 [8].

**6.2. TOR Signaling Pathway.** TOR, a highly conserved serine/threonine protein kinase, plays an important role in the regulating growth and proliferation of cells [50]. According to its different functions, TOR can be divided into TORC1 and TORC2. The former is sensitive to rapamycin, participating in the biological process of transcription and translation in cells. The latter is insensitive to rapamycin and mainly regulates remodeling the cytoskeleton [51]. The TOR signaling pathway regulates longevity. When TOR signaling is decreased or inactivated, the lifespan of wireworms and *Drosophila* is extended. Similarly, the lifespan of yeast can be increased by exposure to a low dose of rapamycin [52]. At present, it is believed that TORC1 participates in regulating aging. S6K is a positive regulation target of TORC1. In the mouse knockout of the S6K gene, lifespan is extended. 4E-BP is a gene that is necessary to lifecycle and is a negative regulator of TORC1. When 4E-BP is overexpressed, lifespan is prolonged [53].

In terms of the physiological function of TOR signal regulation, TOR regulates growth during embryonic development, and in maturity TOR regulates metabolism. However, in old age TOR signaling regulation is excessively activated and is associated with many age-related diseases. The excessive production of cytokines and inflammatory factors induces aging and the changes to the local microenvironment, causing age-related diseases. TOR regulates inflamm-aging by activating NF- $\kappa$ B [54].

**6.3. RIG-I Signaling Pathway.** Retinoic-acid-inducible gene-I (RIG-I) may be involved in inflamm-aging. RIG-I is induced via the ataxia telangiectasia mutated interferon regulatory factor-1 (ATM-IRF1) axis in senescent cells and interacts with increased levels of IL-6 and IL-8. The activation of RIG-I signaling pathway upregulates IL-6 expression [55]. RIG-I is a caspase recruitment domain- (CARD-) containing protein that functions as a cytoplasmic RNA sensor [55]. Liu et al. [55] showed that IL-6 and IL-8 levels increase in replicating senescent cells. They reported that senescent cells transfected with RIG-I show increased secretion of IL-6. However, knockdown of RIG-I in senescent cells leads to

the extension of the lifespan of cells, which shows that RIG-I-induced inflammation plays a role in promoting and maintaining aging. Interfering with RIG-I expression significantly decreases the levels of inflammatory cytokines in senescent cells [55]. This imbalance in the inflammatory process may cause chronic inflammation during aging.

**6.4. Notch Signaling Pathway.** The Notch signaling pathway is a major intercellular communication pathway that is highly conserved through evolution. Notch signaling plays an essential role in aging [56]. At the cellular level, aging of vascular endothelial cells (EC) leads to senescence. Senescent EC secrete proinflammatory cytokines and this is often accompanied by a low-grade chronic upregulation of certain proinflammatory responses [56]. Constitutive activation of Notch signaling induces EC senescence. Consistent with these results, HeyL, a Notch downstream target, is elevated in aged compared to young EC. Notch activation also triggers EC inflammatory responses by upregulating expression of a panel of proinflammatory cytokines/chemokines and adhesion molecules in EC. This has revealed a novel function of Notch1 signaling in EC biology and may shed light on the mechanism whereby Notch signaling may contribute to some age-related vascular diseases characterized by chronic inflammation.

**6.5. Sirtuin Signaling Pathway.** Silent information regulator (Sir) proteins regulate lifespan in multiple model organisms [57]. Sir2 (SIRT1-7 in mammals) is a NAD-dependent deacetylase that has been implicated in aging and inflammation in yeast, worms, and flies. SIRT1, the most extensively studied in mammals, has a highly conserved NAD-dependent sirtuin core domain and is a good candidate lifespan regulator along with the other six homologs. Recent studies showed that SIRT1 is a potent anti-inflammatory protein and inhibits the COX-2/MMP pathway via suppression of the potent proinflammatory factor NF- $\kappa$ B. NF- $\kappa$ B signaling is limited by SIRT6, which is recruited to NF- $\kappa$ B target gene promoters by a physical interaction with the NF- $\kappa$ B subunit RelA. SIRT6 deacetylates histone H3 lysine 9 on target gene promoters, thereby altering the chromatin structure to facilitate NF- $\kappa$ B destabilization and signal termination. SIRT1 activation decreases the proinflammatory effects induced by TNF- $\alpha$ . In addition, treatment with SIRT1 activators such as resveratrol, or overexpression of SIRT1, inhibits the expression and activation of the main proinflammatory regulator NF- $\kappa$ B, which is increased by TNF- $\alpha$ . When SIRT1 is overexpressed, the anti-inflammatory action of SIRT1 is similar to that exerted by resveratrol. Resveratrol, as an SIRT1 activator, inhibits TNF- $\alpha$ -induced inflammatory factor release. Resveratrol effectively inhibits the activation of proinflammatory factors by activating SIRT1, leading to the deacetylation of NF- $\kappa$ B p-65 and subsequent downregulation of TNF- $\alpha$ -induced COX-2 and MMP expression [58].

**6.6. TGF- $\beta$  Signaling Pathway.** Sequence variations in a variety of pro- or anti-inflammatory cytokine genes have been found to influence successful aging and longevity. TGF- $\beta$ 1

has been shown to have an essential role in inflammation and in the maintenance of immune response homeostasis. TGF- $\beta$ 1 belongs to the group of cytokines with anti-inflammatory effects and is a deactivating factor of macrophages with potent anti-inflammatory properties. Because of the role played by the transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1) in inflammation and the regulation of immune responses, variability of the TGF- $\beta$ 1 gene may affect longevity by playing a role in inflamm-aging. The potential role of TGF- $\beta$ 1 in aging and longevity has been suggested by many *in vitro* and *in vivo* studies. In particular, TGF- $\beta$ 1 gene overexpression has been observed in human fibroblasts that display a senescent-like phenotype following exposure to oxidative stress, which may help to seek a new proposal to treat disease-related aging [59].

**6.7. Ras Signaling Pathway.** Ras is an important signaling molecule involved in atherogenesis and is a proinflammatory molecule involved in inflammation and aging. Ras promotes aging in yeast and cellular senescence in primary human fibroblasts. Activation of Ras drastically increases the expression of proinflammatory cytokines, in part through extracellular signal-regulated kinase activation. Introduction of Ras into arteries enhances vascular inflammation and senescence [60].

Ang II promotes reactive oxygen species (ROS) production, cell growth, apoptosis, cell migration and differentiation, and extracellular matrix remodeling. Ang II regulates gene expression and can activate multiple intracellular signaling pathways leading to tissue injury. Ang II also mediates several key events in the inflammatory process. Blocking Ang II signaling protects against neurodegenerative processes and promotes longevity in rodents. Ang II-induced ROS production via the AT1 receptor promotes the onset of vascular senescence associated with functional and structural changes to blood vessels that contribute to age-related vascular diseases [60].

At present, the mechanisms of inflamm-aging remain unclear, because the methods and tools used for research into the mechanisms of inflamm-aging are not sufficiently sophisticated to explain inflammatory reactions caused by complex inflammatory cytokine cascades during inflamm-aging. Unfortunately, an adequate and reliable assessment system for aging has not yet been established. Furthermore, the causal relationships between inflammation and aging have not yet been elucidated. Moreover, the mechanisms referred to above need to be further verified. Inflamm-aging influences all levels of function, from cells to tissues, organs, and the whole body. Inflamm-aging also involves aberrant gene regulation and an imbalance in energy metabolism as well as interactions between these two factors. The mechanisms of inflamm-aging are very complicated and require multidisciplinary research to further investigate the interactions at multiple levels from cells to the whole body (Figure 2 and Table 1).

## 7. Potential Markers of Inflamm-Aging

One of the constraining factors of aging research is the lack of recognized, accurate, and reliable biological markers.

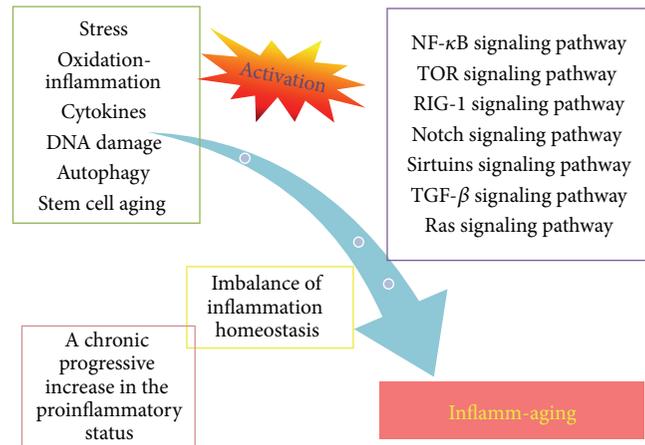


FIGURE 2: The mechanisms and regulated signaling pathways of inflamm-aging.

The main biological markers of aging can be categorized as follows: (1) the marker is related to age; (2) the marker does not change with disease; (3) the marker does not change with metabolic and nutrient conditions; (4) the marker is influenced by the process of aging; (5) the marker does not change in immortalized cells. Unfortunately, the biological markers of aging have not yet been defined and need to be further investigated. This will facilitate the evaluation of the degree of inflamm-aging and assist in identifying the molecular mechanisms underlying inflamm-aging. The potential markers of inflamm-aging may include *immune cell* markers, *serum cytokine* markers, and microRNAs.

**7.1. Immune Cell Markers.** A main characteristic of the immune system in the elderly is antigenic T cell accumulation. The shortage of naive CD8<sup>+</sup> T lymphocytes is regarded as a reliable biological marker related to the risk of death. The increase in CD8<sup>+</sup> T cells, a decrease in CD4<sup>+</sup> T cells and CD19<sup>+</sup> B cells, and inhibition of mitogen-induced proliferation of T cells may be predictors of inflamm-aging [3].

**7.2. Serum Cytokine Markers.** The increase in serum IL-6 in the elderly is related to diseases, disability, and mortality. A study on a large cohort showed that IL-6 in the serum is a reliable marker of disability in the elderly and contributes to the predictive index of disability and mortality [3]. The functions of some cytokines, such as IL-10 and TNF- $\alpha$ , are complex and play opposing roles in the systemic inflammatory reaction. IL-10 inhibits inflammatory reactions, while TNF- $\alpha$  activates inflammatory reaction locally and systemically. Different regulation of IL-10 and TNF- $\alpha$  may be essential to the final outcome of the inflammatory reaction. Therefore, levels of IL-6, TNF- $\alpha$ , and IL-10 may be regarded as serum markers of inflamm-aging [3].

**7.3. MicroRNA Markers.** MicroRNAs (miRs) are a class of molecules involved in the regulation of gene expression and

TABLE 1: Inflamm-aging-related mechanisms and regulated signaling pathways.

Mechanisms	Effects	Signaling pathways	References
Stress	Chronic antigen stressors lead to excessive stress response and contribute to inflamm-aging	Ras	[19, 20]
Oxidation-inflammation	Oxidative stress and inflammation influences the homeostasis and health of body	NF- $\kappa$ B, Notch, TGF- $\beta$ , sirtuin	[10]
Cytokines	High levels of proinflammatory cytokines result in inflamm-aging and age-related diseases	mTOR, RIG-I, Notch	[29, 30, 52, 53]
DNA damage	DNA damage response increases proinflammatory cytokines	NF- $\kappa$ B	[36]
Autophagy	Autophagic function dysfunction leads to increased oxidative products and oxidative stress	NF- $\kappa$ B	[40, 41]
Stem cell aging	Chronic inflammation induces stem cell aging and inhibits the regenerative capacity of stem cells	NF- $\kappa$ B, mTOR, RIG-I	[42]

are emerging as modulators of biological pathways including NF- $\kappa$ B, mTOR, sirtuins, TGF- $\beta$ , and Wnt. miRs may be associated with inflammation, cellular senescence, and age-related diseases and they can be classified as inflammation-associated (inflamm-miRs) and senescence-associated (SA-miRs) [61]. miR-based anti-inflammatory mechanisms may play a crucial role during aging, where a chronic, low-level proinflammatory status is likely sustained by the cell senescence secretome and by progressive activation of immune cells over time. Circulating miRs seem to be promising biomarkers of major age-related diseases [60]. Some miRs are found in plasma and leukocytes in centenarians. Some miRs, such as miR-21, miR-126, and miR-146a, which target mRNAs belonging to the NF- $\kappa$ B pathway can be considered as both SA-miRs and inflamm-miRs [61]. Thus specific inflamm-miRs may be regarded as biomarkers of inflamm-aging [62].

## 8. Intervention in Inflamm-Aging

A significant feature of inflamm-aging is that there is a chronic, low-grade, microinflammatory state in the body. Therefore, drugs used for the treatment of inflamm-aging must be effective, safe, nontoxic, and appropriate for long-term use. Calorie restriction, zinc (Zn), resveratrol, *Epimedium* total flavonoids, and icariin have these characteristics and may be candidate drugs to treat inflamm-aging.

**8.1. Calorie Restriction.** Calorie restriction (CR), also known as dietary restriction (DR), has been regarded as the gold standard of many aging interventions to counteract aging. CR together with adequate nutrient intake prolongs maximum lifespan possibly through beneficial metabolic, hormonal, and functional alterations [63]. The antiaging action of CR may be based largely on its ability to suppress oxidative stress related alterations and oxidative stress induced age-related diseases [64, 65]. CR can modulate many important inflammatory signaling pathways involved in aging and inflammation such as NF- $\kappa$ B, mTOR, and MAPK [66]. The age-related upregulation of NF- $\kappa$ B, IL- $\beta$ , IL-6, and TNF- $\alpha$  in the proinflammatory states of the aging process is attenuated by CR [67].

**8.2. Zn.** Zn is an especially important, necessary microelement for the human body and has an important impact on regulating the balance between the genetic expression of metalloproteinases (MPs) and MPs inhibitors, on maintaining inducible nitric-oxide synthase (iNOS) activity, and on many biochemical functions. The interaction between Zn and IL-6, TNF- $\alpha$ , or heat shock protein 70 (Hsp70) regulates the immune-inflammatory reaction. The elderly frequently lack adequate levels of Zn. A moderate amount of Zn added to the diet may expand the lifespan of the elderly, which suggests that Zn may intervene in inflamm-aging [68].

**8.3. Resveratrol.** Some studies have found that resveratrol affects aging and lifespan in mammals [22]. Researchers reported that this potent natural compound is a SIRT1 activator and may help in preventing the aging-related decline in heart function and neuronal loss through stimulating SIRT1 activation [69]. Moreover, resveratrol decreases ovarian inflammation via inhibition of NF- $\kappa$ B by upregulation of PPAR- $\gamma$  and SIRT1 expression [70]. Furthermore, resveratrol can suppress tumorigenesis at least in part by targeting Sirt1 and suppressing NF- $\kappa$ B activation [71]. Resveratrol suppresses the upregulation of proinflammatory molecules (such as IL-1 $\beta$  and IL-6) by TNF- $\alpha$  in 3T3 cells in a dose-dependent manner. However, knockdown of Sirt1 by RNA interference makes 3T3 cells susceptible to TNF- $\alpha$  stimulation and decreases the anti-inflammatory effect of resveratrol. The potential anti-inflammatory mechanisms of resveratrol involve a reduction in NF- $\kappa$ B subunit RelA/p65 acetylation, which is notably Sirt1 dependent. Resveratrol also attenuates the phosphorylation of the mammalian target of rapamycin (mTOR) and S6 ribosomal protein (S6RP) while ameliorating inflammation [72]. These data demonstrate that resveratrol may have inhibitory effect on inflamm-aging.

**8.4. Epimedium Total Flavonoids and Icarin.** In our previous studies, based on the neuroendocrine-immunological network, we used an inflammatory cytokine and genetic receptor chip-based assay to detect critical genes in the hippocampus, hypothalamus, hypophysis, adrenal gland, and spleen of elderly rats. We also detected the proteins corresponding

to the genes referred to above. The findings showed that overexpression of some proinflammatory cytokines at the transcription and protein level may be involved in the highly proinflammatory reactive state during inflamm-aging. Additionally, our experiments showed that *Epimedium* total flavonoids (EF) and icariin (Ica) reduced the proinflammatory response, enhanced the anti-inflammatory response, and reestablished the equilibrium between proinflammatory and anti-inflammatory reactions in the process of inflamm-aging [73, 74].

**8.5. Metformin.** The biguanide drug metformin, a type of hypoglycemic drug, is widely prescribed to treat type 2 diabetes and metabolic syndrome. Researchers have also noted the effect of metformin on delaying aging, an effect validated in rodents [75] and in the nematode *Caenorhabditis elegans* [76]. Recently, Hall conducted a clinic trial called “Metformin, Anti-aging,” which was supported by the U.S. Food and Drug Administration (FDA) [77]. This was a landmark event in the history of aging research and showed that metformin may be used as an antiaging drug to improve the health span of humans. The mechanisms underlying the antiaging effects of metformin remain unclear. Several lines of evidence support that metformin may act by inducing metabolism associated with dietary restriction (DR) to increase lifespan and thereby limit the onset of age-associated diseases across species [78, 79]. A potential mediator of metformin benefits is the AMP-activated kinase (AMPK) and metformin can be viewed as DR-like compound [76]. Cabreiro et al. reported that metformin disrupts the bacterial folate cycle, leading to reduced levels of S-adenosylmethionine (SAME) and decelerated aging in *C. elegans* [80]. Meanwhile, Moiseeva et al. showed that metformin inhibits the expression of genes coding for multiple inflammatory cytokines seen during cellular senescence, and metformin blocks the activity of NF- $\kappa$ B [81]. The effects of metformin on anti-inflammation and antiaging imply its potential on inflamm-aging.

## 9. Novel Research Strategies in Inflamm-Aging

Research into inflamm-aging is still at an early stage. The mechanisms, biomarkers, evaluation method, research models, and intervention methods of inflamm-aging have not been fully elucidated. Moreover, inflamm-aging involves cells, organs, and the whole body and this requires an extensive and varied range of research investigations.

Based on the essential effects and our understanding of inflammatory cytokine pathways in the process of inflamm-aging, we can begin to explore the inflammatory cytokine network and perform a quantitative evaluation of inflamm-aging. Inflammatory cytokines, including interleukins, tumor necrosis factor, and interferon, mediate their effects by binding to their receptors and competing in a complex cell-cell network. These cytokines act in both paracrine and autocrine ways to exert direct effects on the microenvironment. This plays an important regulatory role by activating inflammatory and immune cells and by releasing cytokines. In addition, inflammatory cytokines induce the

systemic inflammatory response in the circulation. Interactions between many inflammatory cytokines comprise the inflammatory cytokine network, which features polyphyletism, pleiotropy, and overlapping effects. Inflammatory cytokines form a complex network which extends in all directions and throughout the whole body. The inflammatory cytokine network can be divided into the proinflammatory cytokine network and anti-inflammatory cytokine network. As with the immune reaction, the inflammatory reaction is also a normal defense function. A moderate inflammatory reaction is advantageous to the body, whereas a high reaction is harmful and the outcome of these reactions is determined by changes in the inflammatory cytokine network. The dynamic balance between the proinflammatory cytokine network and the anti-inflammatory cytokine network maintains the normal function of inflammation in body. Once the balance is broken, pathological inflammation occurs [82, 83]. Therefore, we infer that the cause of inflamm-aging is an imbalance in the proinflammatory cytokine and anti-inflammatory cytokine networks, which leads to a proinflammatory status with increasing age. This may be the mechanism of inflamm-aging.

The changes in the inflammatory cytokine network have three characteristics: parallelity, multilevel action, and non-linearity. Unraveling the inflammatory cytokine network will require nonlinear research methods such as systems biology methods. The inflammatory cytokine network is a typical systems biology issue, which needs to replace the model involving individual genes with a systems biology model.

Systems biology is an interdisciplinary field integrating multiple disciplines such as biology, medicine, mathematics, physics, and chemistry. It integrates many kinds of experimental data and biological information to build a mathematical model tested and verified by experimental data and finally predicts the behavior of biological systems [84]. Systems biology provides an excellent opportunity to elucidate the essential features of the inflammatory cytokine network. It also provides a theoretical guide and new ways to illustrate the relevant mechanisms and build a quantitative evaluation system for the inflammatory cytokine network, which may provide a breakthrough in research on inflamm-aging.

Aging (including inflamm-aging) is also a systems biology issue involving a complex process that results from the combined effects of many factors. Cellular senescence is the basic unit of biological aging. Organ aging is not only the macropresentation of cellular senescence but also a bridge that connects cellular senescence and integral aging. Cellular senescence, organ aging, and integral aging form a chain of aging, and cellular senescence is the critical link in the chain of aging. In the aging process, at all levels (i.e., genes, proteins, metabolites, cells, and tissues), the organism undergoes varying degrees of change in these structures, such that the body's systems (e.g., the nervous system, endocrine system, cardiovascular system, respiratory system, digestive system, urinary system, and motor system) undergo a significant functional decline. Aging is not the result of a unilateral factor, but a systemic decline in body function, and the distinctive features of aging are systemic [85]. The systemic

features of aging strongly reflect the gradual changes in body function. Functional changes in the process of aging appear gradually with age, and all the changes are the results of progressive accumulation; time is the driving factor. The body is composed of various tissues and organs, so aging is a gradual process, not a point but an evolution of tissues and organs over time.

Multidisciplinary research including the fields of medicine, biology, mathematics, computer science, and systems biology should be applied and developed to investigate the mechanisms of inflamm-aging and the relationship between inflamm-aging and age-related diseases. Specifically, the important scientific problems that need to be addressed are the following: (1) the regulatory mechanisms responsible for the development of inflamm-aging and (2) the molecular mechanisms, the regulatory network, and the key role of the transformation from inflammation to age-related diseases in the process of inflamm-aging.

In summary, inflamm-aging and the inflammatory cytokine network are both classical systems biology issues. The inflammatory cytokine network is involved in the process of inflammation and senescence and may be the ideal breakthrough point of research into inflamm-aging. Omics, such as genomics, transcriptomics, proteomics, and metabolomics, are excellent methods to solve systemic biology problems. Therefore, under the guidance of systems biology, it would be novel strategy to conduct basic research into inflamm-aging using omics methods to identify characteristic inflammatory cytokine genes in the process of aging and to uncover new mechanisms to regulate inflammatory cytokines during inflamm-aging. This will also illustrate the mechanism of inflamm-aging and provide new ways to assess inflamm-aging.

## 10. A Novel Concept of Immuno-Inflamm-Aging

There is an essential relationship between inflammation and immunity. Both the inflammatory steady state and immune steady state have defense functions, protecting the body from injury [86]. However, when the inflammatory steady state and immune steady state are broken, excessive inflammation and pathological immunity ensue and the normal physiological function of the body is compromised, which causes immune-inflammatory diseases. Inflammation and immunity coexist in the same pathological process, the two sides of a whole, and are inseparable. In a sense, inflammatory cells are immune cells [87]; therefore, they have the same cellular foundation. Many inflammatory and immune cells have the same cytokine receptors [88], which mediate cell-cell and cell-cytokine interactions. The internal relationship between inflammation and immunity is not known, and the causes and pathological mechanisms of immune-inflammatory diseases are not understood and this limits effective treatments for immune-inflammatory diseases [86].

EF and Ica, anti-inflammatory immunomodulatory Chinese traditional medicines, not only reduce inflammation but also regulate immunity. As anti-inflammatory, immunomodulatory Chinese traditional medicines, EF and Ica have the

double effects of intervening in immunosenescence and inflamm-aging, suggesting an intimate relationship between immunosenescence and inflamm-aging [73, 74].

Based on the integrated relationship between oxidative stress and inflammatory stress, De La Fuente and Miquel proposed an innovative oxidation-inflammation theory of aging (oxi-inflamm-aging) [10]. The oxi-inflamm-aging theory posits that chronic oxidative stress affects all immune cells, and particularly regulatory systems such as neural, endocrine, and immune systems, as well as the mutual interactions among these systems. These events lead to stable, internal environment disorders that are harmful to health. The relationship between the redox state and immune function affects the speed of aging and lifespan. A diet with sufficient antioxidants improves immune function, reduces oxidative stress, and prolongs lifespan, which supports the notion that the inflammatory response of immune cells and the immune system play an important role in oxi-inflamm-aging [10]. Inflamm-aging and immunosenescence are connected, and they cause and affect each other. This can result in a vicious cycle that further aggravates the occurrence and development of age-related diseases including atherosclerosis, metabolic syndrome, type 2 diabetes, insulin resistance, osteoporosis, bone arthritis, muscle mass and muscle weakness, cancer, and neurodegenerative diseases.

It is currently not possible to distinguish whether diseases are caused by inflamm-aging alone or immunosenescence alone [30, 89]. Therefore, we propose that inflamm-aging is accompanied by immunosenescence, and they occur together. We propose the novel concept of immune/inflammatory aging (immuno-inflamm-aging), instead of the individual concepts of inflamm-aging and immunosenescence.

## Competing Interests

The authors declare that they have no competing interests regarding the publication of this paper.

## Authors' Contributions

Shijin Xia and Xinyan Zhang contributed equally to this work and should be considered the first authors.

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## Research Article

# CD3<sup>+</sup>CD8<sup>+</sup>CD28<sup>-</sup> T Lymphocytes in Patients with Lupus Nephritis

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The results of studies on the CD3<sup>+</sup>CD8<sup>+</sup>CD28<sup>-</sup> cells in SLE are inconsistent since several analyses describe CD3<sup>+</sup>CD8<sup>+</sup>CD28<sup>-</sup> as either immunosuppressive or cytotoxic. The aim of this study is to inquire whether the quantitative changes of CD3<sup>+</sup>CD8<sup>+</sup>CD28<sup>-</sup> T lymphocytes subpopulation are related to the clinical status of patients with lupus nephritis. Evaluation of Foxp3 expression on CD3<sup>+</sup>CD8<sup>+</sup>CD28<sup>-</sup> cells may shed some light on functional properties of these cells. 54 adult SLE patients and 19 sex and age matched healthy volunteers were enrolled in the study. There were 15 patients in inactive (SLEDAI ≤ 5) and 39 in active (SLEDAI > 5) phase of disease. We determined absolute count of CD3<sup>+</sup>CD8<sup>+</sup>CD28<sup>-</sup> and CD3<sup>+</sup>CD8<sup>+</sup>CD28<sup>-</sup> Foxp3<sup>+</sup> subpopulations by flow cytometry. We observed a statistically significant increase in absolute count and percentage of CD3<sup>+</sup>CD8<sup>+</sup>CD28<sup>-</sup> in SLE patients compared to HC ( $p < 0.001$ ). Moreover there was significant positive correlation between increasing absolute count of CD3<sup>+</sup>CD8<sup>+</sup>CD28<sup>-</sup> cells and disease activity measured by SLEDAI ( $r_s = 0.281$ ,  $p = 0.038$ ). Active LN patients had increased absolute count of CD3<sup>+</sup>CD8<sup>+</sup>CD28<sup>-</sup> cells compared to HC. Positive correlation of CD3<sup>+</sup>CD8<sup>+</sup>CD28<sup>-</sup> number with disease activity, and lack of Foxp3 expression on these cells, suggests that CD3<sup>+</sup>CD8<sup>+</sup>CD28<sup>-</sup> lymphocytes might be responsible for an increased proinflammatory response in the exacerbation of SLE.

## 1. Introduction

T CD8<sup>+</sup> cells play a key role in the recognition and removal of cells infected by intracellular pathogens [1] and also in antitumor response [2]. Binding of T CD8<sup>+</sup> surface receptor TCR and MHC-I-bound antigen, found on the professional antigen presenting cell's (pAPC) surface, leads to T CD8<sup>+</sup> activation [3]. Since stimulation only through the TCR receptor is unable to maintain optimum T CD8<sup>+</sup> activation, the second costimulatory signal is essential for full activation and survival of these cells [4]. The best known costimulatory signal is provided by the interaction of CD28 molecules presented on the T lymphocyte as well as the CD86 and CD80 molecules expressed on the pAPC's surface [3]. Adequate signal power delivered to naive T CD8<sup>+</sup> results in the proliferation and differentiation of two cell types. One of these is cytotoxic T lymphocytes (CTL), which

after reaching maturity and fulfilling their effector function undergo apoptosis. The second type is T CD8<sup>+</sup> memory cells, both central and effector. Their continuous presence in the circulation is essential to control another potential exposure to the same antigen in a faster and more effective way [5].

Under chronic antigen stimulation, repeated cycles of activation occur and lead to progressive and irreversible reduction in CD28 molecule expression on the lymphocyte surface. This results in accumulation of "highly antigen-experienced" T-cell with CD8<sup>+</sup>CD28<sup>-</sup> phenotype characterized by extremely shortened telomeres [6].

There is a close relationship between CD28 molecules presence and degeneration of the telomeres/telomerase. Telomerase activity is necessary for cell proliferation, cytokines and chemokines production, and antiviral activity. However, lack of CD28 molecules leads to loss of ability to increase telomerase activity in activated cells. Maintaining

the presence of CD28 molecule through gene transduction *in vitro* slows down the “immune aging” and improves the efficiency of the immune system [7]. Telomeres are set up on chromosomes ends and ensure their stability. Unprotected chromosomes ends are exposed to a high risk of degradation. Such degradation processes lead to the genetic information loss and cell death [8]. This process functions as a mitotic clock, while telomere length represents the number of cell divisions [9].

Relationship has been demonstrated between the average telomere length in peripheral blood cells and autoimmune diseases, such as SLE [10, 11], rheumatoid arthritis [12], systemic sclerosis (SSc) [13], ANCA-associated vasculitis (AAV) [14], psoriasis, and atopic dermatitis [15].

It is now believed that one of the major causes of abnormal immune response is the telomere properties dysfunction that leads to autoimmunity [16]. Nonetheless some authors did not confirm the relationship between telomere dysfunction and the development of SLE [17]. It has been also observed that the loss of CD28 is associated with increased surface expression of the CD57 molecule. T CD8<sup>+</sup>CD28<sup>-</sup> cells (CD8<sup>+</sup>CD57<sup>+</sup>) are referred to as antigen-specific, terminally differentiated, but also as functionally competent memory or effector T-cells which have gone through many cycles of cell division. Decrease or even loss of telomerase 5 activity and a low level of expression of genes involved in cell cycle regulation are characteristics for these cells. T CD8<sup>+</sup>CD28<sup>-</sup> (CD8<sup>+</sup>CD57<sup>+</sup>) cells have often limited ability to proliferate upon stimulation and it is believed that they have reached a state of “replicative old age” or “clonal exhaustion” [3, 18].

Data on the sensitivity to apoptosis of CD8<sup>+</sup>CD28<sup>-</sup> (CD8<sup>+</sup>CD57<sup>+</sup>) lymphocytes are contradictory. Some of researchers [19, 20] argue that these cells are highly susceptible to activation induced apoptosis. This hypothesis is evidenced by increased expression of Fas and caspase-3 and decreased expression of antiapoptotic molecules such as survivin or heat shock protein 27 (HSP 27). Others maintain that T CD8<sup>+</sup>CD28<sup>-</sup> (CD8<sup>+</sup>CD57<sup>+</sup>) lymphocytes show a high resistance to apoptosis and thus gradually accumulate throughout life [21, 22].

Most of autoimmune diseases are associated with an increase in T CD8<sup>+</sup>CD28<sup>-</sup> (CD8<sup>+</sup>CD57<sup>+</sup>) cells, which exhibit the highly cytotoxic activity and can be related to more severe manifestations of the disease. Quantitative changes in CD8<sup>+</sup>CD57<sup>+</sup> population were observed, among others, in multiple sclerosis [23], type 1 diabetes [23], Graves' disease [24], and rheumatoid arthritis [25]. The decreased number of CD8<sup>+</sup>CD28<sup>-</sup> T-cells correlates with clinical response to abatacept in patients with rheumatoid arthritis [26]. Some researchers have reported that lymphocytes with CD8<sup>+</sup>CD28<sup>-</sup> phenotype show regulatory properties. There are analyses which confirmed the presence of Foxp3 molecule in these cells [27, 28]; however there are analyses which showed no expression of this factor [29, 30]. Moreover, aside from lack of Foxp3 expression, markers characteristic for cytotoxic cells, such as granzyme A or perforin, were detected on CD8<sup>+</sup>CD28<sup>-</sup> surface [31].

So far, few studies addressing the size of the CD8<sup>+</sup>CD28<sup>-</sup> subpopulation in patients with SLE have been conducted. It

TABLE 1: Characteristics of the study group in terms of age, gender, and disease activity measured by SLEDAI scale.

	SLEDAI	
	≤5	>5
SLEDAI score	≤5	>5
Group size	15	39
Mean SLEDAI	3.2	13
Median SLEDAI	4	12
Min–Max SLEDAI	0–5	6–28
Mean age	32.7 ± 9.1	37.9 ± 14.9
Median age	32	33
Sex	♀: 13 (86.7%)	♀: 39 (100%)
	♂: 2 (13.3%)	♂: 0 (0%)

has been shown that the number of CD8<sup>+</sup>CD28<sup>-</sup> cells might be reduced or unchanged as compared to the control group [32, 33].

The main goal of the study was to investigate whether the quantitative changes of CD8<sup>+</sup>CD28<sup>-</sup> T lymphocytes subpopulation are related to clinical status of patients with LN. Detection of Foxp3 molecule expression in CD8<sup>+</sup>CD28<sup>-</sup> cells may shed some light on functional properties of these cells.

## 2. Material

54 adult SLE patients (96.3% female, mean age 36.5 ± 13.7) in the various phases of disease activity were enrolled into the study. Disease activity at the time of evaluation was scored according to the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) [34]. Patients were divided into two groups according to their SLEDAI score and there were 15 patients in inactive (SLEDAI ≤ 5) and 39 in active (SLEDAI > 5) phase of disease [35]. Additionally patients were split into two groups according to their renal SLEDAI (refers to the total of all renal components used to calculate the SLEDAI) and there were 14 (rSLEDAI < 4) and 40 (rSLEDAI 4–16) patients, respectively. Demographic characteristics and clinical data of the study group have been presented in Table 1. Additionally, 19 sex and age matched healthy volunteers (89.5% female, mean age 38.3 ± 14.1) served as the control group.

Patients were treated at the Department of Nephrology and Transplantation Medicine, Wrocław Medical University, in accordance with the current guidelines for lupus nephropathy.

Patients with newly diagnosed renal disease in course of SLE used steroids infusion, with the conversion to the oral steroids at tapering doses, or were given only oral steroids. Furthermore, the immunosuppressive therapy included cyclophosphamide, azathioprine, cyclosporin A, mycophenolate mofetil, and chloroquine. In order to maintain remission, patients used oral steroids or oral steroids combined with mycophenolate mofetil or azathioprine.

Exclusion criteria of the study were presence of an active malignancy and inflammatory processes.

The study was approved by the Wroclaw Medical University Bioethics Committee.

### 3. Methods

**3.1. Determination of  $CD3^+CD8^+CD28^-$  Subpopulation.** 300  $\mu$ L of heparinized blood was stained with 20  $\mu$ L of the following antibodies: anti-CD3APC, anti-CD8FITC, and anti-CD28PE. All were purchased from Becton Dickinson (BD, San Jose, California, USA). After 30 minutes of incubation at 4°C in the dark, the red blood cells were lysed with BD FACS Lysing Solution (Becton Dickinson). The cells were washed twice with PBS 2% FBS and flow cytometry-analyzed (Figures 1(a) and 1(b)).

The measurement was accompanied with BD multitest TBNK (Becton Dickinson) for absolute cell count determination. For each sample, the absolute cell count of the population of  $CD3^+CD8^+CD28^-$  and their percentage in the population of T-cells were determined in relation to the number of  $CD3^+$  lymphocytes.

**3.2. Determination of  $CD3^+CD8^+CD28^-Foxp3^+$  Subpopulation.** 300  $\mu$ L of heparinized blood was stained with 20  $\mu$ L of the following antibodies: anti-CD3APC, anti-CD8FITC, and anti-CD28PerCPCy5.5. All were purchased from Becton Dickinson (BD, San Jose, California, USA). After 30 minutes of incubation at 4°C in the dark, the red blood cells were lysed with BD FACS Lysing Solution (Becton Dickinson). The cells were washed with PBS 2% FBS and permeabilized with the Fixation/Permeabilization Concentrate (eBioscience) in Fixation/Permeabilization Diluent (eBioscience) for 30 minutes at 4°C in the dark. After two washing steps in Permeabilization Buffer (eBioscience) the cell pellet was stained with 5  $\mu$ L of Anti-Human Foxp3 PE clone 236A/E7 (eBioscience, San Diego, CA, USA) for 30 minutes at 4°C in the dark. The samples were then washed twice in Permeabilization Buffer (eBioscience) and flow cytometry-analyzed (Figures 1(a)–1(e)). For each sample, the absolute cell number of  $CD3^+CD8^+CD28^-Foxp3^+$  and their percentage in the population of T-cells were determined in relation to the number of  $CD3^+$  lymphocytes.

**3.3. Statistical Analysis.** The experimental and clinical data were combined and statistically analyzed using STATISTICA 10 software. The results of statistical analysis are presented with interquartile range. Correlation analysis was performed using the Spearman procedure. The Mann-Whitney *U* test (for independent samples) was applied, and differences with *p* less than 0.05 were considered statistically significant.

### 4. Results

The parameters measured regarding  $CD3^+CD8^+CD28^-$  cells were the percentage of the total T  $CD3^+$  lymphocytes population and the absolute number of  $CD3^+CD8^+CD28^-$  cells in whole blood (values given per microliter). All blood samples revealed the presence of  $CD3^+CD8^+CD28^-$  cells. Statistically significant differences in both the percentage of

$CD3^+CD8^+CD28^-$  cells and their absolute numbers between the study group and the control group have been demonstrated. Patients with SLE presented significantly higher absolute count and percentage of  $CD3^+CD8^+CD28^-$  cells compared to HC ( $p < 0.001$ ). Study group had more than three times higher absolute number ( $p < 0.001$ ) and more than two times higher percentage of  $CD3^+CD8^+CD28^-$  cells ( $p < 0.001$ ) compared to the control group.

Additionally, variability in the number of  $CD3^+CD8^+CD28^-$  lymphocytes depending on the activity of the disease measured by SLEDAI scale has been observed (Figures 2 and 3).

The lower percentage of these cells was seen in the group with inactive disease compared to active LN patients ( $p = 0.022$ ). There was also a significant difference in the absolute number of  $CD3^+CD8^+CD28^-$  cells, which was characterized by a lower frequency in patients with low disease activity compared to the group with high activity ( $p = 0.039$ ).

Statistical analysis showed a significantly higher percentage and absolute values of  $CD3^+CD8^+CD28^-$  in patients with high disease activity compared with the control group ( $p < 0.001$ ). Interestingly, the percentages and absolute count of  $CD3^+CD8^+CD28^-$  cells did not differ significantly between patients with inactive disease and the control group (Figure 3).

There were no significant differences in the percentage and absolute count of  $CD3^+CD8^+CD28^-$  between the groups according to rSLEDAI, but in the group with active nephritis (rSLEDAI 4–16) higher values of  $CD3^+CD8^+CD28^-$  have been observed compared to the group with inactive LN.

Significant ( $p = 0.038$ ) positive correlation between increasing percentage of  $CD3^+CD8^+CD28^-$  and disease activity measured by SLEDAI (correlation coefficient 0.281) was also demonstrated (Figure 4).

Lack of Foxp3 expression on  $CD3^+CD8^+CD28^-$  cells in any of the tested blood samples was observed (Figure 1(e)).

### 5. Discussion

There are conflicting reports concerning Foxp3 expression on  $CD3^+CD8^+CD28^-$  lymphocytes. Some of researchers indicate lack of this factor [29, 30] or, on the contrary, others have reported presence of this molecule [27, 28] in T  $CD8^+CD28^-$  cells. In our study, there was no expression of Foxp3 in  $CD3^+CD8^+CD28^-$  cells in any of the tested blood samples, both in the control and in the study group. The potential methodological error regarding Foxp3 detection was eliminated as presence of Foxp3 molecule was demonstrated on non- $CD8^+$  cells (Figure 1(d)). The results of our work suggest nonsuppressive and nonregulative properties of the  $CD3^+CD8^+CD28^-$  subpopulation [36].

In the present study almost three times higher number of  $CD3^+CD8^+CD28^-$  lymphocytes in the study group compared with the control group was demonstrated. Most of autoimmune diseases are associated with an increase in T  $CD8^+CD28^-$  ( $CD8^+CD57^+$ ) cells which exhibit cytotoxic properties and can play an active role in the autoimmune response [36]. The majority of literature reports indicate an

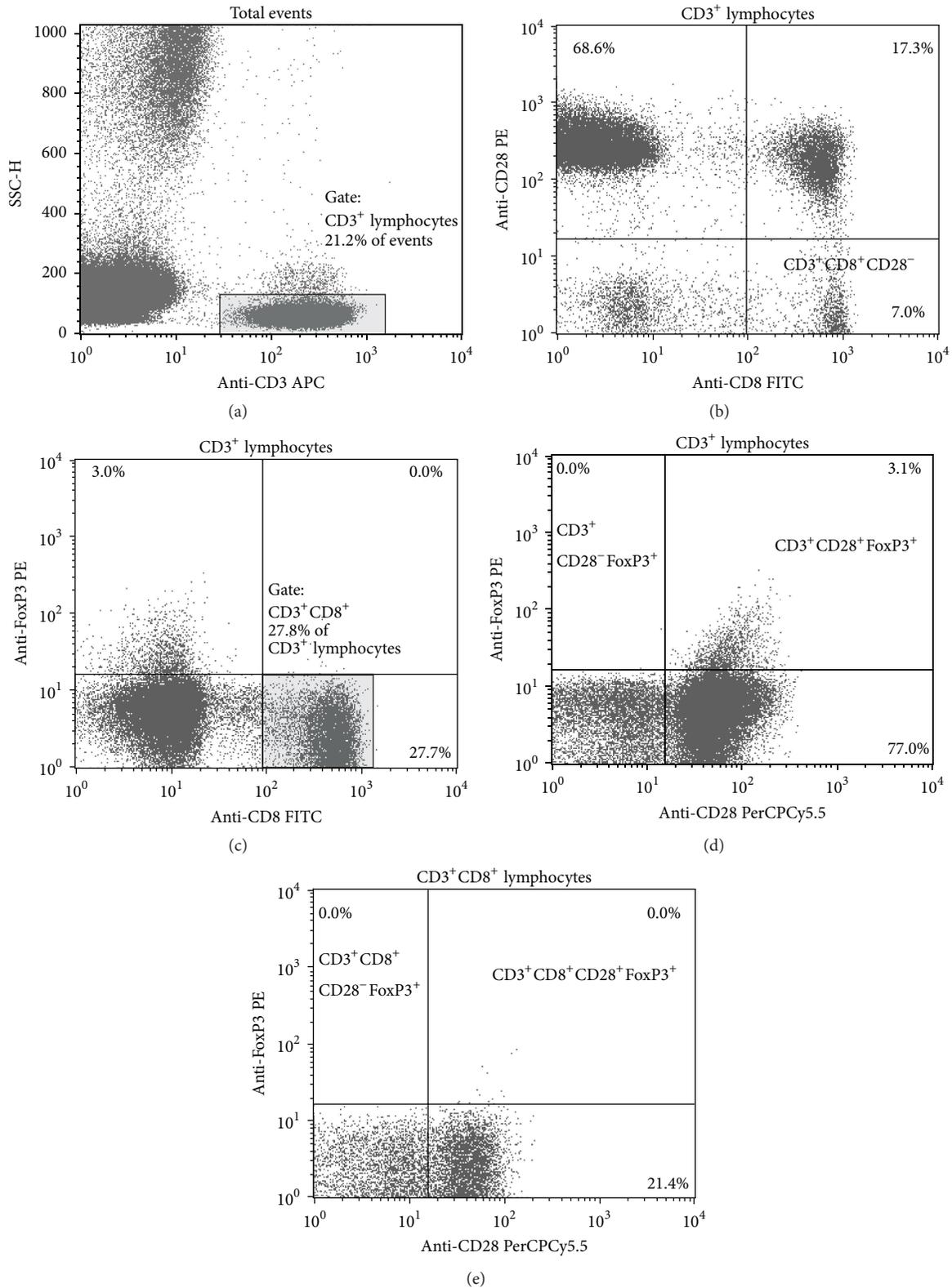


FIGURE 1: Flow cytometry analysis of CD3<sup>+</sup>CD8<sup>+</sup>CD28<sup>-</sup> and CD3<sup>+</sup>CD8<sup>+</sup>CD28<sup>-</sup>Foxp3<sup>+</sup> subpopulations. Gating strategy: (a) SSC versus anti-CD3 APC fluorescence plot. CD3<sup>+</sup> lymphocytes are shown in the gate. (b) Anti-CD28 PE versus anti-CD8 FITC fluorescence plot. CD3<sup>+</sup>CD8<sup>+</sup>CD28<sup>-</sup> cells are shown in lower right part of the chart. (c) Anti-FoxP3 PE versus anti-CD8 FITC fluorescence plot. CD3<sup>+</sup>CD8<sup>+</sup> cells are shown in the gate. (d) Anti-FoxP3 PE versus anti-CD28 PerCPCy5.5 fluorescence plot. Foxp3 expression is present on non-CD8<sup>+</sup> cells in upper right part of the chart. (e) Anti-FoxP3 PE versus anti-CD28 PerCPCy5.5 fluorescence plot. Lack of Foxp3 expression is shown on CD3<sup>+</sup>CD8<sup>+</sup>CD28<sup>-</sup> cells in upper left part of the chart.

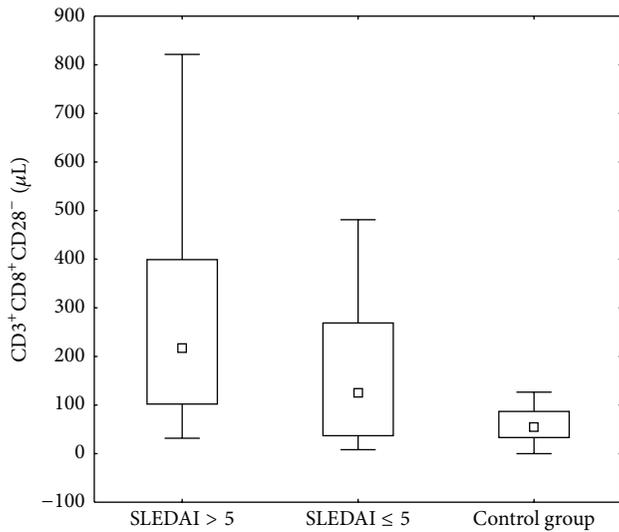


FIGURE 2: Comparison of the absolute number of  $CD3^+CD8^+CD28^-$  cells in the patients divided into two groups according to disease activity measured by SLEDAI scale and the control group.

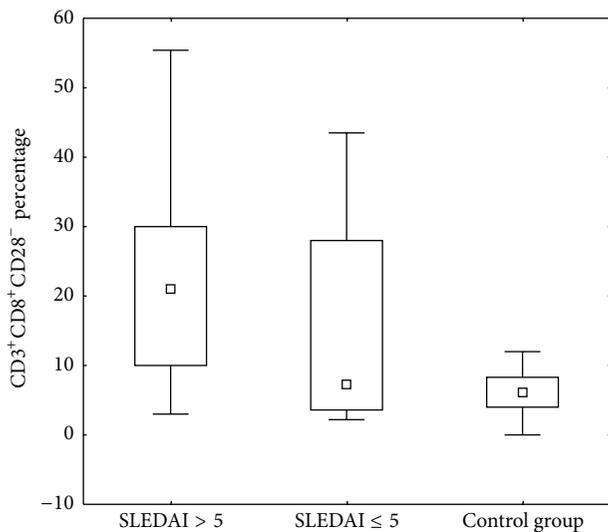


FIGURE 3: Comparison of the percentage of  $CD3^+CD8^+CD28^-$  cells in the patients divided into two groups according to disease activity measured by SLEDAI scale and the control group.

increased number of these cells in autoimmune diseases and define them as a cytotoxic subpopulation, having a negative impact on the development of the immune response. Quantitative changes of a  $CD8^+CD28^-$  ( $CD8^+CD57^+$ ) lymphocytes population have been observed in autoimmune diseases such as multiple sclerosis [23], type 1 diabetes [23], Graves' disease [24], and rheumatoid arthritis [25]. Only a few literature reports regarding assessment of  $CD3^+CD8^+CD28^-$  lymphocyte in SLE presented different results. In one publication, authors showed no significant differences in the percentage of these cells in the PBMC from patients with SLE compared to healthy controls. However, their data showed that three patients with SLE had high levels of  $CD3^+CD8^+CD28^-$

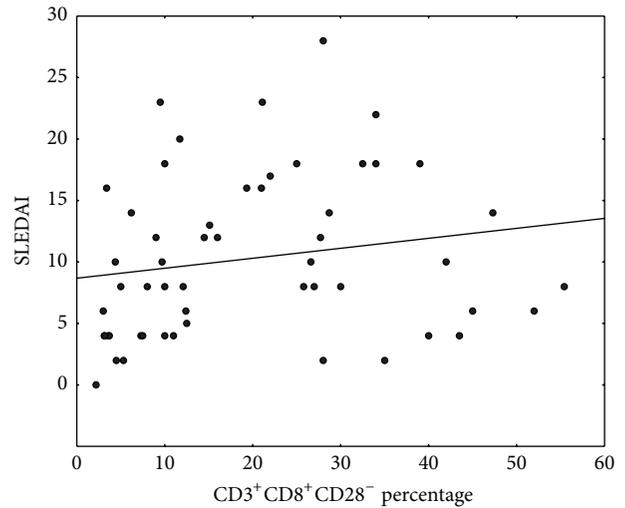


FIGURE 4: Correlation between the SLEDAI and  $CD3^+CD8^+CD28^-$  cells percentage.

lymphocytes, which is in line with our findings. Additional analysis demonstrated that two of these patients had active disease and that another one was inactive, but analysis of these data revealed that there was no significant association between the levels of  $CD3^+CD8^+CD28^-$  cells and disease activity [32]. The second publication demonstrated lower absolute number of  $CD3^+CD8^+CD28^-$  cells in patients with SLE than in healthy controls although no significant difference was found. However, when authors evaluated the distribution of CD28 molecule within the CD8 T-cell population, the  $CD3^+CD8^+CD28^-$  T-cell population was significantly lower in patients with SLE compared to healthy individuals [33]. Moreover, authors found no association between the absolute numbers of  $CD3^+CD8^+CD28^-$  T-cell population and SLEDAI [33].

Under the influence of chronic antigen stimulation in SLE repeating cycles of activation, stimulation and proliferation lead to progressive and irreversible reduction in expression of CD28 molecules on the surface of cells [6]. The result is the accumulation of "antigen-experienced" T-cell phenotype  $CD8^+CD28^-$ . This observation was confirmed in our study. It is also suggested that persistent antigenic stimulation is accompanied by abnormal apoptosis of  $CD3^+CD8^+CD28^-$  [22, 37] which may be confirmed by increased number of these cells in our analysis. Similar results were observed in patients with HIV infection who also have chronic activation of T lymphocytes, particularly in the late stages of infection [37]. In the present study a significant correlation between the number of  $CD3^+CD8^+CD28^-$  cells and disease activity measured by SLEDAI scale was demonstrated. In patients with active disease we observed almost twice the number of these cells compared to patients with inactive SLE. Furthermore, the number of  $CD3^+CD8^+CD28^-$  cells in patients with inactive disease did not differ from the control group; consequently it was increased only in patients with active disease. This observation indicates that the accumulation of cells with a phenotype of  $CD3^+CD8^+CD28^-$  is linked to

the exacerbation of disease activity. Our analysis is the first study which has proved such correlation. The relationship between the number of CD3<sup>+</sup>CD8<sup>+</sup>CD28<sup>-</sup> cells and SLEDAI makes it an attractive target for research in SLE. We believe that further tests in larger groups of patients are required to fully elucidate the mechanisms involved in pathogenesis of the disease, as well as nature of CD3<sup>+</sup>CD8<sup>+</sup>CD28<sup>-</sup> lymphocytes.

## 6. Limitations of the Study

Lack of immunosuppressive therapy impact assessment on determined subpopulations is one of the limitations of the study. Such analysis was not possible due to small size of the study group. It seems, however, that the possible impact of immunosuppressive therapy on the results was at least partially eliminated by comparing groups of different disease activity proven by well known indicator. This research was not designed as prospective cohort study. Its aim was to evaluate number of CD3<sup>+</sup>CD8<sup>+</sup>CD28<sup>-</sup> and CD3<sup>+</sup>CD8<sup>+</sup>CD28<sup>-</sup>Foxp3<sup>+</sup> subpopulations in one point in groups of different disease activity, and thus lack of follow-up can be considered a limitation of this study.

## 7. Conclusions

In conclusion, our analysis does not confirm expression of Foxp3 molecule in CD3<sup>+</sup>CD8<sup>+</sup>CD28<sup>-</sup> cells which suggests nonsuppressive and nonregulative properties of the CD3<sup>+</sup>CD8<sup>+</sup>CD28<sup>-</sup> subpopulation. Our data is the first study to indicate increase in percentage and absolute count of CD3<sup>+</sup>CD8<sup>+</sup>CD28<sup>-</sup> lymphocytes along with an increase in disease activity. That indicates importance of CD3<sup>+</sup>CD8<sup>+</sup>CD28<sup>-</sup> lymphocytes in the inflammatory process and suggests that the extension of the CD3<sup>+</sup>CD8<sup>+</sup>CD28<sup>-</sup> subpopulation is associated with the exacerbation of the disease. Highlighting aspects of the immune imbalances and autoimmunity, the results of present study are a part of a discussion on the significance of CD3<sup>+</sup>CD8<sup>+</sup>CD28<sup>-</sup> cells in the pathogenesis of SLE.

## Abbreviations

AAV:	ANCA-associated vasculitis
ANCA:	Antineutrophil cytoplasmic antibody
APC:	Antigen presenting cell
CD:	Cluster of differentiation
CTL:	Cytotoxic T lymphocyte
FBS:	Fetal bovine serum
Foxp3:	Transcription factor forkhead box P3
HIV:	Human immunodeficiency virus
HSP 27:	Heat shock protein 27
LN:	Lupus nephritis
MHC:	Major histocompatibility complex
pAPCs:	Professional antigen presenting cells
PBS:	Phosphate buffered saline

rSLEDAI:	Renal Systemic Lupus Erythematosus Disease Activity Index
SLE:	Systemic lupus erythematosus
SLEDAI:	Systemic Lupus Erythematosus Disease Activity Index
SSc:	Systemic sclerosis
TCR:	T-cell receptor.

## Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

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## Research Article

# Tumor-Derived CXCL1 Promotes Lung Cancer Growth via Recruitment of Tumor-Associated Neutrophils

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Neutrophils have a traditional role in inflammatory process and act as the first line of defense against infections. Although their contribution to tumorigenesis and progression is still controversial, accumulating evidence recently has demonstrated that tumor-associated neutrophils (TANs) play a key role in multiple aspects of cancer biology. Here, we detected that chemokine CXCL1 was dramatically elevated in serum from 3LL tumor-bearing mice. *In vitro*, 3LL cells constitutively expressed and secreted higher level of CXCL1. Furthermore, knocking down CXCL1 expression in 3LL cells significantly hindered tumor growth by inhibiting recruitment of neutrophils from peripheral blood into tumor tissues. Additionally, tumor-infiltrated neutrophils expressed higher levels of MPO and Fas/FasL, which may be involved in TAN-mediated inhibition of CD4<sup>+</sup> and CD8<sup>+</sup> T cells. These results demonstrate that tumor-derived CXCL1 contributes to TANs infiltration in lung cancer which promotes tumor growth.

## 1. Introduction

Lung cancer is the most commonly diagnosed malignancy with approximately 224,000 new cases in 2014 in western world [1]. Although great advances have been made in the early detection and therapeutic approaches, the morbidity of lung cancer is still raising and leading to the first cause of death in cancer [1]. Chronic inflammation is strongly linked to lung cancer initiation and progression. In lung cancer patients or experimental animal models, the composition and phenotype of neutrophils/granulocytic cells in blood are dramatically altered [2, 3]. Multiple evidence shows that neutrophils are presented in the tumor sites or paratumor tissue in various types of tumors, and the density of neutrophils is an independent factor for predicting the prognosis of cancer patients [4]. Tumor-derived chemokines and cytokines recruit myeloid cells into tumor microenvironment and educate them into protumor phenotype [5]. However, the mechanisms that are involved in the recruitment of neutrophils to the tumor microenvironment are far from clear. In hepatocellular carcinoma (HCC), tumor-derived chemokine

CXCL5 mediate the recruitment of neutrophils, and the density of TANs is associated with poor prognosis of cancer patients [6]. The chemokine receptor CXCR2 is expressed in neutrophils/granulocytic cells. When CXCR2 interacts with its ligand, neutrophils are recruited to the inflamed site [7]. Additionally, Lewis lung carcinoma cells-derived oxysterol plays a key role in the recruitment of CXCR2<sup>+</sup> tumor-promoting neutrophils into tumor tissues [8]. Tumor-expressed chemokines CXCL8 and CXCL6 are also involved in the neutrophils infiltration [9].

Chemokine (C-X-C motif) ligand 1 CXCL1 (also referred to as GRO-1) binds to CXCR2, which is highly expressed on the surface of neutrophils [10]. In both infection and cancer microenvironment, CXCL1 is elevated by various stress-inducing factors, including PGE2 [11]. Elevated levels of CXCL1 and CXCR2 positively correlate with the poorer prognosis of cancer patients [12, 13]. It has been reported that CXCL1/CXCR2 density is strongly associated with the number of neutrophils in the tumor microenvironment and can be an independent factor for predicting the prognosis of patients with hepatocellular carcinomas [14]. In animal

studies, CXCL1 receptor CXCR2 deficiency prevents from tumorigenesis in colitis-associated cancer [15] and colorectal cancers [16] by inhibiting myeloid-derived suppressive cells (MDSCs) infiltration. Furthermore, CXCL1-mediated myeloid cells infiltration is associated with therapeutic response in breast cancer [17]. Recently, it has been reported that stably silencing of CXCL1 can inhibit tumor growth in HCC [18], and knocking down of CXCL1 expression can inhibit tumor growth in colorectal liver metastasis [19]. Additionally, autocrine and paracrine of CXCL1 can also promote tumor invasion and metastasis [17, 20, 21].

In this study, we found that the level of CXCL1 in serum was significantly upregulated in 3LL lung cancer bearing mice. Knocking down CXCL1 expression in 3LL cells significantly inhibited neutrophils infiltration, resulting in reducing tumor growth *in vivo*. Tumor-infiltrated neutrophils in tumor tissues expressed higher levels of MPO and Fas/FasL, which may be involved in TAN-mediated inhibition of CD4<sup>+</sup> and CD8<sup>+</sup> T cells. In conclusion, tumor-derived CXCL1 contributes to neutrophils infiltration in lung cancer which promotes tumor growth.

## 2. Materials and Methods

**2.1. Mice and Cell Lines.** C57BL/6J mice (6–8 weeks) were obtained from Joint Ventures Sipper BK Experimental Animal Company (Shanghai, China). All experimental manipulations were undertaken in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, with the approval of the Scientific Investigation Board of the Second Military Medical University, Shanghai, China. A Lewis lung carcinoma sub-line 3LL was maintained as described previously [22]. A mouse epithelial cell line MLE and a human non-small lung carcinoma cell line A549 were purchased from ATCC.

**2.2. CXCL1 Knockdown.** 3LL cells ( $2 \times 10^5$ /well) were plated into 6-well plates and transfected with negative control (NC) or CXCL1-specific shRNA plasmids (design by Gene Pharma, China) using jetPEI (Polyplus-transfection, France) as described previously [23]. Stably silenced transfected cell clones were selected in 800  $\mu$ g/mL G418 for 14 days; GFP-expressing cells were selected by FACS sorting system and analyzed by real-time PCR and ELISA for CXCL1 expression and secretion.

**2.3. Flow Cytometric Analysis.** For analysis of tumor-infiltrating cells, tumor-infiltrating lymphocytes (TILs) were isolated from transplanted tumors and suspended into single cells. For analysis of the compartment of immune cells in the peripheral blood, blood was collected in a tube with heparin and incubated with Tris-NH<sub>4</sub>Cl to remove erythrocytes. Cells were stained with monoclonal anti-mouse antibodies as follows: CD45-BV510, CD3 $\epsilon$ -PE-Cy7, CD8 $\alpha$ -PE, CD4-PE-Cy5, CXCR2-PerpCy5.5, CD11b-APC, Ly6G-PE, Ly6C-FITC, Fas-PE, FasL-PE, CD11b-PerCP-Cy5.5, CD8a-FITC, 7-AAD, and Annexin-V-FITC. Flow cytometry analysis was carried out using a BD FACS (BD Biosciences).

**2.4. ELISA.** Mouse and human CXCL1 ELISA kit (R&D systems) were used to determine the concentrations of CXCL1 in cell culture supernatant or serum from tumor-bearing mice according to the manufacturer's instructions.

**2.5. qRT-PCR.** Total mRNA of 3LL cells was extracted with Trizol reagent (Invitrogen) and then subjected to reverse transcribed by using M-MLV reverse transcribed kit (Takara). cDNA was amplified by ABI7300 Detection System (Applied Biosystems) using SYBR Green PCR kit (Takara). Levels of CXCL1 were normalized by the levels of  $\beta$ -actin in each individual sample. Specific primers were as follows:

m $\beta$ -actin: forward (5'-AGTGTGACGTTGACATCCGT-3'),

m $\beta$ -actin: reverse (5'-GCAGCTCAGTAACAGTCCGC-3'),

mCXCL1: forward (5'-GTCATAGCCACACTCAAGAA-3'),

mCXCL1: reverse (5'-AGACAGGTGCCATCAGAG-3').

**2.6. Chemotaxis Assay.** Cell migration was estimated by using a pore size of 3  $\mu$ m transwell chambers Matrigel (BD Biosciences, Bedford, MA, USA).  $2 \times 10^6$  cells isolated from spleen and peripheral blood in 200  $\mu$ L of serum free medium were added into the upper chamber, and 600  $\mu$ L of cell culture supernatant from 3LL/NC, 3LL/shCXCL1, or medium control was added into bottom chamber. After incubating for 4 h, the cells phenotype on the bottom was analyzed by flow cytometric analysis.

**2.7. Tumor-Bearing Model.**  $5 \times 10^5$  3LL/shCXCL1 cells or 3LL/NC cells were injected subcutaneously in the abdomen of C57BJ/6L mice, tumor volume (V) was measured twice a week and calculated by using the formula  $0.5 \times [\text{Length} \times \text{Width}^2]$ .

**2.8. Immunofluorescence (IF).** CD11b<sup>+</sup>Ly6G<sup>+</sup> neutrophils in tumor tissue sections from tumor-bearing mice were analyzed by immunofluorescence staining. Ly6G antibody was used at the dilution of 1:50. The fluorescent-labeled second antibodies were used at the dilution of 1:100. Digital imaging was carried out using the software LASV4.5 (Leica DM 2000).

**2.9. Immunohistochemistry (IHC).** Five-micrometer thick sections were cut from formalin-fixed and paraffin-embedded tissue specimens and put onto slides; sections were deparaffinized through alcohol gradients and rehydrated to water. Antigens were retrieved by using Citrate pH 6.0 buffer in thermostatic bath at 100°C for 5 minutes. Tissue sections were incubated with primary anti-mouse Ly6G (Biolegend) at the dilution of 1:50 or anti-mouse MPO (Abcam) at the dilution of 1:200. Rabbit polyclonal IgG (DAKO) was used as negative control instead of primary antibody. Digital imaging was examined using the software LAS V4.5 (Leica DM 2000).

**2.10. Coculture System.** TANs were isolated from tumor tissues derived from 3LL/NC tumor-bearing mice; CD4<sup>+</sup> T cells were isolated from the spleens of naïve mice. Cell sorting were carried out by using MACS system (Miltenyi Biotech). Splenic CD4<sup>+</sup> T cells ( $5 \times 10^4$ /well) with or without neutrophils ( $5 \times 10^4$ /well) were cocultured in precoated CD3 ( $2 \mu\text{g/mL}$ ) 24-well plates with soluble CD28 ( $2 \mu\text{g/mL}$ ) and murine recombinant IL-2 ( $1 \text{ ng/mL}$ ) for 3 days. CD4<sup>+</sup> T cells number was counted by flow cytometry.

**2.11. Statistic Analysis.** All the statistics were analyzed with the assistance of Graphpad Prism 5.0. The comparisons between two groups were analyzed by unpaired Student's *t*-test.

### 3. Results

**3.1. Increased Level of CXCL1 in Serum Is Mainly Derived from 3LL Cancer Cells In Vivo.** Compared to naïve mice, serum levels of CXCL1 in 3LL lung cancer bearing mice were significantly increased (Figure 1(a)). To examine the source of serum CXCL1 in 3LL tumor-bearing mice, we first examined the expression and secretion of CXCL1 in 3LL cells and found that 3LL cells constitutively secreted and expressed high level of CXCL1 (Figures 1(b) and 1(c)). In addition, we found the human non-small lung carcinoma cell A549 also secreted higher levels of CXCL1 (Figure 1(d)). However, the mouse epithelial cell line MLE did not secrete CXCL1 (Figure 1(d)), suggesting lung cancer cells could express and secrete higher levels of CXCL1. It has been reported that neutrophils are also able to express and produce significant amounts of CXCL1 when activated [24, 25]. Larger amounts of Ly6G<sup>+</sup>MPO<sup>+</sup> neutrophils were found in 3LL tumor tissues (Figure 1(e)). Then, we analyzed CXCL1 expression in TANs and 3LL tumor. The results showed that the expression of CXCL1 in 3LL tumor was higher than that in TANs or LPS-stimulated TANs (~9-fold) (Figure 1(f)). Furthermore, we knocked down CXCL1 expression in 3LL cells using CXCL1 targeted shRNA construct. The results showed that compared to negative control (NC), CXCL1 mRNA expression and secretion in CXCL1-silencing 3LL cells (shCXCL1) were significantly decreased (Figures 2(a) and 2(b)). Serum levels of CXCL1 were markedly decreased in the mice inoculated with shCXCL1 cells (Figure 2(c)). Taken together, these results demonstrate that 3LL-derived CXCL1 is the main source that contributes to the increased serum level of CXCL1 *in vivo*.

**3.2. Knockdown of CXCL1 Expression in 3LL Cells Inhibits Tumor Growth In Vivo.** Then, to investigate the roles of CXCL1 in lung cancer growth *in vivo*, 3LL transfected cells (NC or shCXCL1) were subcutaneously inoculated into C57BL/6J mice. The results showed that 3LL negative control cells (NC) grew aggressively *in vivo*, while knockdown CXCL1 expression in 3LL cells (shCXCL1) significantly attenuated tumor growth (Figures 2(d) and 2(e)). Furthermore, quantitative PCR analysis showed that CXCL1 expression in 3LL/shCXCL1 tumor tissues was significantly lower than that in 3LL/NC tumor tissues (Figure 2(f)). These results indicate

that tumor-derived CXCL1 contributes to lung cancer growth *in vivo*.

**3.3. Tumor-Derived CXCL1 Mediates TANs Infiltration.** CXCL1 is an important chemokine that contributes to recruitment of CXCR2 expressing myeloid cells and neutrophils [7]. Myeloid cells in mice consist of CD11b<sup>+</sup>Ly6C<sup>+</sup> monocytic cells and CD11b<sup>+</sup>Ly6G<sup>+</sup> neutrophils/granulocytic cells [26]. Flow cytometric analysis showed that the frequency of CD11b<sup>+</sup>Ly6G<sup>+</sup> neutrophils in the circulation of mice inoculated 3LL/shCXCL1 ( $44.6\% \pm 4.45\%$ ) was significantly decreased compared to mice inoculated with 3LL/NC ( $57.7\% \pm 1.8\%$ ) (Figures 3(a) and 3(b)). However, CD11b<sup>+</sup>Ly6G<sup>+</sup> neutrophils counts in bone marrow were similar (NC versus shCXCL1;  $41.88\% \pm 3.7\%$  versus  $53\% \pm 3.82\%$ ) (Figure 3(c)). In addition, CD11b<sup>+</sup>Ly6G<sup>+</sup> neutrophils counts in spleen of mice inoculated with 3LL/NC tumor cells did not profoundly change compared to mice inoculated with 3LL/shCXCL1 tumor cells (NC versus shCXCL1;  $6.31\% \pm 1.55\%$  versus  $5.34\% \pm 1.65\%$ ) (Figure 3(d)). IHC staining of Ly6G showed that the number of Ly6G<sup>+</sup> neutrophils in tumor tissues from mice inoculated with 3LL/NC was higher than that in 3LL/shCXCL1 bearing mice (Figures 3(e) and 3(f)). As myeloperoxidase (MPO) is signature marker of neutrophils existing in the granules of neutrophils [27], the result showed that there were more MPO<sup>+</sup> TANs from mice inoculated with 3LL/NC than that from mice inoculated with 3LL/shCXCL1 (Figures 3(g) and 3(h)). Furthermore, using a transwell system, the accumulation of CD11b<sup>+</sup>Ly6G<sup>+</sup> neutrophils by CXCL1 *in vitro* was detected. The results showed that knockdown of CXCL1 expression in 3LL cells markedly reduced the number of chemoattracted neutrophils (chemoattracted neutrophils counts isolated from spleen: 3LL/NC medium versus 3LL/shCXCL1 medium  $31784 \pm 5904$  versus  $1804 \pm 582$ ) (chemoattracted neutrophils counts isolated from blood: 3LL/NC medium versus 3LL/shCXCL1 medium  $182502 \pm 13119$  versus  $7992 \pm 5763$ ) (Figure 3(i)). These results demonstrate that tumor-derived CXCL1 promotes neutrophils recruitment *in vitro* and *in vivo*.

**3.4. Tumor-Infiltrated Neutrophils Inhibit T Cell-Mediated Anti-Tumor Function In Vitro and In Vivo.** Previous study reported that TANs exhibited protumor phenotype by suppressing activation of CD8<sup>+</sup> T cells in lung cancer [28]. So, we detected the number of effector T cells in spleen and tumor tissues derived from tumor-bearing mice. The results showed increased number of both CD3<sup>+</sup>CD4<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup> T cells in spleen and tumor tissues in 3LL/shCXCL1 group compared to 3LL/NC control group (Figures 4(a) and 4(b)), suggesting that the higher density of neutrophils was paralleled with the decreased T cells number in lung cancer. It has been reported that Fas/FasL expression by neutrophils can directly regulate CD8<sup>+</sup> T cells infiltration [29]. We analyzed the expression of Fas/FasL in splenic neutrophils or TANs. As shown in Figure 4(c), splenic neutrophils expressed lower levels of Fas/FasL; however, tumor infiltrated neutrophils derived from both 3LL/NC and 3LL/shCXCL1 bearing mice

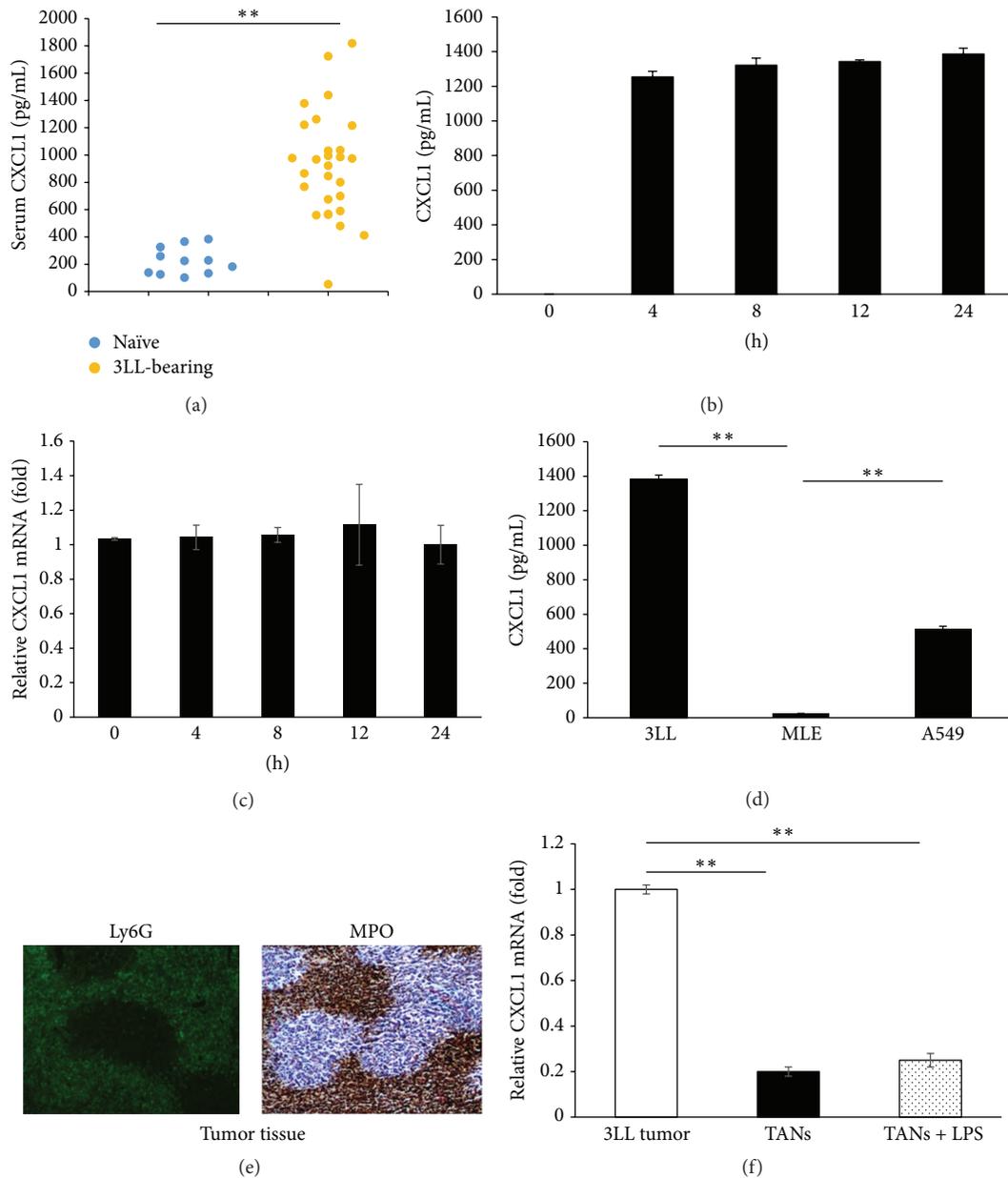


FIGURE 1: Expression and secretion of CXCL1 *in vitro* and *in vivo*. (a) Levels of CXCL1 in the serum of naïve mice ( $n = 11$ ) or 3LL tumor-bearing mice ( $n = 27$ ) were measured by ELISA at 14 days after tumor inoculation. Each dot represents an individual mouse. (b) CXCL1 protein levels in the 3LL culture supernatants were determined by ELISA. (c) mRNA expression of CXCL1 in 3LL cells was measured by quantitative RT-PCR. (d) ELISA analysis of CXCL1 production in Lewis lung carcinoma cell line 3LL, mouse epithelial cell line MLE, and human non-small lung carcinoma cell line A549. (e) Ly6G<sup>+</sup>MPO<sup>+</sup> neutrophils in 3LL tumor tissues were determined by IF and IHC. (f) Quantitative RT-PCR analysis of CXCL1 expression in 3LL tumor cells and TANs (sorted from 3LL tumor tissues) stimulated with or without LPS (100 ng/mL) for 6 hours. \*\*  $p < 0.01$ ; data are shown as mean  $\pm$  SD.

expressed higher levels of Fas/FasL. Flow cytometric analysis demonstrated that the number of apoptotic/necrotic CD4<sup>+</sup> T cells and CD8<sup>+</sup> T was lower in 3LL/shCXCL1 group compared to 3LL/NC group (Figure 4(c)). Furthermore, to determine the effect of TANs on proliferation of T cells, we isolated TANs in tumor tissues from 3LL tumor-bearing mice. TANs significantly inhibited mature dendritic

cells- (mDC-) mediated CD4<sup>+</sup> T proliferation *in vitro* (Figure 4(e)). Consistently, TANs suppressed CD4<sup>+</sup> T proliferation primed by anti-CD3 and CD28 (N/T cells, TANs/T cells versus T cells;  $5612.3 \pm 1487.4$ ,  $3592 \pm 1122.5$ , versus  $6652.7 \pm 764.9$ ) (Figure 4(f)). These results suggest that tumor infiltrated neutrophils could inhibit the proliferation and induce apoptosis/necrosis of T cells.

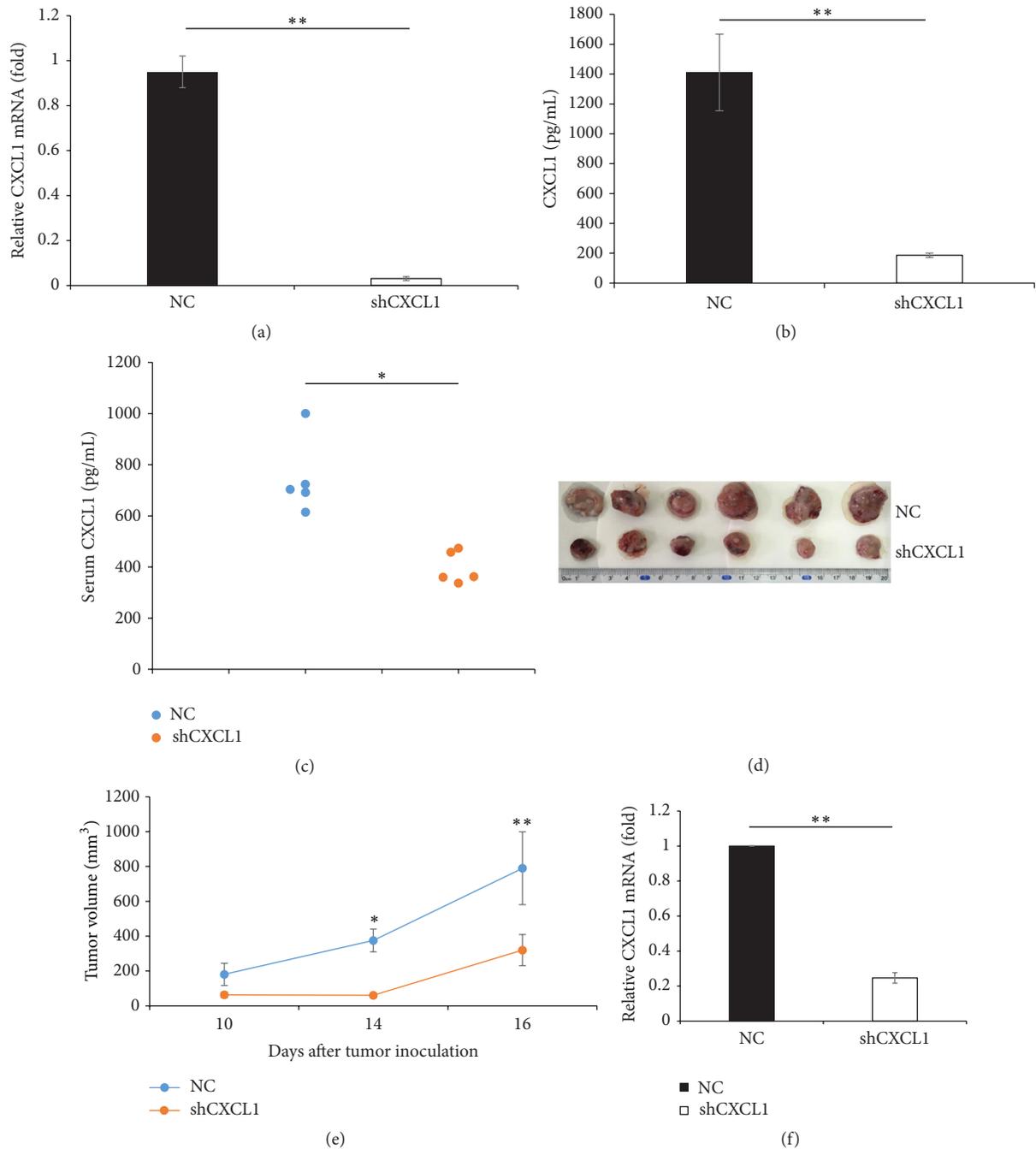


FIGURE 2: Ablation of CXCL1 in 3LL cells limits tumor growth *in vivo*. (a) 3LL cells were stable transfected with negative control shRNA (NC) or specific CXCL1 shRNA (shCXCL1); CXCL1 mRNA expression was measured by quantitative RT-PCR. (b) Protein levels of CXCL1 in the 3LL/NC and 3LL/shCXCL1 supernatants were measured by ELISA. (c) Serum levels of CXCL1 from 3LL/NC or 3LL/shCXCL1 bearing mice were measured by ELISA. Each dot represents an individual mouse. (d) Primary tumors excised from 3LL/NC bearing mice and 3LL/shCXCL1 bearing mice at day 16 after tumor inoculation. (e) Growth of NC and shCXCL1 tumors was monitored for 16 days after cell inoculations. (f) CXCL1 mRNA expression in tumor tissues derived from 3LL/NC or 3LL/shCXCL1 tumor-bearing mice was examined by quantitative RT-PCR. \*  $p < 0.05$ , \*\*  $p < 0.01$ . Results are shown as mean  $\pm$  SD.

#### 4. Discussion

Proinflammatory and chemokine factors play a prominent role in lymphocyte homing and migration [30–32]. Accumulating evidence demonstrated that tumor-derived

chemokines modified the compartment of myeloid cells in the tumor microenvironment. CCL2 induced infiltration of regulatory dendritic cells and regulatory T cells in the tumor microenvironment [33], CXCL5/CXCR2 axis mediates the accumulation of CXCR2<sup>+</sup> MDSCs [34], CXCR2<sup>-/-</sup> mice

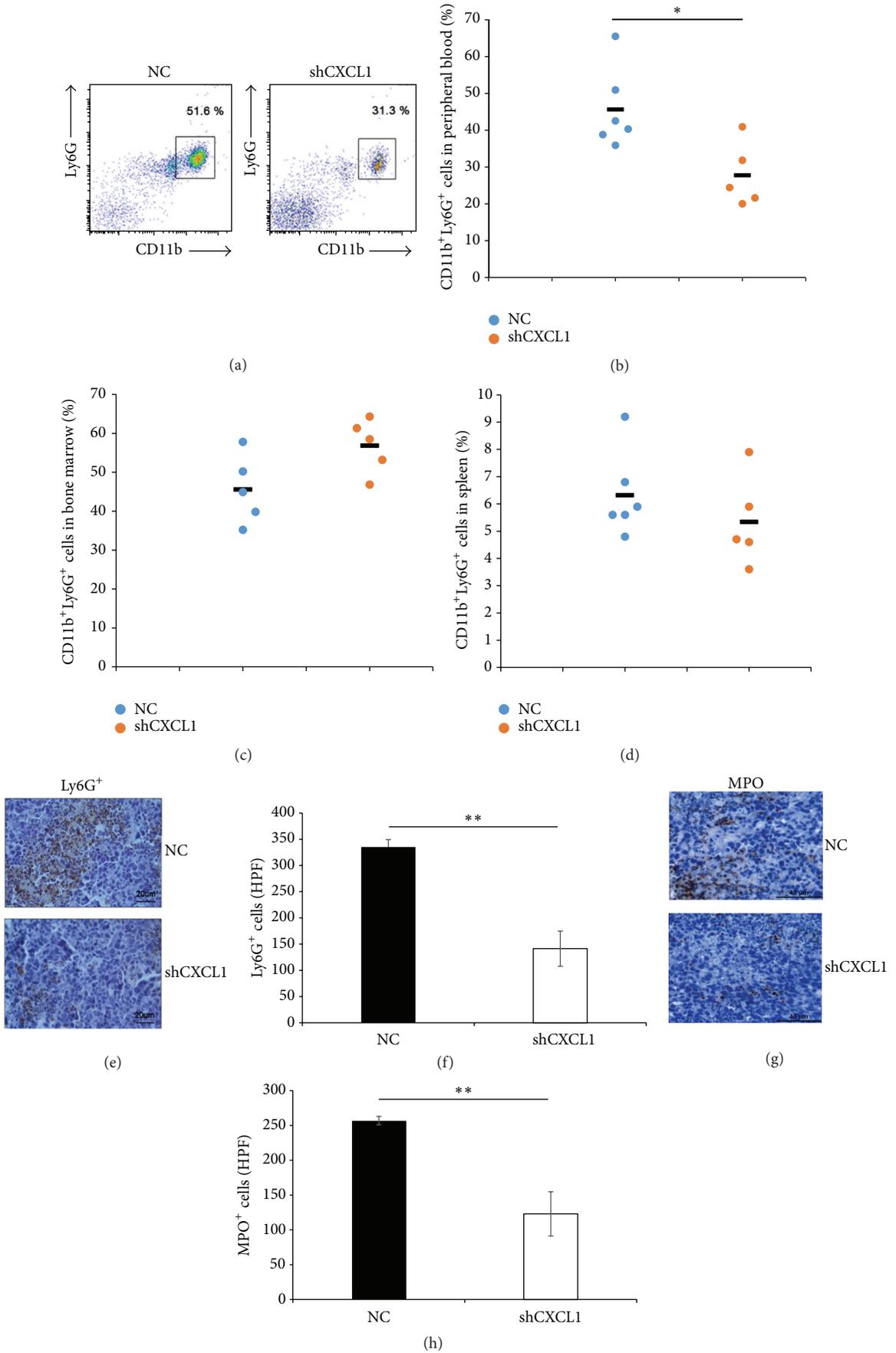


FIGURE 3: Continued.

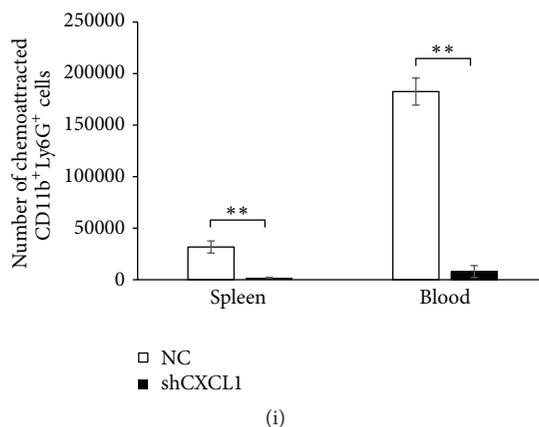


FIGURE 3: Knockdown of CXCL1 expression in 3LL cells decreases the number of neutrophils *in vivo*.  $5 \times 10^5$  3LL/NC or 3LL/shCXCL1 cells were inoculated subcutaneously (s.c.) into C57BL/6J mice. At day 16, mice were sacrificed. The CD11b<sup>+</sup>Ly6G<sup>+</sup> neutrophils in peripheral blood were detected as described in the materials and methods. Representative flow cytometric graph (a) and percentage of CD11b<sup>+</sup>Ly6G<sup>+</sup> neutrophils in peripheral blood (b) were analyzed. Each dot plot represents an individual mice; horizontal lines are shown as mean of each group. (c) Flow cytometric analysis of the frequency of CD11b<sup>+</sup>Ly6G<sup>+</sup> neutrophils in bone marrow from mice inoculated with 3LL/NC or 3LL/shCXCL1. (d) Flow cytometric analysis of the frequency of CD11b<sup>+</sup>Ly6G<sup>+</sup> neutrophils in spleen from mice inoculated with 3LL/NC or 3LL/shCXCL1 tumor cells. (e, f, g, and h) IHC staining of Ly6G<sup>+</sup> or MPO<sup>+</sup> cells in the tumor tissues from mice inoculated with 3LL/NC or 3LL/shCXCL1 tumor cells. IHC staining quantification of Ly6G<sup>+</sup> (f) or MPO<sup>+</sup> (h) cells (per high-power field, HPF). Scale bars, 20  $\mu$ m or 40  $\mu$ m. (i) Neutrophils were isolated from spleen or peripheral blood derived from tumor-bearing mice and the chemoattracted CD11b<sup>+</sup>Ly6G<sup>+</sup> neutrophils were analyzed towards the condition supernatant from NC and shCXCL1 tumor cells by *in vitro* transwell assay. \*  $p < 0.05$ , \*\*  $p < 0.01$ .

were resistance to colitis-associated cancer formation by inhibiting MDSCs accumulation in the mucosa [15], and silence of CXCL1/2 expression in breast cancer decreased the infiltration of myeloid cells in the tumor microenvironment [17]. However, there is little evidence directly elucidating the function of tumor-derived CXCL1 on the migration of neutrophils/granulocytic cells. In this study, we demonstrated that 3LL tumor-derived chemokine CXCL1 regulated the composition of immune cells in tumor microenvironment. Knockdown of CXCL1 in 3LL cells resulted in decreased number of TANs, paralleled by increased number of CD4<sup>+</sup> T and CD8<sup>+</sup> T cells. So, we demonstrated that tumor-derived CXCL1 increased the number of Ly6G<sup>+</sup> neutrophils in the peripheral blood and tumor tissues. Silencing CXCL1 expression and secretion in 3LL cells markedly reduced the number of Ly6G<sup>+</sup> neutrophils. Accordingly, CXCL1 deficiency in tumor significantly inhibited tumor growth *in vivo*. It has been well known that the increased number of neutrophils in peripheral blood is due to increased egress of bone marrow or prolonged life span [35]. So, we further examined the number and proportion of neutrophils in bone marrow. The results showed that there was no statistical significant difference of density of neutrophils in bone marrow between mice inoculated with 3LL/NC and inoculated with 3LL/shCXCL1; however, there is approximately 1.3-fold increase in neutrophil counts in the bone marrow of 3LL/shCXCL1 tumor-bearing mice, suggesting that CXCL1 maybe determine the efficiency of neutrophil egress from bone marrow. Although more evidence has identified the potential role of neutrophils in cancer progression. The roles of neutrophils in tumor microenvironment have not been well demonstrated [36]. Matrix metalloproteinases (MMPs),

such as MMP9, mainly produced by neutrophils, have the ability to degrade the extracellular matrix (ECM), which contribute to carcinogenesis and tumor progression [37, 38]. However, neutrophils-derived MMP-8 was regarded as playing a protective role in tumor progression [39]. In a spontaneous metastasis model, deletion of neutrophils in tumor microenvironment decreased tumor angiogenesis and intravasation [40]. In addition, TANs promoted tumor invasion by releasing of ROS [41], growth factors such as hepatocyte growth factor (HGF) [42], and cytokines like oncostatin M [43]. Orchestrating antitumor immunity in the tumor environment is another important function of TANs. It was proven that neutrophils inhibited T cell proliferation by releasing intracellular arginase I in non-small lung cancer [44]. In mouse lung tumors, neutrophils depletion led to more activated CD8<sup>+</sup> T cells intratumorally [28]. Multiple studies have demonstrated that TANs and lymphocytes ratio in tumor microenvironment could be a prognostic predictor in patients with various types of cancer, including lung carcinoma [45, 46] and esophageal carcinoma [47]. Our results demonstrated that the increased number of neutrophils was associated with decreased T cells number in tumor microenvironment. CD11b<sup>+</sup>Ly6G<sup>+</sup> neutrophils isolated from tumor-bearing mice expressed higher levels of Fas/FasL, which may be involved in T cells apoptosis. However, the expression levels of Fas/FasL in TANs did not depend on the CXCL1 expression in primary tumor cells. Which factor(s) in tumor microenvironment could enhance Fas/FasL expression in TANs needs to be further investigated. Beside Fas/FasL, TANs also expressed higher levels of MPO, and the number of MPO<sup>+</sup> neutrophils was less in 3LL/shCXCL1 tumor tissues than that in 3LL/NC tumor tissues. It had been

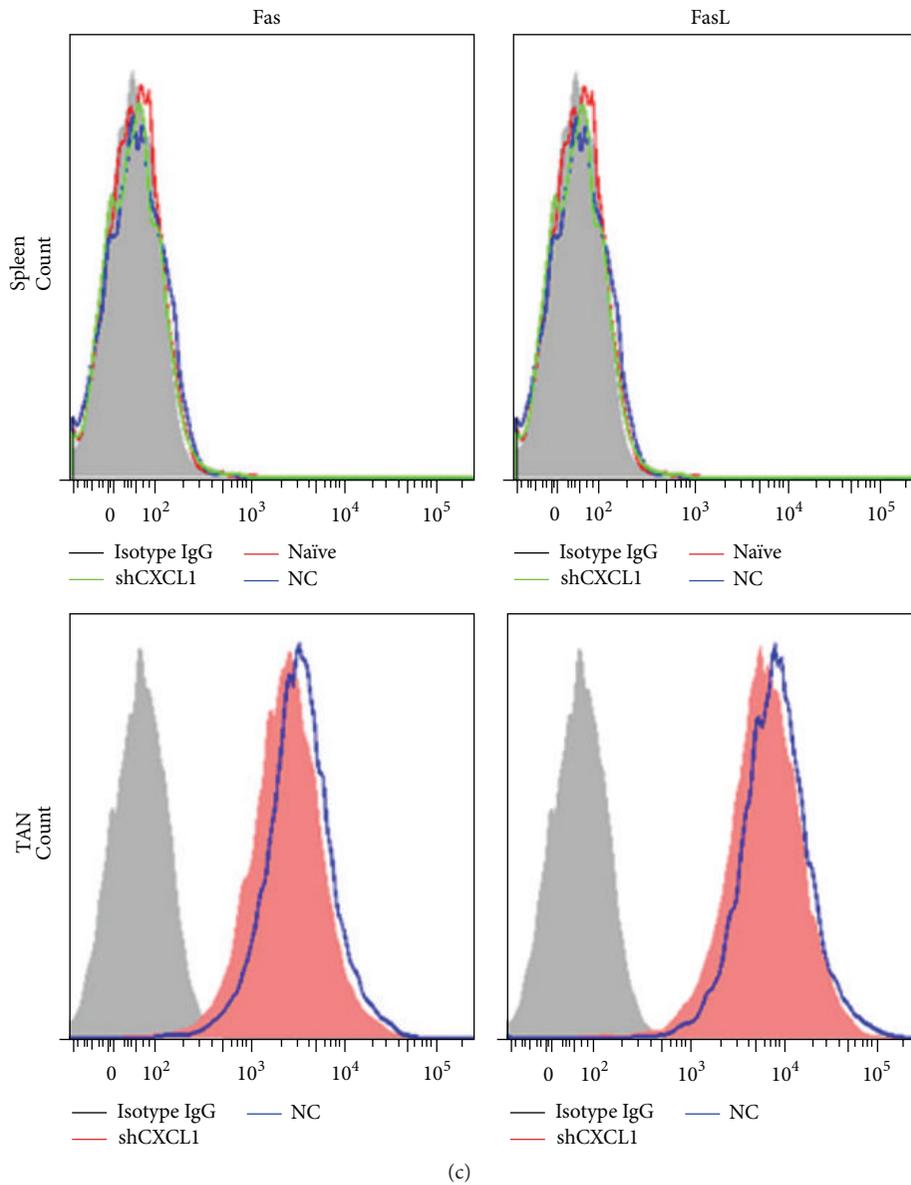
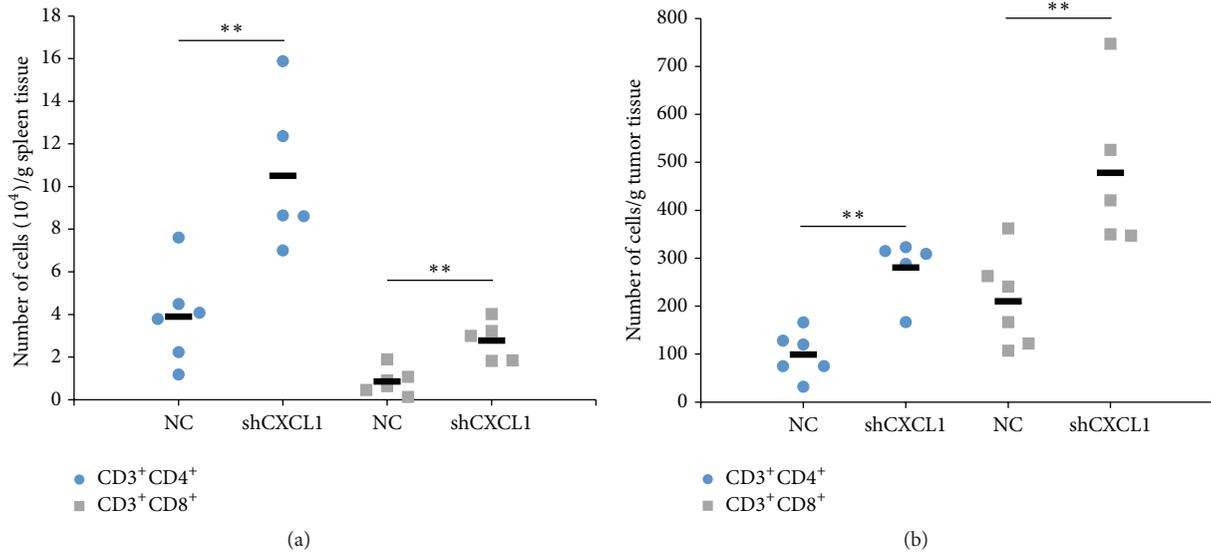
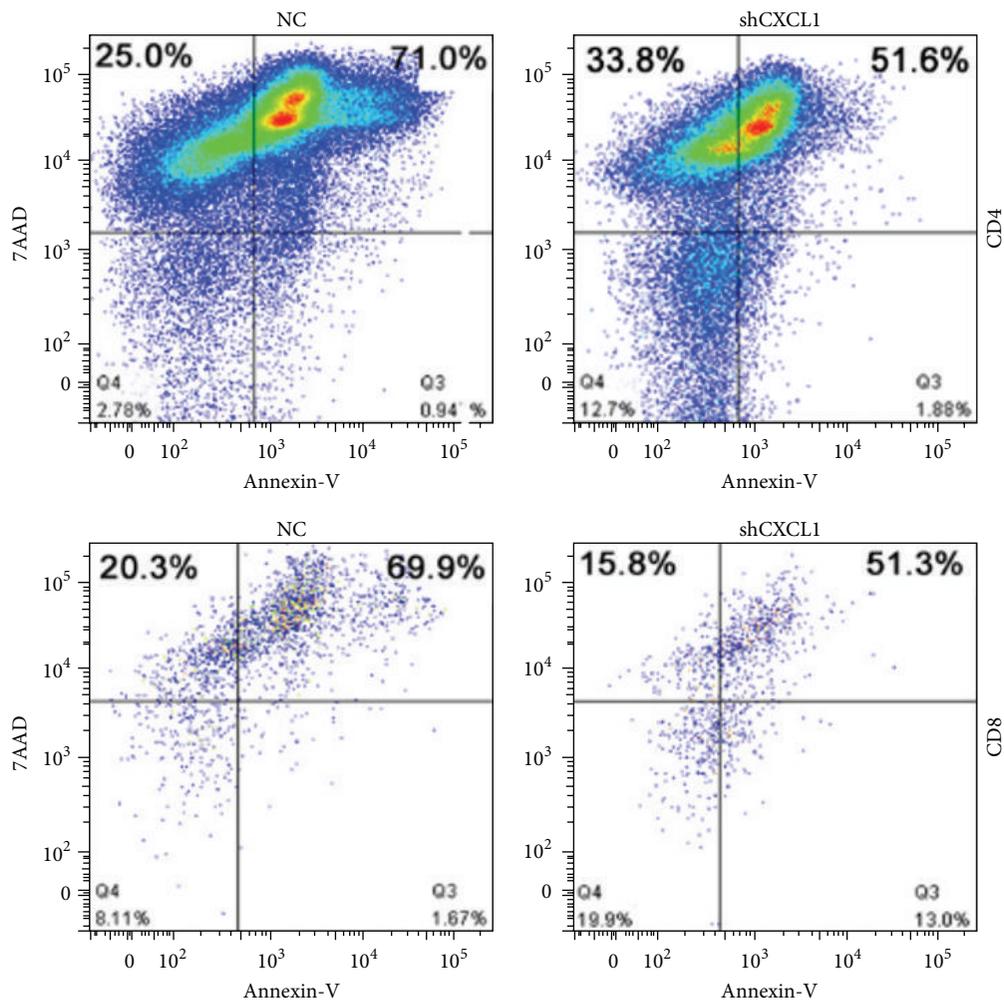
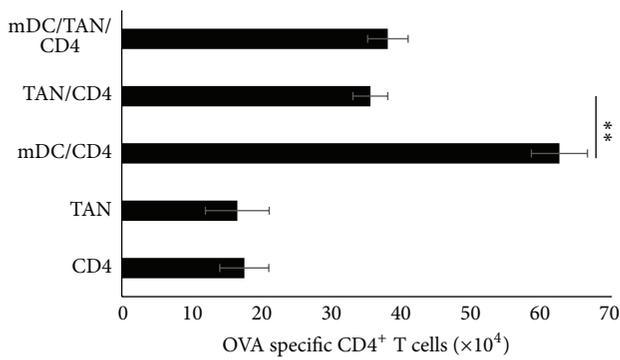


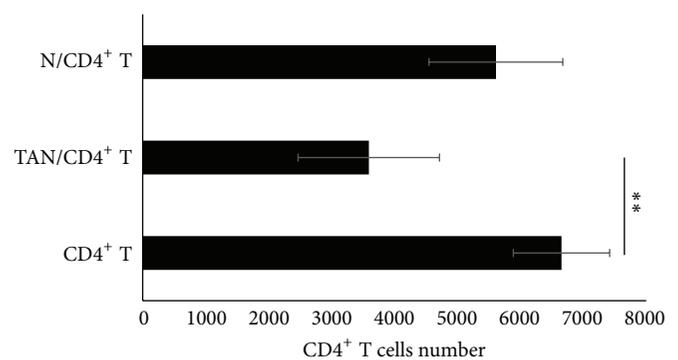
FIGURE 4: Continued.



(d)



(e)



(f)

FIGURE 4: Tumor-infiltrating neutrophils in tumor inhibit T cell proliferation. Number of CD3<sup>+</sup>CD4<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup> T cells in the spleen (a) and tumor tissues (b) at day 14 after tumor inoculation were analyzed by flow cytometric. (c) Fas/FasL expression on splenic CD11b<sup>+</sup>Ly6G<sup>+</sup> neutrophils or TANs from 3LL/NC bearing mice, 3LL/shCXCL1 bearing, or naive mice were analyzed by cytometric analysis. (d) CD4<sup>+</sup> T or CD8<sup>+</sup> T cells derived from tumor tissues were stained with 7AAD and Annexin-V, flow cytometric analysis of apoptotic/necrotic CD4<sup>+</sup> T, and CD8<sup>+</sup> T cells. (e) Splenic CD4<sup>+</sup> T plus conventional dendritic cells together with or without CD11b<sup>+</sup>Ly6G<sup>+</sup> neutrophils were cocultured for 3 days with 10 μg/mL OVA; CD4<sup>+</sup> T cells were counted by flow cytometry. (f) Splenic CD4<sup>+</sup> T and TANs were cocultured for 3 days with anti-CD3 and anti-CD28 and IL-2, CD4<sup>+</sup> T cells numbers were counted by flow cytometry. N (splenic neutrophils); TAN (tumor-associated neutrophils). \*\* *p* < 0.01. Data are presented as means ± SD.

demonstrated that High MPO<sup>+</sup> cell infiltration in colorectal cancer was an independent favorable prognostic factor [48] and MPO inhibitor reduced lung carcinoma growth during the early stages of tumor progression [49], suggesting that MPO<sup>+</sup> neutrophil involved promoting lung cancer growth. Additionally, in our unpublished studies we demonstrated that TANs expressed higher levels of PDL1, which exhibited immunosuppressive function via inhibiting CD4<sup>+</sup> T and CD8<sup>+</sup> T cell proliferation, which maybe contribute to TAN-mediated suppression of T cells proliferation. In conclusion, 3LL tumor-derived CXCL1 contributes to TANs infiltration in lung cancer which promotes tumor growth.

## Abbreviations

MPO: Myeloperoxidase  
 TANs: Tumor-associated neutrophils  
 MDSCs: Myeloid-derived suppressive cells  
 mDC: Mature dendritic cells  
 HCC: Hepatocellular carcinoma  
 IF: Immunofluorescence  
 IHC: Immunohistochemistry.

## Competing Interests

All the authors declare that there is no conflict of interests.

## Authors' Contributions

Ming Yuan and Ha Zhu contributed equally to this work.

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## Research Article

# Low-Dose Oxygen Enhances Macrophage-Derived Bacterial Clearance following Cigarette Smoke Exposure

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**Background.** Chronic obstructive pulmonary disease (COPD) is a common, smoking-related lung disease. Patients with COPD frequently suffer disease exacerbations induced by bacterial respiratory infections, suggestive of impaired innate immunity. Low-dose oxygen is a mainstay of therapy during COPD exacerbations; yet we understand little about whether oxygen can modulate the effects of cigarette smoke on lung immunity. **Methods.** Wild-type mice were exposed to cigarette smoke for 5 weeks, followed by intratracheal instillation of *Pseudomonas aeruginosa* (PAO1) and 21% or 35–40% oxygen. After two days, lungs were harvested for PAO1 CFUs, and bronchoalveolar fluid was sampled for inflammatory markers. In culture, macrophages were exposed to cigarette smoke and oxygen (40%) for 24 hours and then incubated with PAO1, followed by quantification of bacterial phagocytosis and inflammatory markers. **Results.** Mice exposed to 35–40% oxygen after cigarette smoke and PAO1 had improved survival and reduced lung CFUs and inflammation. Macrophages from these mice expressed less TNF- $\alpha$  and more scavenger receptors. In culture, macrophages exposed to cigarette smoke and oxygen also demonstrated decreased TNF- $\alpha$  secretion and enhanced phagocytosis of PAO1 bacteria. **Conclusions.** Our findings demonstrate a novel, protective role for low-dose oxygen following cigarette smoke and bacteria exposure that may be mediated by enhanced macrophage phagocytosis.

## 1. Background

Chronic obstructive pulmonary disease (COPD) is primarily a smoking-related lung disease and afflicts more than 200 million people worldwide. Pathologically, COPD is characterized by chronic bronchitis or emphysema. In chronic bronchitis, airway inflammation leads to increased mucus production and reduced mucociliary clearance, causing bronchoconstriction and airflow limitation. Emphysema is hallmarked by destruction of lung parenchyma. Patients with COPD frequently suffer disease exacerbations often induced by bacterial or viral respiratory infections, suggestive of impaired innate immunity. During disease exacerbation, supplemental oxygen is a mainstay of therapy for COPD patients. Although there is clear benefit to continuous low-level oxygen therapy in chronic, stable COPD disease [1, 2], excessive amounts of nontitrated oxygen may in fact be

harmful during COPD exacerbation [3, 4]. Therefore, how oxygen modulates the effects of cigarette smoke on lung immunity may be relevant for patients with COPD exacerbation and other smoke-induced lung diseases.

The immunomodulatory effects of moderate and high levels of oxygen exposure (FiO<sub>2</sub> 0.6–1.0) have been well described in experimental models. Mice exposed to four days of 95% oxygen had increased mortality with impaired macrophage phagocytosis in a *Klebsiella pneumoniae* model [5]. 60% oxygen exposure shortly following LPS-induced lung injury markedly exacerbated lung inflammation in part mediated by macrophage-induced recruitment of alveolar neutrophils [6]. Although higher levels of oxygen appear to impair lung immunity, there is limited data as to how lower levels of oxygen supplementation (FiO<sub>2</sub> 0.30–0.4) can modulate lung immunity, particularly with coexisting cigarette smoke exposure.

Macrophages are prominent resident cells of the alveolar space and are critical for regulation of immune responses in the lung [7–9]. With smoke-induced COPD, macrophage numbers can increase 5- to 10-fold in the lungs and alveolar space [10, 11] and can correlate with disease severity [12], although they do not conform to the classic M1/M2 dichotomy [13]. Alveolar macrophage dysfunction induced by cigarette smoke exposure leads to excess oxidative stress and may contribute to higher bacterial colonization and increased susceptibility to exacerbations. Alveolar macrophage phenotype and function can be modulated by exposure to varying levels of supplemental oxygen [5, 14].

As a model to help understanding oxygen effects on the lung immune response to bacteria subsequent to cigarette smoke exposure, we challenged mice or macrophages in isolation with subacute durations of cigarette smoke (CS) followed by exposure to low-dose oxygen and *Pseudomonas aeruginosa* bacteria. We chose a shorter cigarette smoke exposure to focus on the effects CS and oxygen-induced changes in inflammatory cell populations [15], similar to bronchitis, and without emphysema-like changes in lung architecture. We present here the results of our findings.

## 2. Methods

**2.1. Animal Use, Care, Smoke, Bacteria, and Oxygen Exposure.** C57Bl/6 mice were purchased (Jackson Labs, Bar Harbor, ME) and housed at the Johns Hopkins University Asthma and Allergy Center. Experiments were conducted under a protocol approved by the Johns Hopkins Animal Care and Use Committee. C57Bl/6 mice were exposed in the Johns Hopkins smoke exposure core. Mice were exposed to cigarette smoke 5 hours/day, 5 days/week for 5 weeks, by burning 3R4F reference cigarettes (2.45 mg nicotine/cigarette; Tobacco Research Institute, University of Kentucky) using a smoking machine (Model TE-10, Teague Enterprises).

For specified groups, the morning after a 5-week cigarette smoke (CS) exposure, we instilled *Pseudomonas aeruginosa* (PAOI, ATCC, Manassas, VA) ( $3 \times 10^6$  CFUs, in 50  $\mu$ L PBS) or vehicle control (PBS) via an intratracheal (i.t.) route through a 20-gauge endotracheal catheter as before [16]. Mice were anesthetized with intraperitoneal ketamine/acetylpromazine (100/2.5  $\mu$ g/g) prior to exposure of the trachea. Within 2 hours of instillation of i.t. PAOI or vehicle control, mice were exposed to 35–40% oxygen or 21% oxygen for up to five days. For oxygen exposure, mice were placed in customized and sealed cages with ad libitum food and water. 35–40% oxygen was achieved with a mixture of air and medical grade oxygen (Roberts Oxygen, Rockville, MD) at adjustable flow rates and constant pressure, with continuous measurements via an oxygen analyzer with a feedback loop to automatically adjust oxygen concentrations (model 65, Advanced Micro Instruments, Huntington Beach, CA). Oxygen exposure was uninterrupted except for 5 min every other day for cage cleaning.

**2.2. Animal Harvesting and Bronchoalveolar Lavage (BAL).** Mice were harvested after 5 weeks of cigarette smoke exposure (or air exposure as a control) and 3 days of 35–40% oxygen (or 21% oxygen as a control). In addition, following

5 weeks of CS exposure (or air exposure), designated mice were exposed to i.t. PAOI (or vehicle control) and 2 days of 35–40% oxygen (or 21% oxygen as control) until sacrifice and harvest of lungs for assessment of CFUs and inflammatory parameters or up to 5 days for assessment of mortality. Mice were anesthetized with intraperitoneal ketamine/acetylpromazine (150/13.5 mg/kg) prior to harvest and killed by exsanguination from the inferior vena cava. The lungs were perfused free of blood with 1 mL of phosphate-buffered saline (PBS). BAL was obtained by cannulating the trachea with a 20-gauge catheter. The right lung was lavaged with two aliquots of 0.7 mL calcium-free PBS. For quantitative measures of bacteria, whole lungs were homogenized without prior lavage, and the lysates were diluted in PBS and streaked on agar plates. After 24 hours at 37°C, colonies were counted.

**2.3. BAL Processing and Analysis.** BAL fluid was centrifuged (700  $\times$ g, 10 min at 4°C), and cell-free supernatants were stored at –80°C. The cell pellet was diluted in PBS, and the total cell number was counted with a hemocytometer after staining with trypan blue. Cell populations were determined by counting 300 cells/sample, and a percentage was calculated based on a minimum of three mice per group. Total protein was measured in the cell-free supernatant using the Lowry method [17].

**2.4. Cell Culture.** MH-S alveolar mouse macrophage cells were obtained from ATCC (Manassas, VA). Cells from passages 4–10 were maintained in Dulbecco's modified Eagle's media with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin/streptomycin mixture at 37°C in 5% CO<sub>2</sub>. Prior to experiments, cells were scraped from culture, collected, and centrifuged at 300 RCF for 5 minutes and then counted via trypan blue exclusion. MH-S cells were added in media to 0.4 nM-tissue culture PET-membrane inserts (Falcon) and placed in media and in six-well plates (Falcon). Cells were maintained at 37°C in 5% CO<sub>2</sub> between 2 and 24 hours to allow adherence to tissue culture inserts prior to experiments, which was verified by light microscopy. In experiments utilizing serum-free media, media was removed from inserts and wells.

**2.5. In Vitro Smoke, Oxygen, and Bacteria Exposure.** MH-S alveolar macrophage cells cultured on inserts were placed into a Vitrocell chamber (Waldkirch, Germany) for exposure to whole cigarette smoke (2 cigarettes, 7 min/cigarette) or room air sham for 2 exposures over 24 hours [15]. Cells were treated with 40% O<sub>2</sub>/5% CO<sub>2</sub> or 21% O<sub>2</sub>/5% CO<sub>2</sub> (control) between CS exposures. Cell-free supernatants were collected after the 24-hour CS exposure.

PAOI-GFP was obtained from ATCC. Bacteria was harvested from agar plates following 24-hour incubation at 37°C, inoculated in LB broth, and then incubated at 37°C and 200 RPM until in log phase of growth. Bacteria were then diluted to OD = 0.1 at 600 nm, approximating  $1 \times 10^8$  CFU/mL of live bacteria. Bacteria were washed in PBS and used immediately. MH-S cells were incubated with GFP-labeled *Pseudomonas aeruginosa* (PAOI-GFP) in phosphate-buffered

saline (PBS) for 3 hours, during which time exposure to 40% oxygen/5% CO<sub>2</sub> (or control, 21% O<sub>2</sub>/5% CO<sub>2</sub>) was continued.

**2.6. Macrophage Bacteria (PAOI) Phagocytosis.** After 3 hours of incubation, the media were then removed and plated to determine extracellular PAOI-GFP CFU counts or collected for quantification of cytokines. After removal of PAOI-GFP inoculum, the MH-S cells on inserts were washed in warm PBS, treated with gentamicin (30–60 minutes) to kill adherent bacteria, and then washed twice in warm PBS and scraped from the inserts into detergent solution (1% Triton-X) that was plated for intracellular CFU counts using serial dilution.

**2.7. Analysis of Cytokines.** Using cell-free BAL fluid from mice or supernatants from cell culture, cytokine analysis of TNF- $\alpha$  and IL-6 was performed by standard ELISA kits following the manufacturer's recommendations (R&D Systems, Minneapolis, MN). All samples were run in duplicate.

**2.8. Flow Cytometry (FACS).** For surface staining, primary lung cells or MH-S cells were incubated with Fc Block-2.4G2 (BD Pharmingen) Ab to block Fc $\gamma$  III/IIRs before staining with a specific Ab. The following antibodies were purchased from BD Pharmingen (San Diego, CA) and BioLegend (San Diego, CA): anti-Gr1-BV570, anti-CD11b-PETR, anti-CD86-BV421, anti-MMR-Ax647, anti-Dectin-1-Ax700, and anti-F4/80-allophycocyanin-Cy7, along with relevant isotype antibodies. The FITC/Ax488 channel was left open for PAOI-GFP. For intracellular staining of cytokines, cells were isolated and resuspended ( $0.5 \times 10^6$  cells/mL) in RPMI 1640/FCS/penicillin/streptomycin/Golgi Plug (unstimulated) or with additional leukocyte activation mixture (BD Biosciences, San Jose, CA; PMA + ionomycin + brefeldin A; 2 mL/mL, stimulated, to enhance intracellular cytokine signal) for 4 hrs. Live-dead discrimination was performed with Fixable UV-Excitable Blue Dead Cell Stain (Invitrogen). Cells were Fc blocked; surface stained for macrophage, neutrophil, and lymphocyte markers; and fixed/permeabilized (Cytofix/Cytoperm, BD Pharmingen, San Jose, CA) and intracellularly stained  $\times$  30 min for cytokines including anti-TNF- $\alpha$ -PerCP. Monocytes, alveolar macrophages, neutrophils, and lymphocytes were gated with characteristic forward scatter/side scatter using a FACSaria instrument, CellDiva for data acquisition (BD Biosciences, San Jose, CA), and FlowJo for analysis (Tree Star, San Carlos, CA).

**2.9. Statistical Analysis.** Analysis was performed using GraphPad Prism 6.0 (La Jolla, CA) software. Student's *t*-test was used for comparisons between two variables with significance determined using the Holm-Sidak method. Multiple comparisons were performed using ordinary one-way ANOVA with Bonferroni or Tukey's correction for multiple comparisons. Survival analysis was performed using Kaplan-Meier curve with Mantel-Wilcox test. *p* < 0.05 was used as a cut-off to determine statistical significance.

### 3. Results

**3.1. Low-Dose Oxygen after Subacute Cigarette Smoke Exposure Does Not Alter Lung Inflammation.** The *in vivo* model

consisted of wild-type C57Bl/6 mice exposure to 5 weeks of cigarette smoke via chamber as before [15]—controls were age-matched and exposed to room air also via chamber. We have shown that a subacute duration (4–6 weeks) of CS exposure was associated with increased alveolar epithelial permeability and increased accumulation of inflammatory cells in the alveolar space but did not induce changes in lung architecture [15]. In the current study, we observed a twofold increase in alveolar macrophages recovered by bronchoalveolar lavage (BAL) from cigarette-smoke-exposed mice compared to sham-exposed controls (Figure 1(a)). The addition of 3 days of continuous 35–40% oxygen exposure did not change the BAL macrophage count.

**3.2. Low-Dose Supplemental Oxygen Promotes Clearance of Bacteria and Reduces Lung Injury in CS-Exposed Mice.** CS- or air-exposed mice were exposed to *Pseudomonas aeruginosa* (i.t. PAOI,  $3 \times 10^6$  CFUs) followed by low-dose oxygen (35–40% O<sub>2</sub>) or control (21% O<sub>2</sub>, room air) for 2 days. Compared to air-exposed controls, smoke-exposed mice had increased PAOI CFUs recovered from the lung, as others have also shown [18]. However, exposure to 35–40% oxygen (CS + O<sub>2</sub>) resulted in a significant decrease in PAOI CFUs recovered from the whole lung compared to smoke-exposed mice exposed to room air (CS + room air) (Figure 1(b)). Low-dose supplemental oxygen also appeared to reduce bacteremia in CS-exposed mice as the CS + O<sub>2</sub> mice had no evidence of bacteremia compared to ~500 PAOI CFUs recovered from the blood of mice exposed to room air. Furthermore, between groups of CS-exposed mice, mice that received 35–40% oxygen for up to 5 days after PAOI exposure had significantly reduced mortality compared to control (room air) exposure for the same period (Figure 1(c), *p* = 0.0357 by Mantel-Cox). There was no mortality after i.t. PAOI in either non-CS-exposed group of mice (not shown).

To determine whether the benefits on bacterial clearance translated to other relevant endpoints, we also quantified lung injury parameters. At day 2 after i.t. PAOI, BAL protein was significantly increased in CS-exposed mice treated with 21% oxygen (CS + room air) compared to non-CS-exposed mice (Figure 1(d)). However, exposure to supplemental oxygen after i.t. PAOI (CS + O<sub>2</sub>) reduced BAL protein to levels observed in non-CS-exposed mice. In contrast, the BAL total cell count at day 2 was not different between groups irrespective of CS or oxygen exposure (Figure 1(e)). Therefore, these data suggest that exposing mice to 35–40% oxygen following cigarette smoke and PAOI exposure markedly improves bacterial clearance to improve survival, with some associated changes in lung injury parameters.

**3.3. Low-Dose Oxygen Modulates Inflammation and Lung Macrophages in CS- and Bacteria-Exposed Mice.** With the significant mortality benefit in O<sub>2</sub>-exposed mice following CS and i.t. PAOI exposure, we measured BAL cytokine and cellular profiles to assess for other phenotypic differences (Figure 2). The addition of 35–40% O<sub>2</sub> to CS and PAOI-exposed mice did not change BAL IL-6 levels but appeared to reduce BAL TNF- $\alpha$  at day 2 after i.t. PAOI (Figure 2(a)). In addition, 35–40% oxygen exposure did not change the

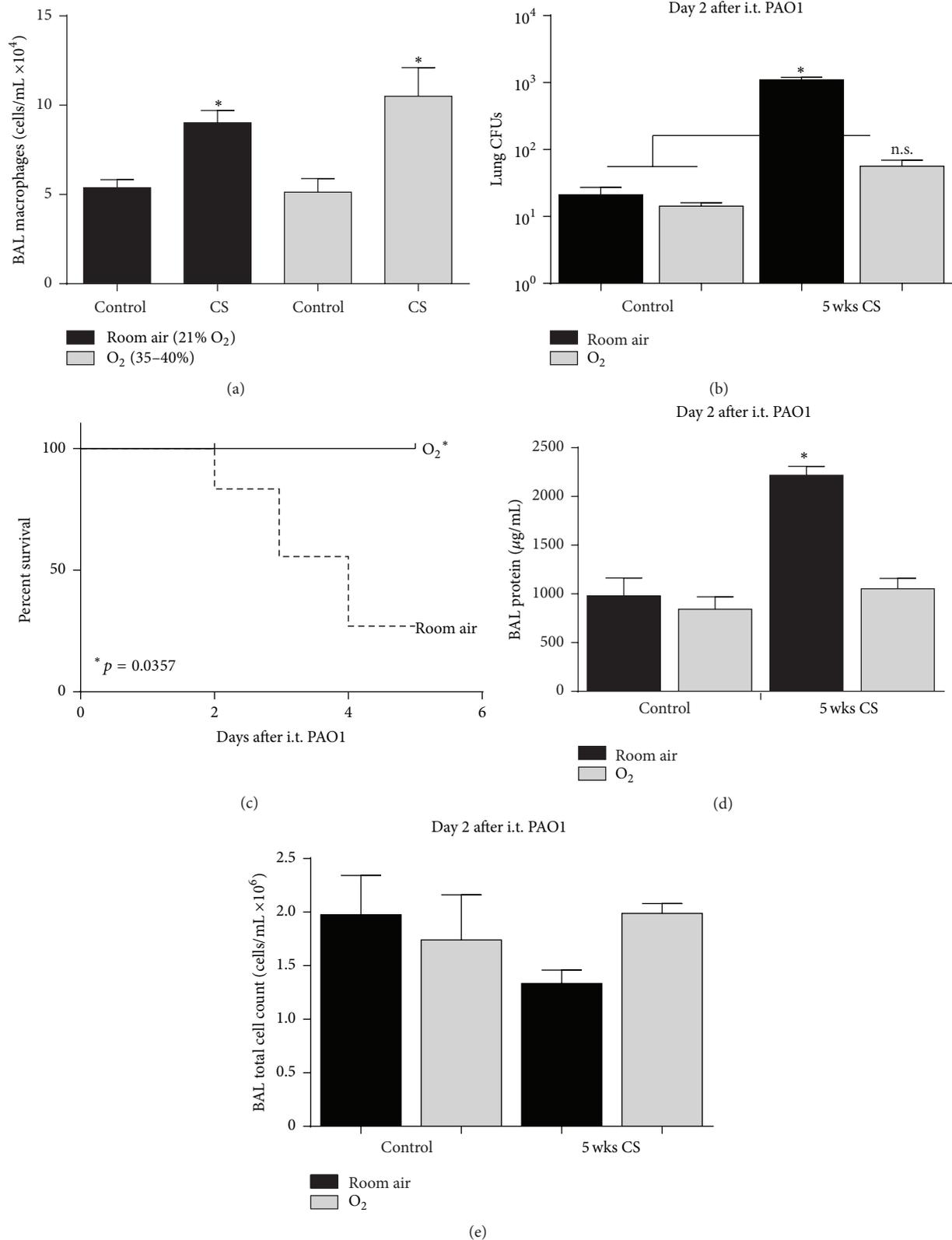


FIGURE 1: Low-dose oxygen is beneficial in CS- and PAO1-exposed mice. (a) Macrophages recovered by BAL from mice exposed to 5 weeks of cigarette smoke followed by 35–40% oxygen or room air for an additional 3 days ( $n = 4-5$ ,  $*p < 0.05$  by one-way ANOVA). (b–d) Following 5-week CS or room air exposure, mice were exposed to PAO1 bacteria by intratracheal (i.t.) injection and either low-dose oxygen (35–40%) or room air (control), followed by up to 5 days of oxygen or room air exposure. (b) At day 2 after PAO1 exposure, mice were harvested and lung CFUs were quantified ( $n = 2-3$ ,  $*p < 0.001$  against all other groups by one-way ANOVA, and n.s. compared to both control groups). (c) Among CS-exposed mice, Kaplan-Meier survival curve following i.t. PAO1 exposure ( $n = 6-7$  mice,  $p = 0.0357$  by log-rank Mantel-Cox). (d) Following bronchoalveolar lavage (BAL) on day 2 after PAO1 exposure, total protein was quantified ( $n = 2-4$ ,  $*p < 0.01$  against all other groups by one-way ANOVA). (e) Following day 2 BAL, total cell count was quantified ( $n = 2-4$ ).

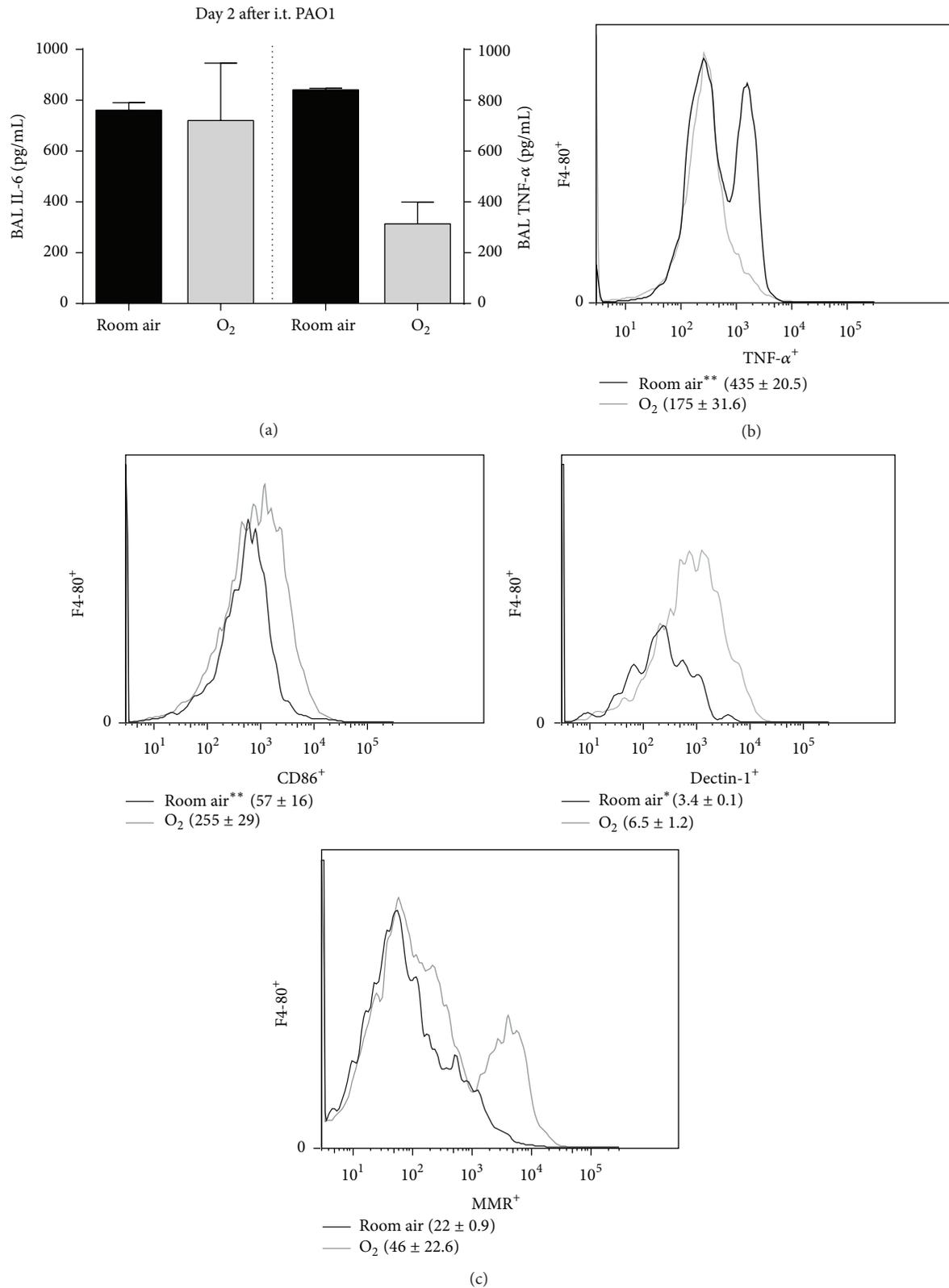


FIGURE 2: Oxygen alters the inflammatory profile and alveolar macrophage phenotype in CS and i.t. PAO1-exposed mice. (a) BAL cytokines were quantified following 2 days of PAO1 exposure in CS-exposed mice ( $n = 2-4$ ). (b) Representative histogram of the mean fluorescent intensity (MFI) of expression of intracellular TNF- $\alpha$  using FACS among BAL F4-80<sup>+</sup> macrophages (\*\* $p < 0.01$  by unpaired  $t$ -test; MFI  $\pm$  SD,  $n = 3$ ). (c) Representative histogram of the MFI of the surface markers CD86, Dectin-1, and MMR on BAL F4-80<sup>+</sup> macrophages (\* $p < 0.05$ , \*\* $p < 0.01$  by unpaired  $t$ -test; MFI  $\pm$  SD,  $n = 3$ ).

percentage of alveolar neutrophils (Gr1<sup>+</sup>) among CS- and PAOI-exposed mice at day 2 after i.t. PAOI (not shown).

To further investigate how 35–40% oxygen exposure may induce macrophage-specific effects in CS- and bacteria-exposed mice, we used FACS analysis to measure TNF- $\alpha$  expression in macrophages as well as expression of macrophage surface receptors. After gating on a similar percentage and number of macrophages (F4-80<sup>+</sup>) between groups, mice exposed to low-dose oxygen had significantly decreased expression of TNF- $\alpha$  by FACS compared to room air exposure (Figure 2(b)). CD86, a proinflammatory M1 macrophage marker involved in cellular cosignaling [19], was significantly increased on macrophages derived from oxygen-exposed mice following CS and i.t. PAOI exposure (Figure 2(c)). Furthermore, low-dose oxygen following CS and i.t. PAOI exposure significantly increased expression of Dectin-1 and trended towards increased expression of mannose receptor, both M2, anti-inflammatory markers involved in phagocytosis. Collectively, these data suggest that low-dose oxygen modulates proinflammatory cytokine production and upregulates expression of macrophage receptors that may be important for bacterial clearance.

**3.4. 40% Oxygen Exposure Enhances Bacterial Clearance by CS-Exposed Macrophages in Culture.** To further understand macrophage-specific effects induced by oxygen exposure, we adapted our *in vivo* model for cell culture. After 24 hours of exposure of MH-S alveolar macrophages to CS (or control) and 40% O<sub>2</sub> (or 21% O<sub>2</sub>), IL-6 and TNF- $\alpha$  cytokines were quantified in the cell-free media, and macrophage marker expression was evaluated by FACS. CS exposure significantly increased IL-6 in the cell-free media compared to control (air) exposure (Figure 3(a)) but did not induce a difference in TNF- $\alpha$  at 24 hours, consistent with prior work [20]. The addition of 40% oxygen did not significantly modify IL-6 or TNF- $\alpha$  secretion. CS exposure significantly increased CD86 expression compared to air (control) (Figure 3(b)), but 40% oxygen did not further augment CD86 expression. In addition, CS also appeared to increase MMR expression on MH-S cells.

Following 24 hours of CS (or control) exposure, MH-S cells were incubated with *P. aeruginosa* (PAOI) with concurrent exposure to 40% oxygen (or 21% oxygen). After 3 hours, we quantified intracellular PAOI CFUs. Exposure to 40% oxygen after CS and PAOI resulted in a significant, greater than twofold increase in intracellular PAOI by CFU counts when compared to air or CS-exposed groups that did not receive 40% oxygen (Figure 3(c)). Because PAOI was GFP-tagged, we were also able to quantify the association of bacteria with macrophages using FACS (Figure 3(d)). Similar to the pattern observed with intracellular CFU counts, the addition of 40% oxygen exposure appeared to increase the GFP signal associated with MH-S macrophages both by mean fluorescence intensity (MFI) among GFP-positive macrophages, as well as the phagocytosis index (GFP MFI  $\times$  % PAOI-GFP<sup>+</sup>) [5].

As a potential confounding factor, oxygen levels have been shown to influence the growth of *P. aeruginosa* [21]. To address this possibility, we quantified CFUs among extracellular bacteria not adherent to or phagocytosed by

macrophages. Among all 4 exposure groups, we observed a strong inverse correlation ( $R^2 = 0.085$ ) of extracellular CFUs with intracellular CFUs from the same well of MH-S cells (Figure 3(e)), suggesting that differences in intracellular bacteria were not attributable to oxygen-induced differences in bacterial growth in the media. Collectively, these data demonstrate that isolated macrophages exposed to cigarette smoke can augment bacterial clearance when treated with low-dose supplemental oxygen.

**3.5. 40% Oxygen Modulates the Inflammatory Profile of CS-Exposed Macrophages in Culture.** We also examined whether oxygen-enhanced bacterial phagocytosis by MH-S cells was associated with changes in its inflammatory profile by measuring selected proinflammatory cytokines and macrophage M1/M2 marker expression following PAOI exposure. Although IL-6 was not different between groups (not shown), the addition of 40% oxygen to either CS or control-exposed MH-S cells significantly reduced TNF- $\alpha$  levels (Figure 4(a)). Expression of the M1 marker CD86 was increased on MH-S following CS and PAOI exposure; the addition of 40% oxygen did not further augment CD86 expression (Figure 4(b)). Among the scavenger, M2 receptors, Dectin-1 showed a possible oxygen-mediated effect following PAOI exposure, as the fold change of MFI expression was highest on MH-S cells exposed to CS and 40% oxygen; in contrast, MMR was not different between the groups. Using FACS to assess PAOI-GFP association with MH-S cells, we observed a strong correlation between Dectin-1 MFI and PAOI-GFP MFI ( $R^2 = 0.7905$ ) (Figure 4(c)). This data suggests that cells expressing higher levels of Dectin-1 also had higher bacterial association and also supports that 40% oxygen was an important modifier of both markers. In contrast, the correlation between CD86 MFI and PAOI-GFP MFI or MMR MFI and PAOI-GFP MFI was not nearly as strong.

**3.6. 40% Oxygen Regulates Expression of Other Macrophage Phagocytic Receptors.** We also measured oxygen-induced effects on other macrophage surface receptors including MARCO and CD200R. CS-exposed MH-S cells demonstrated increased expression of MARCO; 40% oxygen did not further regulate MARCO expression (Figure 5(a)). Following subsequent incubation with PAOI-GFP, MARCO expression was not statistically different between groups, although trended towards an increase on macrophages exposed to CS + 40% oxygen. In contrast, the combination of cigarette smoke and 40% oxygen exposure significantly increased CD200R expression (Figure 5(b)) compared to control and 21% oxygen. Following incubation with PAOI-GFP, however, macrophages exposed to CS + 40% oxygen did not significantly increase CD200 expression but did trend in that direction. Collectively, this data suggests that low-dose oxygen can regulate expression of multiple scavenger or inhibitory receptors that may be important for clearance of bacteria and other immune-mediated functions.

## 4. Discussion

In this study, we sought to understand how oxygen therapy may modulate cigarette smoke-induced immune dysfunction

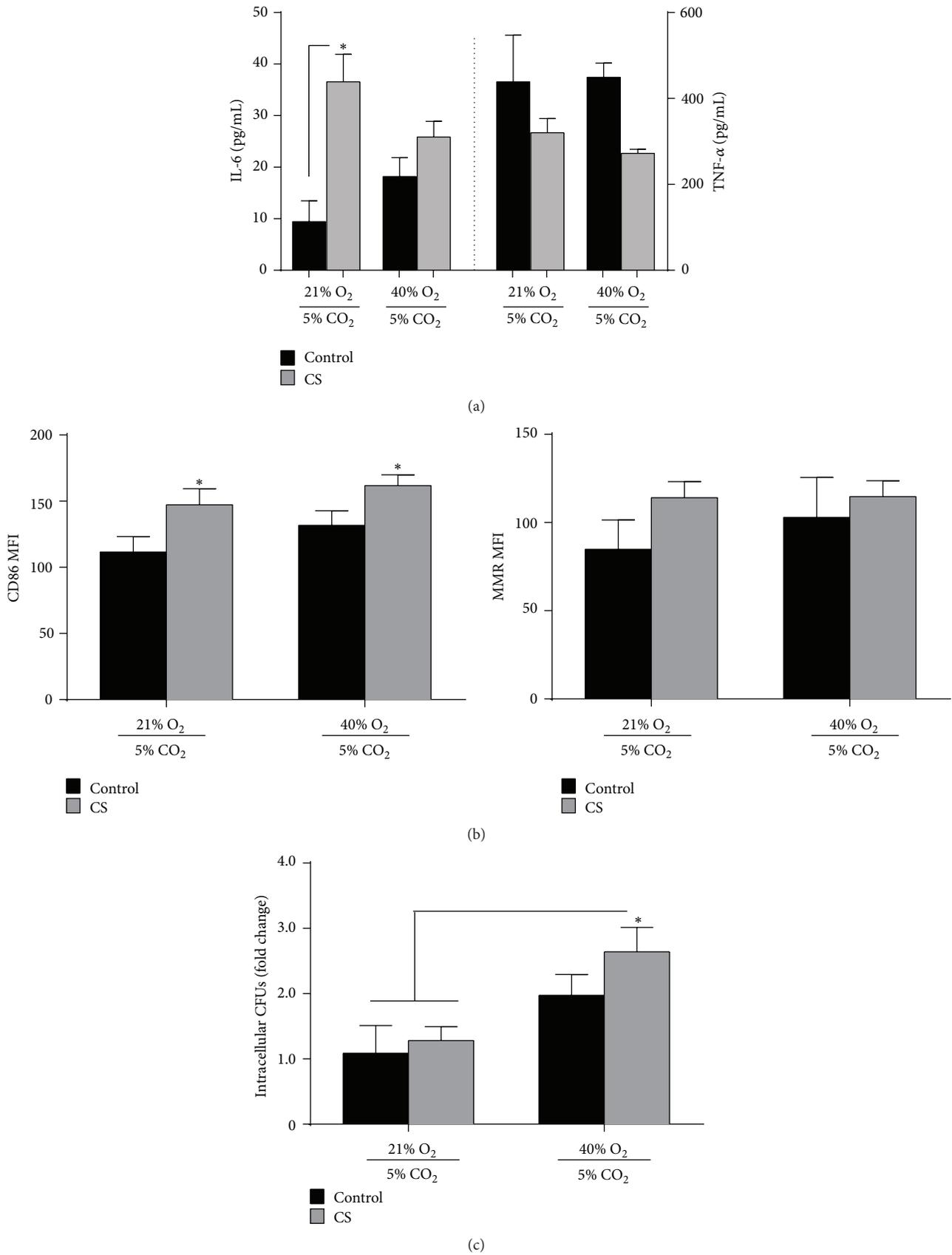


FIGURE 3: Continued.



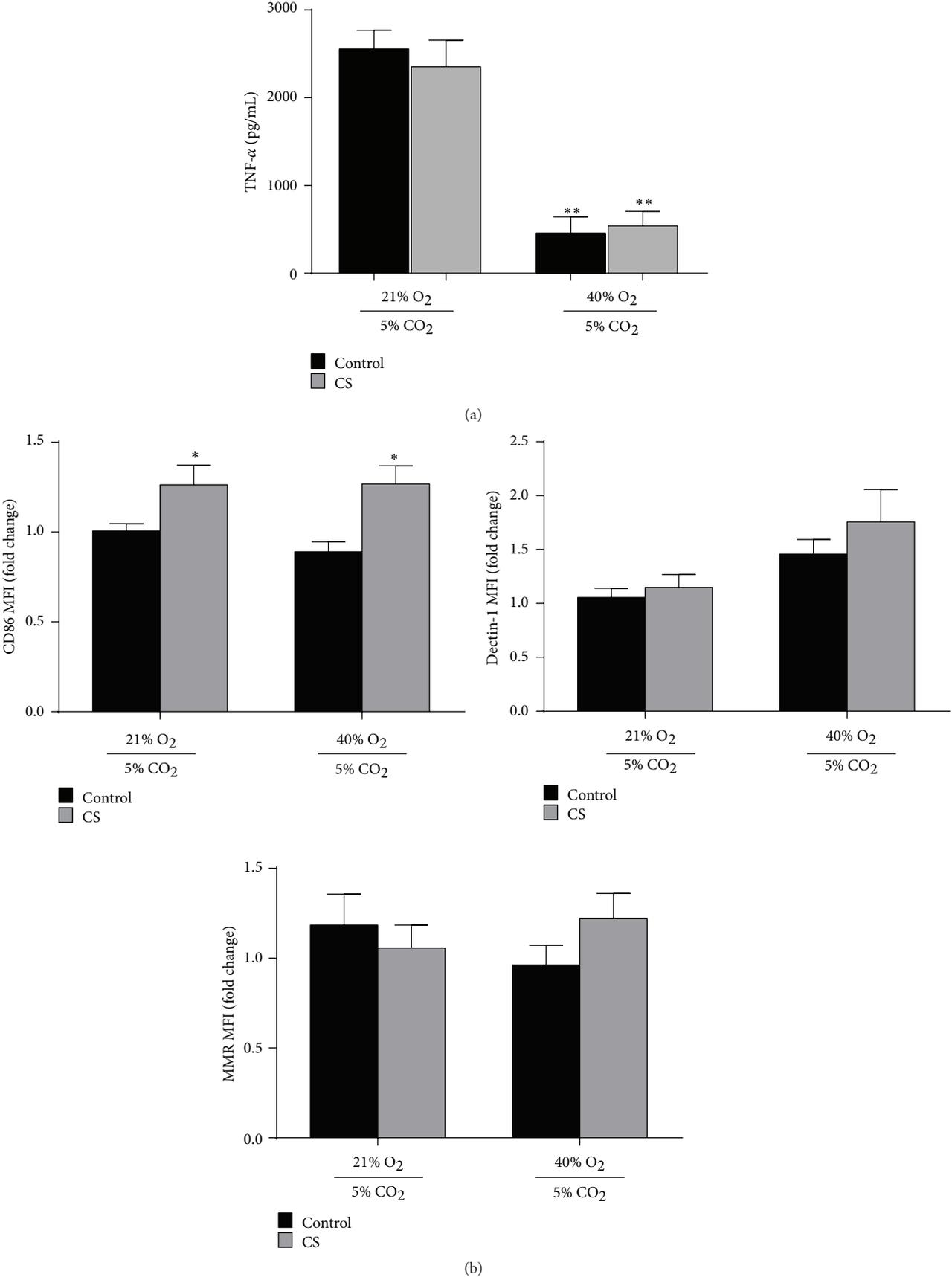


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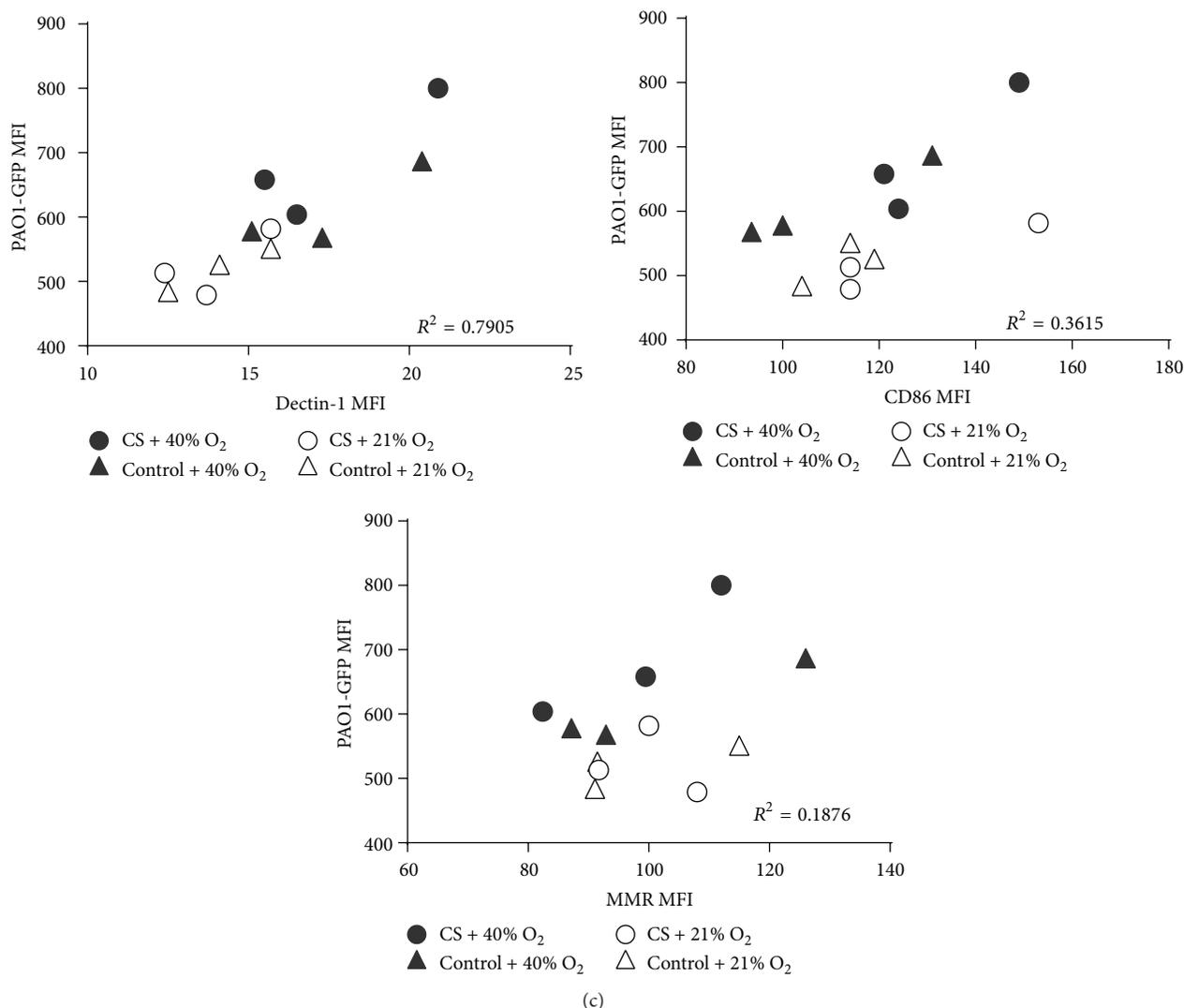


FIGURE 4: Oxygen modulation of macrophage phenotype may support PAOI phagocytosis. (a) Following *in vitro* CS, oxygen, and PAOI exposure, we measured TNF- $\alpha$  in the supernatant ( $n = 3$ , mean  $\pm$  SEM, \*\* $p < 0.01$  by one-way ANOVA compared to both 21% oxygen-exposed groups). (b) Following *in vitro* CS, oxygen, and PAOI-GFP exposure, we measured the MFI of cell surface expression of macrophage markers CD86, Dectin-1, and MMR using flow cytometry and expressed values normalized to control across trials (mean  $\pm$  SEM,  $n = 6$ , \* $p < 0.05$  compared to control + 40% O<sub>2</sub> group by one-way ANOVA with Bonferroni correction). (c) Using FACS, we demonstrate scatter plots of macrophage GFP MFI expression (to denote PAOI macrophage binding or engulfment) with macrophage MFI expression for Dectin-1 ( $R^2 = 0.7905$ ), CD86 ( $R^2 = 0.3615$ ), and MMR ( $R^2 = 0.1876$ ) surface markers.

with emphysema. If similar benefits of low-dose oxygen on promoting bacterial clearance were found in humans with COPD or other smoking-related lung diseases, these findings may provide some basis on the benefit of oxygen therapy for patients with smoking-related lung disease during pathogen-induced disease exacerbation [1, 2]. In that context, low-dose oxygen therapy may also limit disease progression by enhancing macrophage phagocytosis and thereby limiting the severity of bacteria-induced disease exacerbations [23].

There are limited studies analyzing the immunomodulatory effects of supplemental oxygen. Most prior studies demonstrate a detrimental effect of high oxygen levels (60–100%) on lung immunity. Our findings somewhat contrast the work of Baleeiro and colleagues who demonstrated a

detrimental effect of sublethal oxygen exposure on host defense against gram-negative pneumonia [5]. However, important differences in study design include the level of oxygen exposure (95% versus 40%) and the use of cigarette smoke. Their work identified a reduction in macrophage toll-like receptor 4 (TLR4) expression following 95% oxygen exposure resulting in impaired recognition of gram-negative bacteria. However, in our study, with the addition of 40% oxygen following CS exposure, we did not observe differences in macrophage surface TLR4 expression by flow cytometry (unpublished observations). We have also demonstrated that moderate levels of oxygen exposure (60%) ~12 hours after LPS-induced lung inflammation were sufficient to significantly exacerbate lung damage [6]. In one of the few studies

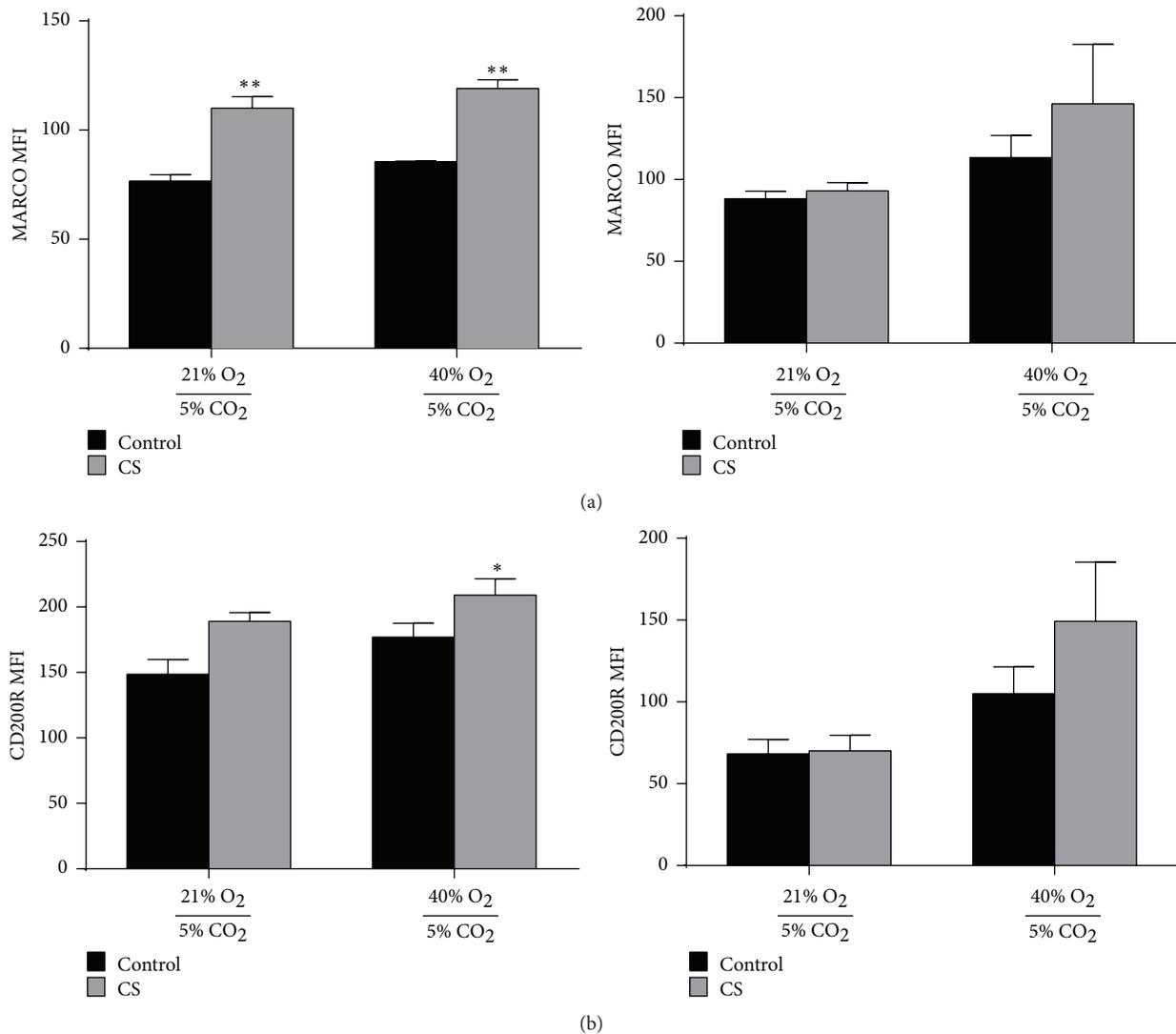


FIGURE 5: Oxygen regulates additional phagocytic receptors on CS-exposed macrophages. (a) We quantified MFI of MARCO expression on MH-S macrophages following CS and oxygen exposure prior to (left) and after (right) incubation with PAO1 bacteria (mean  $\pm$  SEM,  $n = 3$ ,  $**p < 0.01$  compared to both control + 21% O<sub>2</sub> and control + 40% O<sub>2</sub> groups by one-way ANOVA with Bonferroni correction). (b) We also quantified CD200R expression by MFI on MH-S macrophages prior to (left) and after (right) incubation with PAO1 bacteria (mean  $\pm$  SEM,  $n = 3$ ,  $*p < 0.05$  compared to control + 21% O<sub>2</sub> group by one-way ANOVA with Bonferroni correction).

of host defense at lower levels of supplemental oxygen, Knighton and colleagues showed that 45% oxygen decreased tissue necrosis and increased bacterial clearance compared to 12% oxygen following skin infection with *E. coli* in Guinea pigs [24]. Therefore, in addition to the impact of cigarette smoke, these studies also suggest that oxygen-induced effects on lung immunity may be dependent on the level of oxygen exposure.

We noted marked changes in macrophage cell surface phagocytic and scavenger receptor expression in response to cigarette smoke and 40% oxygen. Interestingly, Dectin-1 expression was increased following low-dose oxygen exposure in CS-exposed macrophages, and this increase strongly correlated with increased PAO1 uptake by macrophages.

Dectin-1 is a type II transmembrane receptor involved in  $\beta$ -glucan-derived fungal pathogen immune responses [25, 26]. Macrophage Dectin-1 expression has been shown to be regulated by leukotriene B<sub>4</sub> as a part of the GM-CSF/PU.1 axis [27], and leukotriene B<sub>4</sub> levels were elevated in human BAL fluid following exposure to 50% oxygen [28]. Dectin-1 is critical for clearance of fungal pathogens via recognition of beta-glucans [26, 29, 30]. However, beta-glucans are present in the cell walls of other nonfungal pathogens including *P. aeruginosa* [31, 32] and *H. influenzae*; for the latter, beta-glucan recognition by epithelial Dectin-1 was critical to generate an inflammatory response [33, 34]. With the strong correlation between macrophage Dectin-1 expression and PAO1 phagocytosis among MH-S macrophages in culture,

oxygen-induced upregulation of Dectin-1 may be contributory towards clearance of PAOI via beta-glucan recognition and binding.

We suggest that oxygen improves bacterial phagocytosis through upregulation of cell surface phagocytic and scavenger receptors. However, there are other possible mechanisms for the protective effects of oxygen. One consideration is that room-air-exposed mice were more hypoxic following bacteria exposure and supplemental oxygen may have protected the mice from hypoxia-induced complications. We did not assess for hypoxia *in vivo*, but we did demonstrate a significant increase in macrophage phagocytosis of bacteria with low-dose oxygen exposure in our cell culture system where hypoxia was not a factor. Another potential mechanism by which low-dose supplemental oxygen may improve bacterial phagocytosis is through enhanced production of reactive oxygen species (ROS). NADPH oxidase, a key enzyme in macrophages for generation of superoxide that requires molecular oxygen, modulated bacterial overgrowth [35]. However, unregulated ROS can also be detrimental to bacterial control, as a loss of macrophage-generated extracellular superoxide dismutase (EC-SOD), an antioxidant enzyme, was found to impair phagocytosis of *E. coli* [36]. Interestingly, hyperoxia (100% oxygen) in EC-SOD knockout mice leads to increased lung edema and diminished survival [37], a further evidence that unopposed ROS may augment lung damage. Overall, our findings suggest that oxygen-induced modulation of macrophage function is complex and likely associated with both the underlying lung substrate and the level of supplemental oxygen that is administered.

Our study also highlights the complex regulation of cell surface signaling receptors that is influenced by supplemental oxygen. To our knowledge, this is the first report of macrophage CD200R expression in response to oxygen and cigarette smoke exposure. CD200R demonstrates an inhibitory effect on inflammatory signaling when engaged by the CD200 ligand that is expressed by the respiratory epithelium and other immunomodulating cells (e.g., T cells) [7]. Prior studies have shown decreased expression of CD200R in human monocytes after treatment with diesel emission particles [38]. In contrast, we showed that the combination of cigarette smoke and 40% oxygen exposure increased alveolar macrophage CD200R expression. CD200R may protect against excess CS- and bacteria-induced damage, as it did in a murine influenza model [8]. In addition, due to the important immunomodulatory effects of macrophage CD200R tethering to epithelial CD200, we would anticipate a synergistic effect in terms of limiting inflammation in a macrophage-epithelial coculture system.

Our study has a few limitations. One, because we did not directly measure bacterial killing by macrophages, it is conceivable that low-dose oxygen exposure did not enhance phagocytosis, but instead impaired bacterial killing. If true, we would not anticipate a benefit of low-dose oxygen on murine mortality and lung inflammation. Two, we did not evaluate for an oxygen-mediated effect on neutrophil clearance of bacteria. Although neutrophil numbers in the alveolar space did not appear to be influenced by oxygen

following CS and PAOI exposure, we did not directly assess neutrophil function. Macrophage CD86 and Dectin-1, both modulated by CS and oxygen exposure in our studies, can promote neutrophil costimulation [39] and pathogen clearance [40]. However, since low-dose oxygen-enhanced macrophage phagocytosis of bacteria in isolated macrophages in culture, any oxygen-mediated effects on neutrophils would likely enhance, not diminish, our findings. Three, although the *in vitro* model for CS delivery induced IL-6 secretion by alveolar macrophages, we did not observe a reduction in PAOI phagocytosis amongst CS-exposed MH-S cells as compared to control exposure. However, prior study of cell culture systems investigating bacteria phagocytosis have primarily used cigarette smoke extract (CSE) and not direct cigarette smoke exposure [20, 41, 42]; thus little is known about what duration and intensity of direct CS exposure to macrophages are required to induce a change in phagocytosis. Four, because we have not proven that upregulation of one or several scavenger receptors is critical for low-dose oxygen to enhance macrophage phagocytosis, it is possible that receptor upregulation is correlative and not causal for oxygen-enhanced phagocytosis. Given the concomitant use of diverse pattern recognition receptors including Dectin-1, MMR, and the TLRs by immune cells, we would anticipate synergy between these receptors towards antimicrobial immunity [43]. Our results are similar to that of Hodge and colleagues who demonstrated a clear benefit of azithromycin exposure on human alveolar macrophage phagocytosis with a correlative increase in MMR expression, yet specific cellular mechanisms were not ascertained [44].

## 5. Conclusion

Our findings demonstrate a novel, protective role for low-dose oxygen in cigarette smoke and bacteria-exposed mice that appears to be mediated by enhanced macrophage phagocytosis of bacteria. Upregulation of scavenger and other pattern recognition receptors also denotes a unique cellular phenotype with coinduction of M1 and M2 macrophage markers. The individual role of these receptors is not yet clear, but our work has identified additional potential therapeutic targets to support enhanced bacterial clearance and decreased inflammation seen in response to 35–40% oxygen treatment in the lungs of cigarette-smoke-exposed mice.

## Competing Interests

The authors declare that they have no competing interests.

## Authors' Contributions

William G. Bain and Neil R. Aggarwal conceived and designed experiments; William G. Bain, Ashutosh Tripathi, Pooja Mandke, Jonathan H. Gans, and Neil R. Aggarwal performed experiments and analysis; and William G. Bain, Venkataramana K. Sidhaye, Franco R. D'Alessio, and Neil R. Aggarwal wrote the paper and provided creative input.

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## Clinical Study

# New Alternatives for Autoimmune Disease Treatments: Physicochemical and Clinical Comparability of Biosimilar Etanercept

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Etanercept is a recombinant fusion protein approved for the treatment of TNF- $\alpha$  mediated diseases such as rheumatoid arthritis (RA), psoriasis, psoriatic arthritis, and ankylosing spondylitis. Herein, we present an evaluation of the physicochemical and biological properties of a biosimilar etanercept and its reference product followed by a clinical study in patients diagnosed with RA intended to demonstrate comparability of their immunomodulatory activity. Identity analyses showed a total correspondence of the primary and higher-order structure between the two products. In regard to intrinsic heterogeneity, both products showed to be highly heterogeneous; however the biosimilar etanercept exhibited similar charge and glycan heterogeneity intervals compared to the reference product. Apoptosis inhibition assay also showed that, despite the high degree of heterogeneity exhibited by both products, no significant differences exist in their *in vitro* activity. Finally, the clinical assessment conducted in RA-diagnosed patients did not show significant differences in the evaluated pharmacodynamic markers of both products. Collectively, the results from the comparability exercise provide convincing evidence that the evaluated biosimilar etanercept can be considered an effective alternative for the treatment of RA.

## 1. Introduction

Autoimmune disorders are the consequence of the loss of ability of the immune system to differentiate between self- and non-self-antigens. Their incidence in the worldwide population is around 5% [1, 2]; these disorders are chronic and degenerative, being a major cause of disability resulting in an impact in the quality of life of the patients.

Dysregulation of several inflammatory pathways might be related to the pathogenesis of several autoimmune disorders, specifically immune-mediated inflammatory diseases

(IMID). Although IMID occur in different organs or tissues, they seem to have in common those pathways where the tumor necrosis factor (TNF) is involved. TNF has been associated with rheumatoid arthritis (RA), psoriasis, psoriatic arthritis, and ankylosing spondylitis.

Nonresponding patients treated with nonsteroidal anti-inflammatory agents (NSAIDs), steroids, and common disease-modifying antirheumatic drugs (DMARDs) are prescribed with a newer class of DMARDs [3]. Recently, the development of novel DMARDs has been focused on specific

TNF antagonists that block the interaction between TNF and its receptors. These biological agents include adalimumab, infliximab, certolizumab, golimumab, and etanercept, which were demonstrated to be more effective than traditional treatments in reducing the symptoms and preventing the progression of the disease [4].

Etanercept, in combination with methotrexate, has proved to be a successful treatment for RA [5]. Unlike monoclonal antibodies-TNF antagonists, etanercept is a recombinant dimeric fusion protein that contains two identical chains of the recombinant human TNF-receptor p75 monomer fused with a Fc domain of a human IgG1. This therapeutic protein was approved in 1998 by the Food and Drug Administration (FDA) as the first biologic response modifier (BRM) for the treatment of RA. It has also been prescribed for the treatment of other TNF- $\alpha$  mediated diseases [6]. The patent expiration date of the originator (in 2015 in Europe and 2028 in the US) has led to the development of etanercept's biosimilars in different countries. The advent of biosimilars will increase the health coverage, while improving the quality of life of patients that are unable to afford the cost of BMR therapies, especially in developing countries.

In order to assess the immunomodulatory activity comparability of biosimilar etanercept (Infinitam<sup>®</sup>) with respect to the reference product, we performed a study that included physicochemical and biological evaluations and a confirmatory pharmacodynamic clinical study in RA patients. All the studies presented herein were conducted in accordance with regulatory guidelines [7–9].

## 2. Materials and Methods

**2.1. Materials.** Biosimilar etanercept: Infinitam 25 mg vials were acquired from Probiomed S.A. de C.V., (Mexico, DF). Reference product: Enbrel<sup>®</sup> 25 mg vials were acquired from Amgen (Thousand Oaks, CA).

**2.2. Physicochemical Properties.** Identity was verified through tryptic peptide mappings analyzed by reverse phase ultra-performance-liquid-chromatography coupled to a tandem quadrupole/time-of-flight mass spectrometer (RP-UPLC-MS/MS). Three-dimensional structure was assessed by circular dichroism (CD) and fluorescence lifetime using the time correlated single photon counting technique (TCSPC). Heterogeneity was evaluated by intact mass by mass spectrometry (MS). Charge heterogeneity was assessed by capillary isoelectrofocusing (cIEF) of the whole molecule. Glycan microheterogeneity was studied using hydrophilic interaction ultra-performance-liquid-chromatography (HILI-UPLC). Sample treatment and analysis conditions were performed as previously described by Flores-Ortiz et al., 2014 [10] (MS, RP-UPLC-MS/MS, CD, and CEX-UPLC); Pérez Medina-Martínez et al., 2014 [11] (TCSPC); Espinosa-de la Garza et al. [12] (cIEF); and Miranda-Hernández et al., 2015 [13] (HILI-UPLC).

**2.3. In Vitro Assay.** Apoptosis inhibition assay was performed in U937 cells treated with TNF- $\alpha$  in the presence of different concentrations of etanercept. After 4 hours of treatment, Caspase 3/7-assay reagent was added and samples were incubated for 2–4 more hours. Luminescence emission was measured after 2–4 hours of incubation. The result is expressed as the ED<sub>50</sub> value, calculated by four-parameter logistic curve fit using the Soft-MaxPro<sup>®</sup> software.

**2.4. Clinical Study.** A double-blinded, randomized, three-arm and prospective study was designed to evaluate the pharmacodynamic profile of etanercept. The three arms were combined, continuing the treatment with Infinitam after three cycles of treatment in order to evaluate interchangeability of Infinitam and the possible impact on the efficacy, as suggested by the Mexican health authorities.

The study protocol was approved by the Institutional Review Board/Independent Committee (IRB/IEC) of the participating research centers and by the Mexican health authorities. The study was conducted in accordance with the regulations and ethical principles stated on the Declaration of Helsinki, the principles of the International Conference on Harmonization (ICH), and the Guidelines for Good Clinical Practice (GCP). All patients signed an informed consent prior to the study; all procedures were explained in detail and all questions were resolved.

The aim of the study was to evaluate the biological effects of Infinitam compared to the reference product at 12 and 24 weeks of treatment with methotrexate therapy in patients with RA. Patients received either Infinitam or the reference product, according to their treatment group, at a dose of 25 mg twice a week by subcutaneous administration. 59 patients diagnosed with moderate to high degree RA were randomly assigned into three groups. Groups 1 and 3 were treated with Infinitam for 24 weeks. Conversely, group 2 was initially treated with the reference product for 12 weeks and then with Infinitam for the next 12 weeks. All patients received concomitant methotrexate. A three-month observational period was included after the completion of the treatments. Blood samples were collected from all patients for the determination of C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), B-cell activating factor (BLyS), rheumatoid factor (RF), and TNF. Additionally Disease Activity Score (DAS28) was evaluated based on EULAR criteria with scores ranging from 0 to 9.4.

**2.5. Statistical Analysis.** Biological effect variables were compared with baseline values to those observed throughout the study in both treatment groups (group 2 versus group 1 + 3) to complete the 24 weeks of treatment. The statistical significance was  $p \leq 0.05$ , two-tailed if appropriate unless otherwise noted. The analysis was performed with the SAS statistical package JMP<sup>®</sup>.

## 3. Results and Discussion

**3.1. Physicochemical Analysis.** The first approach to ensure the adequate immunomodulatory response of biosimilar

etanercept in the treatment of RA patients is to demonstrate the identity of the molecule. The protein's primary structure is essential to derive an appropriate higher-order structure, determined by amino acid side chains interactions influenced by the environment, allowing the exposure of the appropriate domains to recognize its target molecule: TNF alpha. Primary structure comparability was confirmed by the superimposition of peptide mapping chromatograms of Inifinitam and the reference product (Figure 1(a)). The analysis was followed by the verification of the sequence coverage with respect to the theoretical sequence, being 98.3% and 97.0% for Inifinitam and the reference product, respectively (Figures 1(b) and 1(c)). Overall, these results confirmed that both products contain etanercept as active pharmaceutical ingredient (API).

Once the amino acid sequence of Inifinitam was verified, spectroscopic techniques were used to compare the higher-order structure, as an indicator of an appropriate folding, between Inifinitam and the reference product. For instance, CD analyses were performed to evaluate the secondary and tertiary structure. The obtained spectra in both far and near UV regions were superimposable (Figure 2) suggesting that both products possess comparable secondary and tertiary structures, respectively. On the other hand, the spatial disposition of the aromatic amino acids in etanercept, which is intrinsically correlated with its fluorescence lifetime ( $\tau$ ), was assessed by TCSPC [14–17]. The obtained results showed that the averaged  $\tau$  of Inifinitam was  $1.56E - 09 \pm 0.02E - 10$  s ( $n = 9$ , CI 95%), while the averaged  $\tau$  for the reference product was  $1.57E - 09 \pm 0.04E - 11$  s ( $n = 9$ , CI 95%). Collectively CD and TCSPC analyses determined that the higher-order structure of Inifinitam was comparable to the reference product. These results are supported by a previous report of the three-dimensional structure comparability between Inifinitam and its reference product. Particularly, CD and TCSPC responses under native, denaturing, and denaturing-reduced conditions revealed similar structural features in both molecules, including their ordered and disordered regions that determine specific steric hindrances, as evidenced by the accessibility of free-thiols [11]. Accordingly, comparable target recognition is expected as long as charge heterogeneity ranges for both molecules overlap.

Furthermore, the evaluation of the biotherapeutic protein heterogeneity must be included to ensure that a biosimilar candidate possesses the same degree of heterogeneity with respect to the reference product. The inherent heterogeneity is the result of the protein's chemical and structural modifications that occur during its lifecycle, resulting in a group of closely related species (i.e., isoforms) that altogether constitute the protein's identity [18].

The heterogeneity in monoclonal antibodies has been widely characterized, studied, and correlated to the biological activity of the molecule [19–23]. Nonetheless, the heterogeneity of etanercept and its implications on the functionality of the molecule are still gaining knowledge.

Mass spectrometry analyses of intact molecule of etanercept and a monoclonal antibody evidenced a high degree

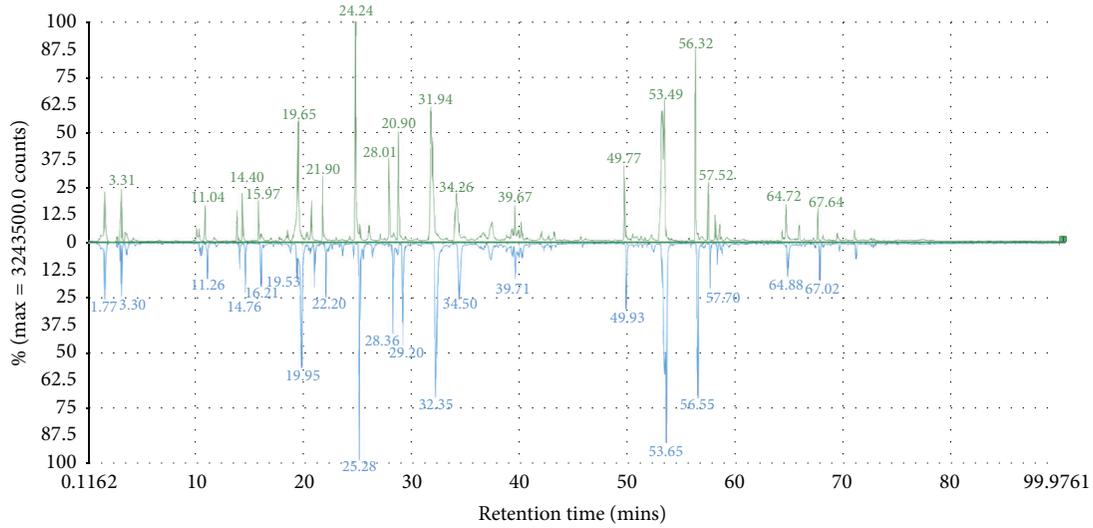
of heterogeneity in the fusion protein in comparison to the monoclonal antibody (Figure 3(a)). As it can be observed, all the charge states of etanercept's isoforms cannot be resolved, developing a continuum profile. Thus, further analyses were performed in order to exhaustively characterize the heterogeneity of Inifinitam in comparison to the reference product.

Charge heterogeneity of Inifinitam and the reference product was evaluated through cIEF analysis (Figure 3(c)). The observed averaged pI values (weighted by isoform abundance) revealed a similar charge heterogeneity among products, being  $5.50 \pm 0.01$  ( $n = 9$ , CI 95%) for Inifinitam and  $5.53 \pm 0.32$  ( $n = 9$ , CI 95%) for the reference product. Furthermore, pI isoforms ranged from  $4.35 \pm 0.07$  to  $6.57 \pm 0.04$  ( $n = 9$ , CI 95%) for Inifinitam and  $4.41 \pm 0.20$  to  $6.68 \pm 0.19$  ( $n = 9$ , CI 95%) for the reference product, confirming similarity. It is worth to notice that typically pI ranges width is less than one pI unit for mAbs [13].

The glycan microheterogeneity of Inifinitam and the reference product was also evaluated by HILIC-UPLC since it is a relevant attribute on the immunomodulatory activity of biotherapeutic proteins. Chromatograms of different analyzed batches of the reference product showed several glycoforms with variable abundance (Figure 3(b)); Inifinitam glycoforms lied within this wide heterogeneity, revealing comparability. It is worth to mention that for monoclonal antibodies, it has been reported that specific glycan isoforms could affect the affinity to the receptors involved in their effector functions and stability due to charge and steric hindrances [20, 22–25]. However, for fusion proteins, the impact on the global charge, stability, and steric hindrances of specific glycan isoforms need to be assessed, either experimentally or theoretically [26], considering that, for etanercept, those glycans do not lie within the recognition sites of its target molecule. In this sense, the demonstration of a similar biological activity supports that the observed heterogeneity in Inifinitam constitutes a basis for a biosimilar as efficient as the reference product.

**3.2. Biological Characterization.** The assessment of the biological activity, after the physicochemical characterization, confirms that the analyzed product has the same identity, higher-order structure, and posttranslational modifications as the reference product. The potency assay evaluated the ability of etanercept to prevent the interaction of TNF with cellular TNFR and can be used as a first indicator of its pharmacological activity.

The results confirmed a comparable neutralization of TNF $\alpha$  activity between Inifinitam and the reference product (Figure 4), thus reducing the uncertainty of presenting different pharmacodynamics profiles. In this regard, the relative content of all etanercept isoforms (i.e., acidic, basic, and glycan isoforms) seems to be determinant for a comparable clinical profile. However, further studies are required to understand the direct correlation between specific physicochemical properties (charge, glycosylation) and their impact on the pharmacological behavior of etanercept. Accordingly,



(a)

Control coverage (%): 97.9      Combined coverage (%): 97.9      Analyte coverage (%): 0.0  
 Control unique coverage (%): 97.9      Common coverage (%): 0.0      Analyte unique coverage (%): 0.0

1:1 to 50	LPAQVAFTPY	APEPGSTCRL	REYYDQTAQM	CCSKCSPGQH	AKVFC TKTS D
1:51 to 100	TVCDS CEDST	YTQLWNWVPE	CLS CGSR CSS	DQVETQACTR	EQNRICTCRP
1:101 to 150	GWYCALSKQE	GCRLCAPLRK	CRPGFGVARP	GTETS DVVCK	PCAPGTF SN T
1:151 to 200	TSSTDICRPH	QICNVVAIPG	NASMDAVCTS	TSPTRSMAPG	AVHLPQP VST
1:201 to 250	RSQHTQPTPE	PSTAPSTSFL	LPMPGSPPAE	GSTGDEPKSC	DKHTC P P C P
1:251 to 300	APELLGGPSV	FLFPPKPKDT	LMISRTPEVT	CVVVDVSHED	PEVKFNWYVD
1:301 to 350	GVEVHNAKTK	PREEQYNSTY	RVVSVLTVLH	QDWLNGKEYK	CKVSNKALPA
1:351 to 400	PIEKTISKAK	GQPREPQVYT	LPPSREEMTK	NQVSLTCLVK	GFYPSDIAVE
1:401 to 450	WESNGQPEN N	YKTPPVLDS	DGSFFLYSKL	TVDKSRWQQG	NVFSCSVMHE
1:451 to 467	ALHNHYTQKS	LSLSPGK			

(b)

Control coverage (%): 98.9      Combined coverage (%): 98.9      Analyte coverage (%): 0.0  
 Control unique coverage (%): 98.9      Common coverage (%): 0.0      Analyte unique coverage (%): 0.0

1:1 to 50	LPAQVAFTPY	APEPGSTCRL	REYYDQTAQM	CCSKCSPGQH	AKVFC TKTS D
1:51 to 100	TVCDS CEDST	YTQLWNWVPE	CLS CGSR CSS	DQVETQACTR	EQNRICTCRP
1:101 to 150	GWYCALSKQE	GCRLCAPLRK	CRPGFGVARP	GTETS DVVCK	PCAPGTF SN T
1:151 to 200	TSSTDICRPH	QICNVVAIPG	NASMDAVCTS	TSPTRSMAPG	AVHLPQP VST
1:201 to 250	RSQHTQPTPE	PSTAPSTSFL	LPMPGSPPAE	GSTGDEPKSC	DKHTC P P C P
1:251 to 300	APELLGGPSV	FLFPPKPKDT	LMISRTPEVT	CVVVDVSHED	PEVKFNWYVD
1:301 to 350	GVEVHNAKTK	PREEQYNSTY	RVVSVLTVLH	QDWLNGKEYK	CKVSNKALPA
1:351 to 400	PIEKTISKAK	GQPREPQVYT	LPPSREEMTK	NQVSLTCLVK	GFYPSDIAVE
1:401 to 450	WESNGQPEN N	YKTPPVLDS	DGSFFLYSKL	TVDKSRWQQG	NVFSCSVMHE
1:451 to 467	ALHNHYTQKS	LSLSPGK			

(c)

FIGURE 1: Confirmation of the primary structure by peptide mapping of (a) Infinitam (upper chromatogram in green) and the reference product (lower chromatogram in blue), and sequence coverages of (b) Infinitam and (c) the reference product with respect to the theoretical sequence of etanercept.

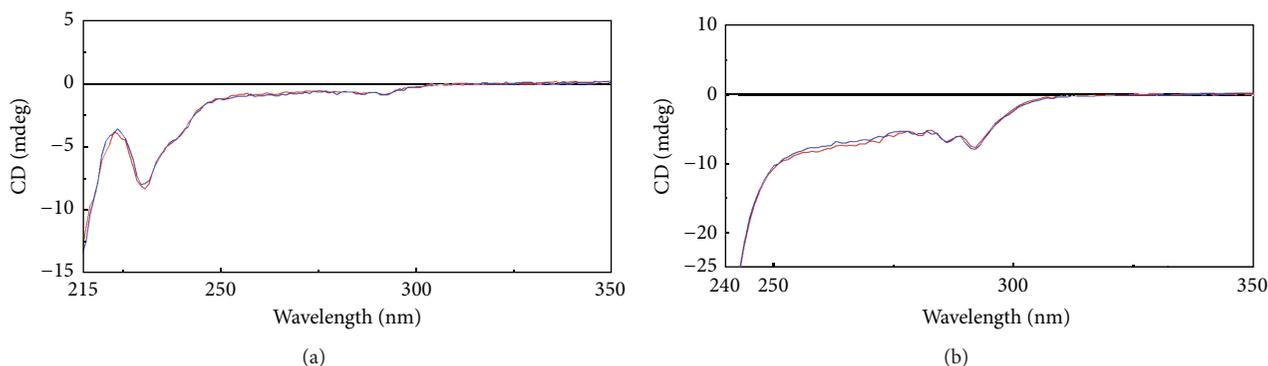


FIGURE 2: CD spectra of Infinitam (blue) and the reference product (red). (a) Far UV region and (b) near UV region.

a clinical assessment was performed to show a comparable modulation of the inflammatory response with Infinitam and the reference product.

**3.3. Clinical Assessment.** The comparison of the biological effect of methotrexate-associated Infinitam and reference product in patients with RA was evaluated based on the seric levels of the BLYS protein, ESR, CRP, TNF, and RF. Additionally, efficacy was assessed by the response of the DAS28 using EULAR criteria [27] at 12 and 24 weeks of treatment. DAS28 consist in the number of painful and swollen joints, ESR, and overall disease activity. The results showed that 70% of the patients had a reduction of at least 1.2 points and reached a DAS28 score lower than 3.2, representing a moderate response on disease activity (Figure 5(a)).

ESR is an indicator of inflammation whose value is increased because of various factors, including RA. This indicator is useful to assess the treatment response based on EULAR criteria. A reduction was observed in all patients in ESR levels in comparison with their ESR basal values (Figure 5(b)). The results showed a similar behavior between groups at 12 and 24 weeks ( $p = 0.116$  and  $p = 0.389$ , resp.).

CRP is also an indicator of inflammation. There are few factors that modify production levels of CRP besides liver failure. Overall, there was a reduction in CRP levels, maintained at weeks 12 and 24, when compared to basal levels. In spite of the values dispersion, a trend towards improvement on acute inflammation of the affected joints was observed till the end of the study. Finally, increasing CRP levels in all treatment groups during the observational period should be a consequence of etanercept depletion (Figure 5(c)).

BLYS is an important protein for regulatory functions in survival, maturation, and differentiation of B cells. It has been reported that after initiating therapy with anti-TNF drugs, patients with RA whose plasma levels of BLYS protein are reduced have a better prognosis than those patients whose BLYS levels are not reduced. The reduction of BLYS levels in both groups was statistically significant and similar in both

groups of treatment ( $p = 0.946$  and  $p = 0.865$ , weeks 12 and 24) (Figure 5(d)).

Serum levels of TNF showed an upward trend, since etanercept prevents the association to the TNF-R and its further internalization and degradation, thereby increasing the circulating levels of this cytokine. Accordingly, no statistically significant differences between treatment groups at weeks 12 and 24 were observed ( $p = 0.178$  and  $p = 0.178$ , resp.).

Finally, as a diagnostic measure, levels of RF were part of the initial evaluation of patients with RA included in the protocol. Although there is no consensus of the correlation levels with the disease status, it is well known that a modification of RF could be used as a biomarker of treatment response.

The observed results can be explained by the inhibition effect on TNF on both Infinitam and the reference product. The clinical response was rapidly achieved within the first four weeks of treatment. The behavior of the two products containing etanercept was similar. The tendency of clinical response can be considered satisfactory according to data published by other authors [28, 29].

## 4. Conclusions

The physicochemical and biological characterization studies revealed no differences in the identity and higher-order structure between Infinitam and the reference product. Regarding etanercept's heterogeneity a major diversity of charge and glycan isoforms was observed, even among batches of the reference product. For this reason, the establishment of acceptable ranges for these isoforms content is still unclear, since no significant effect was observable in the immunomodulatory activity of etanercept during biological assays. Therefore, a narrowed clinical study capable of demonstrating and confirming that both products have similar immunomodulatory response becomes critical. Altogether, the physicochemical, biological, and clinical comparability studies resulted in a similar immunomodulatory activity between the evaluated products.

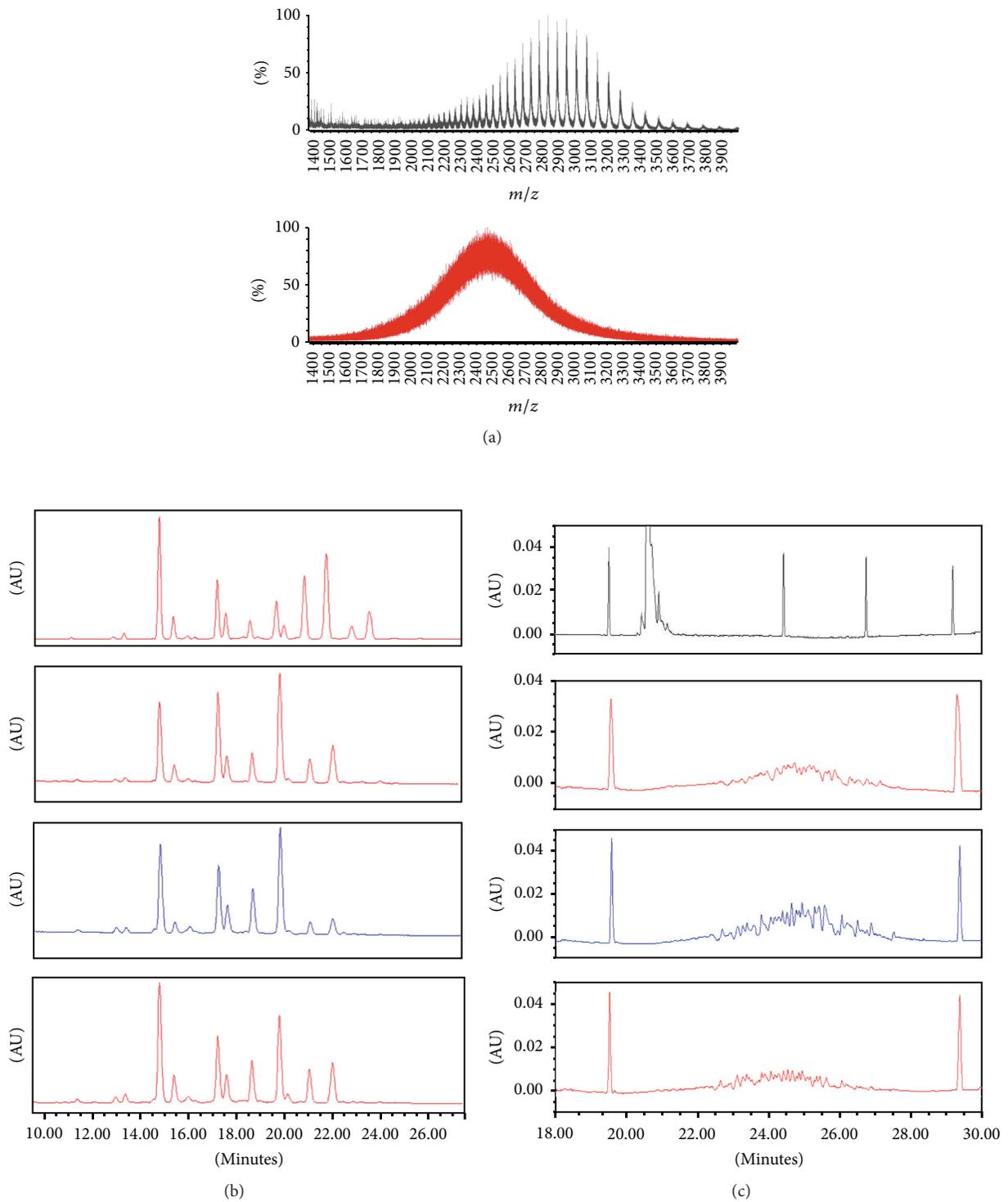


FIGURE 3: Heterogeneity analyses. (a) MS ( $m/z$ ) analyses of etanercept (red) and rituximab (black), (b) glycan heterogeneity by HILI-UPLC of Infinitam (blue) and the reference product (red), and (c) charge heterogeneity by cIEF of Infinitam (blue), reference product (red), and rituximab (black).

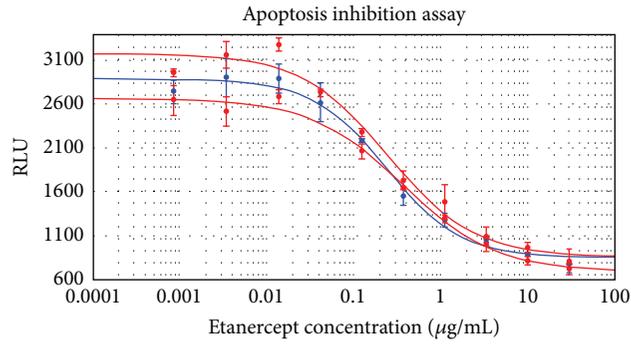


FIGURE 4: Dose-response curves of Infinitam (blue) and the reference product (red).

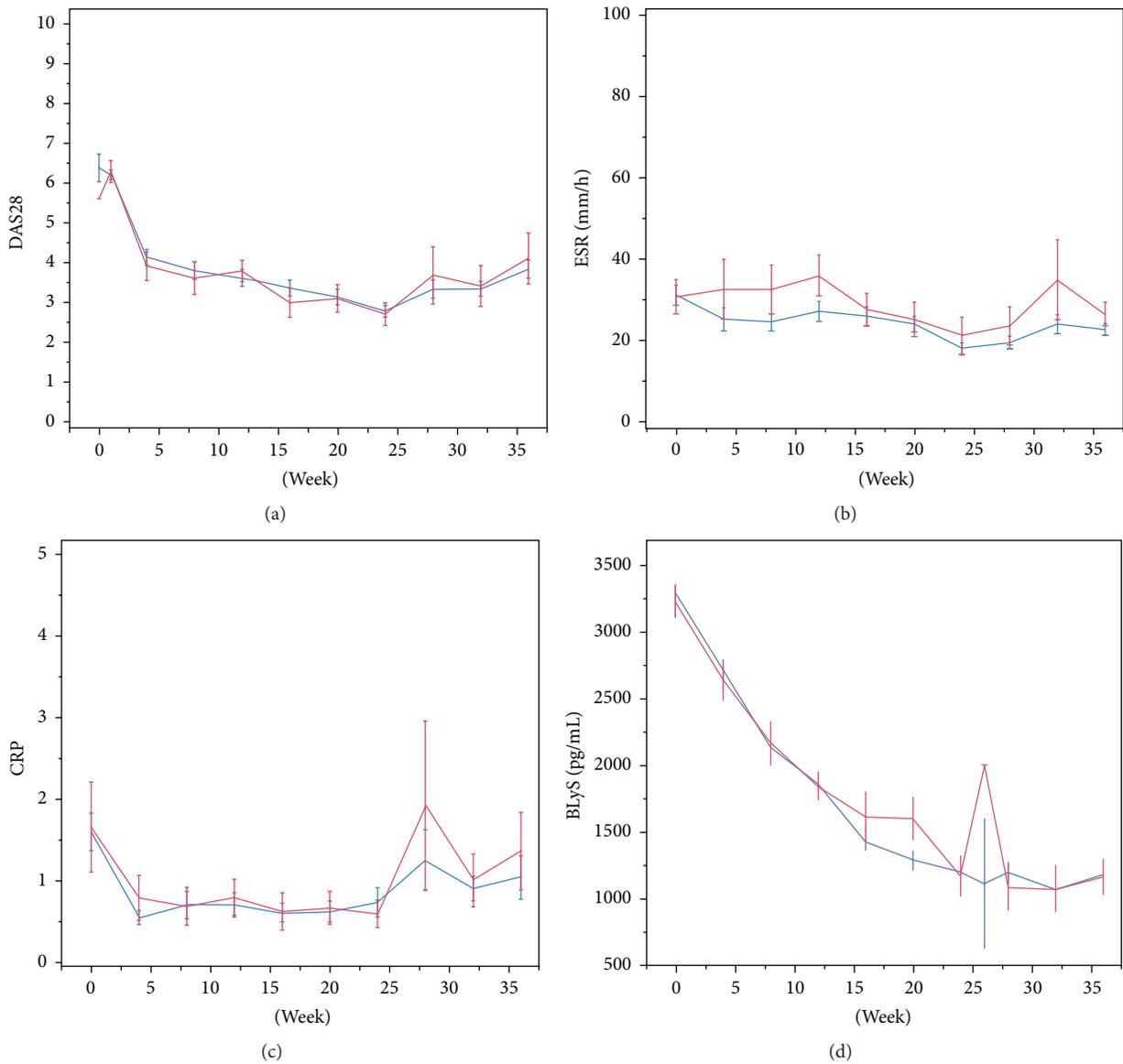


FIGURE 5: Pharmacodynamic parameters of groups treated with Infinitam (blue) and the reference product (red). (a) DAS28 per visit, (b) ESR per visit, (c) CRP per visit, and (d) BLyS per visit.

## Competing Interests

The authors are employees of Probiomed S.A. de C.V., which is developing, manufacturing, and marketing biosimilar products. All authors are involved in the development of biosimilar products for Probiomed.

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## Research Article

# Monocyte Differentiation towards Protumor Activity Does Not Correlate with M1 or M2 Phenotypes

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Macrophages facilitate breast cancer progression. Macrophages were initially classified as M1 or M2 based on their distinct metabolic programs and then expanded to include antitumoral (M1) and protumoral (M2) activities. However, it is still uncertain what markers define the pro- and antitumoral phenotypes and what conditions lead to their formation. In this study, monocytic cell lines and primary monocytes were subjected to commonly reported protocols of M1/M2 polarization and conditions known to engage monocytes into protumoral functions. The results showed that only IDO enzyme and CD86 M1 markers were upregulated correlating with M1 polarization. TNF- $\alpha$ , CCR7, IL-10, arginase I, CD36, and CD163 were expressed indistinguishably from M1 or M2 polarization. Similarly, protumoral engaging resulted in upregulation of both M1 and M2 markers, with conditioned media from the most aggressive breast cancer cell line promoting the greatest changes. In spite of the mixed phenotype, M1-polarized macrophages exhibited the highest expression/secretion of inflammatory mediators, many of which have previously been associated with breast cancer aggressiveness. These data argue that although the existence of protumoral macrophages is unquestionable, their associated phenotypes and the precise conditions driving their formation are still unclear, and those conditions may need both M1 and M2 stimuli.

## 1. Introduction

The study of macrophage behavior in both pathological and normal conditions has shown the versatility of these cells beyond their basic well-known immune effector functions. Although macrophages were first understood as potent removers of invading pathogens through their phagocytic and antigen presenting activities, these cells are most often engaged in clearing of aged cells in nonpathologic conditions. Moreover, macrophages express a battery of bioactive molecules that promote tissue remodeling/healing, support cell proliferation and angiogenesis, and mediate immunosuppression under certain microenvironmental conditions [1, 2].

Mills and colleagues in 2000 [3] were the first to separate macrophages into M1 and M2 subclasses based on their antagonistic metabolic programs and to mirror the T cell literature. This group noticed that macrophages derived from mouse strains with preferential Th1-responses (e.g., C57BL/6 or B10D2) yielded larger quantities of end-products of the induced nitric oxide synthase (iNOS), while macrophages derived from Th2-strains (e.g., BALB/c or DBA/2) preferentially synthesized ornithine, a product of arginase. Macrophages were then referred to as M1 or M2 to relate them with their associated Th1 or Th2 immune responses. Mills and colleagues pointed out very clearly that their classification was mainly useful to explain their findings

and that the M1 and M2 phenotypes might not correspond to “clonally separable cells.” Still, those observations led to the conclusion that there may be macrophages in a spectrum of different phenotypes and functions [4].

M1 and M2 macrophages have also been identified as pro- and anti-inflammatory macrophages, respectively. Moreover, according to the aforementioned functional profiles, M1 proinflammatory macrophages are considered to have anti-tumoral responses, while macrophages that display anti-inflammatory responses are thought to support protumoral functions and are included in the M2 classification, thus evidencing how widely the M1 and M2 terms have been used. Particularly in cancer, there is conflicting evidence of the role that macrophages play during cancer initiation and progression. On one hand macrophages are efficient killers of tumor cells, and on the other, increasing evidence places macrophages as powerful drivers of oncogenesis and promotion towards aggressive tumors. Colony-stimulating factor-1 (CSF-1) has been well documented as a powerful regulator of macrophage proliferation, differentiation, and survival [5], and high levels of CSF-1 and its receptor were later found to point out to human cancers with poor prognosis [6, 7]. In the pioneer study by Lin et al., impediment of peripheral monocytes arrival to the tumor stroma after CSF-1 knockout reduced tumor growth and delayed invasion and metastasis [8]. It was later found in humans that high density of macrophages in the tumor stroma significantly correlates with cancers of poor prognosis, and in histological sections of invasive tumors macrophages were preferentially located in areas of active protease secretion and increased basement membrane degradation [9–13]. Today, it is well accepted that tumor associated macrophages (TAMs) can be an important component of the pathogenic responses that drive the most aggressive tumors. TAMs favor invasion, angiogenesis, intravasation, extravasation, and metastasis through secretion of interleukins, chemokines, growth factors, and proteases [14–16]. Because these responses are more attuned to tissue remodeling or wound healing functions, TAMs have generally been described as M2 macrophages. Nevertheless, macrophages polarized in tumor stroma-like experimental conditions coexpress M1 and M2 markers [1, 11, 17, 18]. Thus, there is still discrepancy regarding the markers to define the TAMs profile.

We recently found that monocytes cocultured in 3-dimensional (3D) conditions with breast cancer cell lines are instructed to switch gene expression to a pattern more attuned to tumor-promoting activities; for instance, we observed increased expression of the COX-2 inflammatory mediator and its metabolite prostaglandin E2 and increased levels of metalloproteinases that correlated with increased collagen degradation [19]. In this study, we have tested the most commonly used protocols to polarize macrophages into M1 and M2 subtypes together with culturing them with conditioned media obtained from aggressive and nonaggressive breast cancer cell lines. Macrophage subtype classification was then addressed phenotypically, as well as through their phagocytic activity and profile of cytokine expression.

## 2. Material and Methods

**2.1. Cell Culture.** All cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and culture media and supplements from Gibco BRL Life Technologies (Grand Island, NY, USA) unless specified. Human monocytic cell lines THP-1 and U937 were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 U/mL streptomycin, at 37°C in 5% CO<sub>2</sub>. Cell stocks were frozen at a density of 2–3 million cells per mL, with RPMI 1640 medium supplemented with 20% FBS and 10% dimethylsulfoxide (DMSO, Sigma Life Science, St. Louis, MO, USA). Breast cancer cells MCF-7 and MDA-MB-231 were cultured in DMEM/F12 medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 U/mL streptomycin, at 37°C in 5% CO<sub>2</sub>.

**2.2. Isolation of Peripheral Blood Primary Monocytes.** Peripheral blood mononuclear cells (PBMCs) were isolated according to the following protocol: 35–40 mL of blood of at least two healthy volunteers was extracted, diluted in a 1 : 3 proportion with sterile Phosphate Buffered Saline (PBS, Gibco BRL Life Technologies, Grand Island, NY, USA), and subjected to density gradient centrifugation with Histopaque®-1077 (Sigma Aldrich Inc., St. Louis, MO, USA) per 30 minutes at 2000 rpm. PBMCs were then carefully retrieved from the gradient and washed 3 times with PBS, each time followed by slower centrifugation (1500, 1250, and 1000 rpm). To obtain the monocyte-enriched fraction, PBMCs were subjected to negative selection with the Monocyte Isolation Kit II Human (Miltenyi Biotec Inc., Auburn, CA, USA) following the manufacturer's recommendations as we briefly describe next. PBMCs were washed once with diluted 1 : 20 MACS BSA Stock Solution (Miltenyi Biotec Inc., Auburn, CA, USA), counted, and adjusted to a density of 10<sup>7</sup> cells per 30 μL of buffered solution; 10 μL of FcR blocking reagent and 10 μL of monocyte biotin-antibody cocktail were then added for every 10<sup>7</sup> cells to be labeled; cells were mixed and incubated for 15 minutes at 4°C. An additional 30 μL of buffered solution was added plus 20 μL of antibiotin microbeads for every 10<sup>7</sup> cells to be labeled; cells were mixed and incubated for 20 minutes at 4°C. Cells were then washed once with buffered solution, centrifuged at 1500 rpm for 5 minutes, and resuspended in 1–1.5 mL of buffered solution for magnetic separation. Suspension of cells was passed through a prerinsed LS column (Miltenyi Biotec Inc., Auburn, CA, USA) and 6–7 mL of buffered solution was added. Entire effluent was collected in a conical 15 mL tube identified as the monocyte-enriched fraction. Collected cells were counted and if not cultured immediately they were frozen at a density of 2 × 10<sup>6</sup> with DMEM/F12 medium supplemented with 50% FBS and 10% DMSO at –80°C. Frozen monocytes were thawed and cultured after no more than 2 months of freezing. Cultures were done in DMEM/F12 medium supplemented with 6% FBS, 100 U/mL penicillin, and 100 U/mL streptomycin, at 37°C in 5% CO<sub>2</sub>. Each set of experiments was performed utilizing primary monocytes of at least two different donors, and the number and phenotype of purified monocytes were very homogeneous.

**2.3. Activation of Monocytes.** According to the literature consulted, each source of monocytes used as a model of activation (THP-1, U937, and primary monocytes) has different requirements to achieve an activated state, including different incubation periods and cytokines; therefore treatments were adapted for each case as follows. THP-1 cells were subjected to different stimulation treatments in RPMI 1640 medium supplemented with a low FBS concentration (2%), at a density of  $2 \times 10^5$  cells per well in 24-well flat-bottom culture plates [17, 20–22]. Activation treatments consisted of (1) no stimulation control (mock); (2) phorbol 12-myristate 13-acetate (PMA, Sigma Aldrich Inc., St. Louis, MO, USA) 30 ng/mL for 4 h (PMA-only control); (3) pretreatment with PMA 30 ng/mL for 4 h, followed by LPS (Sigma Aldrich Inc., St. Louis, MO, USA) 10 ng/mL and INF- $\gamma$  (R&D Systems Inc., Minneapolis, MN, USA) 5 ng/mL for 72 h (condition favoring M1 polarization); (4) pretreatment with PMA 30 ng/mL for 4 h, followed by IL-4 (Sigma Aldrich Inc., St. Louis, MO, USA) 25 ng/mL and IL-13 (Sigma Aldrich Inc., St. Louis, MO, USA) 25 ng/mL for 72 h (condition favoring M2 polarization); and (5) IL-4 25 ng/mL and IL-13 25 ng/mL for 72 h (condition favoring M2 polarization without PMA).

The activation of U937 cells highly resembles the activation conditions of THP-1 cells; however incubation times differ. Also, for these monocytes an additional treatment with M-CSF was included based on the references consulted. Although M-CSF has also been used to stimulate THP-1 cells, it has not been specified whether it achieved an M2 polarization [23–25]. U937 cells were also treated in RPMI 1640 medium supplemented with 2% FBS, at a density of  $2 \times 10^5$  cells per well in 24-well flat-bottom culture plates. Activation treatments consisted of (1) no stimulation control (mock); (2) PMA 20 ng/mL for 48 h (PMA-only control); (3) pretreatment with PMA 20 ng/mL for 48 h, followed by LPS 50 ng/mL and INF- $\gamma$  10 ng/mL for 96 h (condition favoring M1 polarization); (4) pretreatment with PMA 20 ng/mL for 48 h, followed by IL-4 25 ng/mL and IL-13 25 ng/mL for 96 h (condition favoring M2 polarization (M2-A)); (5) pretreatment with PMA 20 ng/mL for 48 h followed by M-CSF 20 ng/mL for 72 h (condition favoring M2 polarization (M2-B)); and (6) IL-4 25 ng/mL and IL-13 25 ng/mL for 96 h (condition favoring M2 polarization without PMA (M2-C)) [26–29].

For primary monocyte activation, cells were treated in DMEM/F12 medium supplemented with 6% FBS, at a density of  $2 \times 10^5$  cells per well in 24-well flat-bottom culture plates in the following conditions: (1) no stimulation control (mock); (2) pretreatment with GM-CSF (PeproTech Inc., Rocky Hill, NJ, USA) 100 ng/mL for 6 days followed by LPS 100 ng/mL and INF- $\gamma$  25 ng/mL for 48 h (condition favoring M1 polarization); and (3) pretreatment with M-CSF (PeproTech Inc., Rocky Hill, NJ, USA) 100 ng/mL for 6 days followed by IL-4 25 ng/mL and IL-13 25 ng/mL for 48 h (condition favoring M2 polarization) [17, 30, 31]. Treated cells were carefully harvested by rinsing with PBS and mild trypsinization when needed.

**2.4. Monocyte Treatment with Conditioned Media Obtained from Breast Cancer Cell Lines.** THP-1, U937, and primary

monocytes were plated at a density of  $2 \times 10^5$  cells/mL/well in 24-well flat-bottom culture plates in a 1:1 mix of RPMI 1640 medium (2% FBS) and either MCF-7 or MDA-MB-231 supernatant. A control with a 1:1 mix of RPMI 1640 medium (2% FBS) and no supplemented DMEM/12 was included. After incubation for 5 days (with one replacement of correspondent media after 48 h) cells were harvested as indicated above.

**2.5. Harvest of Cell Culture Supernatants.** Two  $\times 10^6$  MCF-7 and MDA-MB-231 cells were plated in 182 cm<sup>2</sup> cell culture flasks in standard supplemented medium. When cultures reached 80% confluence supernatants were discarded, cells were rinsed with PBS, and then 20 mL of DMEM/F12 without FBS was added. Supernatants were harvested after incubation for an additional 48 h, centrifuged at 1500 rpm/5 min, aliquoted, and stored at  $-20^\circ\text{C}$  until use. Supernatants from treated monocytes were also collected for analysis of cytokine secretion. For this, supernatants were collected after finishing treatment and centrifuged at 1500 rpm/5 min, aliquoted, and stored at  $-20^\circ\text{C}$  until use.

**2.6. Flow Cytometry.** Initial characterization of monocytes: all three types of monocytes were washed twice with washing buffer (3% FBS in PBS) and incubated in 100  $\mu\text{L}$  of blocking solution (50% FBS in PBS) at room temperature (RT) for 15 minutes. After incubation in blocking solution 50  $\mu\text{L}$  of a 1:50 dilution in washing buffer of antibodies was added (for panel 1 staining: mouse anti-human anti-CD34-allophycocyanin (APC), mouse anti-human anti-CD11b-phycoerythrin (PE), and mouse anti-human anti-CD14-fluorescein isothiocyanate (FITC); for panel 2 staining: mouse anti-human anti-CD64-APC, mouse anti-human anti-CD68-PE, and mouse anti-human anti-CD16-FITC; all antibodies from BioLegend, San Diego, CA, USA) and incubated at RT for 30 minutes. Cells were then washed once with 1 mL of washing buffer, centrifuged, and resuspended in 150  $\mu\text{L}$  of PBS for acquisition in BD FACS CANTO Flow Cytometer and analyzed with the FlowJo V10 Software.

Phenotyping of polarized monocytes: monocytes were phenotyped by flow cytometry after the various treatments as follows. Monocytes were washed twice with washing buffer (1% FBS 0.01% NaN<sub>3</sub> PBS) and incubated in 100  $\mu\text{L}$  of blocking solution (25% FBS 15% human serum PBS) at  $4^\circ\text{C}$  for 20 minutes. After incubation in the blocking solution 100  $\mu\text{L}$  of a 1:100 dilution in blocking solution of antibodies was added (for panel 1 staining: mouse anti-human anti-CD86-PE and anti-CD163-peridininchlorophyll protein complex (PerCP); for panel 2 staining: rat anti-human anti-CD36-fluorescein and mouse anti-human anti-CCR7-PerCP; all antibodies from R&D Systems) and incubated at  $4^\circ\text{C}$  for 30 minutes. Cells were then washed once with 1 mL of washing buffer, fixed, and permeated with 200  $\mu\text{L}$  of Fixation/Permeabilization Solution (BD Biosciences, San Jose, CA, USA) and incubated at  $4^\circ\text{C}$  for 20 minutes. Cells were then washed with the BD Perm/Wash™ buffer (BD Biosciences, San Jose, CA, USA), centrifuged, and resuspended in 100  $\mu\text{L}$  of Perm/Wash buffer. 100  $\mu\text{L}$  of a 1:100 dilution in Perm/Wash buffer of antibodies against intracellular antigens

was added (for panel 1 staining: mouse anti-human anti-TNF- $\alpha$ -fluorescein and anti-IL-10-APC; for panel 2 staining: mouse anti-human anti-arginase I-PE and mouse anti-human anti-indoleamine-pyrrole 2,3-dioxygenase- (IDO-) APC; all antibodies from R&D Systems Inc., Minneapolis, MN, USA) and incubated at 4°C for 30 minutes. After incubation cells were washed with 1 mL of Perm/Wash buffer, centrifuged, and resuspended in 150  $\mu$ L of PBS for acquisition in BD FACS CANTO Flow Cytometer and analyzed with the FlowJo V10 Software.

**2.7. Analysis of Cytokine Profiles.** To determine the cytokine profiles present in monocytes supernatants after the various polarization treatments, concentrations (pg/mL) of G-CSF (granulocyte-colony-stimulating factor), GM-CSF (granulocyte-macrophage-colony-stimulating factor), IL-1 $\beta$  (interleukin-1 beta), IL-8 (interleukin-8), IL-12p70 (interleukin-12p70), INF- $\alpha$ 2 (interferon-alpha 2), MCP-1 (monocyte chemoattractant protein-1, also known as CCL2), EGF (Epidermal Growth Factor), VEGF (Vascular Endothelial Growth Factor), and RANTES (regulated on activation, normal T cell expressed and secreted, also known as chemokine CCL5) were measured with the MILLIPLEX HCYTOMAG-60 K Kit (EMD Millipore Corporation, Billerica, MA, USA) following the manufacturer's recommended procedure. Briefly, in each well of a 96-well flat-bottom culture plate 25  $\mu$ L of assay buffer was mixed with 25  $\mu$ L of supernatants or controls and 25  $\mu$ L of the detection microbeads cocktail. The mixture was incubated at 4°C overnight with orbital agitation. Wells were then washed twice with washing buffer (included in the kit) and 25  $\mu$ L of the detection antibodies mix was added to each well and the plate was incubated at RT with orbital agitation for 1 h. After incubation, 25  $\mu$ L of streptavidin-PE was added to each well followed by 30 more minutes of incubation at RT with orbital agitation. The wells were then washed twice with washing buffer and 150  $\mu$ L of PBS was added to each well to proceed with the analysis in Luminex MAGPIX multiplexing instrument and the analysis of data was performed in the xPONENT® Software.

**2.8. Phagocytosis Assay.** Monocyte suspensions after the various treatments were counted and  $1 \times 10^5$  cells were plated per well of a 96-well flat-bottom culture plate in 100  $\mu$ L of medium. Cells were allowed to settle at the bottom of the wells, the supernatant was carefully aspirated from each well, and 100  $\mu$ L of fluorescein-labeled *Escherichia coli* K-12 BioParticles (Vybrant Phagocytosis Assay Kit, Molecular Probes Inc., Eugene, OR, USA) was added; monocytes were then incubated at 37°C in 5% CO<sub>2</sub> for 2 h. After incubation the BioParticles were carefully aspirated from each well, 100  $\mu$ L of trypan blue (Vybrant Phagocytosis Assay Kit, Molecular Probes Inc., Eugene, OR, USA) was added to each well, and the plate was incubated for 1 minute at RT; trypan blue was then aspirated and fluorescence present within the cells was detected in an Ascent fluorometer with an excitation wavelength of 480 nm and emission of 520 nm. A series of at least 3 blank wells were included to subtract background fluorescence to the sample's emissions. Phagocytic activity was expressed as mean fluorescence intensity of at least five

technical replicates after subtraction of the average fluorescence intensity of the group of blank wells. Three independent experiments were performed.

**2.9. Statistical Analysis.** Statistical comparison of values from the different conditions tested was performed with the GraphPad Prism 5 Software, using one-way analysis of variance (ANOVA) test and Tukey's posttest to compare all pairs of data columns. Significance  $\leq 0.05$  was indicated with \*,  $\leq 0.01$  was indicated with \*\*, and  $\leq 0.0005$  was indicated with \*\*\*.

### 3. Results

**3.1. Phenotypic Characterization of Monocytic Cell Lines and Primary Monocytes.** U937 and THP-1 are myeloid cells derived from patients with a histiocytic lymphoma and monocytic leukemia, respectively, which are often used to study monocyte differentiation. The phenotypes of both monocytic cell lines together with primary monocytes derived from peripheral blood of healthy donors were characterized by flow cytometry to know their stage of differentiation. U937 cells were almost entirely CD34<sup>neg</sup> (99.8%) CD11b<sup>pos</sup> CD14<sup>pos</sup>, which outlines their myeloid lineage, although already compromised to monocytes. 99.7% of these cells were also CD64<sup>pos</sup> CD68<sup>neg</sup> CD16<sup>neg</sup>, which shows that U937 cells are mostly in an undifferentiated inactive monocyte state (Figure 1(a)). THP-1 cells were very similar; >99% of the population consisted of CD34<sup>neg</sup> CD11b<sup>pos</sup> CD14<sup>pos</sup> CD64<sup>pos</sup> CD68<sup>neg</sup> CD16<sup>neg</sup>. These features along with their effortless maintenance in culture make U937 and THP-1 cells a suitable *in vitro* experimental model for macrophage differentiation and activation. One slight difference found was that THP-1 cells had a very small fraction (about 0.1% of the total population) of CD68<sup>pos</sup> CD16<sup>pos</sup> cells, which indicates the presence of activated monocytes (Figure 1(b)). Figure 1(c) shows a representative characterization of one of the isolates of primary monocytes. There was more heterogeneity in the cell population found in primary monocytes; 99.4% of these cells were CD34<sup>neg</sup>, from which 81.1% were CD11b<sup>pos</sup> CD14<sup>pos</sup> myeloid cells. 99.8% of primary monocytes were also CD68<sup>neg</sup> CD64<sup>pos</sup> CD16<sup>neg</sup> and the small fraction (0.16%) of CD68<sup>pos</sup> cells was also CD16<sup>neg</sup>. This profile denotes also a classical monocytic profile.

**3.2. Morphological Characterization of M1- or M2-Polarized Monocytes.** Differentiation from monocyte to macrophage is accompanied by cell morphological changes. Figure 2 shows that THP-1 and U937 cells had very similar basal morphology and exhibited very similar changes after culture in M1- or M2-polarizing conditions. Both cell lines presented a rounded nonadherent basal morphology, which upon treatment with PMA alone or with M2-polarizing conditions became elongated with some cells displaying adherence. These changes were more sizable after treatment with M1-polarizing conditions. Cell aggregates were also observed in the THP-1 cells, which became larger after treatment with PMA alone or with M2-polarizing conditions. Primary

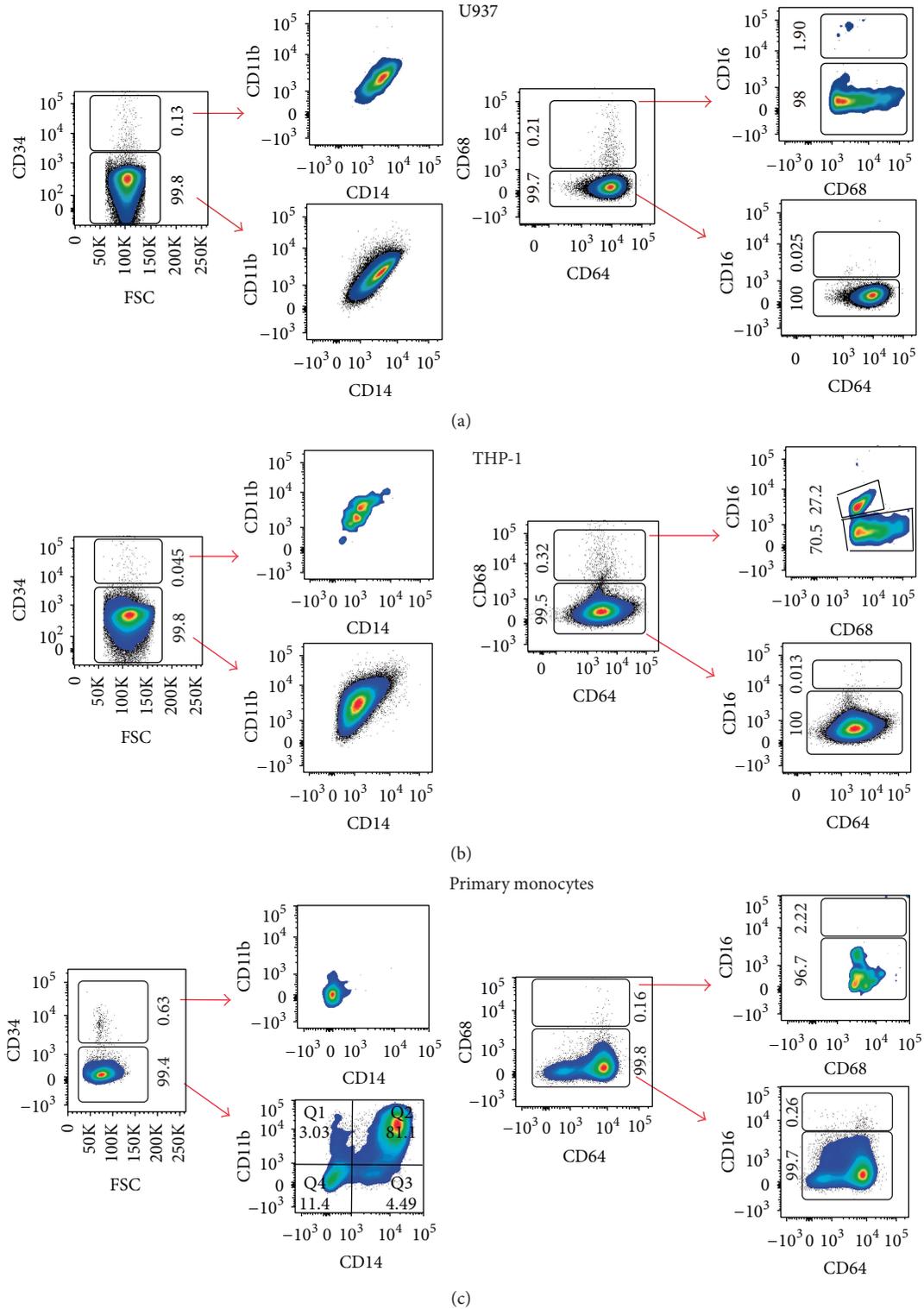


FIGURE 1: Phenotypic characterization of monocytic cell lines and primary monocytes. Dot blots representative of the flow cytometry analysis. Depicted are population percentages based on two marker panels: CD34, CD11b, and CD14 (left) and CD68, CD64, and CD16 (right) markers for (a) U937 cells, (b) THP-1 cells, and (c) primary monocytes. One donor is used as an example for primary monocytes although similar results were obtained with different donors and with different isolation batches. The phenotype found in most of the cells analyzed is basically the same for all three types of monocytes: CD34<sup>neg</sup>, CD11b<sup>pos</sup>, CD14<sup>pos</sup>, CD68<sup>neg</sup>, CD64<sup>pos</sup>, and CD16<sup>neg</sup>, which denotes a classical inactivated monocyte pattern.

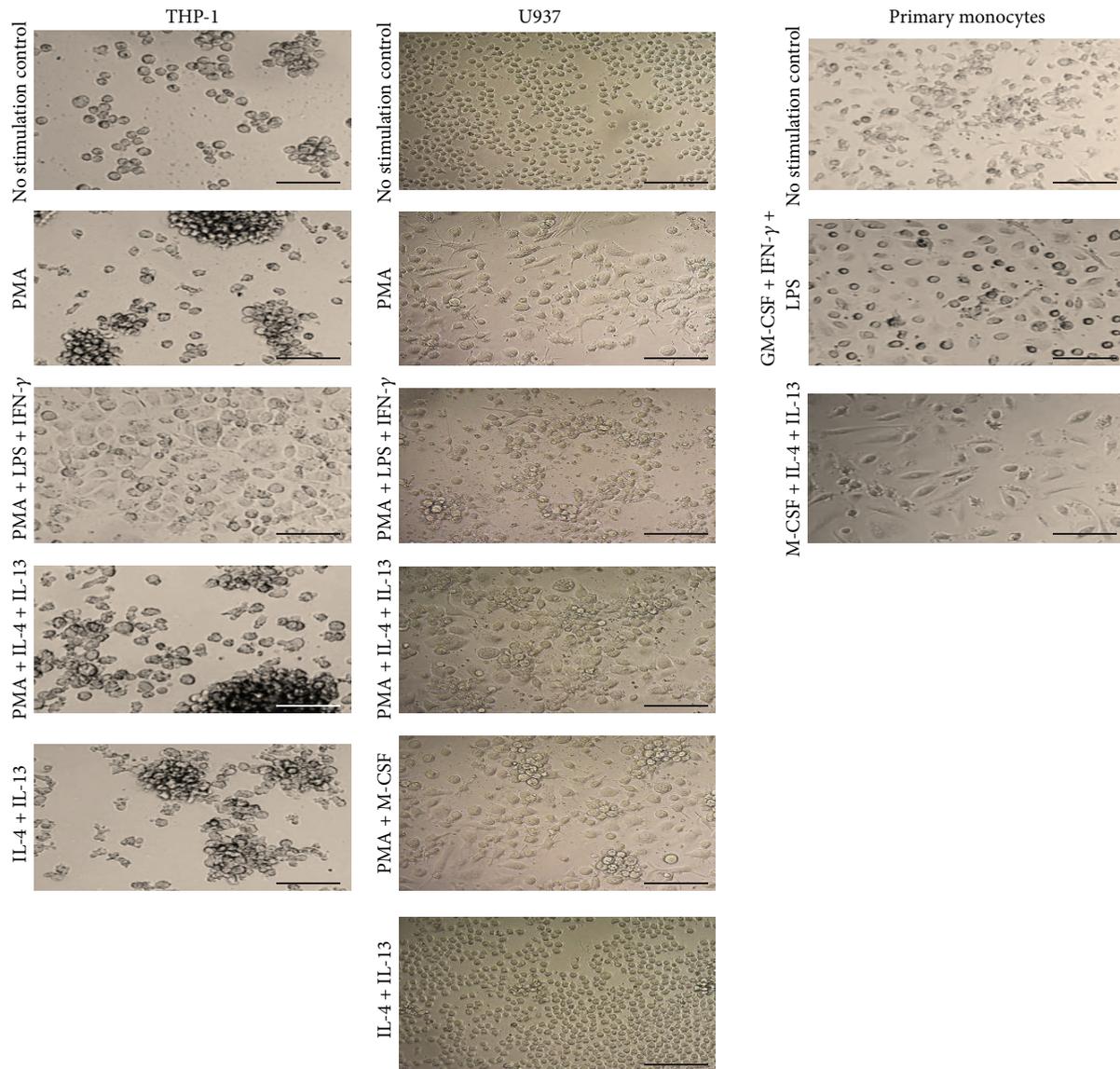


FIGURE 2: Morphological characterization of M1- or M2-polarized monocytes. Morphology observed for THP-1, U937, and primary monocytes under the indicated treatments. Images were taken with Motic Microscope at 200x magnification; scale bar indicates 50  $\mu\text{m}$ . The most relevant features of differentiation were adherence, stronger in M1-polarizing treatments, and several cell shape changes in most cases presenting mixed shapes (round and spindle-like and with several membrane projections) and cell sizes.

monocytes were characterized by a mixed morphology in conditions of no stimulation; cells were observed in variable sizes and shapes with some of them showing spindle-like forms and membrane projections. Cells were also moderately adherent and became highly adherent after treatment with GM-CSF + INF- $\gamma$  + LPS (M1 conditions), showing also evident nuclei. In contrast, primary monocytes treated with M-CSF + IL-4 + IL-13 (M2 conditions) were mostly spindle-like shaped and less adherent.

**3.3. Characterization of M1- and M2-Related Phenotypes.** Several markers previously reported as being M1- or M2-related were analyzed in untreated or polarized cells by flow cytometry. For M1 the markers considered were TNF- $\alpha$ ,

IDO enzyme, CCR7, and CD86 and for M2 they were IL-10, arginase I enzyme, CD36, and CD163. Cells were analyzed after polarization by flow cytometry and Figures 3(a) and 3(b) show the results expressed as the median fluorescence intensity (MFI). Contrary to what is expected, we found that the levels of all markers of M1 or M2 were very variable impeding assignment of a clear M1 or M2 phenotype. Also, the basal expression levels were very variable between cells, in spite of the highly similar differentiation stage. For instance, THP-1 cells express more of the M1 markers than U937 and primary monocytes and of IL-10 and CD36 M2 markers, while primary monocytes express higher levels of arginase I and CD163. Interestingly, even the basal levels of expression were highly variable within one type of cell suggesting very

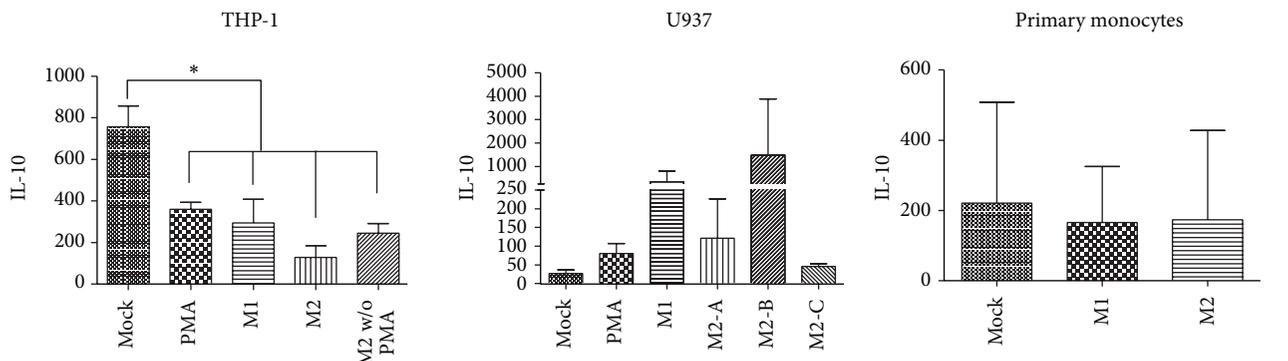
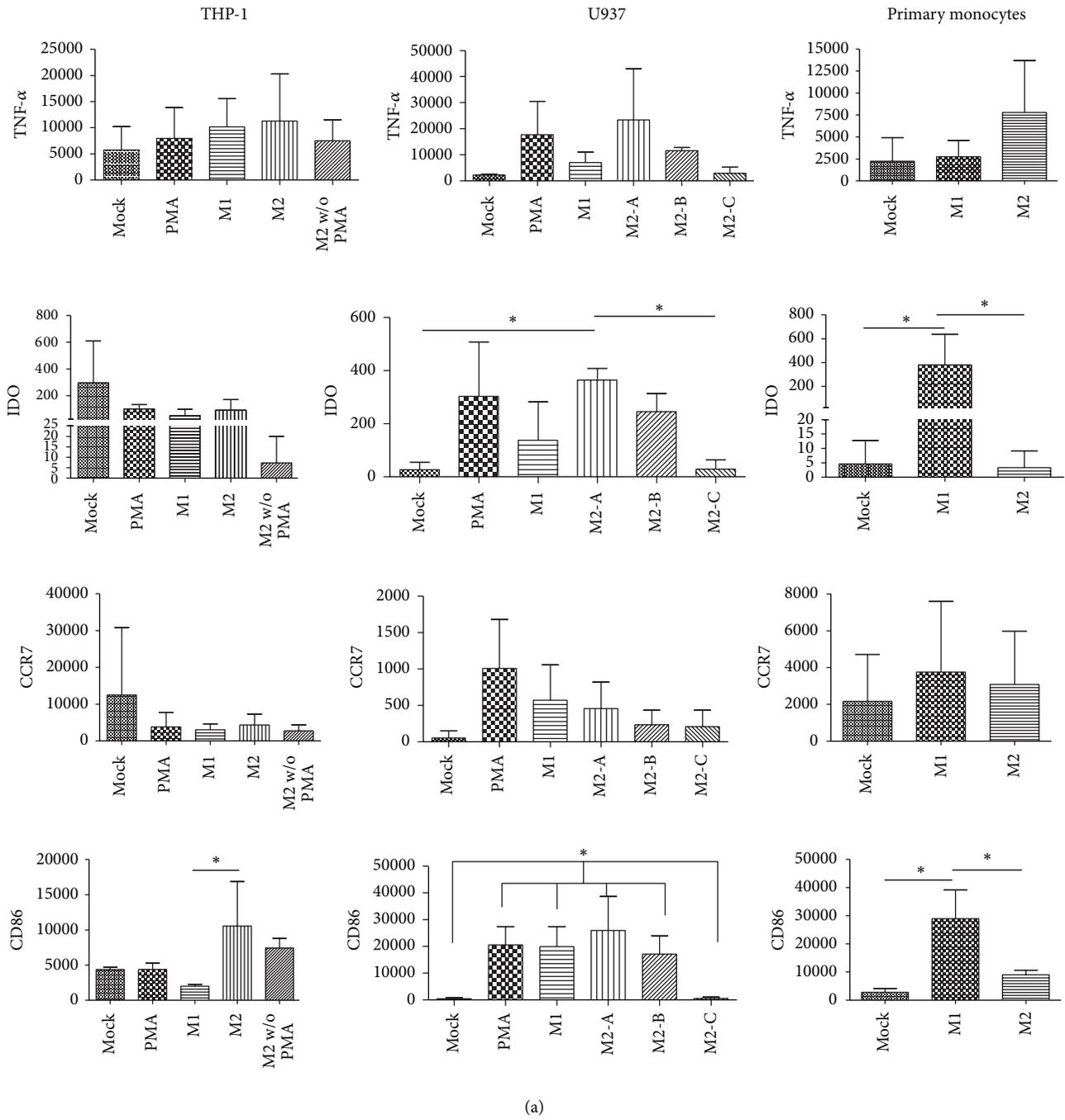


FIGURE 3: Continued.

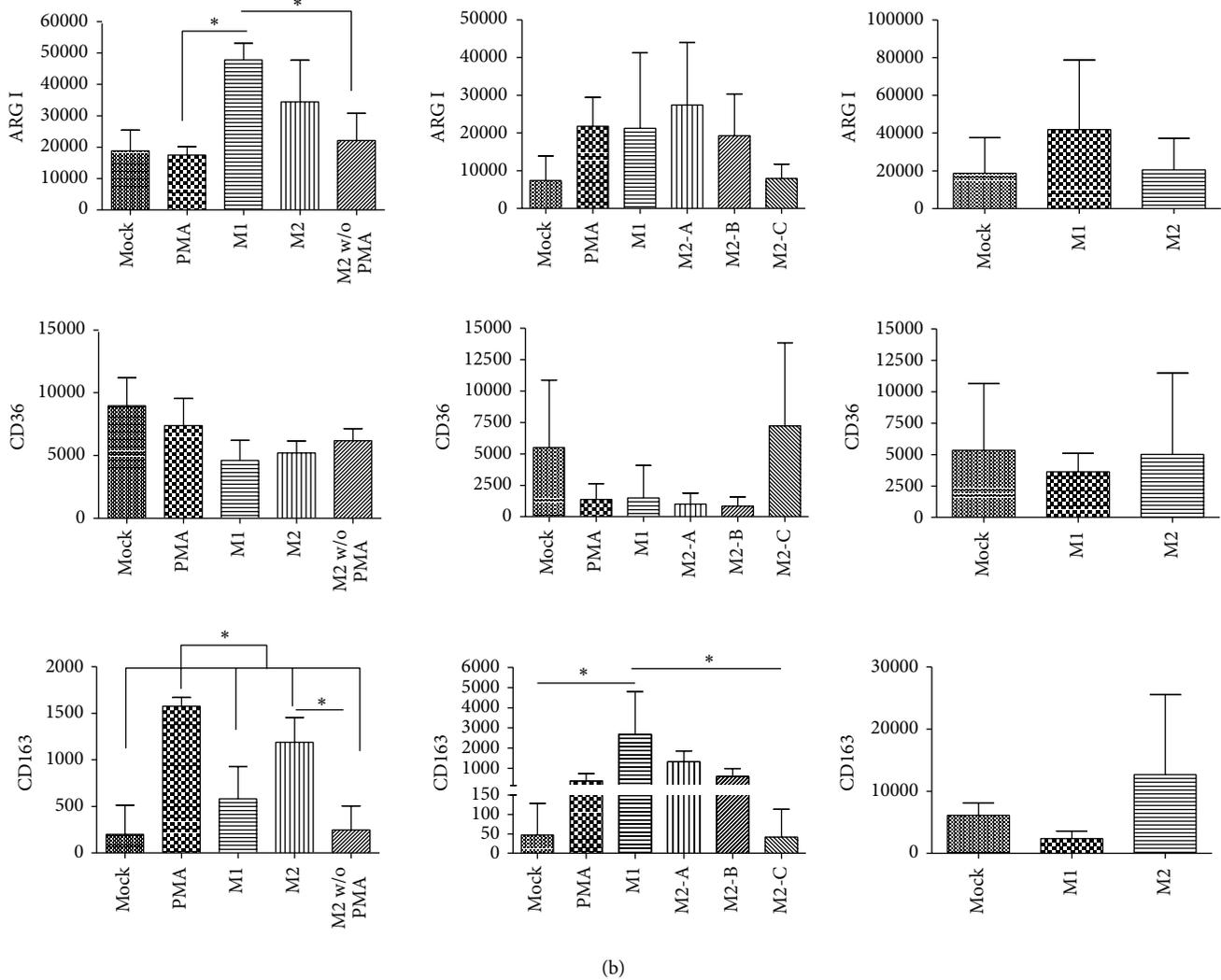


FIGURE 3: Characterization of M1- and M2-related phenotypes. Median fluorescence intensity corresponding to (a) M1-related markers TNF- $\alpha$ , IDO enzyme, CCR7, and CD86 and (b) M2-related markers IL-10, arginase I, CD36, and CD163, found in cells harvested from all different polarizing treatments and controls and analyzed by flow cytometry. For THP-1, mock: no stimulated cells; PMA: PMA-only control; M1: PMA + LPS + INF- $\gamma$ ; M2: PMA + IL-4 + IL-13; and M2 w/o PMA: IL-4 + IL-13. For U937, mock: no stimulated cells; PMA: PMA-only control; M1: PMA + LPS + INF- $\gamma$ ; M2-A: PMA + IL-4 + IL-13; M2-B: PMA + M-CSF; and M2-C: IL-4 + IL-13. For primary monocytes, mock: no stimulated cells; M1: GM-CSF + LPS + INF- $\gamma$ ; and M2: M-CSF + IL-4 + IL-13. Asterisks and bars denote statistical significance between conditions. Significant differences ( $p \leq 0.05$ ) found were as follows: in THP-1 cells CD86, M1 versus M2; IL-10, mock versus all the rest of the conditions; arginase I, M1 versus PMA and versus M2 without PMA; CD163, PMA versus all the rest of the conditions and M2 versus M2 without PMA. In U937 cells IDO, M2-A versus mock and versus M2-C; CD86, (PMA, M1, M2-A, and M2-B) versus mock and M2-C; CD163, M1 versus mock and versus M2-C. In primary monocytes IDO and CD86 were significantly different in M1 versus mock and versus M2.

responsive cellular stages. In agreement with that, PMA treatment alone often resulted in levels of M1 or M2 markers as high as after M1- or M2-polarizing conditions.

Analyses of M1 markers (Figure 3(a)) showed that only primary monocytes displayed a correlative significant increased expression of M1 markers IDO and CD86 after polarization with M1 conditions. CD86 behaved contrary to what is expected in THP-1 cells, since it was higher after treatment with PMA + IL-4 + IL-13 M2-polarizing conditions. In U937 cells only the IL-4 + IL-13 M2-polarizing conditions (M2-C) did not result in upregulation of CD86. Since M2-C is the

only condition that does not include PMA, this result suggests that PMA is a potent inducer of CD86 in these cells. IDO was significantly more expressed after PMA + IL-4 + IL-13 (M2-A) treatment than after IL-4 + IL-13 (M2-C), while M1 treatment did not significantly increase IDO expression.

Meanwhile, for M2-related markers, we did not find a *bona fide* marker whose expression varied specifically according to the M2-polarizing conditions (Figure 3(b)). If we had only compared M2 against mock conditions, U937 would have been the cells closer to the expected behavior. In these cells IL-10, arginase I, and CD163 were higher after M2

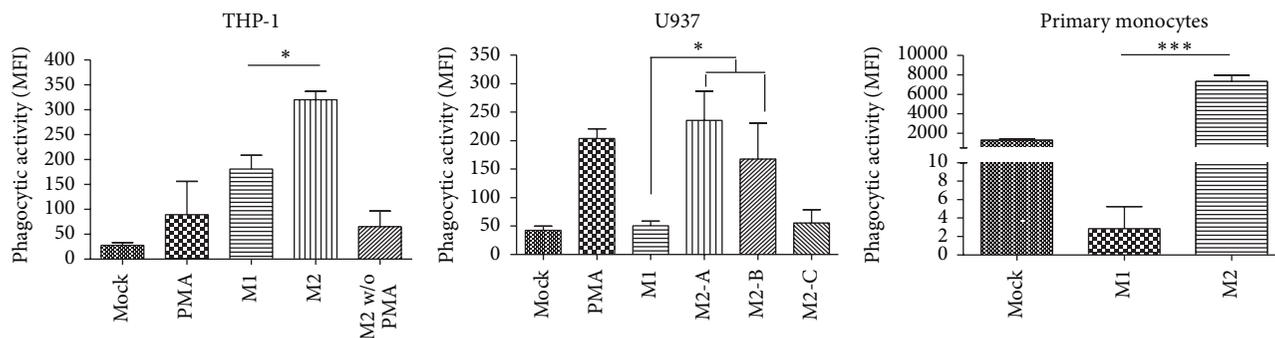


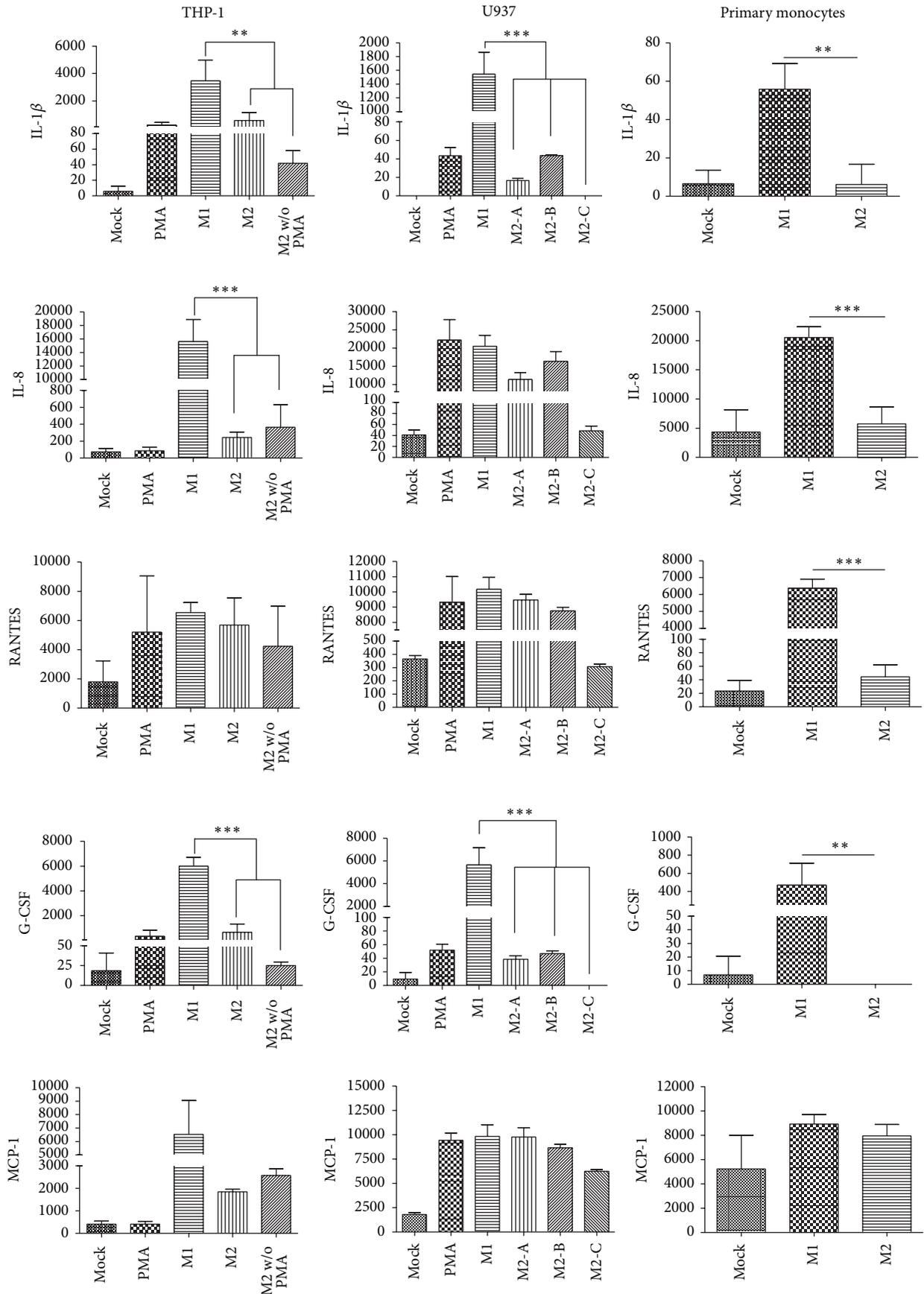
FIGURE 4: Determination of phagocytosis. Phagocytic activity of THP-1, U937, and primary monocytes expressed as the median fluorescence intensity of fluorescein-labeled *E. coli* K-12 BioParticles engulfed by cells. Mean of three independent experiments is shown. For THP-1, mock: no stimulated cells; PMA: PMA-only control; M1: PMA + LPS + INF- $\gamma$ ; M2: PMA + IL-4 + IL-13; and M2 w/o PMA: IL-4 + IL-13. For U937, mock: no stimulated cells; PMA: PMA-only control; M1: PMA + LPS + INF- $\gamma$ ; M2-A: PMA + IL-4 + IL-13; M2-B: PMA + M-CSF; and M2-C: IL-4 + IL-13. For primary monocytes, mock: no stimulated cells; M1: GM-CSF + LPS + INF- $\gamma$ ; and M2: M-CSF + IL-4 + IL-13. Asterisks and bars denote statistical significance between conditions. Significance  $\leq 0.05$  was indicated with \*, and  $\leq 0.0005$  was indicated with \*\*\*. In THP-1 cells and primary monocytes there was statistically significant differences between M1 and M2 conditions and in U937 between M1 and M2-A and M2-B.

conditions, although nonsignificant. In THP-1 cells, arginase I and CD163 showed a nonsignificant increased expression between M2-polarization and mock. However, M2 markers were also upregulated after treatment with M1-polarizing conditions. In U937 cells CD163 was significantly higher in M1 conditions than after IL-4 + IL-13 (M2-C) treatment, and in THP-1 cells IL-10 was significantly higher in mock than in any other condition. Primary monocytes untreated or treated showed very similar levels of M2 markers.

**3.4. Determination of Phagocytosis.** In order to better understand the functional activity of M1- and M2-polarized monocytes/macrophages we performed a phagocytosis assay. This assay consisted of measuring the fluorescence intensity of engulfed fluorescein-labeled *E. coli* K-12 BioParticles. Both M1 and M2 macrophages are highly phagocytic although to different substrates; while M1 macrophages are in charge of pathogen clearing, M2 macrophages remove aged or damaged cells. Because the assay was based on *E. coli*, we were expecting an increased activity given by M1-polarized monocytes. Strikingly, we found that all three types of monocytes tested showed the highest phagocytic activity in M2 conditions (Figure 4). In the cases of THP-1 and U937 cells this elevated phagocytic capability matched the conditions where prestimulation with PMA was performed, and in concordance with that, PMA treatment alone also showed a significant increased activity compared to mock cells (Figure 4 only shows the M1-versus-M2 statistical analysis). Although M2-polarized THP-1 and U937 cells without PMA (only IL-4 and IL-13) were poorly phagocytic, this activity does not seem to be exclusively mediated by PMA since M1 polarization also uses PMA. Moreover, M2-polarized primary monocytes, in which PMA was not used, were also highly phagocytic.

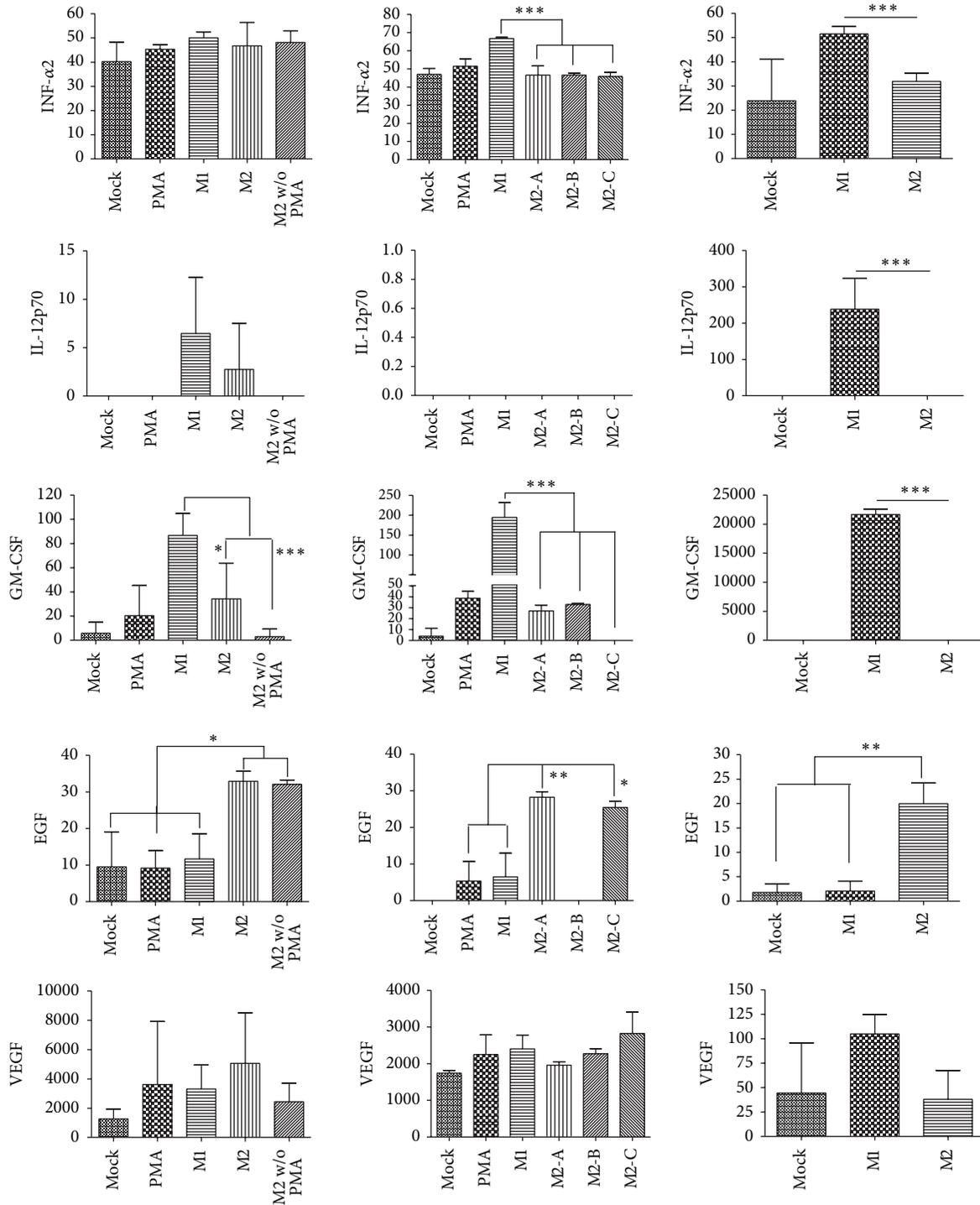
**3.5. Analysis of the Profile of Cytokine Expression.** To fulfill their specific immune effector or remodeling functions, M1

and M2 macrophages should express a battery of specific cytokines, chemokines, and growth factors. To determine whether *in vitro* M1- and M2-polarized monocytes exhibited a specific profile of secreted immune modulators, we analyzed the supernatants of cells after the different treatments (Figure 5). Using a multiplex platform we tested cells for their capability to secrete proinflammatory cytokines, chemokines, and growth factors. Remarkably, in spite of our previous analyses in which we could not assign a specific M1 or M2 phenotype to the *in vitro* polarized monocytes/macrophages, we found a clear expression profile greatly conserved among all cells tested. IL-1 $\beta$ , IL-8, RANTES, G-CSF, IFN- $\alpha 2$ , GM-CSF, and IL-12p70 were significantly increased in M1 conditions at least by one of the monocytes, while EGF clearly identified monocytes differentiated in M2 conditions (Figure 5). M1-polarized THP-1 cells presented a 111-fold increase of IL-1 $\beta$  over the value of M2-A treated cells; for U937 this cytokine was significantly higher in M1-polarizing treatment than all three M2 conditions; however the greatest difference was a 1544.5-fold increase in M1 over M2-C conditions, whereas for primary monocytes the difference was 8.6-fold. The level of IL-8 in supernatants of THP-1 cells was 46.2-fold higher in M1 conditions than in M2 conditions; the same comparison in primary monocytes resulted in a 2.7-fold difference. G-CSF increased 52.4-fold in THP-1 cells, 5656.12-fold in U937 cells (the highest increase seen between M1 and M2-C treatments), and 3.8-fold in primary monocytes in M1 conditions in comparison to M2 conditions. In spite of these numbers in which transformed cell lines seem to be more reactive than primary monocytes, it does not seem that polarized monocyte cell lines are more responsive than polarized primary monocytes. Primary monocytes presented a 1296.4-fold increased expression of GM-CSF and this chemokine only increased 18.6-fold in THP-1 cells and 195-fold in U937 (the greatest difference). The levels of RANTES and IL-12p70 in M1 treated primary monocytes were 77.6-fold and 121.5-fold higher, respectively, than the level found in M2



(a)

FIGURE 5: Continued.



(b)

FIGURE 5: Analysis of the profile of cytokine expression. Concentrations expressed in pg/mL of IL-1β, IL-8, RANTES, G-CSF, MCP-1, INF-α2, IL-12p70, GM-CSF, EGF, and VEGF measured in supernatants harvested from all different polarizing treatments and controls and analyzed in the Luminex MAGPIX® multiplexing instrument. For THP-1, mock: no stimulated cells; PMA: PMA-only control; MI: PMA + LPS + INF-γ; M2: PMA + IL-4 + IL-13; and M2 w/o PMA: IL-4 + IL-13. For U937, mock: no stimulated cells; PMA: PMA-only control; MI: PMA + LPS + INF-γ; M2-A: PMA + IL-4 + IL-13; M2-B: PMA + M-CSF; and M2-C: IL-4 + IL-13. For primary monocytes, mock: no stimulated cells; MI: GM-CSF + LPS + INF-γ; and M2: M-CSF + IL-4 + IL-13. U937 cells did not produce IL-12p70 under any condition. Asterisks and bars denote statistical significance between conditions. Significance ≤0.05 was indicated with \*, ≤0.01 was indicated with \*\*, and ≤0.0005 was indicated with \*\*\*. In THP-1 cells IL-1β, IL-8, G-CSF, and GM-CSF were significantly different between MI versus M2 and M2 without PMA; EGF, (mock, PMA, and MI) versus M2 and M2 without PMA. In U937 cells IL-1β, G-CSF, INF-α2, and GM-CSF, MI versus (M2-A, M2-B, and M2-C); EGF, PMA and MI versus M2-A and M2-C. In primary monocytes IL-1β, IL-8, RANTES, G-CSF, INF-α2, IL-12p70, and GM-CSF, MI versus M2; EGF, mock and MI versus M2.

treated cells, while no significant changes were observed in the monocytic cell lines. If concentrations are considered, the levels of IL-12p70 and GM-CSF are still higher in M1-polarized primary monocytes than in any of the monocytic cell lines. There were no significant changes in the levels of MCP-1 and VEGF mediated by any treatment condition.

### 3.6. Phenotypic Changes Induced by Breast Cancer Cell Lines.

We have previously documented that monocytes grown in the presence of conditioned media from breast cancer cell lines display characteristics of protumoral macrophages. Those monocytes expressed higher levels of COX-2, prostaglandin E2, and metalloproteinases and exhibited an increased extracellular matrix (ECM) remodeling activity [19]. Interestingly, invasive/metastatic breast cancer cells better promoted those changes than noninvasive cells [19]. We hypothesized that engaged monocytes to perform protumoral functions would better correlate with an M2 phenotype. To address that, we cultured the monocytic cell lines and primary monocytes for five days with conditioned media from MCF-7 and MDA-MB-231 breast cancer cell lines and analyzed M1/M2-related markers by flow cytometry. MCF-7 cells have been characterized as poorly aggressive (low invasive and nonmetastatic potential) tumor cells while MDA-MB-231 cells are well known as being highly aggressive because of their invasive and metastatic capacity. The result is shown in Figure 6; first with histograms of CD36 as an example, both primary monocytes and U937 cells exhibited changes in this marker especially after treatment with MDA-MB-231 conditioned media (Figure 6(a)). In Figure 6(b) fold changes are depicted; here basal expression of the marker in mock treated cells was given a value of 1, and expression after treatment with conditioned media was normalized dividing by this basal value. Only changes > 1.5-fold are presented; white bars represent M1 markers and black bars M2 markers. In general, we found that the aggressive MDA-MB-231 cell line induced more dramatic changes than the noninvasive cell line in all types of monocytes tested. Interestingly, those changes were not exclusive of M2 markers as we would expect considering the protumor activity conferred to the monocytes after treatment. THP-1 cells showed increased expression of M2 marker arginase I but also of M1 marker CD86. Primary monocytes only changed the expression of the M2 marker CD36. U937 cells changed M1 marker CD86 and M2 markers arginase I and CD36. These results reflect the ambiguity of phenotypes based on M1/M2 marker panels and suggest that protumoral macrophages cannot be defined as M1 or M2 based on these markers.

## 4. Discussion

Monocytes are highly reactive cells that undergo morphological, phenotypic, and functional changes when exposed to different stimuli. Although it is relatively easy to experimentally induce macrophages to perform specific activating functions, to assign a reliable phenotypic stage matching the plethora of functions that they perform remains a challenge. Up to today, the ideal technical guidelines to generate desired specific macrophage phenotypes have not been achieved due

to the heterogeneity in reported experimental procedures and phenotypic markers [32]. We have previously developed experimental conditions in which monocytes can be coaxed to perform protumoral activities [19]. Since TAMs have been thought to perform protumoral activities that better correlate with M2 macrophage functions, we assumed that our experimental conditions would generate M2 phenotypes. However, when we analyzed three sources of monocytes, two cell lines derived from cancer patients and peripheral blood primary monocytes derived from healthy donors, which were subjected to a series of stimuli extensively reported in the literature, plus conditioned media from aggressive breast cancer cells, we found no clear phenotype correlating with M1 or M2 macrophages but a mix of marker expressions.

In this study, we first established the baseline identity of the monocytes confirming that they were undifferentiated monocytes with the classical associated morphology. When these cells were subjected to different stimulating conditions, cells underwent morphological changes that indicated their response to the stimulus; among these changes adherence is considered a feature of differentiation, signaling that monocytes have matured into macrophages, probably reflecting tissue resident macrophages. We then analyzed two panels of markers related to M1 and M2 macrophage programs, according to previous reports [1, 18, 33–35]. M1-related markers were CD86, TNF- $\alpha$ , CCR7, and IDO enzyme. Based on our results, while IDO enzyme and CD86 were significantly associated with M1 treatment in primary monocytes, TNF- $\alpha$  and CCR7 were not associated with any particular condition. When M2 selected markers (CD163, arginase I enzyme, CD36, and IL-10) were analyzed, data were even less correlative with M2 polarization conditions.

It is important to state that if we had only compared a specific monocyte polarizing condition against mock treated cells we would have obtained significant and correlative upregulation of the M1 or M2 markers. It is because we decided to be more rigorous, comparing marker expressions between M1- and M2-polarized cells, that no significant results were obtained for most markers. Our data support that M1 and M2 markers are upregulated by both polarizing conditions and also by protumoral stimuli. Of interest too is the fact that basal expression levels of these markers were very variable between monocyte types, in spite of a highly similar starting differentiation stage. We also observed a high level of variation within the same type of monocyte. We confirmed that variability after multiple series of experiments, in which two conditions were compared first, and then more polarizing conditions were added, plus PMA alone to address whether PMA was a major trigger of M1/M2 marker expression and thus responsible for the ambiguity of the data. We believe that marker upregulation reflects very reactive cellular stages already observed at basal culture conditions. Still, M1- or M2-polarizing conditions resulted in a more dramatic upregulation of marker expression.

Recent data support the notion that tumor progression results not only from genetic changes in the tumor cell itself, but also from the communication that it establishes with surrounding cells. The inflammatory microenvironment in which the tumor develops has been found to be critical

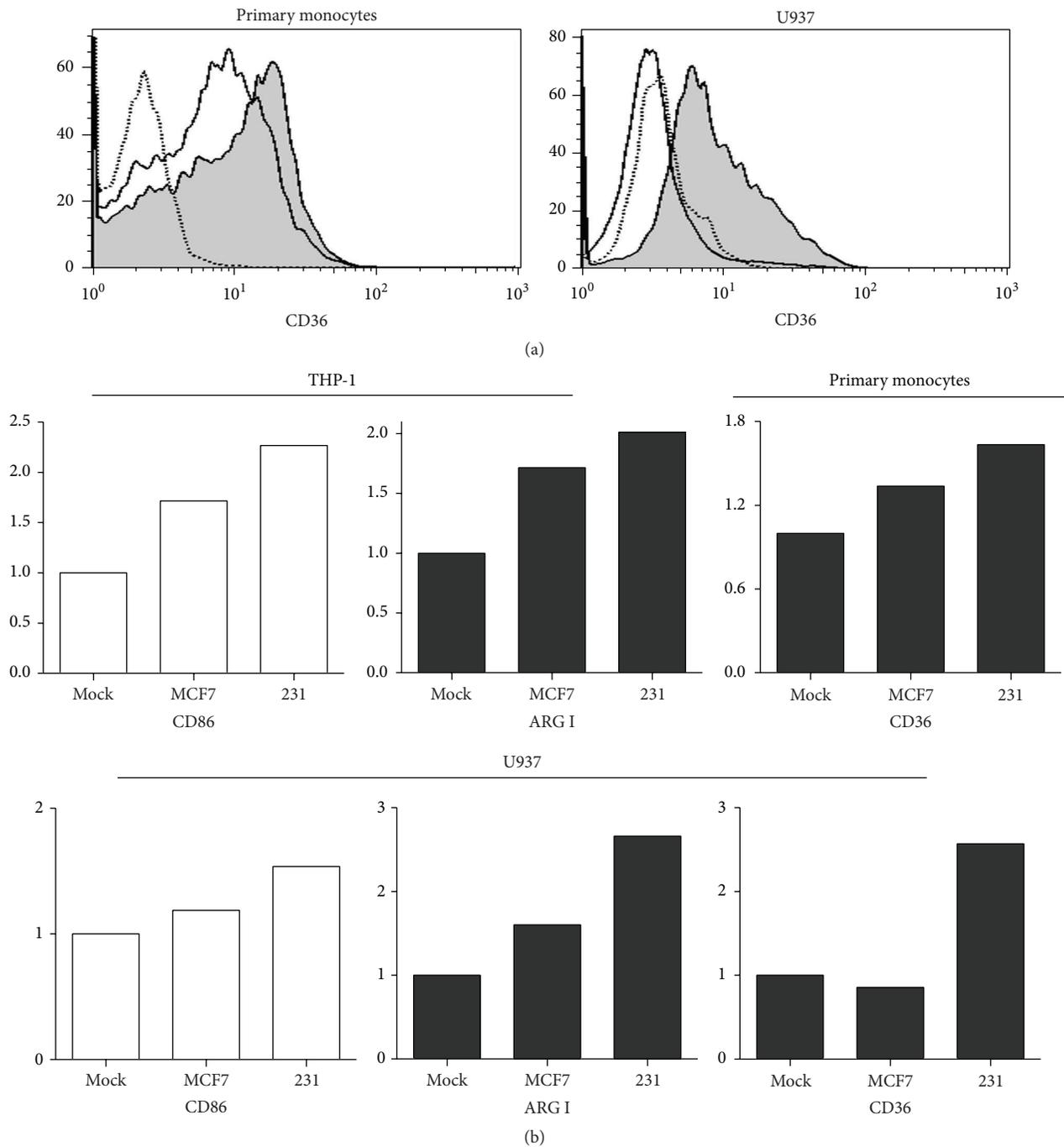


FIGURE 6: M1 and M2 polarization induced by breast cancer cell lines. After treatment of all three types of monocytes with either MCF-7 or MDA-MB-231 conditioned media, cells were harvested and the panel of M1/M2-related markers was analyzed by flow cytometry. In (a) the result for M2-related marker CD36 in primary monocytes and U937 cells is depicted: dotted line histograms represent autofluorescence control and straight line and shaded histograms correspond to stimulation with MCF-7 and MDA-MB-231 conditioned media, respectively. In (b) the autofluorescence was used to normalize marker expression, a value of 1 to the level of autofluorescence, and expression after treatment with conditioned media was normalized dividing by this basal value. Fold changes are depicted and the markers represented are only those with changes  $\geq 1.5$ -fold. White bars represent M1 markers and black bars M2 markers.

for tumor initiation and growth [36]. In breast cancer, macrophages are particularly enriched at the invasive front and in the vascular areas of the tumor, possibly facilitating tumor invasion and metastasis [37]. Because M2

macrophages participate in tissue maintenance, increasing cell survival and proliferation and tissue angiogenesis, they are thought responsible for those protumoral activities [38]. The above observations support a model in which the tumor

microenvironment actively recruits peripheral monocytes promoting their polarization into M2 macrophages and thus coaxing them to perform functions more attuned to the tumor needs [16]. In agreement, a meta-analysis showed that in >80% of breast cancer patients an elevated tumor macrophage density correlates with poor prognosis [39].

It is presently unclear how tumor and macrophages communicate to establish the tumor-promoting conditions. Inflammatory mediators and inflammatory targets with protumor activities have been described. It is thought that protumoral macrophages favor tumor growth through secretion of interleukins, chemokines, growth factors, angiogenic factors, proteases, and immunomodulatory molecules [40, 41]. We have previously observed that an aggressive breast cancer cell line engaged monocytes to express inflammatory mediators COX-2 and prostaglandin E2 and metalloproteinases, which rendered tumor and monocyte cocultures with an increased proteolytic capacity of ECM components. In this study, we found that M1-polarizing conditions result in higher expression/secretion of inflammatory mediators than M2-polarizing conditions. Only EGF secretion was found favored in M2-polarizing conditions, while IL-1 $\beta$ , IL-8, RANTES, G-CSF, MCP-1, IFN- $\alpha$ 2, IL-12p70, and GM-CSF were all favored in M1 conditions. This result agrees that chronic stimulation with LPS and IFN- $\gamma$  is more suitable to establish highly inflammatory stages than those promoted by IL-4 and IL-13 or that monocytes are more reactive to them. It is easy to envision how this inflammatory admixture may contribute to tumor initiation and progression. For instance, clinical studies have found that RANTES promotes progression of the most aggressive triple negative breast cancers [42]. RANTES and MCP-1 belong to the same CC chemokine family and coexpression of RANTES and MCP-1 has been observed in advanced human breast cancers [43]. Concomitant expressions of RANTES and IL-1 $\beta$  have also been observed in breast cancer relapsing patients [44]. These data support important protumoral functions by this mix of inflammatory components arguing that M1-polarizing conditions can also be critical to shape a microenvironment supporting of tumor growth.

Interestingly, although we observed an increased expression/secretion of inflammatory mediators after monocytes were subjected to M1-polarizing conditions, the resulting polarized cells did not match an M1 or M2 exclusive phenotype. Moreover, conditioned media from tumor cells did not result in formation of macrophages with a clearly defined M2 phenotype, an experimental condition with which we have previously coaxed monocytes to perform protumoral activities. Contrary to many immune cells that are terminally differentiated through extensive epigenetic modifications, monocytes/macrophages are highly plastic cells that remain responsive to environmental signals even after polarization into a specific subtype [45]. Since Mills and colleagues pioneering studies [3], it was clear that the M1 and M2 classification was better helpful to explain different metabolic responses than effector functions. Mosser and Edwards [4] stated that macrophages may only exist in a spectrum of different phenotypes and functions in which perfectly separable M1 and M2 subtypes may only exist at the opposite ends. In

agreement, monocyte polarization with a plethora of different signals generated macrophages with at least nine different transcriptional programs [46]. Moreover, TAMs isolated from the MMTV-PyMT murine breast cancer model did not show a transcriptional program related to either M1 or M2 subtypes, and instead a Notch signaling fingerprint was found [47]. Although the existence of protumoral macrophages is unquestionable, their associated phenotypes and the precise conditions driving their formation are still an area in need of extensive research. Importantly, our data support that the macrophage-tumor cooperation may need a mix of inflammatory and anti-inflammatory stimuli together with a mix of M1 and M2 activities, and this has critical implications in the M2-to-M1 reverse polarization as a therapeutic option.

### Competing Interests

The authors declare that the grant, scholarship, and/or funding mentioned in Acknowledgments did not lead to any competing interests. Additionally, the authors declare that there are no competing interests regarding the publication of this paper.

### Authors' Contributions

G. Karina Chimal-Ramírez and Nancy Adriana Espinoza-Sánchez performed the experiments; Luis Chávez-Sánchez helped with monocyte polarization assays and with the phenotypification of monocytes/macrophages; Lourdes Arriaga-Pizano provided support with flow cytometry; Ezequiel M. Fuentes-Pananá designed and supervised the experiments; G. Karina Chimal-Ramírez and Ezequiel M. Fuentes-Pananá wrote the paper; and Nancy Adriana Espinoza-Sánchez, Lourdes Arriaga-Pizano, and Luis Chávez-Sánchez gave critical reviews of the paper.

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## Research Article

# Prolactin Rescues Immature B-Cells from Apoptosis Induced by B-Cell Receptor Cross-Linking

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Prolactin has an immunomodulatory effect and has been associated with B-cell-triggered autoimmune diseases, such as systemic lupus erythematosus (SLE). In mice that develop SLE, the PRL receptor is expressed in early bone marrow B-cells, and increased levels of PRL hasten disease manifestations, which are correlated with a reduction in the absolute number of immature B-cells. The aim of this work was to determine the effect of PRL in an *in vitro* system of B-cell tolerance using WEHI-231 cells and immature B-cells from lupus prone MRL/lpr mice. WEHI-231 cells express the long isoform of the PRL receptor, and PRL rescued the cells from cell death by decreasing the apoptosis induced by the cross-linking of the B-cell antigen receptor (BCR) as measured by Annexin V and active caspase-3. This decrease in apoptosis may have been due to the PRL and receptor interaction, which increased the relative expression of antiapoptotic Bcl-xL and decreased the relative expression of proapoptotic Bad. In immature B-cells from MRL/lpr mice, PRL increased the viability and decreased the apoptosis induced by the cross-linking of BCR, which may favor the maturation of self-reactive B-cells and contribute to the onset of disease.

## 1. Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease that may affect any organ or system in the organism [1, 2]. It is characterized by the presentation of a defect in the tolerance mechanisms (central and peripheral) that give rise to self-reactive T- and B-cell clones, both in patients and in mice that develop SLE [3, 4]. Serum samples from SLE patients characteristically have strong reactivity to a broad spectrum of nuclear components, including DNA, RNA, histones, RNP, Ro, and La. These antibodies form immune complexes that are deposited in the kidneys and may cause proteinuria and kidney failure [5]. SLE is considered a multifactorial disease in which genetic, immunologic,

environmental, and hormonal aspects have a close interaction in the development of the disease. SLE incidence is higher in women than in men, and it increases after puberty and decreases after menopause. The severity of SLE also increases during pregnancy [6, 7] and high serum concentrations of PRL correlate with SLE activity [8, 9]. Therefore, the presence of sexual hormones, such as prolactin (PRL), has been associated with this disease [10–12]. In SLE murine models (NZB × NZW and MRL/lpr), the disease activity is exacerbated after induction of hyperprolactinemia, and increased PRL serum levels correlate with the early detection of autoantibodies, proteinuria, and accelerated death [13, 14]. PRL has different functions (over 300) that depend on the type of cell in which its receptor is expressed. There are

4 known PRL isoforms in mice (one long and three short isoforms) [15, 16]. The isoforms present in the extracellular domain are identical, but they differ in size and composition in the intracellular domain. The signaling pathway depends on the isoform that is expressed [17]. Similarly, the PRL receptor is distributed in different cell types, including cells of the immune system [18, 19]. PRL has been implicated as a modulator of both cellular and humoral immunity [20–22].

It has been reported that different maturation stages of B-cells in bone marrow (pro-B, pre-B, and immature) and in the spleen (transitional, marginal zone, and follicular B-cells) express the PRL receptor in mice. However, the expression of the receptor is higher in mice that develop SLE before presenting manifestations of the disease, and the pattern of receptor expression during B-cell development is different in SLE mice from that in mice that do not develop SLE. Additionally, the increase in the PRL serum levels in mice with SLE correlates with a decrease in the absolute numbers of immature and an increase in transitional-1 B-cells, stages that represent important checkpoints for the elimination of self-reactive clones [14, 23].

One of the mechanisms of central tolerance for the elimination of self-reactive clones is clonal deletion, which consists of elimination by apoptosis of immature B-cells that recognize self-antigens with high affinity [24, 25]. To better understand this mechanism, the murine WEHI-231 immature B-cell line has been used as a model to study apoptosis induced by the cross-linking of the B-cell antigen receptor (BCR) [26, 27].

The aim of this work was to determine the effect of PRL in an *in vitro* model of B-cell tolerance. We found that WEHI-231 cells express the long isoform of the PRL receptor and the presence of PRL rescued WEHI-231 cells from apoptosis-mediated cellular death induced by the cross-linking of BCR. The enhanced survival of WEHI-231 cells correlated with increasing the relative expression of antiapoptotic Bcl-xL and decreasing the expression of proapoptotic Bad. In immature B-cells derived from MRL/lpr mice, PRL also increased the viability and decreased apoptosis induced by BCR cross-linking. Taking together our observations in the *in vitro* model of tolerance and in the lupus prone mice, PRL may favor the maturation of self-reactive B-cell clones and contribute to the onset of disease.

## 2. Materials and Methods

**2.1. Cells.** WEHI-231 cells were derived from a B-cell lymphoma in F1 mice (BALB/c × NZB) and were donated by Dr. Leopoldo Santos' laboratory (CINVESTAV, Mexico). The cells were grown in RPMI medium (Hyclone, Utah, USA) supplemented with 10% fetal bovine serum (SFB, Biowest, Riverside, USA), 2-mercaptoethanol (Sigma, St. Louis, USA), and antibiotics (Invitrogen, CAU, USA) at 37°C in 5% CO<sub>2</sub>.

**2.2. Mice.** All studies were approved by the Animal Care Committee of the Instituto Nacional de Enfermedades Respiratorias "Ismael Cosío Villegas" and the Hospital de Pediatría, Centro Médico Nacional Siglo XXI, IMSS (R-2015-785-037),

and all the mouse measurements were in accordance with approved guidelines established by Mexico (Norma Oficial Mexicana NOM-062-ZOO-1999) and the NIH *Guide for the Care and Use of Laboratory Animals*. MRL/MpJFAS<sup>lpr</sup> (MRL/lpr) mice were purchased from the Jackson Laboratory (Maine, USA), and C57BL/6 mice were purchased from Harlan (Indianapolis, USA). Mice were housed in a pathogen-free barrier facility and were provided with sterile food and water *ad libitum*.

**2.3. Hormones.** Recombinant PRL from mice (National Hormone and Peptide Program, NIH) was used.

**2.4. Antibodies.** The following antibodies were used: PECy5-conjugated anti-mouse CD19 (eBio1D3), FITC-conjugated anti-mouse CD43 (eBioR2160), APC-conjugated anti-mouse IgM (11/41), PE-Cy7-conjugated anti-mouse CD23 (B3B4), and PE-conjugated anti-mouse CD93 (AA4.1) antibodies from eBioscience (California, USA); PE-conjugated anti-mouse B220 (RA3-6B2) antibody from BioLegend (California, USA); goat anti-mouse PRL-receptor (PRL-R) (E20) antibody from Santa Cruz Biotechnology (California, CA, USA); and swine anti-goat-biotinylated antibody from Invitrogen (California, USA). The biotinylated secondary antibody was detected with streptavidin-phycoerythrin-Cy5 from eBioscience. The anti-IgM Fab biotinylated and anti-IgM F(ab')<sub>2</sub> antibodies were from Jackson ImmunoResearch (Pennsylvania, USA).

**2.5. Cell Sorting Using WEHI-231 Cells.** WEHI-231 cells were incubated with fluorescently labeled antibodies specific for CD43, CD19, IgM, CD23, and goat anti-mouse PRL receptor in staining buffer (PBS with 0.5% BSA) for 20 minutes at 4°C. To select live cells, cells were incubated with DAPI, which marks dead cells (DAPI<sup>+</sup>). The cells were washed and isolated according to the expression of the following surface markers: CD43<sup>-</sup>, CD23<sup>-</sup>, CD19<sup>+</sup>, IgM<sup>+</sup>, PRL receptor<sup>+</sup>, and DAPI<sup>-</sup> for live cells. Cell sorting was performed using a FACS Aria sorter with FACSDiva software (BD Biosciences, California, USA). The purity of the sorted cells ranged from 95% to 98%. For the experiments in which the effect of PRL was tested, cells were cultured in TexMACS medium (Miltenyi Biotec, Bergisch Gladbach, Germany) free of serum, supplemented with 2-mercaptoethanol and antibiotics at 37°C in 5% CO<sub>2</sub>.

**2.6. Cell Sorting Using Immature B-Cells.** Bone marrow (BM) cells were collected by flushing femoral shafts with cold RPMI supplemented with 2% bovine serum albumin (BSA, US Biological, Swampscott, MA, USA) and 2 mM EDTA (IBI Scientific, USA). After depleting red blood cells using lysis buffer (Sigma-Aldrich, St. Louis, Missouri, USA), the cells were incubated with anti-B220 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), and the B-cells were isolated using positive selection with a magnetic activated cell-sorting (MACS) system (Miltenyi Biotec, Bergisch Gladbach, Germany). Single-cell suspensions of B220<sup>+</sup> B-cells from BM were incubated with fluorescently labeled antibodies specific for CD43, B220, IgM, and CD23 in staining buffer (PBS with

0.5% BSA) for 20 minutes at 4°C, and cells were incubated with DAPI to select live cells (DAPI<sup>-</sup>). The cells were washed, and the immature B-cells were isolated according to the expression of the following surface markers: B220<sup>+</sup>, CD43<sup>-</sup> CD23<sup>-</sup>, IgM<sup>+</sup>, and DAPI<sup>-</sup>. Cell sorting was performed using a FACS Influx Sorter (BD Biosciences). The purity of the sorted cells ranged from 95% to 98%.

**2.7. Real-Time PCR.** Total RNA was extracted from B-cells using TRIzol reagent (Invitrogen, California, USA) according to the manufacturer's protocol, and the RNA concentration was determined using UV spectrophotometry. Total RNA (0.5 µg) was used to generate cDNA with SuperScript II reverse transcriptase (Invitrogen, California, USA) according to the manufacturer's specifications. Genes of interest were amplified by real-time PCR using a LightCycler TaqMan Master kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's specifications and using hydrolysis probes and primers designed by Roche Diagnostics. The following primers were used: PRL receptor, 5'-CAGTAA-ATGCCACGAACGAA-3' (left) and 5'-GAGGAGGCT-CTGGTTCAACA-3' (right); PRL receptor large, 5'-AGC-AGTTCTTCAGACTTGCCCTT-3' (left) and 5'-AAGCCA-GACCATGGATACTGGAG-3' (right); PRL receptor short, 5'-TTGTATTTGCTTGACAGAGCCAGT-3' (left) and 5'-AAGCCAGACCATGGATACTGGAG-3' (right); Bcl-xL, 5'-GCATTGTTCCCGTAGAG-3' (left) and 5'-GGACCG-CGTATCAGAG-3' (right); Birc5 (survivin), 5'-CCCGAT-GACAACCCGATA-3' (left) and 5'-CATCTGCTTGAC-AGTGAGG-3' (right); Bad, 5'-GGAGCAACATTCATC-AGCAG-3' (left) and 5'-TACGAACTGTGGCGACTCC-3' (right); and β-actin, 5'-AAGGCCAACCCTGAAAAGAT-3' (left) and 5'-GTGGTACGACCAGAGGCATAC-3' (right). The final volume of the reaction was 10 µL, and a LightCycler instrument was used to perform the PCR reaction (Roche Diagnostics). The following PCR conditions were used: 15 minutes at 95°C, followed by 40 cycles of 10 seconds at 95°C, 30 seconds at 60°C, and 1 second at 72°C, and 1 cycle of cooling for 30 seconds at 50°C. The samples were normalized to the β-actin gene. The relative expression was calculated using the  $2^{-\Delta\Delta CT}$  formula.

**2.8. PCR Array.** The Mouse CAPM12814F RT<sup>2</sup> Profiler™ PCR Array (Qiagen, Hilden, Germany) was performed in 96-well plates following the manufacturer's recommendations using the RT<sup>2</sup> SYBR Green ROX qPCR Mastermix (Qiagen). We analyzed 12 genes of interest, 1 constitutive gene, and the following 3 controls to validate each sample: HGDC (DNA genomic control), TRC (retrotranscription efficiency), and PPC (presence of PCR inhibitors). The Mouse PAMM-012ZF RT<sup>2</sup> Profiler™ PCR Array (Qiagen, Hilden, Germany) was performed in 96-well plates following the manufacturer's recommendations using the RT<sup>2</sup> SYBR Green ROX qPCR Mastermix for apoptosis.

**2.9. Viability Assay.** WEHI-231 cells (PRL receptor<sup>+</sup>) and immature B-cells from MRL/lpr mice were incubated with PRL (50 ng/mL) for 1 hour before stimulating the cells with

anti-IgM F(ab')<sub>2</sub> (10 µg/mL) for 48 hours and 18 hours, respectively. Cells cultured with medium, PRL, or anti-IgM F(ab')<sub>2</sub> were used as controls. The cells were washed with PBS and incubated with Ghost-Red (Tonbo Biosciences, California, USA) for 30 minutes at 4°C; Ghost-Red was used to measure viability (live cells do not stain and remain Ghost-Red<sup>-</sup>). Data were acquired using a MACSQuant Analyzer 10 cytometer (Miltenyi Biotec) and analyzed with FlowJo software (Tree Star, Ashland, OR, USA).

**2.10. Apoptosis Assays.** WEHI-231 cells (PRL receptor<sup>+</sup>) and immature B-cells from MRL/lpr mice were incubated with PRL (50 ng/mL) for 1 hour before stimulating the cells with anti-IgM F(ab')<sub>2</sub> (10 µg/mL) for 48 hours and 18 hours, respectively. Cells cultured with medium, PRL, or anti-IgM F(ab')<sub>2</sub> were used as controls. The cells were washed with PBS and incubated with Ghost-Red (Tonbo Biosciences) for 30 minutes at 4°C. The Annexin V assay was performed following the manufacturer's instructions (BD Biosciences). For the caspase-3 assay, the cells were permeated with Cytofix/Cytoperm (BD Biosciences) for 1 hour at 4°C, and the cells were then washed with Perm/wash (BD Biosciences) and incubated with anti-caspase-3-FITC for 1 hour at 4°C. Data were acquired using a MACSQuant Analyzer 10 cytometer (Miltenyi Biotec) and analyzed with FlowJo software (Tree Star, Ashland, OR, USA).

**2.11. Statistical Analysis.** Data were analyzed with standard statistical tests (mean value, SD, Student's *t*-test, and ANOVA), and the results are expressed as the means ± SD. The level of significance was set at *p* < 0.05. All calculations were performed using SPSS 22 software.

### 3. Results

**3.1. PRL Receptor Expression in WEHI-231 Cells.** The expression of the PRL receptor in WEHI-231 cells was determined at both mRNA and protein levels. We first tested whether WEHI-231 cells express the PRL receptor by PCR using primers directed against the extracellular moiety of the receptor, common to all PRL receptor isoforms. After confirming PRL receptor expression (0.51 ± 0.05), primers directed against the intracellular portion of the receptor showed that the WEHI-231 cells only expressed the mRNA of the long isoform (0.51 ± 0.04), as shown in Figure 1(a). With regard to the protein levels, 47.50 ± 5.36% of the cells were positive for expression of the receptor on surface (Figure 1(b)). PRL receptor positive cells were sorted obtaining a 95–98% pure fraction in all cases (Supplementary Figure 1, in Supplementary Material available online at <http://dx.doi.org/10.1155/2016/3219017>). All experiments were carried out with the PRL receptor positive sorted population grown in RPMI medium supplemented with 10% of FBS, conditions in which PRL receptor positive cells always outgrow PRL receptor negative cells (Supplementary Figure 2). To test for PRL function, PRL receptor positive cells were incubated in TexMACS medium free of serum.

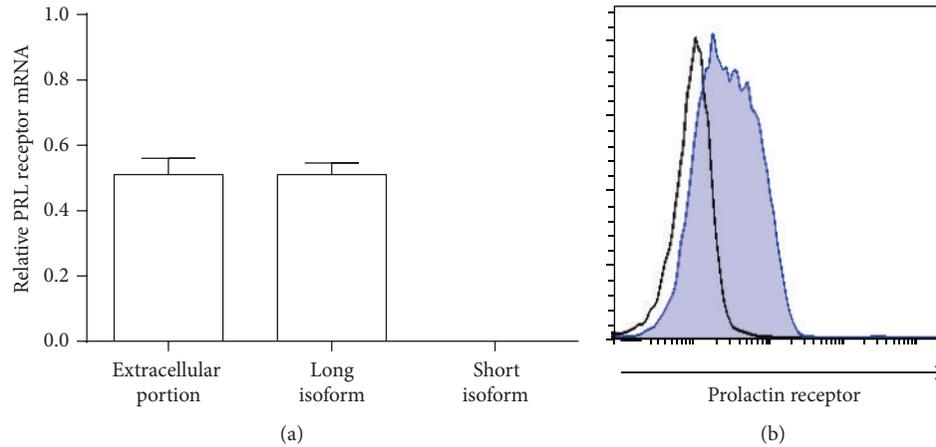


FIGURE 1: Expression of the PRL receptor. mRNA and protein expression levels of the PRL receptor in WEHI-231 cells. (a) The PRL receptor mRNA expression was measured by RT-PCR using primers specific for the extra- and intracellular domains of the receptor in WEHI-231 cells to allow recognition of the different isoforms (long and short). (b) The expression level of the receptor was measured by flow cytometry using goat anti-PRL receptor antibodies. The isotype control was an unrelated goat antibody.

**3.2. Characterization of WEHI-231 Cells.** The phenotype of the WEHI-231 cells was determined by flow cytometry. The WEHI-231 cells were positive for CD93, CD19, and IgM but negative for CD43 and CD23 corresponding to immature bone marrow B-cells (Figure 2). The relative expression of the genes related to the maturation of B-cells was determined by a PCR array, which analyzed the following genes: the constant region of the light kappa chain (Igkc); the Rag1 recombinase; the IL-1 receptor (IL7r); the light subrogated  $\lambda 5$  chain (IgII1); and the transcription factors Ikaros (Ikzf1), E2A (Tcf3), Pax5, Irf4, Foxo1, Stat5b, Ailos (Ikzf3), and Irf8. The results showed that WEHI-231 cells do not express genes that are important for the maturation stages of pro- and pre-B-cells, such as IL7r, Rag1, and IgII1, but the results showed that WEHI-231 cells did express *Ikzf1* ( $7.60 \times 10^{-5}$ ), *Tcf3* ( $2.23 \times 10^{-4}$ ), *Pax5* ( $4.28 \times 10^{-4}$ ), *Irf4* ( $1.30 \times 10^{-4}$ ), *Irf8* ( $1.67 \times 10^{-4}$ ), *Foxo1* ( $4.50 \times 10^{-5}$ ), *Ikzf3* ( $1.49 \times 10^{-4}$ ), and *Stat5b* ( $6.40 \times 10^{-5}$ ), as shown in Figure 3. These data argue that WEHI-231 cells are committed to the B-cell lineage expressing important transcription factors critical for lineage maintenance which are in a post-VDJ rearrangement stage. *Igkc* was negative implying that these cells express a BCR with lambda light chains.

**3.3. PRL Effect on Viability and Apoptosis of WEHI-231 Cells.** Immature B-cells are constantly being subjected to negative selection mechanisms to check whether their BCRs are directed against self-antigens. To measure how PRL influences the viability and apoptosis outcome of WEHI-231 cells, they were preincubated for 1 hour with PRL and for 48 hours with the anti-IgM F(ab')<sub>2</sub> antibody to induce cross-linking of the BCR, a step that mimics self-antigen recognition. The percentage of live and apoptotic cells was measured by flow cytometry. Cells that were incubated with anti-IgM F(ab')<sub>2</sub> showed a significantly decreased percentage of live cells ( $40.93 \pm 0.87\%$ ;  $p < 0.01$ ) compared to the cells incubated with medium ( $65.72 \pm 1.96\%$ )

or PRL ( $67.10 \pm 5.90\%$ ). However, cells that were preincubated with PRL and incubated with anti-IgM F(ab')<sub>2</sub> showed a significantly increased percentage of live cells ( $58.42 \pm 0.82\%$ ;  $p < 0.01$ ) compared to cells that were not preincubated with PRL, as well as a similar percentage of live cells to those incubated only with medium (Figure 4).

Apoptosis measurement was performed using two different parameters as follows: (1) Annexin V and Ghost-Red staining and (2) active caspase-3. The percentage of cells in early apoptosis (Annexin V<sup>+</sup> Ghost-Red<sup>-</sup>) and late apoptosis (Annexin V<sup>+</sup> Ghost-Red<sup>+</sup>) significantly increased ( $23.88 \pm 2.56$  and  $31.62 \pm 2.66\%$ ;  $p < 0.01$ ) for cells incubated with anti-IgM F(ab')<sub>2</sub> compared to cells incubated with medium ( $16.23 \pm 2.02$  and  $14.28 \pm 0.71\%$ ) or PRL ( $15.37 \pm 0.97$  and  $13.0 \pm 0.44\%$ ). A significant decrease ( $14.44 \pm 0.99$  and  $21.92 \pm 2.00\%$ ;  $p < 0.01$ ) was found in early and late apoptosis for cells preincubated with PRL and incubated with anti-IgM F(ab')<sub>2</sub> as compared to cells not preincubated with PRL (Figure 5).

In contrast, the percentage of cells with active caspase-3 significantly increased ( $50.76 \pm 1.35\%$ ;  $p < 0.01$ ) for cells incubated with anti-IgM F(ab')<sub>2</sub> as compared to cells incubated with medium ( $9.99 \pm 0.33\%$ ) or PRL ( $11.0 \pm 0.44\%$ ). The percentage of cells with active caspase-3 significantly decreased ( $29.50 \pm 1.93\%$ ;  $p < 0.01$ ) for cells preincubated with PRL and incubated with anti-IgM F(ab')<sub>2</sub> as compared to cells not preincubated with the hormone. The same differences were found when determining the mean intensity fluorescence (MIF) [(medium  $197.5 \pm 22.4$ ; PRL  $185.3 \pm 11.68$ ; anti-IgM F(ab')<sub>2</sub>  $342.2 \pm 35.9$ ; PRL 1 hour and anti-IgM F(ab')<sub>2</sub>  $292.3 \pm 11.4$ ] (Figure 6).

The expression of genes involved in apoptosis in WEHI-231 cells treated with PRL for 1 hour was determined by a PCR array, finding that PRL modulates the expression of several members of the Bcl2 family, suggesting that PRL specifically targets the intrinsic pathway of apoptosis. In this family, PRL decreased the expression of the proapoptotic *Bad* (0.22) gene and increased the expression of antiapoptotic genes *Bag3* (2.50), *Bcl2l1* (2.98), and *Bcl2l2* (3.22), besides decreasing

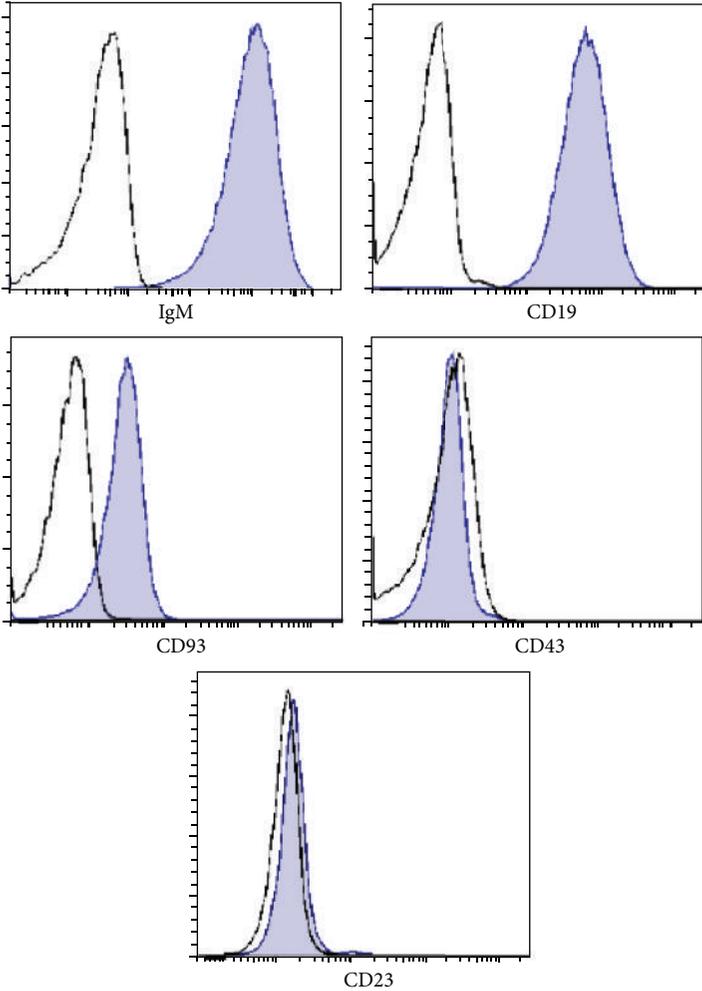


FIGURE 2: Phenotype of WEHI-231 cells. WEHI-231 cells were stained with Ghost-Red (viability marker) and the following antibodies to determine their phenotype: IgM, CD43, CD23, CD19, and CD93.

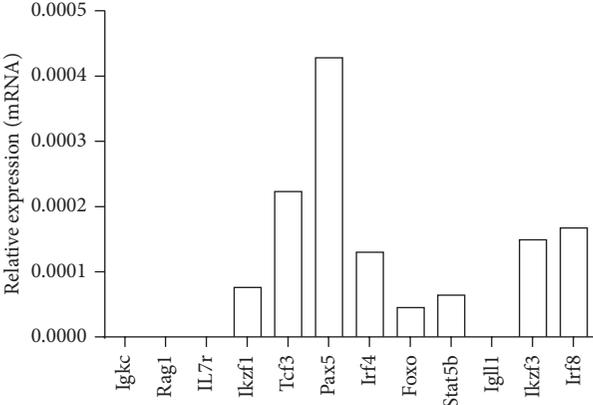


FIGURE 3: Expression of early development genes in WEHI-231 cells. RNA was extracted from WEHI-231 cells, and cDNA was obtained to determine the expression of genes related to the early development of B lymphocytes by a PCR array.

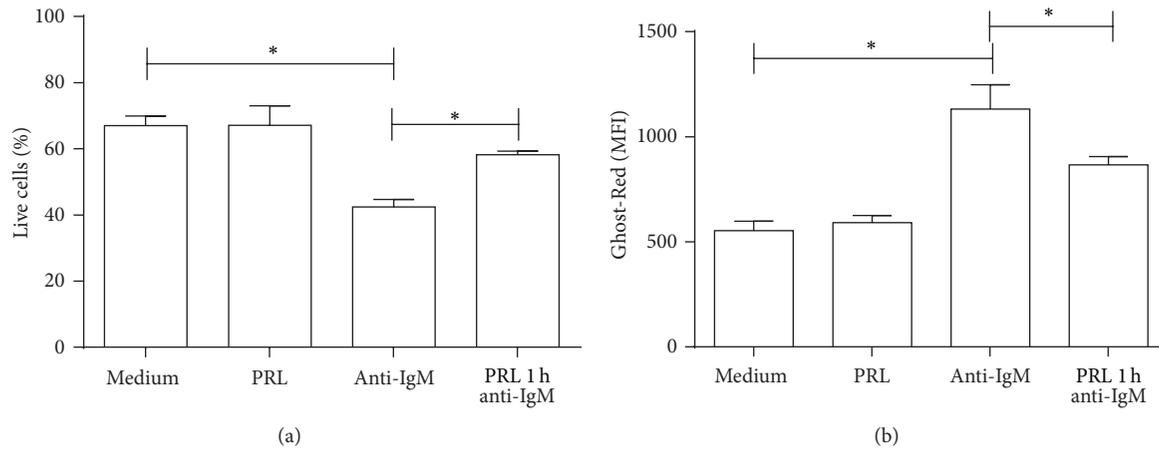


FIGURE 4: Viability of WEHI-231 cells. WEHI-231 cells were preincubated for 1 hour with PRL and incubated with anti-IgM F(ab')<sub>2</sub> antibody for 48 hours. Dead cells were stained with Ghost-Red. (a) Percentage of live cells. (b) Mean intensity of fluorescence (MFI), Ghost-Red. \* $p < 0.01$ .

the expression of *Casp3* (0.29) and *Casp9* (0.43) as shown in Figure 7(a) and in Supplementary Figure 3. The relative expression of some of these genes was confirmed by real-time PCR. PRL significantly increased the relative expression of *Bcl-xL* ( $2.07 \pm 0.30$ ) and decreased the expression of *Bad* ( $0.47 \pm 0.12$ ) as compared to cells incubated with medium alone ( $p < 0.01$ ). No change was observed in the *Birc5* gene ( $0.95 \pm 0.23$ ) (Figure 7(b)).

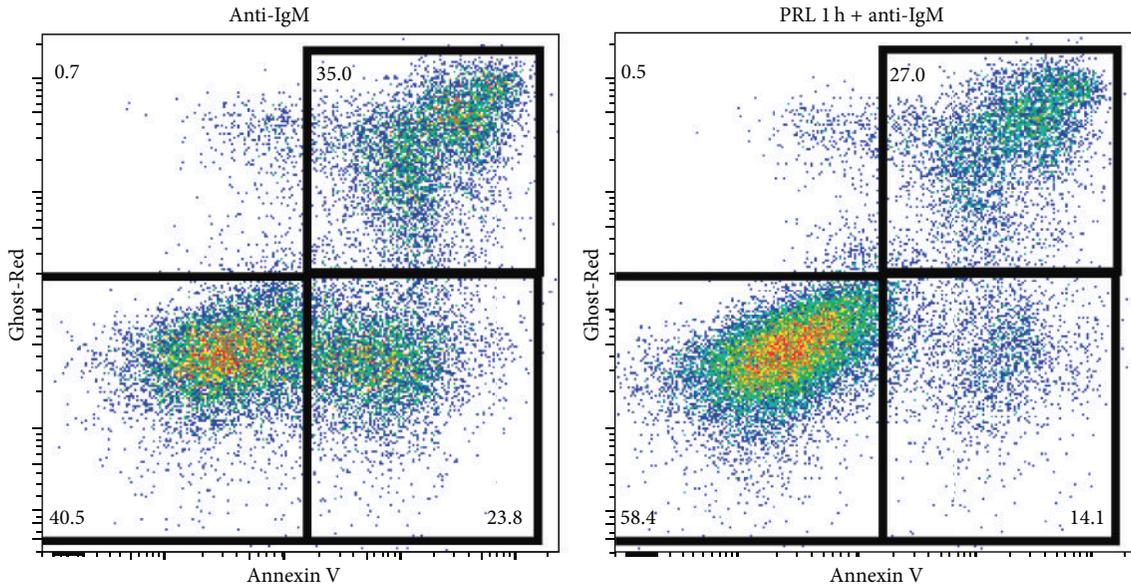
**3.4. PRL Affects Viability and Apoptosis of Immature B-Cells from MRL/lpr Mice.** To measure the effect of PRL on the viability and apoptosis of sorted immature B-cells from MRL/lpr and C57BL/6 mice, Ghost-Red was used to measure viability, and active caspase-3 was used to measure apoptosis. In C57BL/6 control mice, a slight but statistically significant decrease ( $p < 0.01$ ) in the viability of immature B-cells was observed when cells were incubated with anti-IgM F(ab')<sub>2</sub> ( $43.68 \pm 3.01\%$ ) as compared to cells incubated with medium ( $53.42 \pm 1.75\%$ ) or PRL ( $53.40 \pm 1.14\%$ ). However, no difference in the viability was observed in cells preincubated with PRL and incubated with anti-IgM F(ab')<sub>2</sub> ( $43.22 \pm 2.79\%$ ) as compared to cells not preincubated with the hormone ( $p = 0.7864$ ) (Figure 8(a)). On the contrary, more profound changes were observed in the MRL/lpr immature B-cells; in cells incubated with anti-IgM F(ab')<sub>2</sub> for 18 hours ( $25.40 \pm 1.27\%$ ), the percentage of live cells significantly decreased ( $p < 0.01$ ) as compared to cells incubated with medium ( $37.96 \pm 0.50\%$ ) or PRL ( $37.30 \pm 2.43\%$ ). Moreover, cells preincubated with PRL and incubated with anti-IgM F(ab')<sub>2</sub> showed a statistically significant increase in the percentage of live cells ( $41.10 \pm 2.26\%$ ) as compared to cells not preincubated with PRL ( $p < 0.01$ ) (Figure 8(c)).

A similar result was obtained when addressing apoptosis. The percentage of cells with active caspase-3 significantly increased ( $32.40 \pm 0.94\%$ ;  $p < 0.01$ ) for immature B-cells from C57BL/6 mice that were incubated with anti-IgM F(ab')<sub>2</sub> as compared to those incubated with medium ( $26.43 \pm 0.87$ )

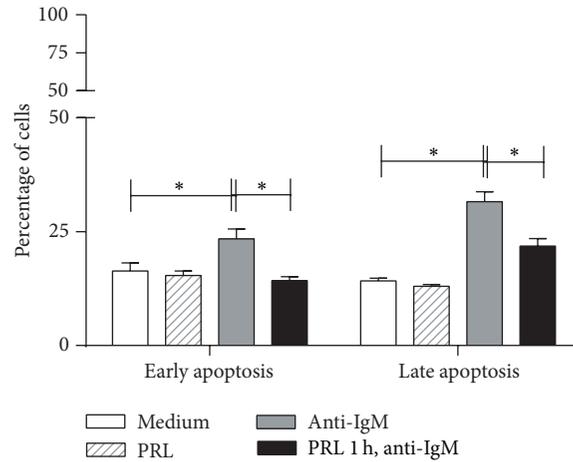
or PRL ( $26.47 \pm 0.70\%$ ). However, no statistically significant difference ( $p = 0.2497$ ) was found in the percentage of apoptotic cells when these cells were preincubated with PRL and incubated with anti-IgM F(ab')<sub>2</sub> ( $30.98 \pm 2.61\%$ ) as compared to those not preincubated with PRL (Figure 8(b)). However, for MRL/lpr immature B-cells, the percentage of cells with active caspase-3 significantly increased ( $49.65 \pm 0.64\%$ ;  $p < 0.01$ ) for cells incubated with anti-IgM F(ab')<sub>2</sub> as compared to cells incubated with medium ( $37.80 \pm 0.57\%$ ) or PRL ( $30.98 \pm 7.39\%$ ). Moreover, the percentage of cells with active caspase-3 ( $34.75 \pm 1.91$ ) significantly decreased for cells preincubated with PRL and incubated with anti-IgM F(ab')<sub>2</sub> compared to cells not preincubated with PRL (Figure 8(d)).

## 4. Discussion

During the maturation of B-cells, elimination of autoreactive clones in bone marrow immature B-cells is a central control of tolerance, a mechanism that serves to avoid humoral self-responses [28–30]. Lack of elimination of B-cell clones with autoreactive BCRs favors the development of autoimmune diseases, such as SLE [3, 4]. In previous studies, we have demonstrated that, in mice that develop SLE, an increase in the serum levels of PRL decreases the absolute number of immature B-cells and increases transitional-1 cells in the spleen, correlating with the exacerbation of the disease [14, 23]. We consider that such observation could be explained by accelerated exit of bone marrow immature B-cells and increased arrival of B-cells to secondary lymphoid organs and that the tolerance mechanisms operating on immature B-cells could be compromised. Thus, the aim of this work was to determine whether PRL can rescue immature B-cells from apoptosis induced by the cross-linking of BCR. We first used the murine WEHI-231 cells, an *in vitro* model of immature cells widely used to study BCR mediated apoptosis, and then we corroborated the *in vitro* results using immature B-cells isolated from MRL/lpr mice, a mouse model of SLE-like disease.



(a)



(b)

FIGURE 5: Apoptosis of WEHI-231 cells as measured by Annexin V. WEHI-231 cells were preincubated for 1 hour with PRL and then incubated with an anti-IgM F(ab')<sub>2</sub> antibody for 48 hours. The cells were stained with Ghost-Red and Annexin V-FITC to measure early apoptosis (Annexin V<sup>+</sup> Ghost-Red<sup>-</sup>) and late apoptosis (Annexin V<sup>+</sup> Ghost-Red<sup>+</sup>). (a) Dot-Plot representative of early and late apoptosis. (b) Percentage of cells in apoptosis. \* *p* < 0.01.

Our results authenticated the notion that mouse WEHI-231 cells have the phenotype of immature B-cells. Moreover, these cells do not express genes that are exclusive of pro-B and pre-B-cells, such as IL7r, Rag1, and Igl11 [31, 32], but they express transcription factors that together with BCR signaling are critical for B-cell lineage commitment and maintenance [33–36]. Our results showed, for the first time, that this cell line expresses the PRL receptor similar to immature B-cells from C57BL/6, MRL, and MRL/lpr mice [14, 23]. In addition, the cells expressing the PRL receptor had better growth than those not expressing the PRL receptor when the cells were separated by the expression of the receptor (PRL receptor<sup>+</sup> and PRL receptor<sup>-</sup>). This result may be attributed to the receptor potentially serving as a growth

factor as reported in mouse B-cell hybridomas [37], or this result may be due to increased expression of antiapoptotic genes.

Different isoforms of the PRL receptor have been reported. In humans, the long isoform has been shown to be involved in the progression and metastasis of breast cancer, promoting the proliferation and viability of cancerous cells; the short isoform has been associated with antiproliferative and proapoptotic effects [38–40]. Our results indicate that WEHI-231 cells only express the mRNA for the long isoform of the PRL receptor. PRL modulates the expression of genes from the Bcl2 family that participate as part of the intrinsic pathway of apoptosis, which correlated with decreased apoptosis induced after cross-linking of the BCR. This provides for

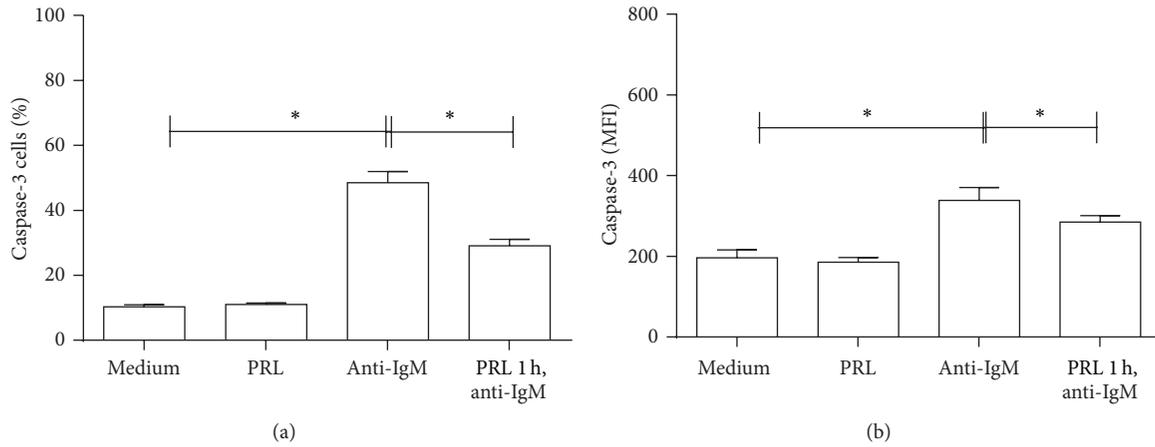


FIGURE 6: Apoptosis of WEHI-231 cells as measured by active caspase-3. WEHI-231 cells were preincubated for 1 hour with PRL and incubated with anti-IgM F(ab')<sub>2</sub> antibody for 48 hours. The cells were stained with Ghost-Red and active caspase-3-FITC to determine apoptosis. (a) Percentage of caspase-3+ cells. (b) IMF of caspase-3. \**p* < 0.01.

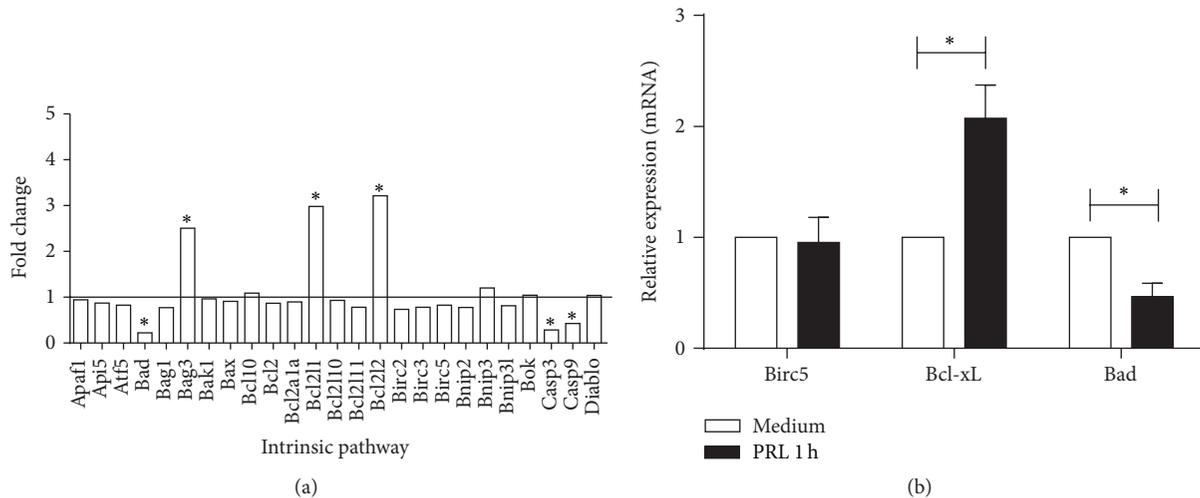


FIGURE 7: Expression of apoptotic genes modulated by PRL. WEHI-231 cells were incubated for 1 hour with PRL, and the expression of apoptosis genes was determined by (a) PCR array (mouse apoptosis) and (b) real-time PCR. \**p* < 0.01.

a potential mechanism of rescuing self-reactive clones from clonal deletion.

Our results and others indicate that PRL protects cells from apoptosis when challenged with different stimuli, an effect in which increasing the expression of antiapoptotic genes of the intrinsic pathway of apoptosis probably has a central role. Prolactin-treated spleen B-cells from B6.Sle3 mice were more resistant to apoptosis in [41]; PRL protected Nb2 cells from apoptosis mediated by dexamethasone through the expression of the Bcl-xL gene [42]; and in breast cancer cells, PRL increased the mRNA and protein expression of Bcl2 [43]. Other studies support the notion that the Jak/Stat signaling pathway modulates the expression of apoptotic genes from the Bcl2 family [44–46]. In an arthritis model, it has been discovered that the Jak2/Stat3 pathway activates the transcription of antiapoptotic genes, such as Bcl2, and rescues chondrocytes from apoptosis [47]. Our studies demonstrated

that PRL increases the expression of *Stat5b* in WEHI-231 cells (Supplementary Figure 4). This suggests that the interaction of PRL with the long isoform of the receptor expressed by the immature B-cells signals through the Stat5b pathway, modulating several Bcl2 family members from the intrinsic pathway of apoptosis to rescue the cells from death. However, it is necessary to perform more experiments to determine the signaling pathway of the long isoform of the PRL receptor in immature B-cells.

In other autoimmune diseases, such as arthritis and multiple sclerosis, it has been described that PRL increases the expression of antiapoptotic genes, such as Bcl2, and decreases the expression of proapoptotic genes, such as Trp63 and Bax, suggesting that this hormone may favor the progression of the disease [47, 48]. Our results showed that PRL promotes the viability of immature B-cells that should be subjected to negative selection, rescuing them from apoptosis, both

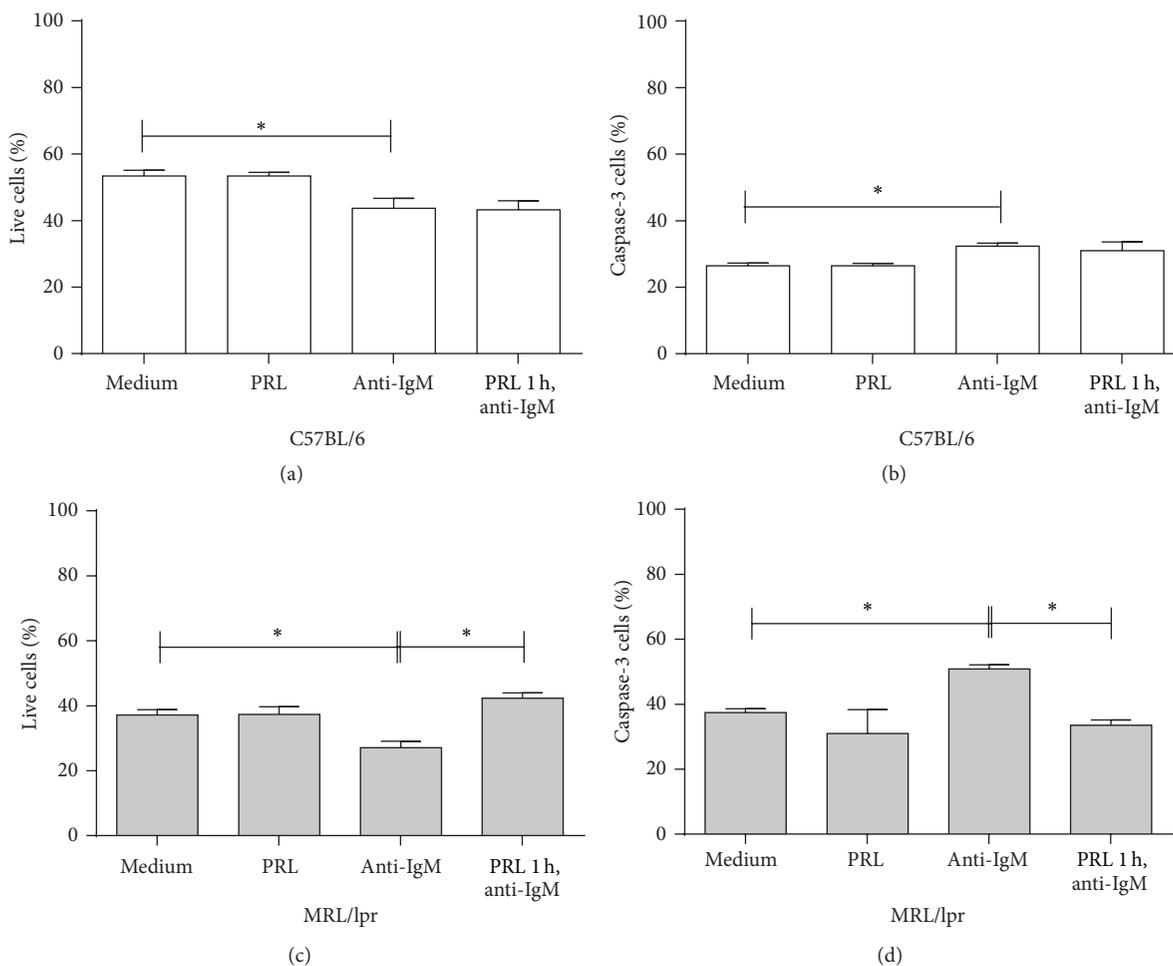


FIGURE 8: Effect of PRL on the viability and apoptosis of immature B-cells. Sorted immature B-cells from C57BL/6 or MRL/lpr mice were preincubated for 1 hour with PRL and incubated with anti-IgM F(ab')<sub>2</sub> antibody for 18 hours. The cells were stained with Ghost-Red and active caspase-3-FITC to determine apoptosis. (a) Percentage of live cells in C57BL/6 mice. (b) Percentage of caspase-3+ cells in C57BL/6 mice. (c) Percentage of live cells in MRL/lpr mice. (d) Percentage of caspase-3+ cells in MRL/lpr mice. \*  $p < 0.01$ .

in a cell line and in a model of SLE (MRL/lpr). PRL may prevent apoptosis of bone marrow immature B-cell clones that recognize self-antigens (potentially autoreactive clones), which may allow maturation of autoreactive B-cell clones, thus increasing the risk of developing autoimmune diseases. These results, together with our previous observations in *in vivo* studies, indicate an important effect of PRL on B-cell maturation and the development of the disease.

## 5. Conclusions

WEHI-231 cells express the long isoform of the PRL receptor associated with induction of resistance to apoptosis. In these cells, PRL modulates the expression of genes from the intrinsic pathway of apoptosis increasing the relative expression of Bcl-xL (antiapoptotic gene) and decreases the expression of Bad (proapoptotic gene), which may prevent the apoptosis of these cells induced by the cross-linking of BCR. Furthermore, PRL increases the viability of immature

B-cells by rescuing them from apoptosis (through BCR cross-linking) preferentially in cells from mice that developed SLE (MRL/lpr). These results suggest that PRL may favor the maturation of self-reactive clones, thus allowing the onset of autoimmune diseases.

## Disclosure

Rocio Flores Fernández is a doctoral student from Programa de Doctorado en Ciencias Biomédicas, Universidad Nacional Autónoma de México, and received Fellowship 472811 from CONACYT and IMSS (99096704).

## Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

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## Review Article

# The Emerging Role of HMGB1 in Neuropathic Pain: A Potential Therapeutic Target for Neuroinflammation

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Neuropathic pain (NPP) is intolerable, persistent, and specific type of long-term pain. It is considered to be a direct consequence of pathological changes affecting the somatosensory system and can be debilitating for affected patients. Despite recent progress and growing interest in understanding the pathogenesis of the disease, NPP still presents a major diagnostic and therapeutic challenge. High mobility group box 1 (HMGB1) mediates inflammatory and immune reactions in nervous system and emerging evidence reveals that HMGB1 plays an essential role in neuroinflammation through receptors such as Toll-like receptors (TLR), receptor for advanced glycation end products (RAGE), C-X-X motif chemokines receptor 4 (CXCR4), and N-methyl-D-aspartate (NMDA) receptor. In this review, we present evidence from studies that address the role of HMGB1 in NPP. First, we review studies aimed at determining the role of HMGB1 in NPP and discuss the possible mechanisms underlying HMGB1-mediated NPP progression where receptors for HMGB1 are involved. Then we review studies that address HMGB1 as a potential therapeutic target for NPP.

## 1. Introduction

Pain is a type of physical experience defined as an unpleasant sensory and mental problem resulting from actual or potential tissue damage, or something that makes people uncomfortable (International Association for the Study of Pain) [1]. Thus, pain is a multidimensional and subjective experience. Peripheral tissue injury or inflammation can cause reversible adaptive changes in the sensory nervous system, such as hyperalgesia, that provide a protective role against further nociceptive stimuli, leading to the promotion of wound healing and the subsidence of inflammation [2], while neuropathic pain (NPP) is intolerable, persistent, and specific type of long-term pain. NPP is considered to be a direct consequence of pathological changes affecting the somatosensory system and can be debilitating in affected patients [3, 4]. NPP is relatively common, occurring in about 8% of

the population, and can impact on the patient's health and many aspects of their quality of life [5, 6]. In the USA, pain severity in patients suffering from NPP significantly correlated with NPP-related issues such as health care resource utilization, personal productivity, and costs [7].

NPP is not a disease, but a syndrome manifested by common and less common signs and symptoms [8]. NPP may originate from the central nervous system (CNS) or periphery and is characterized by both spontaneous and provoked pain, as well as by paresthesia, dysesthesia, and deficits in normal sensation reflecting nerve damage [9]. An innocuous irritation can lead to pain, but it has been determined that the duration and the extent of the response to stimuli can magnify the pain, indicating that the threshold of NPP falls dramatically with pain progression [10]. In general, NPP has a peripheral origin, arising as a consequence of peripheral nerve injury or as a consequence of a metabolic disease

TABLE 1: The cardinal etiologies of neuropathic pain.

Classification	Frequent etiology	Reference
Disease process	Infection/inflammation, immunity, tumor infiltration, metabolic abnormalities, neurotoxicity	[2, 11, 14–16]
Therapeutic intervention	Surgery, chemotherapy, irradiation	[17, 18]
Trauma	External injury, nerve compression	[19–21]
Genetic predisposition	Inherited neurodegeneration, metabolic abnormalities, endocrine abnormalities	[22, 23]

such as diabetes [11]. However, NPP can also be caused by infectious diseases such as postherpetic neuralgia and can be manifested in disorders of various etiologies such as spinal cord injury, local poststroke ischemia, cancer, and complex regional pain syndrome [1, 4, 12, 13] (Table 1).

Accumulating evidence indicates that NPP is not merely a concept, or a single disorder, or neural tissue damage caused by a simple nociception. Indeed NPP is considered to be an evolving collection of established clinical and experimental conditions that share the prolongation of manifested pain symptoms or pain-related behaviors [24]. Abnormalities in neural activity predictably play essential roles in the spontaneous pain typical of NPP states [24]. Although the pathogenesis of NPP has been intensively studied in recent decades, the underlying mechanisms remain to be clarified and currently there are no effective therapies for NPP. Thus, identification of the novel molecular machinery involved in NPP may lead to the development of promising analgesics and is therefore eagerly anticipated.

Recently, high mobility group protein box 1 (HMGB1) was identified as an important inflammatory mediator in various diseases caused by an abnormally enhanced inflammatory immune response [25]. Furthermore, mounting evidence supports the hypothesis that HMGB1 plays an essential role in the pathology of NPP progression. Here, we discuss the evidence from studies that address the role of HMGB1 in NPP. We firstly review studies concerning the role of HMGB1 in NPP and discuss the possible mechanisms underlying HMGB1-mediated NPP progression. Then, we review the evidence indicating that HMGB1 is a potential therapeutic target for NPP.

## 2. HMGB1

In 1973, Goodwin discovered a group of nonhistone nuclear proteins that were characterized by high electrophoretic mobility and called them high mobility group (HMG) proteins. These proteins include three superfamilies designated as HMGB, HMGN, and HMGA [26–28]. HMGB1 (also known as amphoterin) is the most abundant and well-studied HMG protein, which has been highly conserved in evolution and is ubiquitously expressed in most cell types [29–31]. HMGB1 consists of 215 amino acids encoded by a gene on human chromosome 13q12-13. HMGB1 has two positively charged domains (box A and box B) that are involved in binding to the nucleosome structure and govern

gene expression via combination with transcription elements (Figure 1(a)).

Investigations revealed that HMGB1 isoforms in different redox states are involved in activating immune cells [32, 33]. These redox states result from posttranslational modifications dependent on three cysteines at positions 23, 45, and 106 (C23, C45, and C106, as shown in Figure 1(a)). HMGB1 interacts with TLR4 via the disulfide bond at C23 and C45 and the free thiol at C106 [33] and in doing so induces cytokine production. In contrast to its inactive form, which contains disulfide bonds at all three sites, HMGB1 does not interact with TLR4 but interacts with CXCL12 to promote chemotaxis [32]. Therefore, the redox modification of HMGB1 is critical in induction of immune responses.

HMGB1 also has a major role as a nonhistone nucleosomal regulatory nuclear protein that is pivotal in DNA rehabilitation and replication [29, 34]. Under physiological conditions, HMGB1 is located in the nucleus where it acts as a DNA chaperone with a role in repair, recombination, regulation of transcription, replication, and genome stability [35]. HMGB1 also has important extracellular functions either through passive release by necrotic cells following cell disruption and nuclear breach or by spontaneous secretion from monocytes and macrophages, where HMGB1 acts as an amplifier of proinflammatory signals originating from innate immune cells [29, 36, 37]. Once located extracellularly, HMGB1 acts as a prototypical damage-associated molecular pattern molecule (DAMP) and promotes inflammasome activation [32]. This particular DAMP cooperates with other factors such as chemokines, growth factors, and cytokines and orchestrates the inflammatory and immune response [38]. Similarly in the immune system, activated immunocytes release HMGB1 into extracellular locations and the details surrounding this process are starting to emerge [39]. However, the mechanism of how other cells, including sensory neurons and spinal glial cells, regulate HMGB1 release in response to different signals remains largely unknown [40] (Figure 1(b)). HMGB1 released by immune cells is highly proinflammatory via cooperation with several definitive receptors that are involved in the inflammatory reaction. These receptors include the receptor for advanced glycation end products (RAGE) [41, 42], Toll-like receptors (TLR-2, TLR-4, and TLR-9) [43–45], integrin [46], SNCA/ $\alpha$ -Synuclein filaments [47], CD24 [48], and NMDA receptor [49]. Accumulating evidence shows that its role as an “alarmin” is a pivotal biological role for HMGB1 [50]. HMGB1 recruits and stimulates antigen-presenting cells

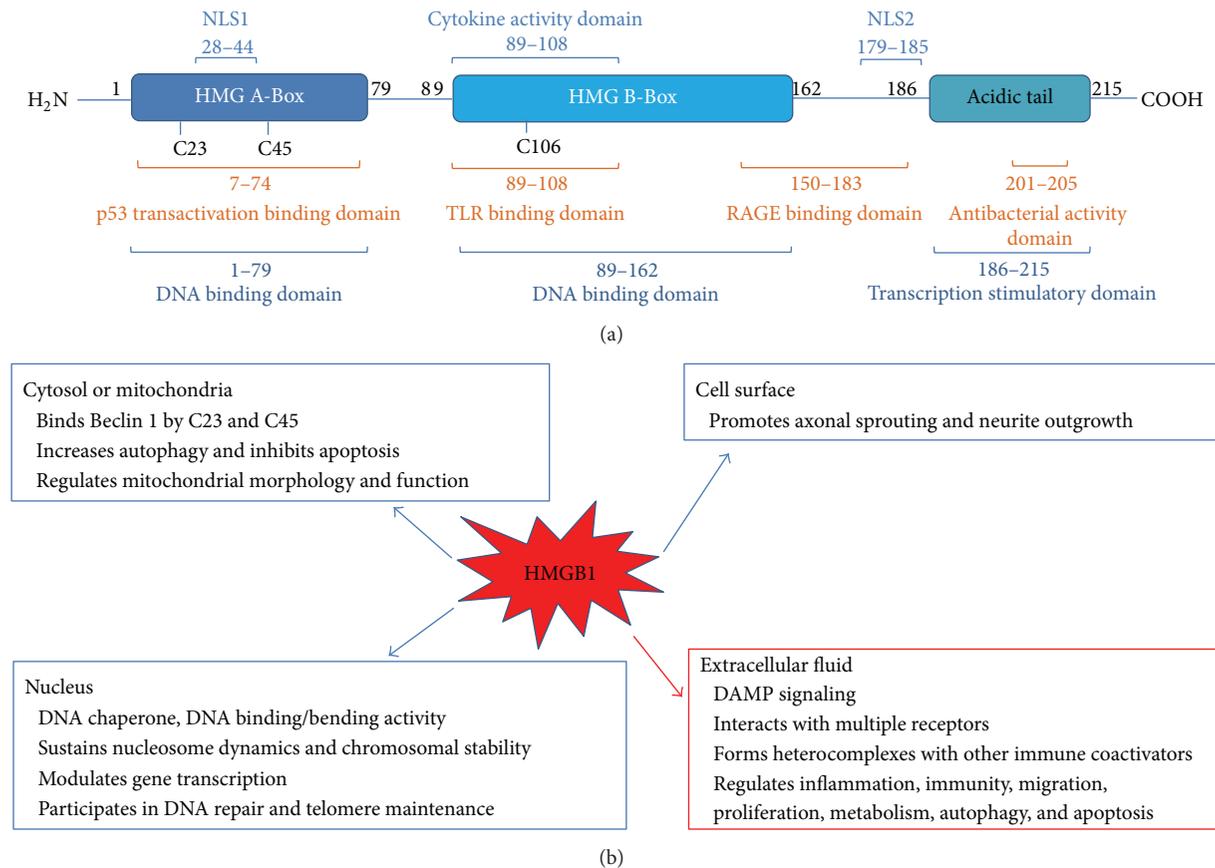


FIGURE 1: Multiple roles of HMGB-1 in cell response.

(APC), such as dendritic cells (DCs). Usually, HMGB1 is an alarm signal that denotes endogenous danger and reports cell damage/necrosis as well as the necessity for repair and induction of a “sterile” immune response by boosting both innate and adaptive immune responses [29, 36, 51]. More importantly, HMGB1 in the cytoplasm plays a role as a cytokine when it is secreted by macrophages in case of injury, inflammation, or disease [52].

### 3. HMGB1 in NPP

The first study that revealed the proinflammatory properties of HMGB1 was conducted more than 10 years ago [53], but given the potentially important implications of the study, there are fewer than expected reports on the relationship between NPP and HMGB1. HMGB1 applied to the sciatic nerve in rats via a preimplanted, indwelling perisciatic catheter induced a dose-dependent reduction in mechanical thresholds against von Frey filament stimulation but no apparent thermal hyperalgesia over a period of 24 h after application [53]. Subsequently, a similar result was observed after the application of HMGB1 to the sciatic nerve after blunt dissection to expose the nerve [54]. More importantly, HMGB1 induced pain-like behavior without prior surgery [54]. Subcutaneous injection of HMGB1 to the plantar side of

the paw, as well as intra-articular injection of HMGB1 to the ankle joint of mice, drives mechanical hypersensitivity [54].

Recently, HMGB1 was shown to be secreted not only by leukocytes but also by irritated or damaged neurons, which release HMGB1 into the extracellular space [55]. Accumulating evidence indicates that HMGB1 is also involved in the pathology of NPP [2, 27, 40, 42, 56]. In the spinal nerve ligation (SNL) disease model, HMGB1 expression was detected in Schwann cells in the spinal nerve, in the primary afferent neurons, and in satellite glial cells (SGCs) in the dorsal root ganglion (DRG) [57, 58]. HMGB1 is involved in the translocation of a damage signal from the nucleus to the cytoplasm. HMGB1 forms complexes with other factors and enhances the effect of its partner molecules (e.g., IL-1 or LPS), and one unexplored possibility is that HMGB1 directly or indirectly facilitates neuronal activity by also potentiating the actions of RAGE or TLRs [59, 60]. In contrast, an HMGB1 neutralizing antibody inhibits pain onset in NPP models [57, 61]. In a DRG study, treatment with an anti-HMGB1 neutralizing antibody significantly depressed TNF- $\alpha$  expression [61]. Furthermore, behavioral tests to investigate the mechanical withdrawal threshold revealed that the administration of anti-HMGB1 neutralizing antibody improved the pain-related behavior [61].

Nuclear HMGB1 immunoreactivity has been detected in various cells such as neurons, satellite cells, Schwann cells,

microglia, and astrocytes from naive rodents [57, 62, 63]. Increased levels of both HMGB1 mRNA and protein have been detected in several pain studies, and this has been interpreted as HMGB1 release and involvement in nociception, since these levels are decreased following the administration of an HMGB1 neutralizing antibody [42, 64]. In a study on the involvement of HMGB1 in mechanical allodynia in a model of type 2 diabetes, the development of mechanical allodynia in the rodent was associated with upregulation of HMGB1 protein in the spinal cord [65]. An intrathecal injection HMGB1 neutralizing antibody inhibited mechanical allodynia [65]. The mRNA levels of inflammatory mediators, including interleukin, TNF- $\alpha$ , and monocyte chemoattractant proteins, were upregulated together with glial fibrillary acidic protein (GFAP) in the spinal dorsal horn in this model and these effects were attenuated by application of the HMGB1 neutralizing antibody [65]. In another study, intravenous treatment with an anti-HMGB1 monoclonal antibody significantly ameliorated partial sciatic nerve ligation- (PSNL-) induced hind paw tactile hypersensitivity and decreased the injury-induced expression of cFos and iba-1 [63]. These data indicate that the synthesis and secretion of HMGB1 from spinal neurons increase in nerve injury, facilitating the activity of multiple type cells including microglia, astrocytes, and neurons, which in turn results in the symptoms of NPP and suggests that HMGB1 could be a potential therapeutic target for NPP.

#### 4. Receptors for HMGB1 in NPP

**4.1. RAGE.** RAGE is a transmembrane cell signaling receptor that belongs to the immunoglobulin superfamily of cell surface multiligand receptors [66, 67]. Mounting evidence reveals that RAGE is a potential contributor to the pathogenesis of many diseases, such as diabetic complications, atherosclerosis, chronic inflammation, cancer, and neurodegeneration [37, 66, 68]. RAGE is widely expressed in human immunocytes, vascular endothelial cells, and neuron and glial cells, but its basal expression is low except in the lungs [37, 69, 70]. RAGE expression leads to the upregulation of proinflammatory factor and cytokines [71]. RAGE was originally identified as a receptor for advanced glycation end products (AGEs). However, RAGE also binds to other structurally multiform ligands, including all-thiol HMGB1, S100 family members, and amyloid- $\beta$  (A $\beta$ ), whereby it regulates multiple physiological and pathological processes [66, 67]. By virtue of its multidomain structure and ability to recognize different classes of ligands, RAGE behaves as a pattern recognition receptor (PRR), analogous to innate immune receptors such as TLRs, and plays a role in orchestrating the immune response [72]. In contrast to other PRRs, RAGE not only binds to exogenous ligands but also interacts with endogenous ligands, especially those considered to be DAMPs, such as HMGB1 [72]. RAGE was the first identified receptor for HMGB1 in neuritis and macrophages [73]. Mounting evidence suggests that RAGE is required for HMGB1-induced injury, inflammation, and immunity and that RAGE activation is essential for this pathology [74–76]. Moreover, HMGB1 located in the extracellular space can lead to the upregulation of RAGE in multiple cell types [77, 78].

Recent studies show that the HMGB1/RAGE signaling axis might be a potential target for diseases such as diabetes [79], neurodegeneration [80], inflammation [81], and NPP [42]. Administration of an HMGB1 neutralizing antibody blocking HMGB1/RAGE signaling depressed the RAGE level and significantly alleviated mechanical allodynia in a SNL model [57]. In a rat NPP model induced by tibial nerve injury (TNI), all-thiol HMGB1 activation of nociceptive neurons was dependent on RAGE [42]. RAGE mRNA and the protein expression in the lumbar dorsal root ganglion (DRG) were substantially increased when compared with sham injured rodents [42]. To distinguish the possible roles of RAGE in NPP, a neutralizing antibody against RAGE (RAGE Ab) was administered. RAGE Ab treatment did not abrogate pain behavior at postinjury day (PID) 7, 14, or 21, but it resulted in the reversal of mechanical hyperalgesia on PID28 [42]. Cyclophosphamide, administered intraperitoneally, caused bladder pain-like nociceptive behavior and referred hyperalgesia accompanying cystitis symptoms [64]. Tanaka et al. found that blocking HMGB1 or RAGE, using neutralizing antibodies, prevented cyclophosphamide-induced bladder pain and referred hyperalgesia [64]. Thus, these data suggest that a RAGE-triggered damage signal is involved in HMGB1 activation and may be responsible for sensory neuron sensitization and mechanical hyperalgesia associated with NPP. Furthermore, these data suggest that targeting HMGB1 or blocking RAGE might serve as a novel therapeutic strategy for the management of NPP.

**4.2. TLR.** The Toll-like receptors (TLRs) are a type I transmembrane superfamily, which is highly evolutionarily conserved in diverse species. TLRs consist of extracellular leucine-rich repeat (LRR) domains, by which pathogen-associated molecular pattern (PAMP) recognition is evoked under conditions of cytopathology [82]. TLRs are extensively expressed in innate immune cells such as macrophages and DCs, as well as in nonimmune cells such as epithelial cells and fibroblasts. Based on location, TLRs are sorted into two subfamilies including cell surface receptors (TLR-2, TLR-4, TLR-5, TLR-6, and TLR-10) and intracellular receptors (TLR-3, TLR-7, TLR-8, TLR-9, TLR-11, TLR-12, and TLR-13) [83, 84]. HMGB1 works together with TLR-2, TLR-4, and TLR-9 and then triggers the NF- $\kappa$ B and IRF pathways, resulting in the elevated expression of inflammation factors [85].

Due to its striking role in inflammatory reactions, attention was focused on TLRs in the CNS. TLRs are expressed in both neuronal and nonneuronal cells in the CNS and contribute to both infectious and noninfectious disorders in the CNS. Following tissue insult and nerve injury, TLRs (such as TLR-2 and TLR-4) induce the activation of microglia and astrocytes and the production of proinflammatory cytokines in the spinal cord, which leads to the development and maintenance of inflammatory pain and NPP [86, 87]. Emerging evidence suggests that TLRs and their associated signaling components contribute to pain hypersensitivity and blockading of TLR signaling reduces pathological pain [88]. Since some members of the TLR subfamily are thought to function as receptors for disulfide HMGB1, HMGB1 could affect NPP by amplifying and maintaining the inflammatory

response via the TLR pathway [2]. Accordingly, nerve injury-induced NPP is impaired after deletion or inhibition of TLR-2 and TLR-4 [89, 90], while application of a TLR-9 antagonist blocks tumor-induced thermal hyperalgesia [91].

In an arthritis induced murine pain model, both the levels of *HMGB1* mRNA and endochylema protein in the lumbar spinal cord were significantly increased [62]. Further investigations indicated that the pronociceptive effect of intrathecal (i.t.) injection of HMGB1 was absent in *TLR-4* deficient mice [62]. In a SNL model of NPP, Ma et al. found that HMGB1 and TLR-4 were increased at both the protein and mRNA levels [44]. Furthermore, mechanical hypersensitivity in collagen antibody-induced arthritis (CAIA) can be reversed by administration of a monoclonal neutralizing antibody against HMGB1 [62]. When using an agent that can produce anti-inflammatory effects via the inhibition of inflammatory cytokines such as IL-1 $\beta$ , IL-6, and TNF- $\alpha$ , the levels of HMGB1 and TLR-4 were downregulated and the paw mechanical withdrawal threshold (PWT) data indicated that the NPP was alleviated after treatment [44, 87]. In another study, NPP was induced in rats by chronic compacting injury of the sciatic nerve (CCI) [19]. In the CCI rat spinal cord, the mRNA and protein levels of HMGB1 and TLR-4, as well as the levels of TNF- $\alpha$  and IL-1 $\beta$ , were upregulated, and the expression of NF- $\kappa$ B in the spinal dorsal horn was significantly increased [19]. Furthermore, the abovementioned effects were reversed by inhibition of the TLR-4, which was accompanied by a dramatic improvement in pain behaviors [19, 44]. These data suggest that the HMGB1/TLR-4 signaling pathway plays a central role in the occurrence and development of NPP, and a therapy targeting HMGB1/TLR-4 might be a novel strategy for the treatment of NPP.

However, very recently, the interaction of HMGB1 with TLRs was identified in another NPP model induced by TNI [42]. HMGB1 activation of nociceptive neurons was shown not to be dependent on TLR-2 and TLR-4 since incubation with small molecule inhibitors of TLR-2 or TLR-4 failed to alter repeated current pulse induced action potential in the presence of HMGB1 [42]. Although the results of this study are different from previous work, there are currently no additional reports on how NPP is affected by HMGB1-induced activation. More detailed studies are thus required to confirm the HMGB1/TLR interaction and to reveal its potential role in the pathogenesis of NPP.

**4.3. Other Receptors.** Besides RAGE and TLR4, NMDA receptor and CXCR4 are also involved in the HMGB1-activated inflammatory response. The disulfide forms of HMGB1 do not bind to TLR4 but can promote cytokine production through cooperation with the chemokine CXCL12 for activation of CXCR4 [32]. CXCL12 is indispensable for HMGB1-induced inflammation via CXCR4 signaling. Cooperation of HMGB1 and CXCL12 leads to stimulation of CXCR4 and recruitment of inflammatory cells around damaged cells [92]. Investigations also revealed that the NMDA receptor is involved in HMGB1-mediated neural damage [93, 94]. Disulfide HMGB1 activates and facilitates NMDA receptor-induced damage responses, such as Ca<sup>2+</sup> influx and nitric oxide synthase, which leads to cell death

[49]. Furthermore, using MK-801, a NMDA channel blocking agent, the effect of HMGB1 was abolished [94]. These data suggest that NMDA receptor is critical for HMGB1-mediated cell responses and might be independent of other signaling pathways in the HMGB1-activated inflammatory response.

## 5. HMGB1 as a Potential Therapeutic Target for NPP

Our recent understanding of the mechanisms and, consequently, the diagnosis of NPP has become progressively clearer in clinical practice. However, the treatment of this condition remains challenging [95]. One of the reasons for this difficulty is the diversity of mechanisms involved in NPP and its persistence [95]. Growing evidence supports a role for HMGB1 as an essential inflammatory pronociceptive factor that acts alone or cooperates with other mediators including RAGE, TLRs, TNF- $\alpha$ , and IL-1 $\beta$  and supports that HMGB1 is involved in the pathology of NPP [32, 44, 62, 72, 74]. As shown by many studies, agents (e.g., HMGB1 neutralizing antibody, HMGB1 inhibitor, and HMGB1 receptor neutralizing antibody or inhibitor) that prevent the action of HMGB1 reduce hypersensitivity in animal models of NPP [44, 72, 75, 78]. Additionally, the level of HMGB1 in samples such as serum, plasma, and cerebrospinal fluid (CSF) has been determined using Western blot or ELISA. The results indicate that HMB1 might be a promising biomarker for human disease diagnosis and therapeutic outcome [27]. However, more studies are needed to elucidate the underlying mechanisms in order to use these agents as pain-relieving drugs in human subjects.

## 6. Concluding Remarks and Future Directions

NPP still presents as a major diagnostic and therapeutic challenge despite considerable progress in the understanding of its mechanisms and the publication of a number of studies that assessed the efficacy and safety of drugs used for symptomatic treatment. HMGB1 plays an essential role in the pathogenesis of a large number of inflammatory conditions and has also been identified as a mediator of neuroinflammation. Accumulating data indicate that HMGB1 plays an important role in NPP progression. Strategies that target HMGB1 and its associated cell signaling pathways markedly depress inflammatory reactions in several models of NPP. This may lead to a promising and efficacious clinical approach for the treatment of NPP. Cell surface receptors, including RAGE, TLR, CXCR4, and NMDA receptor, are important signaling pathways in inflammatory injury. They share common ligands and signaling pathways that are involved in HMGB1-induced cellular pathology, and mounting of evidence points towards their cooperative role in the host immune response. Although much attention has been paid to the association of HMGB1 with RAGE or TLRs, there are still many unknowns underlying the association of HMGB1 with NPP. Furthermore, there are still only a few reports on *in vivo* experiments for investigating HMGB1, with even fewer on the evaluation of pathological mechanisms using either *ex vivo* or *in vitro* tests. Moreover, the mechanisms of RAGE-TLR crosstalk in

response to HMGB1 are still unclear. Given the important role of these pathways in NPP, targeting them may offer new treatments for debilitating and refractory pain. Thus, further investigations are needed to better understand the pathogenesis of NPP.

## Competing Interests

The authors declare that they have no conflict of interests regarding the publication of this paper.

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## Research Article

# Systemic Administration of Proteoglycan Protects BALB/c Retired Breeder Mice from Experimental Arthritis

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This study was undertaken to evaluate the prophylactic potential of proteoglycan (PG) administration in experimental arthritis. Female BALB/c retired breeder mice received two (2xPG50 and 2xPG100 groups) or three (3xPG50 group) intraperitoneal doses of bovine PG (50  $\mu$ g or 100  $\mu$ g) every three days. A week later the animals were submitted to arthritis induction by immunization with three i.p. doses of bovine PG associated with dimethyldioctadecylammonium bromide adjuvant at intervals of 21 days. Disease severity was daily assessed after the third dose by score evaluation. The 3xPG50 group showed significant reduction in prevalence and clinical scores. This protective effect was associated with lower production of IFN- $\gamma$  and IL-17 and increased production of IL-5 and IL-10 by spleen cells restimulated *in vitro* with PG. Even though previous PG administration restrained dendritic cells maturation this procedure did not alter the frequency of regulatory Foxp3<sup>+</sup> T cells. Lower TNF- $\alpha$  and IL-6 levels and higher expression of ROR- $\gamma$  and GATA-3 were detected in the paws of protected animals. A delayed-type hypersensitivity reaction confirmed specific tolerance induction. Taken together, these results indicate that previous PG inoculation determines a specific tolerogenic effect that is able to decrease severity of subsequently induced arthritis.

## 1. Introduction

Approximately 1% of people worldwide are affected by rheumatoid arthritis (RA) that is a systemic autoimmune disease [1]. This is a form of arthropathy characterized by a chronic inflammation that leads to progressive cartilage and bone destruction. The etiology of RA is multifactorial, involving both genetic and environmental factors [2]. The immunopathogenetic pathways implicated in RA are not fully elucidated; however, it is known that they involve the recruitment of several cell types from both innate and adaptive immunity [3]. Cytokines are viewed as the main players

in arthritic lesions, maintaining chronic inflammation and promoting autoimmunity. TNF- $\alpha$  greatly contributes to joint inflammation by stimulating cell proliferation, metalloproteinases and adhesion-molecules expression, secretion of other cytokines, and prostaglandin production by the synovial tissue [4]. IL-6 is another mediator that is found in elevated levels in synovial fluid and serum from RA patients. This cytokine contributes to both local and systemic RA histopathological alterations and clinical manifestations, such as *pannus*, cartilage degradation, fever, fatigue, and weight loss [5].

Some of the autoantigens involved in the pathogenesis of RA include IgG, collagen, proteoglycan, citrullinated proteins, fibrin, fibrinogen, and the shared human leukocyte antigen (HLA) DR epitope [6, 7]. Experimental models induced with some of these antigens, specially collagen and proteoglycan, are being widely employed due to their similarities with the corresponding human disease [8, 9]. We recently demonstrated that bovine PG can also induce typical arthritis when associated with dimethyldioctadecylammonium bromide [10].

It is well known that several autoantigens able to induce autoimmunity have also the potential to induce antigen-specific tolerance depending upon the route, the dose, and the physical form of the antigen [11]. Systemic administration of soluble antigens is a well-accepted approach to induce immunological tolerance [12]. Anergy and clonal deletion of effector cells, induction of regulatory T cells (Tregs), and immune response deviation have been postulated as mechanisms underlying systemic tolerance [11].

To date, RA is not a curable disease and most of the treatments consist in general suppression of the immune response to control synovitis and prevent joint injury worsening [13]. These therapeutic interventions have also side effects including higher susceptibility to infections. Therefore, there is an increasing interest in alternative immunomodulatory strategies, particularly the ones that induce antigen-specific tolerance. Inverse vaccination refers to an antigen-specific immunization protocol aiming at inducing tolerance [14]. These procedures could be, theoretically, the safest measures to avoid or treat autoimmune diseases. In this context, we expected that the systemic administration of proteoglycan (PG) that is a cartilage compound would induce antigen-specific tolerance and therefore restrain arthritis development.

## 2. Material and Methods

**2.1. Animals.** Female BALB/c retired breeder mice aged 8 to 11 months were purchased from CEMIB (Campinas, São Paulo, Brazil). They were maintained in the animal facility of the department of microbiology and immunology under controlled conditions of luminosity (12 h light/12 h dark) and temperature ( $22 \pm 2^\circ\text{C}$ ). Mice were allocated in ventilated cages with sterile pine shavings and received sterile food and filtered water *ad libitum*. The manipulation of the animals was in compliance with the local ethics committee (protocol number 257-CEEA).

**2.2. PG Administration, Arthritis Induction, and Score Evaluation.** Mice received two or three intraperitoneal (i.p.) doses of proteoglycan (PG) ( $50 \mu\text{g}$  or  $100 \mu\text{g}$ ) every three days. One week later, experimental arthritis was induced as described by Ishikawa et al., 2014 [10]. Briefly, mice received three i.p. injections of bovine PG associated with dimethyldioctadecylammonium bromide (DDA) adjuvant at intervals of 21 days. After the third injection, arthritis score was daily evaluated until euthanasia (70 days after the beginning of arthritis induction). Arthritis severity was determined using

a standard visual scoring system based on the degree of edema and erythema ranging from 0 to 4 for each paw. The following system was used: 0 = normal; 1 = mild edema in one joint in the paw; 2 = moderate edema and erythema in one or more joints in the paw; 3 = pronounced edema and erythema in all joints in the paw and ankle; 4 = severe edema and erythema of the entire paw and ankle and movement impairment. This classification resulted in a total score that ranged from 0 to 16 for each animal.

**2.3. Histopathological Analysis.** Mice paws were collected after euthanasia and fixed in 10% formalin phosphate buffer for at least 48 hours at room temperature. The samples were demineralized in 10% Titriplex EDTA disodium salt (Merck Millipore, Darmstadt, Germany) for three months. The decalcified tissues were embedded in paraffin and  $5 \mu\text{m}$  sections were mounted on glass slides and stained with hematoxylin and eosin (HE). The images were acquired by a digital camera attached to the optical microscope (Nikon, Kurobanemuko, Otawara, Japan).

**2.4. Cytokine Production by Spleen Cells.** Spleens were collected after euthanasia, resuspended in RPMI medium containing gentamicin and fetal calf serum ( $5.0 \times 10^6$  cells/mL), and stimulated with PG ( $50 \mu\text{g}/\text{mL}$ ). After 48 hours of incubation at  $37^\circ\text{C}/5\% \text{CO}_2$ , culture supernatants were collected for TNF- $\alpha$ , IL-6, IL-17, IFN- $\gamma$ , IL-5, and IL-10 quantification. These cytokines were assessed by using enzyme linked immunosorbent assay (ELISA), according to manufacturer's instructions (BD Biosciences, San Jose, CA, USA, and RD Systems, Minneapolis, MN, USA). Sensitivity of ELISA kits for these cytokines was 31.25, 19.5, 15.6, 31.25, 7.8, and 15.6 pg/mL, respectively.

**2.5. Frequency of Dendritic Cells and Regulatory T Cells in the Spleen.** Flow cytometry analysis was performed according to manufacturer's instructions (eBiosciences, San Diego, CA, USA). Briefly, for dendritic cells (DCs) immunophenotyping, splenic cells were incubated with FITC-conjugated anti-mouse CD11c (clone N418), APC-conjugated anti-mouse MHCII (clone M5/114.15.2), and PE-conjugated anti-mouse CD80 (clone 16-10A1) antibodies. The cells were washed and resuspended in flow cytometry buffer containing paraformaldehyde solution. For Tregs, splenic cells were first incubated with FITC-conjugated anti-mouse CD4 (clone GK1.5) and APC-conjugated anti-mouse CD25 (clone PC61.5) antibodies. Intracellular Foxp3 transcription factor was detected using Foxp3 PE Staining Set (eBiosciences, San Diego, CA, USA) according to manufacturer's instructions.

**2.6. Local mRNA Expression for T Cell Subsets Transcription Factors.** mRNA was extracted using TRIzol (Life Technologies) and RNeasy Mini Kit (Qiagen, Valencia, CA, USA). DNase treatment followed manufacturer's instructions and purity of the samples was assessed by 260/280 ratio. Single-strand cDNA synthesis was performed from  $100 \text{ ng}/\mu\text{L}$  of extracted RNA, using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA). Aliquots of cDNA ( $3 \mu\text{L}$ ) were subjected to real time PCR

TABLE 1: Arthritis prevalence and severity in mice previously inoculated with proteoglycan. Arthritis score was daily evaluated and disease severity was assessed by a visual scoring system with total score ranging from 0 to 16 for each animal. Score above eight indicates severe arthritis. These parameters were compiled 70 days after arthritis induction. Control (+): arthritic group not previously injected with PG; 2xPG50, 2xPG100, and 3xPG50: arthritic groups previously injected with two (50  $\mu$ g), two (100  $\mu$ g), or three (50  $\mu$ g) PG doses, respectively. Data from two independent experiments were combined.

	Arthritis prevalence	<i>p</i> value	Disease onset (days)	Animals with score > 8	<i>p</i> value
Control (+)	22/22 (100%)	0.042	43	9/22 (41%)	<0.001
2xPG50	12/13 (92%)		44	0/13 (0%)	
2xPG100	7/7 (100%)		44	0/7 (0%)	
3xPG50	12/16 (75%)		43	0/16 (0%)	

reaction using TaqMan system. Each reaction contained 15  $\mu$ L of TaqMan Gene Expression Master mix (Life Technologies, Carlsbad, CA, USA), 0.25  $\mu$ L of the reference gene, and 1.0 to 1.5  $\mu$ L of the target genes. The following inventoried primes/probes tested by Life Technologies were used:  $\beta$ -actin (Mm00607939\_s1), T-bet (Mm00450960\_m1), GATA-3 (Mm00484683\_m1), ROR- $\gamma$  (Mm01261022\_m1), and Foxp3 (Mm00475162\_m1). The reactions were performed in ABI 7300 equipment (Applied Biosystems, Carlsbad, CA, USA) using standard parameters. Data were analyzed in SDS Software System 7300 and relative quantification was determined based on fold difference ( $2^{-\Delta\Delta C_t}$ ) using Ct value of the target gene normalized to the reference gene and the control (-) group as the calibrator.

**2.7. Local Cytokine Production.** Proteins from paws were extracted by homogenization in RIPA (Radioimmunoprecipitation Assay) buffer with an automatic homogenizer (Ultra Turrax Werke, IKA, Staufen, Germany). Homogenates were centrifuged, the supernatants were filtered through a 22  $\mu$ m filter, and protein concentration was determined by Pierce method (Thermo Scientific, Waltham, MA, USA). The samples were stored at -80 until cytokines were quantified by Cytometric Bead Array (CBA). Mouse Th1/Th2/Th17 Cytokine Kit (BD Biosciences, San Jose, CA, USA) was used to quantify these cytokines in all samples. Briefly, 25  $\mu$ L of supernatants was incubated with beads of different sizes conjugated with fluorochromes. The acquisition of the beads was performed in FACS Canto II flow cytometer (BD Biosciences, San Jose, CA, USA) with FACS Diva software and the analysis was done using FCAP 3.0 software (Soft Flow Inc., St. Louis Park, MN, USA).

**2.8. Delayed-Type Hypersensitivity Reaction (DTH).** Mice were injected with three i.p. doses of 50  $\mu$ g of PG or chicken ovalbumin (OVA) (Sigma Aldrich, St. Louis, MO, USA) every three days. One week after the last dose, the animals were immunized with a single i.p. dose of PG associated with the DDA adjuvant. DTH was performed one week later by PG inoculation (10  $\mu$ g) in the hind paw. Paw thickness was measured before and 24 hours after PG inoculation using a caliper (Starrett, Athol, MA, USA).

**2.9. Statistical Analysis.** Results were presented as mean  $\pm$  standard deviation for parametric variables and the comparison among the groups was performed by ANOVA followed

by Tukey's test. For nonparametric variables, the results were presented as median and the comparison among the groups was performed by Kruskal-Wallis followed by Dunn's test. Disease prevalence and severity were compared using Chi-square test. All data were analyzed using SigmaPlot software version 12.0 (Systat Software Inc., San Jose, CA, USA) and  $p < 0.05$  was considered significant.

### 3. Results

**3.1. PG Administration Decreases Arthritis Prevalence and Severity.** Disease prevalence in 2xPG50 and 2xPG100 groups was similar to those in the control (+) group. However, a significant decrease in prevalence was observed in the group previously injected with 3xPG50 as presented in Table 1. Although clinical disease appeared around days 43 or 44 after arthritis induction in all experimental groups, the maximum clinical scores reached in 2xPG50 and 3xPG50 groups were significantly reduced in comparison to control (+) group. Moreover, these two groups presented a much less severe disease, with a significant smaller number of animals with clinical score above 8. Protection in the 3xPG50 group was also confirmed by histopathological analyses that indicated a clear reduction in inflammation. Figure 1(c) shows representative micrographs of mice hind paws in score 0 from control (-), score 3 from control (+), and score 1 from 3xPG50 group, respectively. 70 days after arthritis induction, there were a massive inflammation and *pannus* formation only in the control (+) group. The 3xPG50 group presented well preserved joint structures similar to the control (-) group.

**3.2. PG Administration Reverses the Proportion of Pro- and Anti-Inflammatory Cytokines.** Cytokine profile, which is illustrated in Figure 2, was assessed in both unstimulated and PG stimulated spleen cell cultures. TNF- $\alpha$  was not detected in any experimental group (not shown). Spontaneous production of IFN- $\gamma$ , IL-6, IL-5, and IL-10 was observed in all arthritic groups. However, except IL-6, all other cytokines were produced in much higher levels after PG stimulation. Previous administration of PG reduced IFN- $\gamma$  and IL-17 and increased IL-5 and IL-10 production. Reduction in IFN- $\gamma$  and IL-17 levels was more accentuated in the 3xPG50 whereas IL-5 and IL-10 levels were comparable in the three groups inoculated with PG before disease induction.

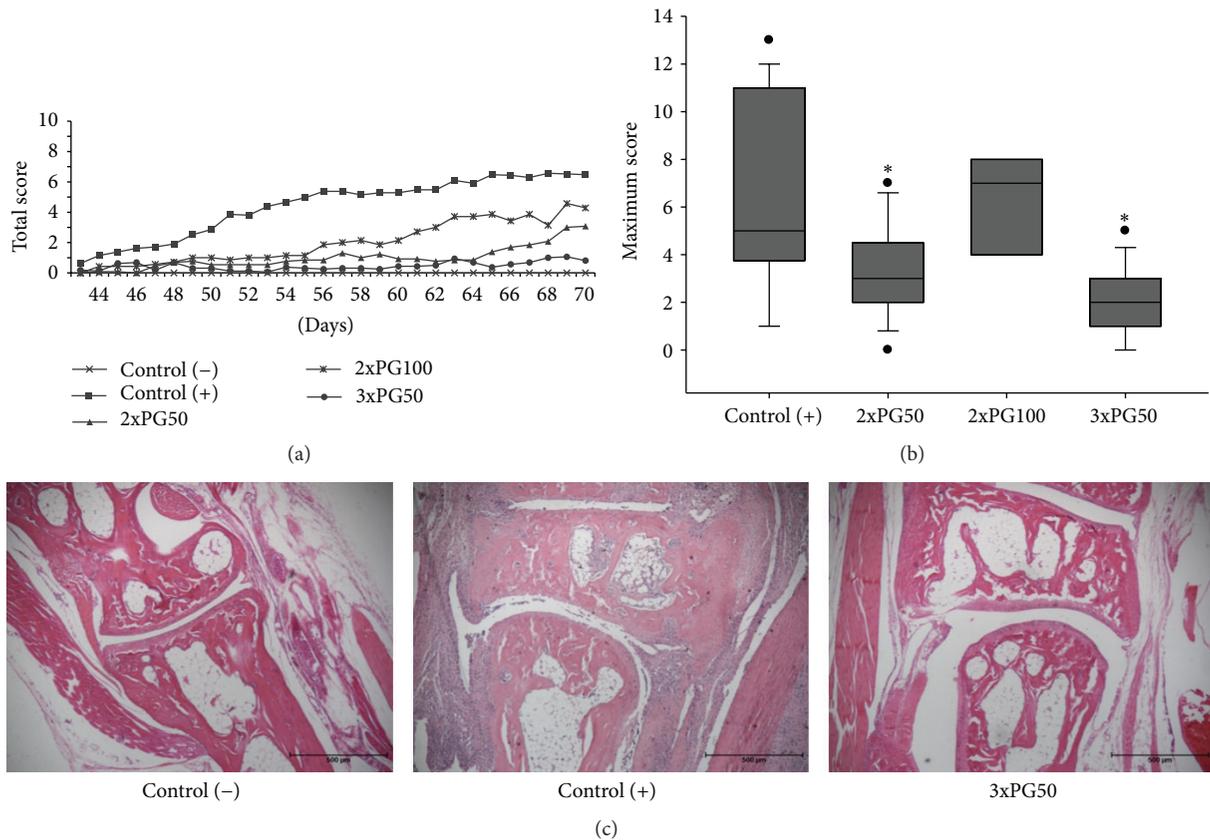


FIGURE 1: Effect of previous PG inoculation on experimental arthritis development. (a) Kinetics of total score, ranging from 0 to 16 for each animal, assessed by a visual scoring system based on the degree of edema and erythema. (b) Maximum score mean per group. (c) Histopathological analysis assessed 70 days after arthritis induction. Control (-): healthy group not previously injected with PG; control (+): arthritic group not previously injected with PG; 2xPG50, 2xPG100, and 3xPG50: arthritic groups previously injected with two (50  $\mu$ g), two (100  $\mu$ g), or three (50  $\mu$ g) PG doses, respectively. Six to ten animals per group from one representative experiment of two performed. \*  $p < 0.05$ .

**3.3. PG Administration Limited DC Maturation.** As DC immaturity has been associated with tolerance induction in rheumatoid arthritis [15], we evaluated the frequency of mature DCs in the spleen of healthy, arthritic, and 3xPG50 experimental groups. The frequency of splenic CD11c+ MHCII+ CD80+ DCs, which is illustrated in Figure 3(c), is significantly higher in the control (+) group whereas the 3xPG50 group presented levels comparable to the control (-) group. Considering that immaturity of DCs is frequently associated with the development of CD4+ CD25+ Foxp3+ T cells [16] and that this is an important mechanism to keep peripheral tolerance, the amount of splenic CD4+ CD25+ Foxp3+ Tregs was also evaluated. However, the frequency of this T cell subset was similar in the three experimental groups (Figure 3(f)).

**3.4. PG Administration Alters the Proportion of Canonical T Cell Transcription Factors and Drops Down TNF- $\alpha$  and IL-6 Levels.** The knowledge of the interplay among T cell subsets has been pivotal to understand arthritis immunopathogenesis and also to suggest new therapeutic modalities [17]. To evaluate if the prophylactic efficacy of PG administration

would affect the proportion of these subsets, the local mRNA expression of their respective putative transcription factors was determined. Relative quantification of T-bet, GATA-3, ROR- $\gamma$ , and Foxp3 expression, which are related to Th1, Th2, Th17, and Treg subsets, respectively, was determined. As illustrated in Figure 4, there was a significant increase in GATA-3 and ROR- $\gamma$  expression in animals from the 3xPG50 group compared with the control (+) one. Furthermore, Foxp3 expression was significantly decreased in this previously immunized group when compared with the control (+) group. IL-2, IL-4, IL-10, IL-17, and IFN- $\gamma$  production was not detected in the arthritic joints. However, TNF- $\alpha$  and IL-6 levels were detected in all the experimental groups. Previous PG immunization determined a significant reduction in the local (paws) production of these cytokines. These data are also illustrated in Figure 4.

**3.5. Tolerogenic Effect of PG Administration Is Specific.** A delayed-type hypersensitivity (DTH) reaction was used to confirm that PG was able to induce tolerance and that this tolerance was specific for PG. As expected, there was no increase in the paw thickness of animals from the control (-) group

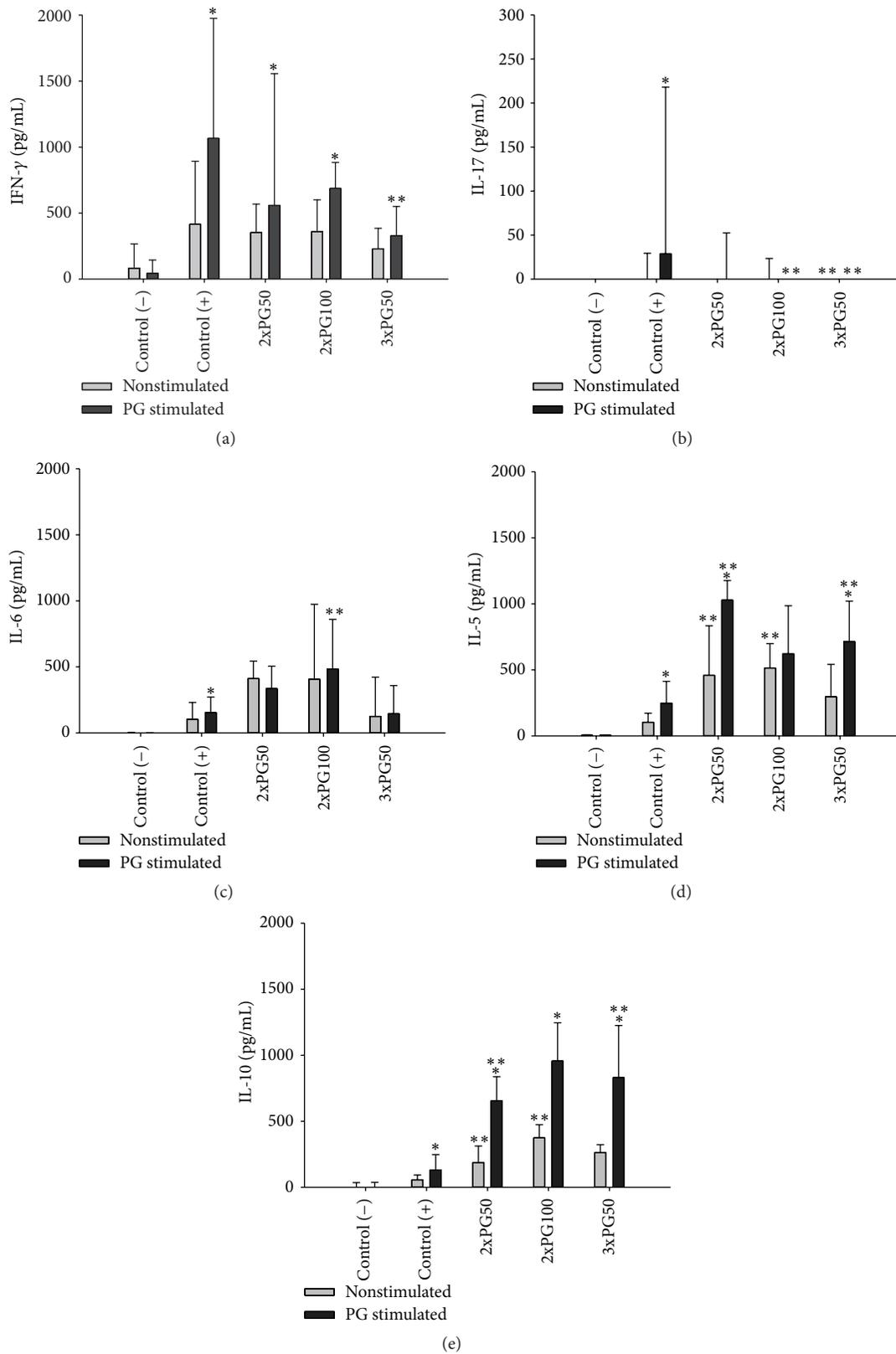
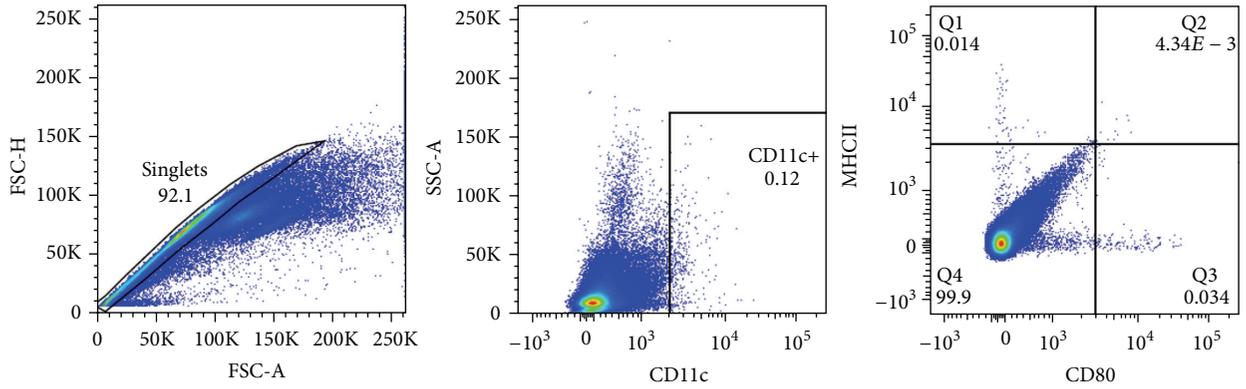
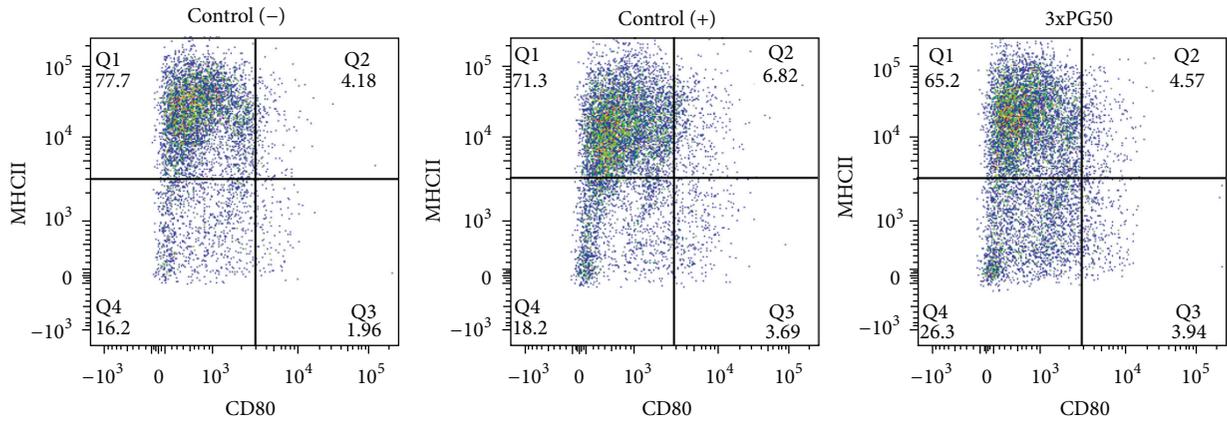


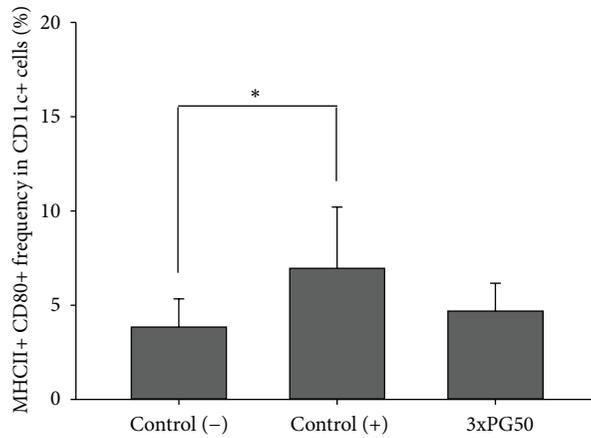
FIGURE 2: Effect of previous PG inoculation on cytokine production by spleen cells from arthritic mice. (a) IFN- $\gamma$ , (b) IL-17, (c) IL-6, (d) IL-5, and (e) IL-10 production by spleen cells assessed by enzyme linked immunosorbent assay. Control (-): healthy group not previously injected with PG; control (+): arthritic group not previously injected with PG; 2xPG50, 2xPG100, and 3xPG50: arthritic groups previously injected with two (50  $\mu$ g), two (100  $\mu$ g), or three (50  $\mu$ g) PG doses, respectively. Six to ten animals per group from one representative experiment of two performed. \*  $p < 0.05$  compared with the unstimulated counterpart and \*\*  $p < 0.05$  compared with the control (+) group.



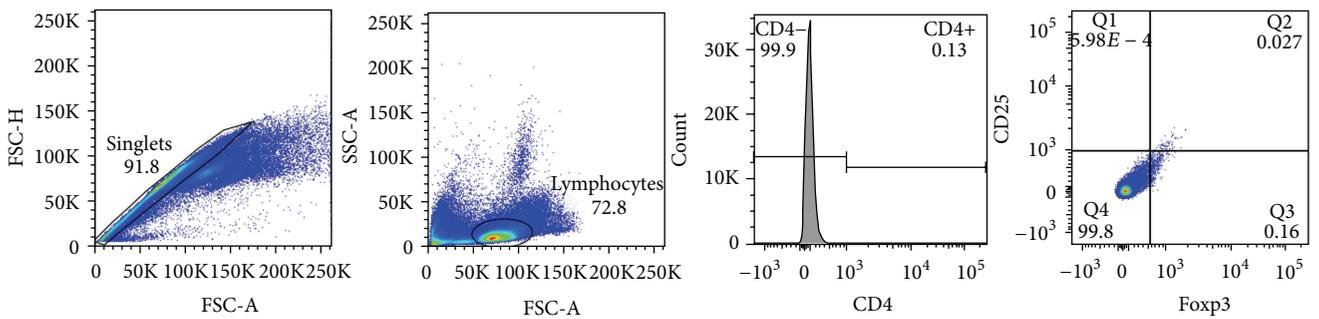
(a)



(b)



(c)



(d)

FIGURE 3: Continued.

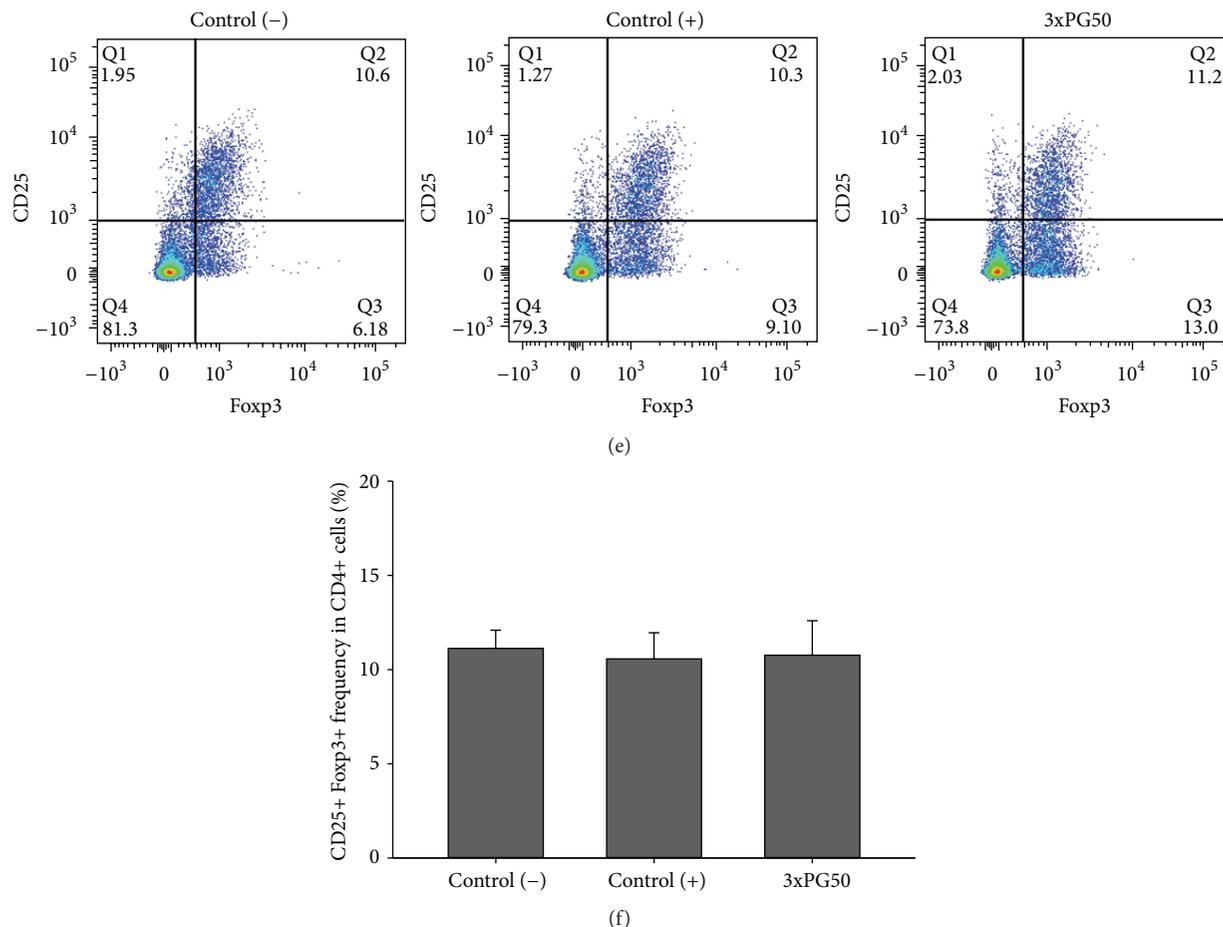


FIGURE 3: Effect of previous PG inoculation on the frequency of splenic dendritic cells (DCs) and regulatory T cells (Tregs). (a) Gate strategy for DCs in a control (-) sample. (b) Representative flow cytometry analysis for DCs frequency in spleen. (c) MHCII+ CD80+ cell frequency in total CD11+ cells. (d) Gate strategy for Tregs. (e) Representative flow cytometry analysis for Tregs frequency in spleen. (f) CD25+ Foxp3+ cell frequency in total CD4+ cells. Control (-): healthy group not previously injected with PG; control (+): arthritic group not previously injected with PG; 3xPG50: arthritic group previously injected with three doses of PG (50  $\mu$ g). Six to ten animals per group from one representative experiment of two performed. \*  $p < 0.05$ .

(not immunized with PG+DDA but challenged with PG). However, all other experimental groups presented elevated paw thickness 24 hours after PG challenge in comparison to the control (-) group. Inoculation of OVA (an irrelevant antigen) before immunization with PG+DDA did not trigger a tolerogenic effect. Animals from this group presented paw thickness similar to the control (+) group when challenged with PG. Previous inoculation of the specific antigen (PG) determined a tolerogenic effect demonstrated by a significant decrease in DTH response in comparison to the control (+) group (Figure 5).

#### 4. Discussion

Autoimmune diseases such as rheumatoid arthritis, multiple sclerosis, and type 1 diabetes are on the list of the most relevant causes of female death [18]. In this context, much effort has been expended to induce tolerance to autoantigens

as an alternative to current and usually global immunosuppressive treatments. Antigen administration by oral route, for example, has been promising by showing a protective effect in experimental diabetes [19] and encephalomyelitis [20]. Intravenous administration of soluble antigen in the absence of adjuvants is another well documented procedure to induce tolerance [21, 22]. The present study was undertaken to evaluate if proteoglycan (PG) injected by intraperitoneal (i.p.) route could induce a tolerogenic state and consequently reduce arthritis development. We initially demonstrated that i.p. PG administration, before arthritis induction by this same antigen, was able to decrease disease prevalence, clinical score, and also the histopathological features. The most effective protocol was the inoculation of three doses of 50  $\mu$ g of PG. Two doses of 50  $\mu$ g of PG triggered a similar but less accentuated protective effect. The prophylactic effect observed in this work is consistent with the literature. Already in 1997, Liblau et al. [12] suggested that systemic inoculation of soluble proteins, synthetic peptides, or peptide analogues

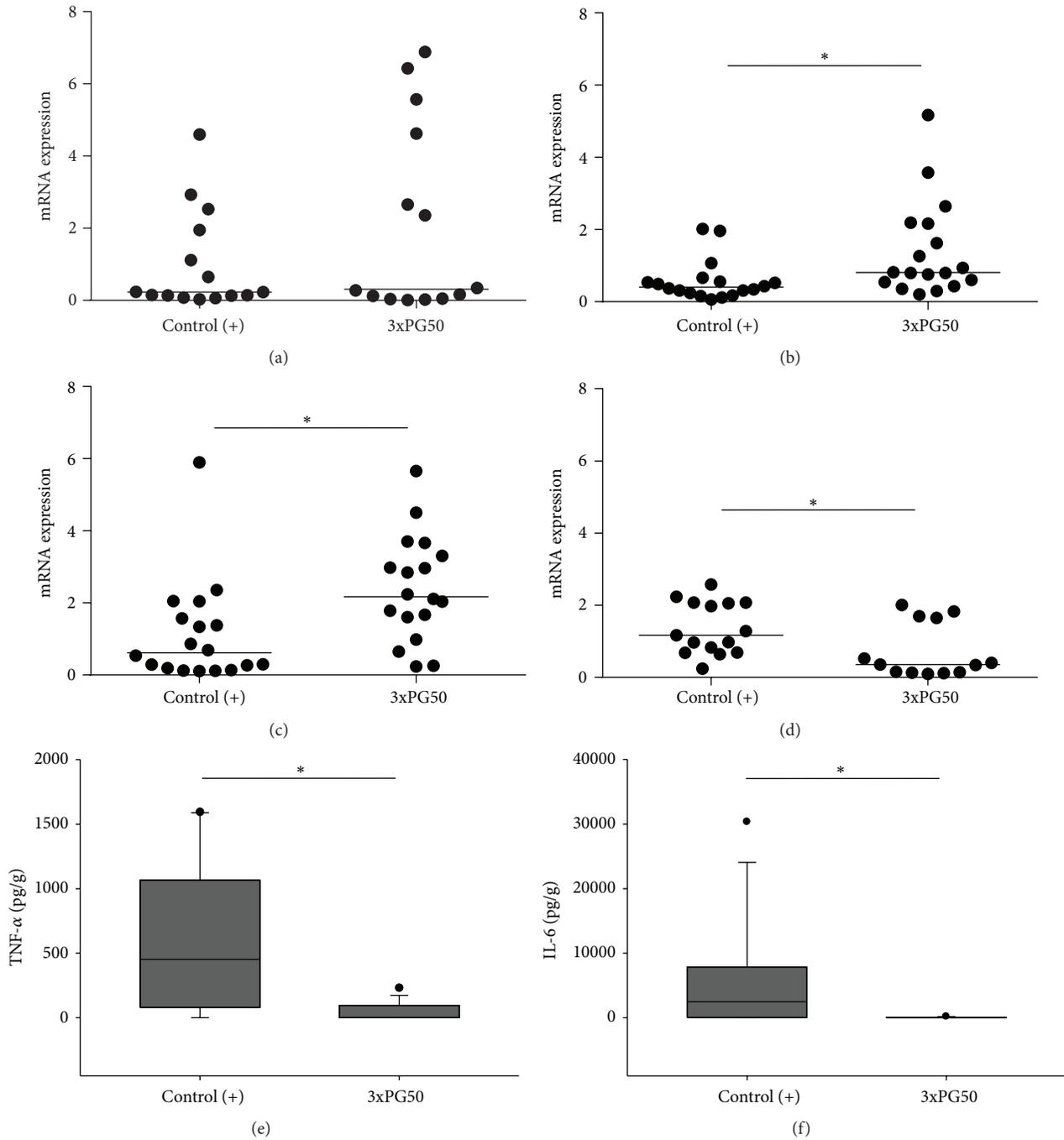


FIGURE 4: Effect of previous PG inoculation on local T cell transcription factors and cytokine levels. (a) T-bet, (b) ROR- $\gamma$ , (c) GATA-3, and (d) Foxp3 mRNA expression in paw homogenates. Quantification was based on fold difference ( $2^{-\Delta\Delta Ct}$ ) between groups, using control (-) group as calibrator. (e) TNF- $\alpha$  and (f) IL-6 levels in paw homogenates assessed by Cytometric Bead Array. Control (+): arthritic control not previously injected with PG; 3xPG50: arthritic group previously injected with three doses of PG (50  $\mu$ g). Hind and forepaws from six to ten animals per group from one representative experiment of two performed. \*  $p < 0.05$ .

could become an acceptable approach to prevent or to treat human autoimmune diseases as multiple sclerosis and type 1 diabetes. The prophylactic effect of a cartilage specific antigen was described, for the first time, by Gumanovskaya et al., 1999 [23]. At first sight, it could appear too complex to translate our findings to human arthritis because, differently from multiple

sclerosis and type 1 diabetes, whose involved autoantigens are better established, arthritis targeted autoantigens are still not so well characterized. However, it was recently reported that oral administration of shark type II collagen suppressed arthritis induced in rats by inoculation of Complete Freund's Adjuvant [24]. It is possible, therefore, that a specific joint

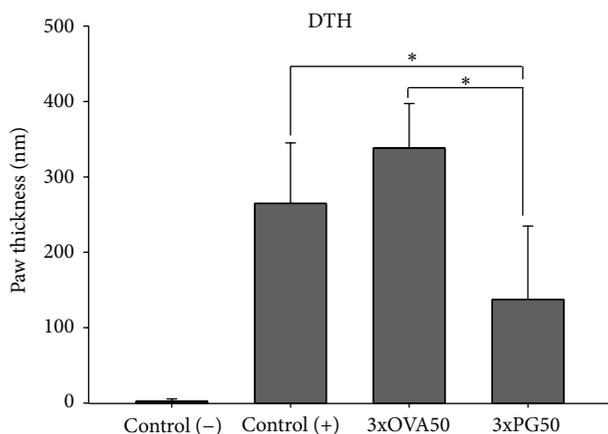


FIGURE 5: Delayed-type hypersensitivity reaction elicited by PG in mice previously inoculated with PG or OVA. Paw thickness was measured before and 24 hours after PG challenge using a caliper. Control (-): healthy group not previously injected with PG; control (+): arthritic group not previously injected with PG; 3xPG50: arthritic group previously injected with three doses of PG (50  $\mu$ g). Four to six animals per group from one representative experiment of two performed. \*  $p < 0.05$ .

antigen, as is the case of PG, can trigger a similar protective effect in human arthritis when administered by a systemic route. This possibility still needs a profound preclinical investigation.

To elucidate the possible protective mechanism triggered by PG, we initially analyzed the production of some pro- and anti-inflammatory cytokines. All experimental groups produced detectable levels of IFN- $\gamma$  and IL-17 but not TNF- $\alpha$ . These findings are in accordance with several studies that reveal the proarthritogenic effect of IFN- $\gamma$  and IL-17 [25]. Previous PG administration prevented the development of a more severe disease and concomitantly reduced the production of IFN- $\gamma$  and IL-17. Analysis of cytokine production by spleen cells stimulated with PG also suggests that the protective effect is mediated by an increased production of IL-5 and IL-10 anti-inflammatory cytokines. As IL-5 is described as a typical Th2 cytokine [26, 27], it is possible that i.p. administration of PG has polarized the immune response towards Th2. The higher expression of GATA-3 in the paws from previously PG injected mice reinforces this possibility. Moreover, IL-10 has been described by some authors as being comparable to IL-4 and IL-5 concerning the Th2 pattern [28]. IL-10 is endowed with a strong anti-inflammatory potential and its contribution to tolerance induction to both self-antigens and foreign antigens has also been described [29]. A similar anti-inflammatory role has been attributed to these cytokines in experimental diabetes [30] and encephalomyelitis [31]. The higher percentage of mature DCs in the positive control group shows that the arthritogenic process is associated with an increased maturation of DCs. Otherwise, the smaller amount of mature DCs in PG inoculated mice suggests that PG effect over DCs could be involved in this protective effect. Demonstration that skewing DCs differentiation towards a tolerogenic state is efficient as

an RA treatment supports this possibility [15]. Unexpectedly, however, there was no increase in the percentage of Tregs. A possible interpretation of these findings is that PG administration by i.p. route is determining immunological tolerance independently of Tregs. Intraperitoneal delivered substances are primarily absorbed by mesenteric vessels which drain into the portal vein and pass to the liver. It is well known that this organ also uses other pathways to establish a state of immunological tolerance [32]. Satpute et al. 2009 [33] that employed the i.p. route did not observe alterations in Tregs frequency and function in rats with adjuvant arthritis after treatment with the 65 kDa heat shock protein. They also demonstrated that this tolerogenic effect was not mediated by indoleamine 2,3-dioxygenase expressing DCs.

Otherwise, the increased expression of GATA-3 mRNA levels suggests higher accumulation of Th2 polarized cells in the joints of PG protected mice. This is consistent with the literature since Th2 polarization has been associated with tolerance induction by systemic immunization [11]. The increased expression of ROR- $\gamma$  and the decreased expression of Foxp3 in the tolerized experimental group were unexpected since Th17 and Treg subsets are usually associated with antagonistic roles in the course of autoimmune inflammatory pathologies [34]. The plasticity phenomenon that characterizes T cell subsets could account for this finding. The extreme complexity of Tregs plasticity is being acknowledged [35] and includes an unpredictable correlation between Foxp3 expression and suppressive ability and also the potential of this subset to acquire the features of effector T cells [36]. Accumulating evidences also indicate that Th17 cells are particularly prone to present functional plasticity. Thus, additional subsets as Th17/Th1, Th17/Th2, and Th17/Treg have been recently described [37]. The concomitant elevated expression of GATA-3 and ROR- $\gamma$  could therefore represent the local presence of a Th17/Th2 subset. Alternatively, the elevated amount of Th cells expressing ROR- $\gamma$  (Th17) could be linked to Tregs plasticity. In this scenario, Tregs that migrated early to the lesions could later express the Th17 transcription factor. This possibility is partially supported by a recent report by Wang et al. 2015 [38]. These authors demonstrated that Tregs from RA patients presented increased plasticity toward Th17. They also observed that IL-17 producing T cells retained suppressive function and were associated with milder inflammatory conditions.

A possible correlation between T cell subsets and their signature cytokines was not found in mice paws, but the prophylactic PG effect clearly downmodulated the local production of TNF- $\alpha$  and IL-6. The relevance of these findings resides in the pivotal role of these two mediators on joint destruction [39]. This knowledge upgraded disease treatment with the adoption of various strategies based, initially, on TNF- $\alpha$  inhibition [40]. More recently, mainly because some RA patients are nonresponsive to anti-TNF therapy, agents targeting IL-6 are viewed as promising biologicals in RA treatment [41].

To determine if the prophylactic activity elicited by PG administration was specific, we compared the effects of previous injection of PG and OVA on the intensity of a DTH reaction. Previous administration of three doses of the

specific antigen (PG) determined a tolerogenic effect characterized by a significant decrease in the DTH reaction in comparison to the control (+) group. This effect was not observed in animals previously inoculated with the non-related cartilage antigen (OVA), indicating, therefore, that the tolerogenic effect was specific for PG. The possible prophylactic use of bovine PG to prevent human arthritis, based on the presence of shared epitopes between human and bovine PG [42], certainly needs further investigation.

Further investigation is also necessary to disclose if this procedure can be translated to RA patients who already present with disease symptoms. Considering that these patients have a compromised self-tolerance, RA putative target autoantigens as PG, collagen, or citrullinated molecules could be associated with tolerogenic adjuvants or with the standard RA therapies to optimize disease treatment. The possible therapeutic use of PG alone or combined with vitamin D3 is being investigated by our research group. We also recently demonstrated that treatment with myelin peptide combined with vitamin D3 controlled experimental encephalomyelitis development in both therapeutic [43] and prophylactic [44] approaches. Other reports reinforce the feasibility of tolerogenic approaches in RA therapy [45]. Combined therapies would also allow decreasing immunosuppressive drugs dosage and, therefore, avoiding a generalized immunosuppression [46]. In addition, these antigen-specific strategies could be efficacious during the preclinical disease phase when immunological abnormalities are already happening without clinical manifestation.

## 5. Conclusion

Together, these results indicate that previous PG inoculation determines a specific tolerogenic effect strong enough to reduce the severity of subsequently induced arthritis.

## Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

## Acknowledgments

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## Research Article

# Immunomodulatory Effects of a Bioactive Compound Isolated from *Dryopteris crassirhizoma* on the Grass Carp *Ctenopharyngodon idella*

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In the present study, we investigated effects of compound kaempferol 3-a-L-(4-O-acetyl)rhamnopyranoside-7-a-L-rhamnopyranoside (SA) isolated from *Dryopteris crassirhizoma* during immune-related gene expression in *Ctenopharyngodon idella* head kidney macrophages (CIHKM). The expression of immune-related genes (*IL-1 $\beta$* , *TNF- $\alpha$* , *MyD88*, and *Mx1*) were investigated using real-time PCR at 2 h, 8 h, 12 h, and 24 h after incubation with 1, 10, and 50  $\mu\text{g mL}^{-1}$  of SA. Furthermore, fish were injected intraperitoneally with 100  $\mu\text{L}$  of SA, and immune parameters such as lysozyme activity, complement C3, SOD, phagocytic activity, and IgM level were examined at 1, 2, and 3 weeks after injection. The differential expression of cytokines was observed after exposure to SA. *IL-1 $\beta$*  genes displayed significant expression at 2 and 8 h after exposure to 1–10  $\mu\text{g mL}^{-1}$  of SA. SA also induced gene expression of cytokines such as *MyD88*, *Mx1*, and *TNF- $\alpha$* . Furthermore, enhanced immune parameters in grass carp confirmed the immunomodulatory activity of SA. Interestingly, this compound has no toxic effect on CIHKM cells as tested by MTT assay. In addition, fish immunised with 10  $\mu\text{g mL}^{-1}$  of SA exhibited maximum resistance against *Aeromonas hydrophila* infection. These results suggest that SA has the potential to stimulate immune responses in grass carp.

## 1. Introduction

Aquaculture is the fastest-growing sector of the animal food production industry [1], and in the last five decades, aquaculture production has grown considerably and production reached 62 million tons in 2011 [2]. However, world aquaculture production is vulnerable, and culture intensification could result in partial or total loss of production because of an increase in disease outbreaks, including infections from parasite, pathogenic bacteria, fungi, and virus diseases [3]. Generally, chemotherapeutics, vaccines, and antibiotics are used for disease control in aquaculture. However, indiscriminate use of antibiotics has resulted in the development of bacterial-resistant strains. For example, trichlorfon or praziquantel in bath treatments for parasites has disadvantages, including resistance development, potential hazards to animal health, and environmental pollution [4]. Furthermore, single vaccine

application is only effective against one type of pathogen, and the vaccination of juvenile fish is labour intensive and expensive. Therefore, application of natural immunostimulants could be a viable alternative to ensure the sustainability of aquaculture. Fish primarily depend on innate immunity, and immunostimulants play a major role in enhancing innate immune responses.

Plant extracts have been reported to promote growth, stimulate appetite, and have antiparasite and antibacterial properties, as well as acting as immunostimulant in fish cultures. Some of herbal immunostimulants are alkaloids, terpenoids, tannins, saponins, glycosides, flavonoids, phenolics, steroids, or essential oils [5, 6]. *Dryopteris crassirhizoma*, a semievergreen pteridophyte, is widely distributed in Japan, Korea, and China. Various plant extracts have a number of pharmacological activities, including antioxidant, tumouricidal, and fatty acid synthase inhibitory activities [7–9].

Furthermore, bioactivities of *D. crassirhizoma* are mainly caused by the presence of phloroglucinol, polyphenols, and flavonoid [10]. The active compound sutchuenoside A (SA) isolated from *D. crassirhizoma* has been shown to exhibit vermifuge activity against *Dactylogyrus intermedius* in goldfish *Carassius auratus* [11]. However, research on the immune function of bioactive components from *D. crassirhizoma* in fish is lacking.

Cytokines are low molecular weight proteins, and they include interleukins (IL), interferons (IFN), chemokines, monokines, and certain growth factors [12]. Among these cytokines, interleukin- $1\beta$  (IL- $1\beta$ ), tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ), and interleukin-8 (IL-8) play a significant role in the recognition, initiation, and regulation of the inflammatory process and serve as an important component of innate immunity [13]. Moreover, myxovirus resistance-1 (Mx1) protein facilitates defense against a diverse range of viruses [14], and myeloid differentiation factor 88 (MyD88) is an important adaptor molecule in the Toll-like receptor (TLR) signalling pathway and plays an important role in host defense against bacterial infections [15]. The numerous roles of cytokines in fish immunity have been previously established. Because cytokines are important regulators of the immune system, investigating their function may provide significant information for the development of vaccines and immunostimulants for fish. Therefore, the goal of the present study was to determine if the compound sutchuenoside A, which was isolated from *D. crassirhizoma*, could influence immune-related gene expression in *C. idella* kidney cells *in vitro* using a quantitative real-time PCR (qRT-PCR) based method. Furthermore, the effect of SA on immune responses and disease resistance in grass carp was investigated to determine its potential as an immunostimulant.

## 2. Materials and Methods

**2.1. Isolation of Bioactive Compound from *Dryopteris crassirhizoma*.** In our previous study, we demonstrated that kaempferol 3-a-L-(4-O-acetyl) rhamnopyranoside-7-a-L-rhamnopyranoside (sutchuenoside A, SA) isolated from *D. crassirhizoma* exhibited anthelmintic activity against *D. intermedius* in goldfish. The compound was isolated and stored as we described [11]. Dried rhizomes (8 kg) were ground into a coarse powder using a mechanical pulveriser and were ultrasound-extracted with methanol (MeOH) (10 L  $\times$  4 times) at 60°C for 40 min. The extract was filtered, concentrated under reduced pressure in a vacuum rotary evaporate, and desiccated to yield 508 g of methanol extract. The MeOH extract was then partitioned using petroleum ether (60–90°C) and ethyl acetate (EtOAc). The concentrated EtOAc phase was applied to silica gel column chromatography using chloroform–methanol (gradient; 1:0  $\rightarrow$  0:1; v/v) solvent mixtures and three fractions were obtained (A–C). All fractions were monitored and combined based on thin-layer chromatography. Compound SA was isolated from fraction B as a yellowish powder by repeated silica gel column chromatography using the chloroform–methanol mixture as the eluent. Stock solutions were prepared by dissolving fractions and pure compounds

in dimethyl sulfoxide (DMSO) at the concentration of 100 mg mL<sup>-1</sup>.

**2.2. Experimental Fish.** Healthy grass carp (average body weight: 103.2  $\pm$  3.4 g) was procured from a local fish farm and acclimatised to laboratory conditions (dissolved oxygen: 5.50  $\pm$  0.68 mg L<sup>-1</sup>; pH: 7.2  $\pm$  0.5; nitrites: 0.022  $\pm$  0.01 mg L<sup>-1</sup>; ammonia: 0.14  $\pm$  0.05 mg L<sup>-1</sup>) for two weeks in 500-L quarantine tanks at 23  $\pm$  1°C. Fish were fed a basal diet [16] during the acclimatisation period. Approximately 20% of the water in all tanks was exchanged daily, and 100% of the water was exchanged once a week. Basic physiochemical parameters of the water were measured every week.

### 2.3. In Vitro Study

**2.3.1. Isolation of *C. idella* Head Kidney Macrophage Cells and Treatment.** Isolation and cultures of *C. idella* headkidney macrophage (CIHKM) cells were performed as described by Secombes [17]. Cell viability was assessed using the trypan blue (Sigma-Aldrich, USA) exclusion test, and cell number was adjusted to 1  $\times$  10<sup>7</sup> cells mL<sup>-1</sup>. CIHKM cells were cultured in an incubator at 28°C within sterile 6-well tissue culture plates containing 5.0 mL Minimum Essential Medium (MEM) supplemented with 10% inactivated foetal calf serum (GIBCO/BRL). For isolated compound treatment, 1  $\times$  10<sup>7</sup> cells were treated with SA (100  $\mu$ L) at three different concentrations of 1, 10, and 50  $\mu$ g mL<sup>-1</sup> for 2, 8, 12, and 24 h at 28°C. For the control, CIHKM cells were incubated with 100  $\mu$ L of DMSO. Incubated cells were harvested at the time points mentioned above for RNA extraction. All the tests were performed in triplicate.

**2.3.2. RNA Extraction and Reverse Transcription.** Total RNA was extracted from CIHKM cells using TRIzol Reagent (Cwbio, China). The quality and purity of RNA were assessed by spectrophotometry, and 260:280 ratios were 1.8–2.0. Afterwards, genomic DNA contamination was removed by the treatment with DNase I (Promega, Madison, WI, USA). DNA was then synthesised using the PrimeScript™ RT reagent Kit (TaKaRa, Osto, Japan) following the manufacturer's instructions. The resulting cDNA was stored at -80°C.

**2.3.3. Real-Time Quantitative PCR Analyses of Gene Expression.** The analysis of expression of immune-related genes *MyD88*, *IL-1 $\beta$* , *TNF- $\alpha$* , and *Mx1* was carried out using real-time quantitative PCR (Qiagen, Germany). All the qRT-PCR reactions were performed using the SYBR Premix Ex Taq™ Perfect Real-Time Kit (TaKaRa, Osto, Japan) and carried out in the Qiagen Rotor-Gene Q Real-Time PCR Detection System (Qiagen, Germany). The  $\beta$ -*actin* gene was used as a house-keeping gene. The PCR primer sequences used for qRT-PCR are listed in Table 1. The reaction mixture included 10  $\mu$ L of SYBR Premix Ex Taq™, 1  $\mu$ L of forward and reverse primer (10 mM), and 1  $\mu$ L of cDNA and was filled with ultra-pure water to a final total volume of 20  $\mu$ L. The reaction conditions and cycle index were 95°C for 10 min followed by 40 cycles of 95°C for 15 s, 56°C for 30 s, and 72°C for

TABLE 1: Primers used for the analysis of mRNA expression by real-time PCR.

Genes	Primer sequence	Accession number
<i>β-actin</i>	F: 5'GATGATGAAATTGCCGCACTG 3' R: 5'ACCGACCATGACGCCCTGATGT 3'	M25013
<i>IL-1β</i>	F: 5'GGAGAATGTGATCGAAGAGCGT 3' R: 5'GCTGATAAACCATCCGGGA 3'	EU047716
<i>TNF-α</i>	F: 5'TGTGCCGCCGCTGTCTGCTTCACGCT 3' R: 5'GATGAGGAAAGACACCTGGCTGTAGA 3'	EU047718
<i>MyD88</i>	F: 5'GAAATGATGGACTTTACCTACCTG 3' R: 5'ACATCTTTCCTTTCGGCTTTT 3'	FJ843088
<i>Mxl1</i>	F: 5'CTGGGGAGGAAGTAAAGTGTCT 3' R: 5'CAGCATGGATTCTGCCTGG 3'	HQ245104

30 s. Negative control without cDNA was included in each assay. The primers used in this study were specific to grass carp; the sequences were obtained from published literature [18]. After the amplification phase, melting curve analysis was conducted to eliminate the possibility of nonspecific amplification or primer dimer formation. A standard curve was created from serial dilutions of sample cDNA. A standard curve was drawn by plotting the natural log of the threshold cycle (Ct) against the number of molecules. Standard curve of each gene was run in duplicate and three times for obtaining reliable amplification efficiency. The correlation coefficients ( $R^2$ ) of all standard curves were  $>0.99$  and the amplification efficiency was between 90 and 110%. The relative expression ratios of target genes in the treatment group versus those in the control group were calculated according to the following formula: fold changes =  $2^{-\Delta\Delta C_t}$ , where  $\Delta\Delta C_t = (C_t [\text{treatment group}] - C_t [\text{treatment } \beta\text{-actin}]) - (C_t [\text{control group}] - C_t [\text{control } \beta\text{-actin}])$  [19]. In all cases, each three replicates of the PCR were performed.

**2.3.4. Cell Viability Assay.** The cell viability assay was performed using the Cell Counting Kit 8 (Dojindo, Tokyo, Japan), according to the manufacturer's protocol, to measure the effect of SA on the viability of CIHKM cells. In each group,  $2 \times 10^4 - 5 \times 10^4$  cells were incubated in a well of a 96-well plate containing 200  $\mu\text{L}$  medium (MEM with 10% foetal calf serum) at 28°C in 5%  $\text{CO}_2$  atmosphere for 24 h. After the culture, the media in all the wells were replaced with 200  $\mu\text{L}$  fresh media containing SA in the concentrations of 0, 1, 10, and 50  $\mu\text{g mL}^{-1}$ , respectively. After 2, 12, 24, 48, 72, and 96 h of incubation, solutions were replaced by 180  $\mu\text{L}$  DMEM without serum, and then 20  $\mu\text{L}$  of sterile filtered MTT (Sigma) solution in phosphate-buffered saline (PBS) at pH 7.4 (5  $\text{mg mL}^{-1}$ ) was added to each well. The final MTT concentration was 0.5  $\text{mg mL}^{-1}$  for 4 h; the unreacted dye was then removed, and the insoluble formazan crystals were dissolved in 200  $\mu\text{L}$  per well dimethylsulphoxide (DMSO) and shaken for 5 min. The OD value of the resulting solution was measured by a multi-well scanning spectrophotometer (ELISA reader) at 570 nm, and the culture medium without cells was considered a blank. The relative cell viability were

calculated by absorbance  $[A]_{\text{test}}/[A]_{\text{control}} \times 100\%$ , where the control group contained culture medium without SA [18]. All the tests were performed in triplicate.

## 2.4. In Vivo Investigation

**2.4.1. Experimental Design.** After acclimatising to culture conditions for a period of 14 days, fish were randomly divided into four groups. Each group consisted of 25 fish with three replicates (i.e., 25 fish  $\times$  3 tanks = 75 fish per group). The fish in the control group were injected intraperitoneally (i.p.) with 100  $\mu\text{L}$  of DMSO and the other three experimental groups were immunised i.p. with 100  $\mu\text{L}$  of DMSO containing either 1, 10, or 50  $\mu\text{g mL}^{-1}$  of SA. Fish were maintained for observation for 21 days and fed a basal diet twice a day (09:00 and 17:00 h). The composition of the basal diet is shown in Table 2.

**2.4.2. Blood Samples for Immunological Measurement.** Three fish were randomly collected from each tank at the end of 1st, 2nd, and 3rd weeks of immunisation to measure immunological parameters. Thus, 9 fish (3 fish  $\times$  3 replicates = 9 fish) were collected from each group for immunological assays. Fish were euthanised with an over dose of MS222. Blood was sampled by caudal venipuncture using a 1-mL syringe and immediately used for leucocytes isolation as description by Cheng et al. [20]. Harvested cells were adjusted to  $1 \times 10^7$  cells  $\text{mL}^{-1}$  by adding an appropriate volume of RPMI 1640 (Sigma-Aldrich, USA) for cellular immunological parameter assays. Another 0.5 mL of blood from each replicate was centrifuged at 3000  $\times g$  for 10 min at 4°C to collect serum and was then stored at -20°C until use.

## 2.4.3. Immunological Parameters Analysis

**Lysozyme Activity.** Lysozyme activity was measured following the turbidimetric method described by Ellis [21]. A unit of lysozyme activity was defined as the amount of serum lysozyme that caused a decrease in absorbency of 0.001  $\text{min}^{-1}$  at 530 nm.

TABLE 2: Ingredient and chemical proximate composition of basic diet (% dry matter).

Ingredient	%
Soybean meal	43
Fish meal	35
Wheat meal	15
Corn meal	5
$\alpha$ -starch	1
Mineral and vitamin mixture <sup>a</sup>	1
<i>Proximate analysis</i>	
Crude protein%	24.8
Crude lipid%	1.9
Ash%	10.8

<sup>a</sup>Every 250 g of mineral-vitamin mixture provided vitamin A, 500,000 IU; vitamin D3, 100,000 IU; vitamin B1, 7 g; vitamin B2, 20 g; vitamin B6, 6 g; vitamin B12, 80 mg; vitamin E, 30 g; vitamin K3, 6 g; vitamin C, 50 g; pantothenate, 15 g; niacin, 65 g; folic acid, 3 g; inositol, 65 g; biotin, 150 mg.

**Complement C3 Assay.** The serum complement C3 level was assayed using the complement C3 assay Kit (Jiancheng, Nanjing, Jiangsu, China) following the manufacturer's instructions [22]. The OD was measured at 340 nm. Methods for complement C3 level analysis included the measurement of the increase in turbidity following the immunity response of complement C3 and the increase in its antibody [23, 24]. Results are presented as complement C3 mg mL<sup>-1</sup>.

**Phagocytic Activity.** Phagocytic activity (PA) of leucocytes was evaluated by the method of Ai et al. [25] with slight modifications. A 100  $\mu$ L of  $1 \times 10^7$  cells mL<sup>-1</sup> suspension of leucocytes was placed on a sterile glass slide and allowed to attach at 25°C for 30 min. Following attachment, 100  $\mu$ L of  $1 \times 10^8$  cells mL<sup>-1</sup> yeast suspension was added to the cell monolayer. The glass slides were incubated at 25°C for 45 min and then were washed with PBS (pH = 6.2) three times to remove uningested yeasts and unattached leucocyte cells. Finally, the slides were fixed with ethanol, redried, and stained with Giemsa. The number of phagocytic cells per 100 adherent cells was microscopically determined. PA was calculated using the formula:

$$PA = \left( \frac{\text{phagocytic leucocytes}}{\text{total leucocytes}} \right) \times 100 \quad (1)$$

**Superoxide Dismutase Activity.** SOD activity was determined with SOD kits (Jiancheng, Nanjing, Jiangsu, China) following the manufacturer's instructions [26]. The OD was measured at 550 nm. One unit of SOD was defined as the amount required for inhibiting the rate of xanthine reduction by 50% in a one mL reaction system. Specific SOD activity was expressed as SOD units per mL of serum [27].

**Serum Immunoglobulin (IgM) Level.** Serum IgM levels were assayed by enzymatic procedures utilising an automatic biochemical analyser (Hitachi 7180, Tokyo, Japan) [26]. Total IgM was expressed as unit mg mL<sup>-1</sup>.

**2.5. Challenge Study.** After 21 days of immunisation, 30 fish ( $10 \times 3 = 30$ ) from each group were injected with 0.2 mL of PBS containing  $1 \times 10^7$  of live *Aeromonas hydrophila* (strain SG 322). Pathogenicity and dose of *A. hydrophila* (strain SG 322) were determined earlier [16]. Another group of 30 fish (fed basal diet during feeding trial) were injected with 0.2 mL of PBS and considered as negative control. The challenged fish were observed for two weeks, and mortality was recorded.

**2.6. Statistical Analysis.** One-way analysis of variance (ANOVA) was used to analyse the data. Multiple comparisons were performed with Tukey's test to analyse differences between treatments. All statistical analyses were performed using the OriginPro software (version 8; OriginLab Corporation, Northampton, USA). The level of significance was set at  $P < 0.05$ . The results are expressed as mean  $\pm$  S.E.M.

### 3. Results

**3.1. Challenge Study.** Two weeks of immunisation with SA enhanced the resistance of fish to *A. hydrophila* infection (Figure 1). The highest postchallenge survival (73.33%) was recorded in the fish group immunised with  $10 \mu\text{g mL}^{-1}$  of SA, whereas the lowest postchallenge survival rate (23.33%) was observed in the control group. Fish immunised with  $1 \mu\text{g mL}^{-1}$  or  $50 \mu\text{g mL}^{-1}$  of SA exhibited survival rates of 56.66% and 40%, respectively. No mortality was observed in the group injected with PBS only. Typical symptoms of haemorrhagic septicaemia were observed in moribund or dead fish. Colonies of *A. hydrophila* were isolated from dead fish.

**3.2. In Vitro Effect of SA on the Viability of CIHKM Cells.** Only a 4.6% decrease ( $P > 0.05$ ) in cell viability was observed in the CIHKM cells treated with  $50 \mu\text{g mL}^{-1}$  of SA for 96 h compared to that of the control. However, 1 and  $10 \mu\text{g mL}^{-1}$  of SA had no cytotoxic effects on CIHKM cells at any time points during the assay (Figure 2).

**3.3. In Vitro Effect of SA on the Expression of Immune-Related Genes in CIHKM Cells.** Expressions of immune-related genes (*MyD88*, *IL-1 $\beta$* , *TNF- $\alpha$* , and *Mx1*) in CIHKM cells treated with SA are shown in Figure 3.

Expression of *MyD88* gene (Figure 3(a)) in CIHKM cells stimulated with 1 or  $10 \mu\text{g mL}^{-1}$  SA was significantly higher at 8 and 12 h poststimulation (hps) as compared to the control. However, SA had no significant effect on *MyD88* expression at 2 and 24 hps.

Exposure of CIHKM cells to SA at any concentration (1, 10, and  $50 \mu\text{g mL}^{-1}$ ) for 2 h and 8 h exhibited striking upregulation of *IL-1 $\beta$*  expression ( $P < 0.05$ ). However, no significant induction in *IL-1 $\beta$*  expression was observed at 12 and 24 hps with SA ( $P > 0.05$ ) (Figure 3(b)).

Expression of *TNF- $\alpha$*  is shown in Figure 3(c). Expression of the *TNF- $\alpha$*  gene was significantly higher in CIHKM cells at 2 to 12 hps with SA and highest expression was at 8 hps with  $10 \mu\text{g mL}^{-1}$  of SA ( $P > 0.05$ ). Longer exposure (i.e., 24 hps)

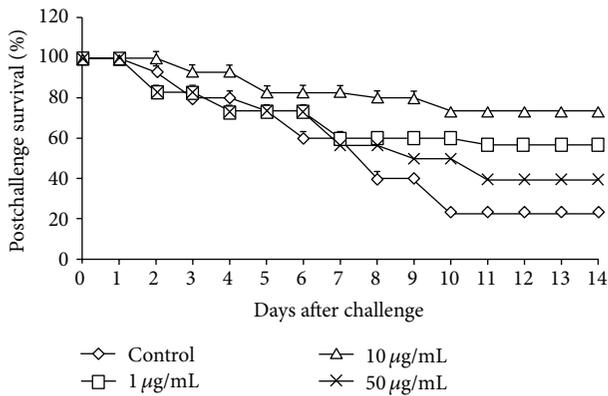


FIGURE 1: Postchallenge survival after artificial challenging with *A. hydrophila* in *C. idella* injected intraperitoneally on different assay days after infection with SA. Bars represent the mean values  $\pm$  S.E.M ( $n = 3$ ).

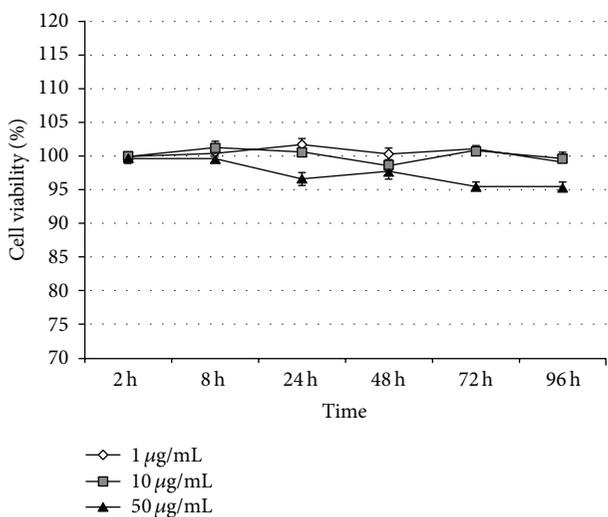


FIGURE 2: Effects of a bioactive compound SA isolated from *D. crassirhizoma* on *C. idella* head kidney macrophages (CIHKM) cells viability measured by MTT assay after 2, 8, 24, 48, 72, and 96 h incubation at 28°C. Bars represent the mean  $\pm$  S.E.M ( $n = 3$ ).

had no significant effect on *TNF- $\alpha$* . Exposure of CIHKM cells to the medium concentration of SA (10  $\mu\text{g mL}^{-1}$ ) had higher expression than the low or high concentrations.

Exposure of CIHKM cells to SA showed a time-dependent induction of *Mxl* expression as shown in Figure 3(d). Two-hour exposure of CIHKM cells to SA did not significantly affect *Mxl* gene expression. However, higher expression ( $P < 0.05$ ) of *Mxl* gene was detected at 8 and 12 hps with SA, and thereafter, expression was noticeably decreased to a nonsignificant level.

**3.4. Effect of SA on Immunological Parameters in *C. idella*.** Results of immunological parameters are shown in Figure 4. A significant increase in serum lysozyme activity was observed in fish after 1-2 weeks of immunisation with 1 or 10  $\mu\text{g mL}^{-1}$  SA, and thereafter it turned to normal level.

However, fish immunised with any concentration of SA exhibited no significant differences in lysozyme activity ( $P > 0.05$ ) at 3 weeks after immunisation (Figure 4(a)).

Fish immunised with SA had significantly higher complement C3 activity at 1 week after immunisation. However, at 2 weeks after immunisation, higher ( $P < 0.05$ ) activity was observed in the 10  $\mu\text{g mL}^{-1}$  SA treated group (Figure 4(b)).

The phagocytic activity was significantly higher in fish groups immunised with 1-10  $\mu\text{g mL}^{-1}$  of SA for 1-2 weeks (Figure 4(c)). However, higher concentration of SA had no significant effect on phagocytic activity at any point of time.

SOD activity was higher ( $P < 0.05$ ) in the treated groups only at 2 weeks after injection (Figure 4(d)). However, at 1 or 3 weeks after immunisation, no significant effects on SOD activities were detected.

Serum IgM levels (Figure 4(e)) showed an increasing ( $P < 0.05$ ) trend up to two weeks after immunisation and turned to normal level subsequently in all treated groups. The highest IgM level recorded in a fish group was after 2 weeks of immunisation with 10  $\mu\text{g mL}^{-1}$  of SA.

#### 4. Discussion

Plant products as ecological sources of medicine have been used widely for disease control in aquaculture. Several bioactive products from plants, which have potential anthelmintic, antibacterial, antiviral, and even immunostimulatory properties, have been reported in numerous studies [2]. Previously, only the anthelmintic effect of SA against *D. intermedius* in goldfish was documented [11]. However, the present study demonstrated the effects of SA on the expression of immune-related genes in CIHKM cells *in vitro* and on immune responses *in vivo* in order to explore the potential of SA as an immunomodulator in fish.

Innate immune responses are triggered upon pathogen recognition by various types of pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), which can recognise conserved pathogen-associated molecular patterns (PAMPs) of foreign intruders, including protozoa, bacteria, fungi, and viruses [28, 29]. TLRs operate by initially recognising corresponding PAMPs via extracellular leucine-rich repeat (LRR) motifs, changing this configuration, recruiting Toll/IL-1 receptor (TIR) adaptors including MyD88 to trigger signalling pathways, inducing the production of proinflammatory cytokines, and developing adaptive immunity [30]. After the TLRs recognise specific PAMPs, TLR signalling can be segregated into the MyD88-dependent pathway, which causes the activation of the nuclear factor-kappa B (NF- $\kappa$ B) and the expression of proinflammatory genes, such as tumour necrosis factor (TNF) or interleukin-1 (IL-1) [29, 31, 32]. The MyD88-independent pathway leads to interferon 3 (IRF3) mediated expression of type I interferons (IFN) and IFN-inducible genes [33]. It was demonstrated that MyD88 played an important role in defense against viral infection and subsequent tissue repair [30]. In the present study, the MyD88 transcript level was found to increase, especially at low concentration (1  $\mu\text{g mL}^{-1}$ ) at 8 hps. Yu et al. [18] reported that stimulation with two compounds (1,5-anhydro-D-glucitol and 3,4,5-trimethoxy cinnamic acid) isolated from

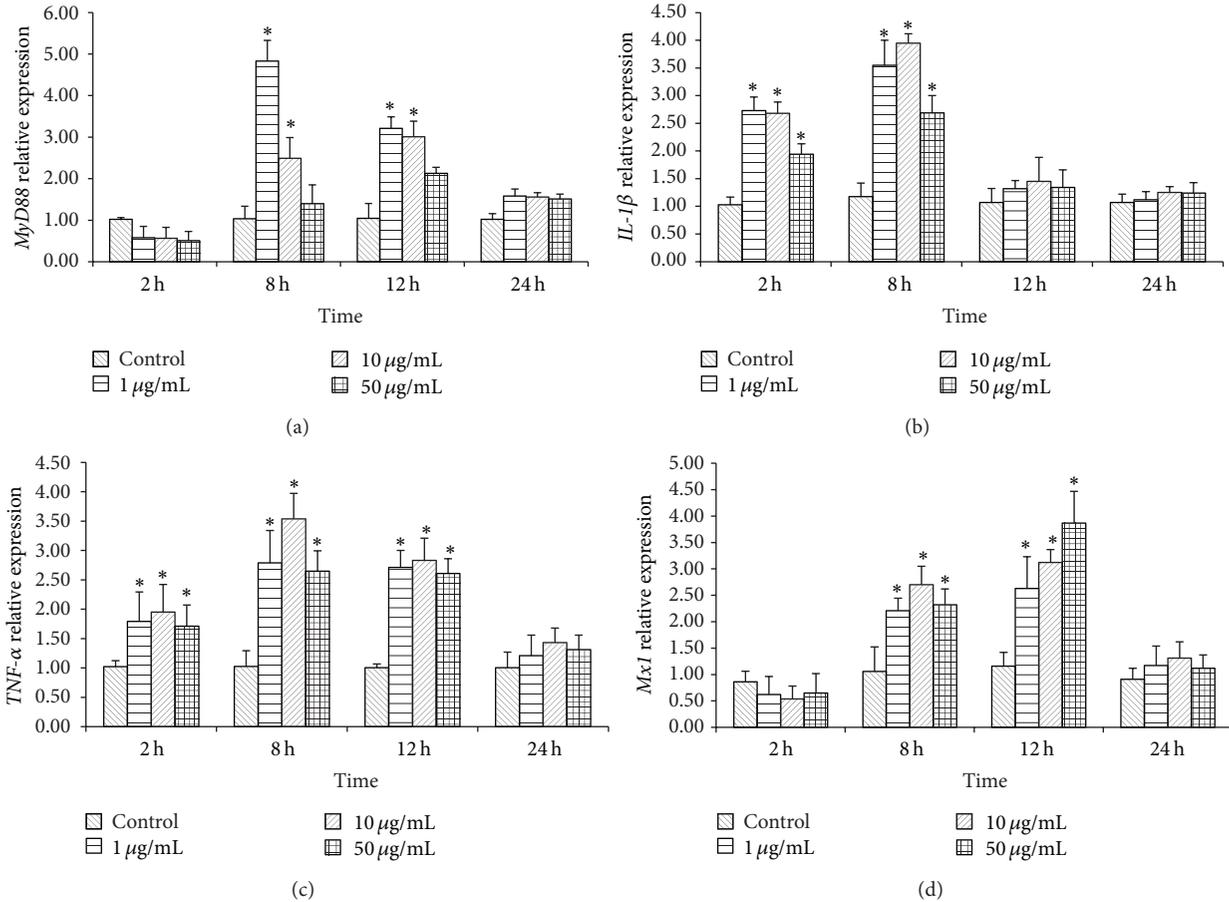


FIGURE 3: Relative expression of immune-related genes at different time points in *C. idella* head kidney macrophages (CIHKM) cells stimulated with SA. (a) Expression of *MyD88* gene; (b) expression of *IL-1 $\beta$*  gene; (c) expression of *TNF- $\alpha$*  gene; (d) expression of *Mx1* gene. Bars represent the mean  $\pm$  S.E.M ( $n = 3$ ). Significant expression levels in SA stimulated cells compared to unstimulated control are indicated by an asterisk (\*) at that time point ( $P < 0.05$ ).

*Polygala tenuifolia* upregulated the expression of the *MyD88* gene in *C. idella* kidney (CIK) cells. Denis and Archambault [34] also reported the induction of *MyD88* expression in CIK cells by secondary metabolites from *Alcaligenes faecalis* FY-3. Moreover, our result was consistent with Su et al. [30] who reported that *MyD88* expression in CIK cells treated with polycytidylic acid was increased at first and then turned to control level at 24 h. These results may be explained by assuming that TLRs can recognise the molecular patterns of SA, or by some other unknown alternative mechanisms related to the immune responses.

The cytokines *IL-1 $\beta$*  and *TNF- $\alpha$*  are produced mainly by monocytes and macrophages, and they regulate several aspects of immune system [35]. *IL-1 $\beta$*  can stimulate immune responses by activating lymphocytes or by inducing the release of other cytokines capable of triggering macrophages, NK cells, and lymphocytes [36, 37]. The upregulation of the expression of cytokine-encoding genes by various immunostimulants in fish has been previously reported [34, 36]. Yu et al. [18] demonstrated that active compounds isolated from *P. tenuifolia* stimulated the expression of *IL-1 $\beta$*  in CIK cells. Previous work demonstrated that *IL-1 $\beta$*  was not constitutively

expressed in carp, *Cyprinus carpio* L., after stimulation with a bioactive compound [36], and this was in accordance with the results of the present study. Our results demonstrated that 1–10  $\mu\text{g mL}^{-1}$  of SA stimulation for 2–8 h resulted in stronger *IL-1 $\beta$*  expression than that in other experimental groups and at other durations. *TNF- $\alpha$*  works together with *IL-1 $\beta$*  in other cells, which, in turn, produce other cytokines and factors that lead to the activation of the acute phase of the immune response [38]. *TNF- $\alpha$*  exerts antiviral effects and may cause direct lysis of tumour cells [38]. In the present study, CIHKM cells stimulated with SA produced significant induction in *TNF- $\alpha$*  expression. Previous studies also reported that stimulation by LPS and flagellin significantly induced the expressions of *TNF- $\alpha$*  in *Oncorhynchus mykiss* [39]. Similar results of enhanced *TNF- $\alpha$*  expression level [18] were also observed in CIK after stimulation with two compounds isolated from *Polygala tenuifolia*.

Mx proteins are key components of the antiviral state, which are interferon (IFN) induced dynamin-like GTPases in many species, and Mx GTPases appear to detect viral infection by sensing nucleocapsid-like structures [40]. Generally, Mx proteins consist of three domains including the

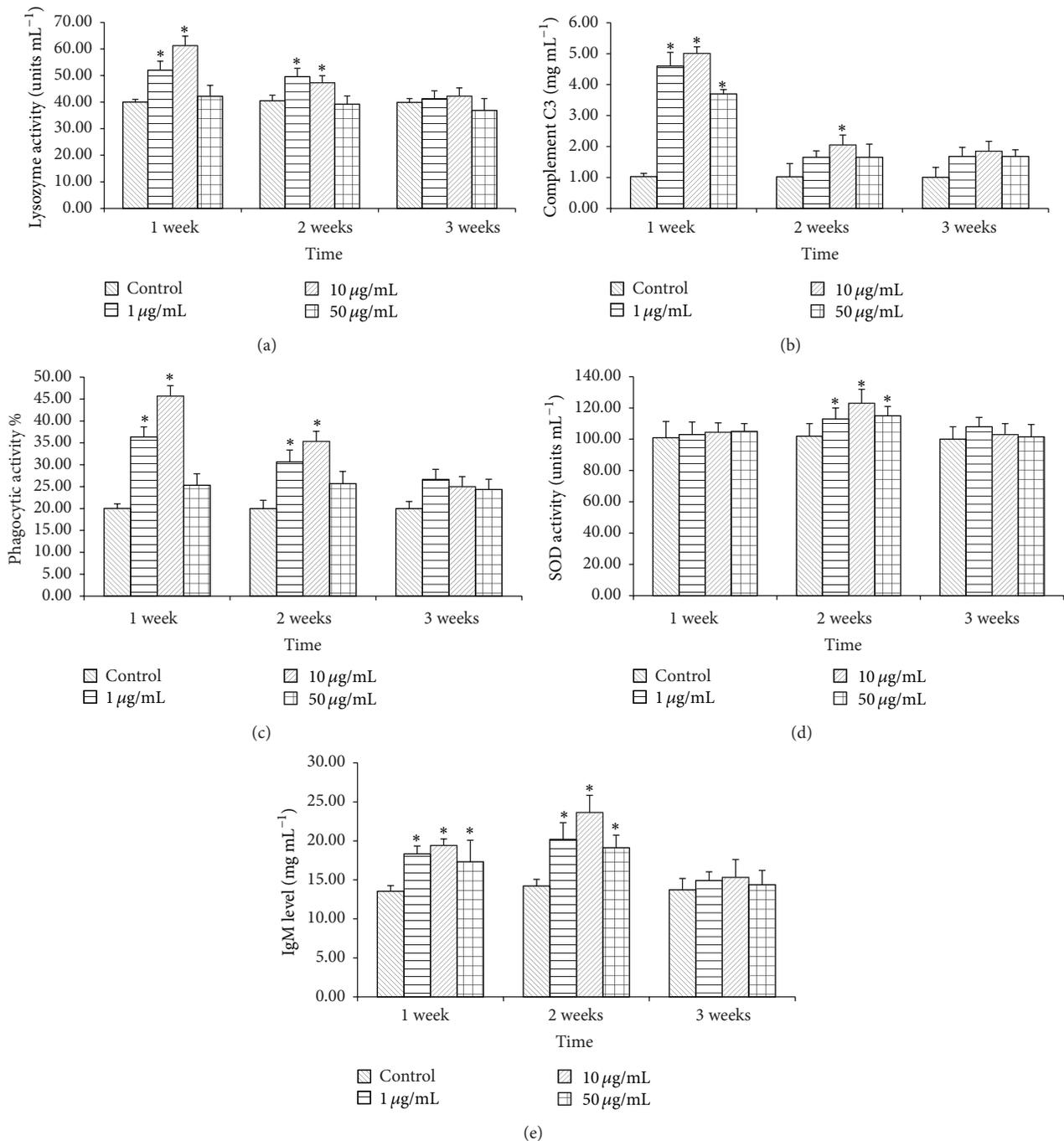


FIGURE 4: Effects of SA on nonspecial immune responses in *C. idella* immunised (i.p) with SA at different time points. (a) Lysozyme activity; (b) complement C3 level; (c) phagocytic activity; (d) SOD activity; (e) IgM level. Bars represent the mean  $\pm$  S.E.M ( $n = 9$ ). A significant difference compared to control on the same sampling week is indicated by an asterisk (\*).

C-terminal region that is divergent and acts as an effector domain, which facilitates defense against a diverse range of viruses [14, 40]. The results of our investigation indicated that the *Mxl* gene was significantly induced after 24 h exposure to SA.

The innate immune system is a fundamental defense mechanism in fish [41]. Lysozyme is a vital antibacterial effector molecule, which can be regarded as an opsonin

by activating phagocytes and the complement system [42]. The increase in serum lysozyme levels suggests elevation of various humoral factors that can protect the host during pathogen invasion [43]. In the present study, the low and medium concentrations of SA revealed a positive influence on the lysozyme activity at 1 to 2 weeks after immunisation with SA. A previous study [44] also reported that dietary administration of herbal extracts enhanced the lysozyme activity

in fish. Several studies have shown that external factors such as nutrients, probiotics, and vitamins influence complement activity in fish [45]. The complement cascade consists of one or a combination of three pathways, namely, the alternate, classical, and lectin pathways [46]. All the three pathways unite at a common amplification step involving complement C3 and proceed through a terminal pathway that leads to the formation of a membrane attack complex, which can lyse pathogens directly [47, 48]. In the present study, serum complement C3 levels were found to be significantly higher in the first week after treatment. Complement activation is usually beneficial to fish, but persistent activation of the complement could cause side effects and immunosuppression in the host [47]. Therefore, short-term activation of serum complement C3 in the present study may provide health benefits to fish.

The present study revealed that phagocytic activity was significantly higher ( $P < 0.05$ ) in fish after 1 to 2 weeks after immunisation with SA. In another study, similar results of higher phagocytic activity were observed in carp after administration of herbal extracts (*A. radix* and *G. lucidum*) [44]. Phagocytosis is an important cellular response in the fish immune system [41], and several studies have confirmed that herbal medicines can enhance phagocytic activity in fish [49, 50]. A recent study suggested that increased *IL-1 $\beta$*  transcripts in the stimulated cells might be a result of enhanced phagocytosis and activation of inflammasomes [48]. Similarly, increased SOD activity was observed in *Labeo rohita* administered herbal extracts [49, 50]. SOD catalyses the dismutation of the highly reactive ( $\cdot\text{O}_2$ ) less reactive  $\text{H}_2\text{O}_2$  and functions in the main antioxidant defense system in response to oxidative stress [51]. Enhanced levels of serum immunoglobulin are thought to be associated with a stronger immune response in fish. In the present study, significant enhancements in serum immunoglobulin level was observed in fish immunised with 1–10  $\mu\text{g mL}^{-1}$  SA for up to 2 weeks. In a recent study, Giri et al. [50] demonstrated that *L. rohita* fed diet supplemented with guava leaf powder exhibited higher serum immunoglobulin activity, which support our result of enhanced serum immunoglobulin after SA stimulation. The increased activities of serum lysozyme, complement C3, PA, SOD, and IgM in the present study indicate that SA plays a significant role in enhancing immune responses in grass carp *C. idella*. Moreover, isolated compound (SA) at low and medium concentrations had no toxicity towards CIHKM cells, and at high concentration, it had a slightly toxic effect on the cells. This noncytotoxic nature of SA (1 to 10  $\mu\text{g mL}^{-1}$ ) ensured that its influence on cytokine responses was not attributed to the production of secondary stimulation induced by inflammatory mediators released by dead cells.

An experimental infection provides an opportunity to evaluate the effectiveness of SA stimulation in terms of protection against pathogens [46]. In the present study, fish immunised with 10  $\mu\text{g mL}^{-1}$  SA exhibited the highest postchallenge survival (73.33%), followed by those immunised with 1  $\mu\text{g mL}^{-1}$  SA (56.66%). Recent studies have reported that dietary administration of guava leaves or *Chlorophytum borvillianum* polysaccharide enhanced the postchallenge survival of *L. rohita* against *A. hydrophila*

infection [49, 50]. The enhanced immune parameters observed in fish immunised with 10  $\mu\text{g mL}^{-1}$  SA might be associated with the elevated resistance of the fish against *A. hydrophila* and the resulting higher postchallenge survival rates.

## 5. Conclusion

In conclusion, the findings of this study demonstrate the immunostimulatory properties of the compound kaempferol 3-a-L-(4-O-acetyl) rhamnopyranoside-7-a-L-rhamnopyranoside (SA) isolated from *D. crassirhizoma*, which increased the immune responses (e.g., lysozyme activity, complement C3, phagocytic activity, SOD activity, and IgM level) of fish *in vivo* and induced expression of immune-related genes (e.g., *MyD88*, *IL-1 $\beta$* , *TNF- $\alpha$* , and *Mx1*) *in vitro*. Further, it enhanced the resistance of fish against pathogen infection. Hence, this natural immunostimulant could be a potential substitute for antibiotics and chemicals in aquaculture practices. However, studies involving use of SA as a feed supplement or oral immunisation on growth promotion and the specific mechanisms controlling immune response modulation are currently under progress in our laboratory.

## Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

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## Review Article

# Clinical Relevance of HLA Gene Variants in HBV Infection

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Host gene variants may influence the natural history of hepatitis B virus (HBV) infection. The human leukocyte antigen (HLA) system, the major histocompatibility complex (MHC) in humans, is one of the most important host factors that are correlated with the clinical course of HBV infection. Genome-wide association studies (GWASs) have shown that single nucleotide polymorphisms (SNPs) near certain HLA gene loci are strongly associated with not only persistent HBV infection but also spontaneous HBV clearance and seroconversion, disease progression, and the development of liver cirrhosis and HBV-related hepatocellular carcinoma (HCC) in chronic hepatitis B (CHB). These variations also influence the efficacy of interferon (IFN) and nucleot(s)ide analogue (NA) treatment and response to HBV vaccines. Meanwhile, discrepant conclusions were reached with different patient cohorts. It is therefore essential to identify the associations of specific HLA allele variants with disease progression and viral clearance in chronic HBV infection among different ethnic populations. A better understanding of HLA polymorphism relevance in HBV infection outcome would enable us to elucidate the roles of HLA SNPs in the pathogenesis and clearance of HBV in different areas and ethnic groups, to improve strategies for the prevention and treatment of chronic HBV infection.

## 1. Introduction

Certain host gene variants, including human leukocyte antigen (HLA) classes I and II alleles as well as non-HLA genes, influence the natural history of hepatitis B virus (HBV) infection. The HLA system, the major histocompatibility complex (MHC) in humans, is one of the most important host factors correlated with the clinical course of HBV infection. The highly polymorphic HLA classes I and II genes can affect the ability of HLA molecules to trigger immune responses, which affects the outcome of infection by any given pathogen [1]. HBV peptides presented by HLA class I molecules to cytotoxic T lymphocytes (CTLs) are critical in the eradication of HBV infection by boosting CTL ability to identify and kill HBV-infected cells [2, 3].

The discordance between HBV-specific CD8<sup>+</sup> T cell repertoires present in different ethnic groups, such as Caucasian and Chinese subjects, was shown to reflect the ability of HLA micropolymorphisms to diversify T cell responses [4]. Previous studies have revealed the associations of certain HLA class I genes with the course of HBV infection [5].

HLA class II molecules are cell surface glycoproteins of antigen presenting cells (APCs), which are responsible for presenting exogenous antigens to CD4<sup>+</sup> T-helper cells. Antigen presentation efficiency may vary due to gene loci [6]. Genome-wide association studies (GWASs) have shown that single nucleotide polymorphisms (SNPs) near HLA-DP, HLA-DQ, and HLA-DR loci are significantly correlated with HBV infection outcomes [7–9]. HLA class II gene variations are strongly associated with not only persistent HBV infection but also spontaneous HBV clearance and seroconversion [10, 11], disease progression, and the development of liver cirrhosis (LC) and HBV-related hepatocellular carcinoma (HCC) in chronic hepatitis B (CHB) [7, 12, 13]. Such variations also affect interferon (IFN) and nucleot(s)ide analogue (NA) treatment efficacy and the response to HBV vaccines. Meanwhile, discrepant conclusions are reached in different cohorts [14]. For instance, the candidate variant rs9277535 (550A/G) in the 3' untranslated region (3'UTR) of the HLA-DPB1 gene, associated most significantly with CHB and HBV infection outcomes in Asians, was shown to have a minimal effect on HBV recovery in European American and African

American subjects [15]. It is therefore critical to identify the associations of specific HLA allele variants with disease progression and viral clearance in chronic HBV infections among different ethnic groups.

A deeper understanding of HLA polymorphism relevance in HBV infection outcome would help elucidate the role of HLA SNPs in the pathogenesis and clearance of HBV in different areas and ethnicities, to improve strategies for the prevention and treatment of chronic HBV infection. This review summarizes the reported associations of HLA polymorphisms with susceptibility to HBV infection, resolution, disease progression, and antiviral treatment efficacy and response to HBV vaccines.

## 2. Associations of HLA Gene Variants with Susceptibility and Persistence of HBV Infection

Though several GWASs have revealed the association of gene variants in the HLA region with chronic HBV infection, the susceptibility gene loci and potential mechanisms have not been fully identified. A comparative review showed that HLA-DRB1\*11/\*12 alleles and DQB1\*0301 are associated with HBV persistence globally [14]. A Chinese study by Zhu et al. [16] identified four loci that independently drive chronic HBV infection, including HLA-DP $\beta$ 1 positions 84–87, HLA-DR $\beta$ 1\*13 sites 71 and rs400488, and HLA-C position 15. In another study [17], after genotyping 140 SNPs within the HLA-DP/DQ genomic region in a total of 1657 HBV-positive patients and 1456 HBV-negative controls, 76 SNPs and 5 LD blocks were identified in HLA-DP/DQ clusters, independent of each other, which are significantly associated with HBV infection; rs9277535 in HLA-DPBI was found to be the most significant locus. Chang et al. [18] found that rs9277535 (HLA-DPBI), rs9276370 (HLA-DQA2), rs7756516 and rs7453920 (HLA-DQB2), and rs9366816 near HLA-DPA3 are significantly associated with persistent HBV infection, especially the “T-T” haplotype composed of rs7756516 and rs9276370 that is more prevalent in severe disease subgroups and associated with nonsustained therapeutic response ( $P = 0.0262$ ) in male Taiwan Han Chinese individuals. DQB1\*0301 and DQB1\*0303 are correlated with continuous infection in Xinjiang Uyghur Chinese subjects [8].

A transethnic association analysis [19] performed among Asian populations, including Japanese, Korean, Hong Kong, and Thai subjects, revealed Asian-specific associations of HLA-DPA1 and HLA-DPBI alleles/haplotypes with HBV infection and disease progression. The latter study identified a new risk allele HLA-DPBI\*09:01 and a new protective allele DPBI\*02:01 in chronic HBV infection. An American study [15] showed that the HLA-DPBI 3'UTR 496GG genotype confers latent susceptibility to persistent HBV infection and is also associated with significantly higher levels of HLA-DP surface protein expression in healthy donors, suggesting that differences in HLA-DP expression may increase the risk for persistent HBV infection in European Americans and African Americans. A prospective study [20] showed that class II alleles DQA1\*0501 ( $P = 0.05$ ) and DQB1\*0301

( $P = 0.01$ ), the two-locus haplotype consisting of the latter two alleles, and the three-locus haplotype DQA1\*0501, DQB1\*0301, and DRB1\*1102 ( $OR = 10.7$ ;  $P = 0.01$ ) are significantly associated with persistent HBV infection in an African American cohort.

In addition, HBV persistence was shown to be associated with class II allelic homozygosity. Interestingly, three SNPs belonging to the HLA-DQ region (rs2856718, rs7453920, and rs9275572), shown to display increased susceptibility to chronic HBV infection, were detected in Saudi Arabian patients [21]. Meanwhile, DRB1\*08 and DRB1\*09 alleles, which are susceptible to HBV infection, were found in Brazilian populations determined in young and male blood donors [9]. A study [22] identified 2 risk alleles in MHC loci, namely, HLA-DPA1 (rs3077) and HLA-DPBI (rs9277535), using salivary DNA extracted with a modified protocol from blood samples in Chinese patients. This provides a new noninvasive screening method for identifying risk loci.

Furthermore, HLA gene variants are also associated with susceptibility to vertical transmission. Multiple factors, including HBV structure and DNA level, placental barrier, the immune status of the mother, and the genetic background of the newborn infant, determine the susceptibility to intrauterine HBV infection. Xu et al. [23] assessed 15 HLA-DR alleles and found HLA-DRB1\*07 to be the only one associated with infant susceptibility to intrauterine HBV infection.

On the other hand, gene polymorphisms of some HLA loci confer protective effects against persistent HBV infection. It was shown that the HLA-DPA1 and HLA-DPBI genes are significantly associated with protective effects against CHB in Japanese, Korean, and other Asian populations, including Chinese and Thai individuals [19]. Cross-sectional studies showed that HLA-DRB1\*1301-02 are associated with protection against persistent HBV infection in Gambia, Germany, and Korea [24–26]. China has the highest HBV prevalence rate in the world, with different ethnic populations. Wang et al. [27] demonstrated that, in two independent case-control studies, HLA-DP A alleles of both rs3077 and rs9277535 significantly decrease the risk for CHB in Chinese Han subjects, while HLA-DP rs9277535 is associated with decreased risk in Chinese Zhuang subjects. In addition, HLA-DQB1\*0201 is HBV resistance gene in Xinjiang Uyghur ethnic groups of China [8]. Although the most significant associations were observed for HLA-DPA1 rs3077 and HLA-DPBI rs9277535 A alleles (decreased risk for HBV infection in Asian populations), only a highly significant association of HLA-DPA1 rs3077 with HBV infection was observed in Caucasians [28].

## 3. Associations of HLA Gene Variants with Spontaneous HBsAg Clearance and HBV Eradication

Spontaneous HBsAg seroclearance occurs in a very small proportion of patients with chronic HBV infection. The mechanisms of spontaneous HBV clearance are determined by the interactions between HBV and the host immune response, including innate and adaptive immune responses,

which are affected by specific HLA gene polymorphisms that alter peptide epitope binding. For instance, HLA-DPA1 and HLA-DPB1, which encode the HLA-DP  $\alpha$  and  $\beta$  chains, may be involved in antigen presentation to CD4<sup>+</sup> positive T lymphocytes, which is important for HBV clearance [27, 29]. HLA-DR13 is consistently associated with HBV clearance globally [14, 24, 30, 31]. A meta-analysis indicated that subjects harboring at least one A allele of HLA-DPB1 rs9277535 and HLA-DPA1 rs3077 variants have increased susceptibility to spontaneous HBV clearance compared with those with G alleles [7]. Three SNPs of the HLA-DP gene, including rs9277535, rs7453920, and rs2856718, confer increased frequency of HBsAg clearance in China [10]. Hu et al. [12] showed a significantly higher proportion of the rs9277534 minor allele A in spontaneous HBV clearance than in the HBV persistent infection group ( $P < 0.0001$ ). Genotypic analyses [5] showed that GA and AA genotypes are associated with spontaneous HBV clearance. In addition, HLA-B\*13:01:01G frequency is associated with spontaneous HBsAg clearance in a Qidong Han Chinese population. HBV carriers with rs9277535 non-G/G genotype and GA haplotype have a higher chance to clear HBsAg in Chinese subjects of Taiwan [32]. The rs3077 and rs9277542 alleles in the HLA-DPA1 in HLA-DPB1 genes, respectively, confer protective effects on HBV infection and clearance in Japanese and Korean populations [19]. In a study assessing European and African American populations, rs9277534 rather than rs9277535 in the HLA-DPB1 3'UTR was shown to be associated with HBV recovery [33]. A meta-analysis of 62,050 subjects from 29 case-control studies showed that rs3077 and rs9277535 in HLA-DP significantly decrease HBV infection risks and increase HBV clearance [34]. The 496A/G variant has a stronger effect than any individual HLA-DPB1 or DPA1 allele, as well as other HLA alleles associated with HBV recovery in European American cohort [15]. Li et al. [35] showed that HLA-B\*15 and DRB1\*11 and DRB1\*14 are associated with spontaneous recovery in patients with HBV subgenotype C2 infection in Northeast China.

HLA-DR2, HLA-DR\*0406, HLA-B\*4001, and HLA-DR7 antigens are associated with protective effect on acute HBV infection [19, 36, 37]. It is known that patients who successfully resolve acute hepatitis B infection develop strong HLA classes I and II restricted T cell response, which is weak or absent in patients with chronic hepatitis B [38, 39]. In Spain, Cotrina et al. [40] found that the HLA-DRB1\*1301 and DRB1\*1302 alleles are associated with infection resolution in acute hepatitis B. Li et al. [35] also showed that HLA-B\*07 and DRB1\*13 may protect subjects from HBV infection. HLA-DQ rs2856718G and rs9275572A are strongly associated with decreased risk of chronic HBV infection and natural clearance [41].

#### **4. Associations of HLA Gene Variants with Early Hepatitis B e Antigen (HBeAg) Seroconversion**

HBeAg seroconversion mainly depends on patient age at infection and the host immune responses. It was shown

that the functional stage of dendritic cells (DCs) plays an important role in HBeAg seroconversion [42]. DCs are the most effective antigen presenting cells and play a pivotal role in antiviral response induction. Exogenous antigens are phagocytized and then loaded on both HLA classes I and II by DCs. Therefore, HLA gene variants may influence host induced early HBeAg seroconversion. A long-term cohort study demonstrated that HLA class I antigen B61 and class II antigen DQB1\*0503 are associated with early HBeAg seroconversion in CHB children in Taiwan [43]. Although the HLA-DPA1 SNP did not show a statistically significant association with early HBeAg seroconversion in Japanese children, it tended to increase the likelihood of achieving early spontaneous HBeAg seroconversion [44]. In an African HIV-positive cohort, it was suggested that HLA-A alleles alone, other than HLA-B or HLA-C, indeed predict HBeAg status (AUC = 0.73,  $P = 0.002$ ) [45]. These results emphasize the role of the CD8<sup>+</sup> T cell response in HBV control.

#### **5. Association of HLA Gene Variants with the Risk for Developing Liver Cirrhosis and HBV-Related Hepatocellular Carcinoma**

HLA gene variations are strongly associated with not only HBV infection persistence or clearance but also disease progression and the development of liver cirrhosis (LC) and HBV-related hepatocellular carcinoma (HCC). HLA-DQ polymorphism analysis [41] using matrix-assisted laser desorption/ionization time of flight mass spectrometry showed that rs9275572A is associated with the development of cirrhosis and HCC (OR = 0.632,  $P = 0.008$ ). Of the SNPs reported in HBV-related HCC GWASs, rs9267673 near C2, rs2647073 and rs3997872 near HLA-DRB1, and rs9275319 near HLA-DQ were found to be significantly associated with the risk for HBV-related LC [46], suggesting that gene variants associated with HBV-related hepatocarcinogenesis may already play an important role in the progression from CHB to LC. Therefore, understanding HLA genetic background would help improve current HCC surveillance programs in HBV-infected patients.

GWAS on genetic susceptibility of HBV-related HCC indicated two consistently identified tagging SNPs around HLA-DQ/DR [47, 48]. A multicenter case-control study including 1,507 HBV-related HCC cases and 1,560 HBV persistent carriers as controls showed that HBV carriers infected with HBV genotype C and carrying HLA-DQ/DR SNPs (rs9272105 AA genotype, rs9275319 AA genotype) have a relatively high risk for HCC [49]. Other studies [10, 13] suggested that HLA-DP rs3077 and rs9277535 polymorphisms are associated with HCC susceptibility in Asian individuals. Four SNPs (rs17875380, rs41557518, rs114465251, and rs115492845), in nonclassical class I alleles, were shown to be associated with altered susceptibility to HBV or HCC, while HLA-F\*01:04, HLA-G\*01:05N, and HLA-E\*01:01 are associated with hepatitis B or hepatitis B complicated with HCC. Six of 16 designated HLA-E, HLA-G, and HLA-F haplotypes were shown to be associated with risk for hepatitis B or HCC [50].

On the other hand, HLA gene variations also decrease the risk for cirrhosis and HCC. Doganay et al. [51] found in a multivariate logistic regression analysis that DRB1\*07 is a significant negative predictor of cirrhosis ( $P = 0.015$ ). This may be due to the fact that a polymorphic amino acid sequence in DRB1\*07 alters interaction with the T cell recognition site. Mohamadkhani et al. [29] revealed that the rs2856718 variant significantly diminishes the host risk for HCC. Zhang et al. [13] also indicated that the HLA-DP SNP rs3077 might act beneficially against HCC susceptibility. In another study, HLA-DP rs3077, rs9277535, and rs7453920 also showed no association with HCC development [52].

Killer cell immunoglobulin-like receptors (KIRs) are involved in the regulation of NK cell activation through recognition of specific HLA class I allotypes. In a multivariate Cox model, Pan et al. [53, 54] suggested that KIR and HLA genetic background can influence the age at HCC onset in male patients and is associated with HCC incidence in patients with HBV infection.

## 6. HLA Gene Variants Associated with Efficacy of Interferon- (IFN-) $\alpha$ and NAs Treatment

Interferon- (IFN-)  $\alpha$  is the first-line therapy for CHB patients but initiates a complete response only in a minority of patients. HLA gene variants have also been shown to be associated with response to IFN- $\alpha$  treatment in CHB patients. Different haplotypes of the same SNP may be associated with different clinical treatment outcomes. It was shown that the "G-C" haplotype of the five SNPs, including rs9277535 (HLA-DPB1), rs9276370 (HLA-DQA2), rs7756516 and rs7453920 (HLA-DQB2), and rs9366816 near HLA-DPA3, is associated with sustained therapeutic response to IFN- $\alpha$  treatment in male Han Taiwanese subjects ( $P = 0.0132$ ; OR = 2.49) [18]. In a large cohort of Caucasian chronic hepatitis B patients infected with the HBV genotype A or D, Brouwer et al. demonstrated that HLA-DPB1 polymorphisms are independently associated with both virological and serological responses to PEG-IFN therapy at 6 months after treatment.

HLA-DPA1 and HLA-DPB1 haplotype block GG showed comparable results for virological and combined response [55]. Han et al. [56] suggested that HLA-DRB1\*14 allele may be associated with a high rate of the response of CHB patients to IFN treatment. Compared with other HLA-DQB1 alleles, HLA-DQB1\*07 may be associated with low response rate to IFN.

Cheng et al. [57] showed by multivariate analysis that, at 6 months of PEG-IFN- $\alpha$  therapy and 6 months after therapy, rs3077-GG genotype is independently associated with higher HBeAg loss and anti-HBeAb seroconversion rates; meanwhile, the rs9277535-GG genotype was independently associated with declined HBV DNA levels in Chinese patients with CHB. Similar results were observed in Taiwan [58].

The HLA-DQ locus rs9275572 is a predictor of viral and biochemical responses to lamivudine (LAM) therapy in Han Chinese subjects [41]. Hosaka et al. [59] demonstrated an association of HLA-DP polymorphisms with  $\geq 2$  A alleles at rs3077 and rs9277535 and decreased HBsAg levels and seroclearance among HBeAg-positive Japanese CHB patients

treated with LAM. Meanwhile, the HLA-DRB1\*010101 allele is closely associated with poor virological response to initial LAM therapy in Korean CHB patients [60]. Chang et al. [18] also showed that, in patients with the TT genotype of rs9276370 (HLA-DQA2), there is a higher nonsustained response rate, especially in the LAM ( $P = 0.0074$ ) and PEG-IFN- $\alpha$ -2a ( $P = 0.0814$ ) groups, rather than in entecavir treated individuals. A randomized clinical trial [61] assessing PEG-IFN- $\alpha$ -2b with or without entecavir in patients with HBeAg-negative CHB revealed the GG genotype of rs3077 (HLA-DPA1) as an independent predictor of therapeutic response.

## 7. HLA Gene Variants Associated with Response to Hepatitis B Vaccination

Accumulating evidence shows that certain HLA types are associated with decreased or increased antibody response to hepatitis B vaccines in different individuals. A meta-analysis [62], including 774 potentially relevant articles and a total of 2308 subjects (1215 responders, 873 nonresponders, and 220 control populations) and assessing the effect of HLA on immunological response to hepatitis B vaccines in healthy individuals, showed that, for DRB1 alleles, the three HLA variants DRB1\*01, DRB1\*1301, and DRB1\*15 are associated with significantly increased antibody response to hepatitis B vaccines, with pooled ORs of 2.73, 5.94, and 2.29, respectively. Meanwhile, DRB1\*03 (DRB1\*0301), DRB1\*04, DRB1\*07, and DRB1\*1302 showed opposite results. For DQB1 alleles, DQB1\*05 (DQB1\*0501), DQB1\*06, and DQB1\*0602 were shown to be associated with markedly increased antibody response to hepatitis B vaccine, with pooled ORs of 1.85, 2.35, 2.34, and 3.32, respectively; DQB1\*02 (pooled OR = 0.27) showed opposite results. Mert et al. [63] found positive correlations between four HLA-DR (HLA-DRB1\*04X, DRB1\*0401X, DRB1\*11/13, and DRB1\*0401X0201) haplotypes and nonresponders but a negative correlation with one class I (HLA-B13) in Turkey. In Korean infants [64] who received HBV vaccination, HLA-A\*33, B62, DRB1\*04, and DRB1\*07 alleles showed positive associations with nonresponsiveness (<10 mIU/mL) or low antibody titers (<100 mIU/mL), while alleles A\*02 and DRB1\*08 showed negative associations. After stratification by other associated alleles at different loci, B62 and DRB1\*07 were still independently associated with nonresponsiveness. So, upon evaluating the response to HBV vaccination, different HLA types of ethnic groups should be taken into consideration; HLA gene frequencies of distinct ethnic groups should be examined in further large-scale population-based studies [65].

## 8. Summary and Perspectives

Overall, the complicated nature history of HBV infection makes it necessary to find clinical and genetic markers to help predict individuals at higher risk to develop CHB and worse outcomes such as LC and HCC. The HLA system is an integral component of the host immune response. The highly polymorphic HLA genes are key factors in the activation of the immune response against HBV infection

through their enormous capacity of attracting and binding viral peptides. HLA gene variations are associated not only with susceptibility or resistance to HBV infection but also with spontaneous HBV clearance, disease progression, efficacy of antiviral treatment, and response to HBV vaccines. Furthermore, specific HLA allele variants may have different impact on clinical outcomes of chronic HBV infections among different ethnic subjects. Identifying the associations of specific HLA allele variants with disease progression or viral clearance in chronic HBV infections among different ethnic populations needs further assessment in larger scale controlled clinical trials.

Finally, upon evaluating the impact of HLA gene variants on HBV infection, SNP-SNP interactions between HLA and other host genes such as granulysin (GNLY) SNPs [66] and polymorphisms in toll-like receptor-interferon (TLR-IFN) [67] pathway genes and HBV mutations [68] should also be kept in mind.

## Competing Interests

The authors declare that they have no competing interests.

## Authors' Contributions

Li Wang and Zhi-Qiang Zou contributed equally to the work.

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## Review Article

# Adjuvants: Classification, *Modus Operandi*, and Licensing

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Vaccination is one of the most efficient strategies for the prevention of infectious diseases. Although safer, subunit vaccines are poorly immunogenic and for this reason the use of adjuvants is strongly recommended. Since their discovery in the beginning of the 20th century, adjuvants have been used to improve immune responses that ultimately lead to protection against disease. The choice of the adjuvant is of utmost importance as it can stimulate protective immunity. Their mechanisms of action have now been revealed. Our increasing understanding of the immune system, and of correlates of protection, is helping in the development of new vaccine formulations for global infections. Nevertheless, few adjuvants are licensed for human vaccines and several formulations are now being evaluated in clinical trials. In this review, we briefly describe the most well known adjuvants used in experimental and clinical settings based on their main mechanisms of action and also highlight the requirements for licensing new vaccine formulations.

## 1. Introduction

Vaccination is one of the most efficient strategies for infectious diseases prevention. According to the World Health Organization (WHO), vaccination saves 5 lives every minute and will save over 25 million lives from 2011 to 2020. Traditional vaccine approaches like inactivated or live-attenuated viruses, although highly effective and immunogenic, present safety concerns. Despite being safer, subunit vaccines are normally less immunogenic/effective and need to be delivered together with an adjuvant. Hence, adjuvants are essential for enhancing and directing the adaptative immune response to vaccine antigens.

The term adjuvant comes from the Latin *adjuvare*, which means to help or aid [1]. Adjuvants can be defined as substances that increase immunogenicity of a vaccine formulation when added/mixed to it. The choice of the adjuvant is of utmost importance as it can stimulate strong humoral and cell mediated immunity indispensable for protection

against some pathogens. In addition, the balance between the adjuvant properties and adverse effects plays a critical role in the selection.

The history of adjuvant discovery begins with Gaston Ramon, a veterinary working at the Pasteur Institute in 1920, that described the term adjuvant after he observed that higher specific antibody titers were detected in horses that developed abscesses at the injection site [2]. To confirm the hypothesis, he induced sterile abscesses at the injection site with starch or breadcrumbs together with inactivated toxin and confirmed that substances capable of inducing inflammation at the injection site also improved the production of antisera [3]. About the same time, Glenn et al. discovered the adjuvant effect of aluminum salts [4], and since then billions of alum-based vaccine doses have been administered to people. Jules Freund developed, in 1930, a powerful adjuvant composed of a water-in-mineral oil emulsion that also contained heat-killed mycobacteria (*Mycobacterium tuberculosis* or others) [5]. Although highly effective, complete Freund's adjuvant

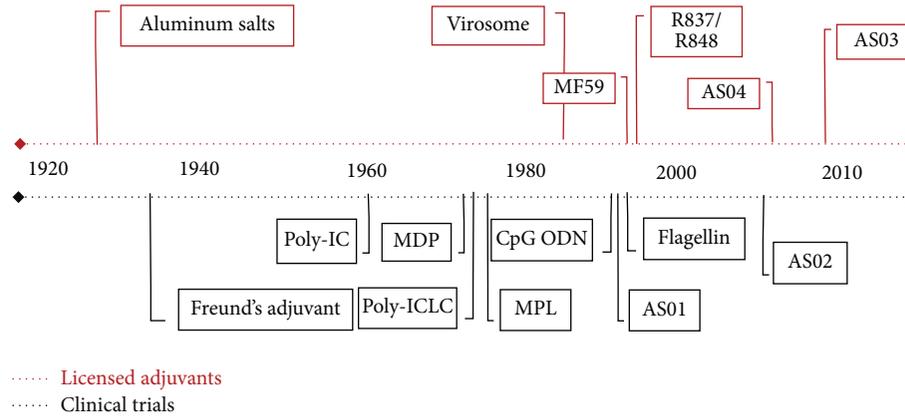


FIGURE 1: Timeline of vaccine adjuvants discovery.

(CFA) is also reactogenic and frequently induces granulomas, sterile abscesses, and ulcerative necrosis at the site of inoculation, which precludes it from being used in human vaccines. Figure 1 shows a timeline of adjuvant discovery.

A variety of compounds with adjuvant properties currently exist, and they seem to exert their functions through different mechanisms of action. Mineral salts, emulsions, microparticles, saponins, cytokines, microbial components/products, and liposomes have all been evaluated as adjuvants [6–8]. Nevertheless, few adjuvants are licensed for human use and several formulations are now being evaluated in clinical trials. In many cases, their use is empirical. Over the past years, many efforts have been made to investigate how and why adjuvants work. Recent advances have shown that adjuvants can (i) increase the biological half-life of vaccines, (ii) increase antigen uptake by antigen presenting cells (APCs), (iii) activate/mature APCs (e.g., dendritic cells), (iv) induce the production of immunoregulatory cytokines, (v) activate inflammasomes, and (vi) induce local inflammation and cellular recruitment [3, 9].

Independently of their mechanism of action, adjuvants have been traditionally used in the formulation of vaccines in an attempt to (i) decrease the amount of antigen, (ii) reduce the number of doses required to induce protective immunity, (iii) induce protective responses more rapidly, and (iv) increase the rate of seroconversion in special populations (the elderly, immunocompromised individuals, individuals with chronic disease, neonates and infants) [9].

## 2. Classification of Adjuvants

Different criteria may be used to group adjuvants in order to allow a rational comparison. Adjuvants can be classified according to their physicochemical properties, origin, and mechanisms of action [10]. Based on their mechanisms of action, adjuvants can be divided into delivery systems (particulate) and immune potentiators (immunostimulatory) [11]. Mucosal adjuvants are a class of compounds that can fit in both of the previously described categories (Table 1).

TABLE 1: Classification of adjuvants.

Type	Adjuvant/formulation
Delivery systems	
Mineral salts	Aluminum salts [alum] Calcium phosphate
Lipid particles	Incomplete Freund's adjuvant MF59 Cochleates Virus-like particles
Microparticles	Virosomes PLA (polylactic acid), PLG (poly[lactide-coglycolide])
Immune potentiators	dsRNA: Poly(I:C), Poly-IC:LC Monophosphoryl lipid A (MPL), LPS Flagellin Imidazoquinolines: imiquimod (R837), resiquimod (848) CpG oligodeoxynucleotides (ODN) Muramyl dipeptide (MDP) Saponins (QS-21)
Mucosal adjuvants	Cholera toxin (CT) Heat-labile enterotoxin (LTk3 and LTR72) Chitosan

Delivery systems can function as carriers to which antigens can be associated. Also, they create local proinflammatory responses that recruit innate immune cells to the site of injection [12]. Hence, it has been proposed that this type of adjuvants can activate innate immunity.

In a simplistic definition, the role of immune potentiators is to activate innate immune responses through pattern-recognition receptors (PRRs) or directly (e.g., cytokines). Pattern-recognition receptors (PRRs) consist of different classes of receptors [Toll-like receptors (TLRs), nucleotide-binding oligomerization domain- (NOD-) like receptors

(NLRs), and the retinoic acid-inducible gene-I- (RIG-I-) like receptors (RLRs)] that are widely expressed on immune cells. Their engagement by pathogen-associated molecular patterns (PAMPs) triggers the activation of such innate cells that can ultimately mature/migrate to other tissues and produce cytokines and chemokines [13].

## 2.1. Delivery Systems

**2.1.1. Mineral Salts.** Delivery systems (particulate adjuvants) cover a wide range of materials such as aluminum salts (alum), lipid particles, and microparticles. Alum is by far the most widely used adjuvant since its introduction in the 1920s [14]. This adjuvant is in the formulation of licensed vaccines against Hepatitis A (HAV), Hepatitis B (HBV), diphtheria/tetanus/pertussis (DTP), human papillomavirus (HPV), *Haemophilus influenzae* type B (HiB), and *Pneumococcus*.

Until recently, alum was believed to owe its adjuvant properties to the slow release of the antigen associated with it [15]. However, several reports demonstrated that if “antigen-alum depot” was removed after immunization, the immune response remained unaltered [16, 17], demonstrating that the depot effect and slow release of the antigen were not responsible for its adjuvant activity. Indeed, recent evidence showed that alum can activate the innate immune response [18, 19]. Aluminum-containing adjuvants are a class of adjuvants that do not use the classical TLRs and MyD88 or TRIF signaling pathways to activate innate immunity. Instead, they are sensed by NOD-like receptors (NLRs) through direct activation of NLRP3/NALP3 inflammasome complex or by the release of uric acid [18, 20, 21]. Another feature of alum is its ability to reduce antigen degradation [22].

However, for some vaccine formulations, alum does not elicit protective and sustained immune responses. This is because aluminum-containing adjuvants preferentially induce Th2 responses (characterized by antibody production), and for some pathogens a Th1 immune response (including cytotoxic CD8 T cells) is required [14, 23]. Hence, for such vaccines alum should not be used, at least not alone.

## 2.1.2. Emulsion Adjuvants

**Freund's Adjuvants.** Complete Freund's adjuvant (CFA) is a water-in-oil emulsion that contains heat-killed mycobacteria and is a classic “gold standard” representative of this group of adjuvants. In general, CFA is used to evaluate the immunogenicity of antigens in mice and on the induction of autoimmune diseases like uveitis and experimental autoimmune encephalomyelitis. In order to induce autoimmunity, evidence suggests that the components of mycobacteria direct T-lymphocytes to acquire a Th1 pattern that mediates delayed type hypersensitivity (DTH). One of the major concerns regarding the use of CFA is the induction of strong long-lasting local inflammation that may be painful to the animal often leading to ulcer at the site of injection [24]. Hence, there are numerous regulatory guidelines to work with CFA in experimental animals [25, 26].

Incomplete Freund's adjuvant (IFA) is also a water-in-oil emulsion, but without mycobacteria. In the 50s, the use

of IFA as an adjuvant in a human influenza vaccine led to higher long-lived antibody titers when compared to the same formulation without the adjuvant [27]. Its adjuvant activity is the result of a continuous release of the antigen from the oily deposit, an increased antigen lifetime, and the stimulation of local innate immunity, as it enhances phagocytosis, leukocyte infiltration, and cytokine production [28]. Although there is a consensus that the use of IFA in humans is hampered by the strong side effects, a survey conducted by the WHO reported that immunization of one million individuals with IFA showed severe side effects, such as sterile abscesses, in 40,000 [29]. Hence, due to the balance between potency and side effects, there are several completed clinical trials using IFA in vaccine candidates for HIV infection (see <https://clinicaltrials.gov/>, access number: NCT00381875), melanoma (NCT00003224, NCT00706992, and NCT00085189), renal carcinoma (NCT00001703), and also multiple sclerosis (NCT02200718).

**MF59.** MF59 is a water-in-oil squalene based emulsion that is currently licensed as part of a flu vaccine (Fluad™, Seqirus) for individuals >65 years old. Initially, the vaccine focused on elderly subjects but was later tested in the second major flu risk group, young children and infants, and was successful in both cases [30, 31]. In addition, it was also approved for the H1N1 pandemic vaccine for pregnant woman and young children [32]. Moreover, infants vaccinated with MF59-adjuvant trivalent inactivated influenza vaccine (TIV) presented higher antibody titers and polyfunctional cytokine producing CD4<sup>+</sup> T cells than children immunized with the nonadjuvant TIV [33, 34]. The inclusion of MF59 enhanced the low effectiveness of this influenza vaccine in children under 2 years of age. Thereafter, MF59 was tested as an adjuvant for an HBV vaccine, and it was able to induce an immune response one hundred times more potent than the one induced with alum [35].

As with the majority of adjuvants, the mechanisms of action of MF59 are not fully understood. Similar to alum, MF59 effect does not rely on depot formation at the injection site, as its half-life is 42 hours [7, 36]. However, MF59 seems to be a powerful adjuvant due to its ability to induce cellular and humoral responses, including high titers of functional antibodies [37]. Indeed, MF59 is able to stimulate macrophages, resident monocytes, and DCs to secrete several chemokines like CCL4, CCL2, CCL5, and CXCL8 that in turn induce leukocyte recruitment and antigen uptake leading to migration to lymph nodes and triggering the adaptive immune response [32, 38, 39]. Systems biology studies also revealed that MF59 increases expression of the leukocyte transendothelial migration gene cluster and recruitment of MHCII<sup>+</sup>CD11b<sup>+</sup> cells at injection site and this profile may be predictive of robust immune responses [40]. Moreover, an elegant paper by Vono and colleagues showed that transient ATP release is required for innate and adaptive immune responses induced by MF59 [41].

**AS03.** AS03 is an oil-in-water adjuvant emulsion that contains  $\alpha$ -tocopherol, squalene, and polysorbate 80 and was developed by GlaxoSmithKline Biologicals [42]. The addition of

$\alpha$ -tocopherol to the formulation differentiated AS03 from other oil-in-water emulsion adjuvants [43]. Its first use in humans was together with a malaria vaccine [44]. More recently, this adjuvant has been included for use in human vaccines especially for influenza. Recent clinical trials have showed that oil-in-water adjuvants as AS03 administered with influenza vaccine induced a more robust immune response [45]. Indeed, children aged from 6 to 35 months immunized with one dose of AS03 adjuvant vaccine developed strong immune response that was observed even 6 months after vaccination [46].

AS03 stimulates the immune system by the activation of NF- $\kappa$ B, proinflammatory cytokine and chemokine production, recruitment of immune cells, mainly monocytes and macrophages, and induction of high antibody titers. An important issue is to administer AS03 with the antigen at the same injection site at the same time to avoid diminished response [42].

### 2.1.3. Microparticles

*Virus-Like Particles.* Virus-like particles (VLPs) are formed by structural viral proteins such as capsid or envelope that mimic intact virus size, shape, and molecule organization with self-assembly properties [47]. Although highly immunogenic because of their self-adjuvant properties, VLPs are noninfective and nonreplicative [48]. The structure of VLPs can be enveloped or nonenveloped depending on the parental virus. Nonenveloped VLPs are only composed by pathogen components with the ability to self-assemble (e.g., HPV) while enveloped VLPs consist of the host cell membrane (an envelope) in combination with the antigen of interest [49]. Other components such as TLRs agonists can also be incorporated into VLPs.

VLPs can induce direct B cell activation, proliferation, and upregulation of genes involved in class switch recombination and somatic hypermutation [50]. In addition, VLPs can bind, activate, and be captured by DCs [51, 52] which in turn lead to T cell immunity. They can also induce cross-presentation to CD8<sup>+</sup> T cells [53]. Hence, VLPs are able to induce broad humoral and cellular immune responses including neutralizing antibodies and specific helper CD4<sup>+</sup> and cytotoxic CD8<sup>+</sup> T cells [54, 55]. There are a few commercially available vaccines that are based on VLPs including Engerix<sup>®</sup>/Recombivax<sup>®</sup> (Hepatitis B), Cervarix<sup>®</sup>/Gardasil<sup>®</sup> (HPV), and Mosquirix<sup>®</sup> (malaria) [49]. Currently, several enveloped and nonenveloped VLPs are in clinical development (Table 2).

*Virosomes.* Virosomes are a type of VLP platform that is composed of reconstituted viral envelopes with membrane lipids and viral glycoproteins that work as a carrier system for antigens or as adjuvants. Although composed of viral proteins, virosomes are not virulent since the genetic material of the native virus is absent and does not replicate [56]. Virosomes are produced by dissolving the envelope of the virus with a detergent followed by a complete removal of the genetic material of the virus and the nonmembranous proteins. The most used virosomal system is the

immunopotentiating reconstituted influenza virosome (IRIV) [57, 58] that contains both the hemagglutinin (HA) and neuraminidase (NA) proteins intercalated within a lipid membrane. Currently, there are five licensed vaccines based on this approach: Inflexal<sup>®</sup> V, Nasalflu<sup>®</sup>, and Invivac<sup>®</sup> for influenza and Epaxal<sup>®</sup> and Epaxal Junior for Hepatitis A virus [58].

Virosomal HA and sialic acid can interact with APCs and induce particle endocytosis. After the acidification of the endosome, HA changes conformation and the fused antigen can either be released into the cytosol and be processed via MHCI or stay in the endosome and be processed via MHCII pathway. Concomitantly, virosomes increase the expression of costimulatory molecules (CD80, CD86, and CD40) on the APC surface. The whole process leads to CD8<sup>+</sup> and CD4<sup>+</sup> T cell activation and cytokine production such as IFN $\gamma$ , TNF $\alpha$ , and GM-CSF [59].

*PLA/PLGA.* Poly(lactic acid) (PLA) and poly(lactic-co-glycolic acid) (PLGA) are biodegradable and biocompatible polymeric micro/nanoparticles that function as a delivery system by encapsulating an antigen or antigen plus adjuvant in the same particle [60, 61]. These particles are produced using techniques such as emulsification/solvent evaporation. Ligands against surface receptors (PRRs, CD1d) have also been loaded in PLGA nanoparticles as an adjuvant to trigger signaling pathways of innate immune responses [62, 63].

The particles are internalized by pinocytosis and clathrin-mediated endocytosis and can rapidly be localized into the cytosol [64]. PLGA can efficiently reach MHCI molecules and cross-present antigens to CD8<sup>+</sup> T cells [65]. PLGA nanoparticle delivery system enhances the uptake by APCs [66] allowing prolonged release of the antigen and induces higher immune responses [67] when compared with the soluble counterpart.

PLGA has been used to deliver antigens from different pathogens including *Bacillus anthracis* [68], *Plasmodium vivax* [69], and Hepatitis B virus (HBV) [70].

*2.2. Immune Potentiators.* As stated before, immune potentiators target innate immunity signaling pathways through PRRs like TLRs, RLRs, and NLRs. In general, activation of PRRs by their agonists induces APC activation/maturation and cytokine/chemokine production that ultimately leads to adaptive immune responses. Examples of PRRs agonists include, but are not limited to, poly(I:C), MPL, flagellin, imiquimod, resiquimod, CpG ODN, and MDP (Figure 2).

*2.2.1. TLR3 Agonists.* Poly(I:C) (polyinosinic:polycytidylic acid) is a synthetic double strand RNA (dsRNA) that mimics viral RNAs and activates TLR3 located within endosomes [71, 72]. Poly(I:C) can also bind to the melanoma differentiation associated gene 5 (MDA5), a cytoplasmic protein that contains two caspase-recruitment domains (CARDs) and a DExD/H-box helicase domain. Results using knockout mice indicate that MDA5 is essential for poly(I:C)-induced IFN $\alpha$  production, while TLR3 signaling is critical for IL-12 production. Both seem to regulate IL-6 production [73]. The administration of poly(I:C) activates DCs that quickly

TABLE 2: Adjuvants in clinical development (for details see <https://www.clinicaltrials.gov/>).

Adjuvant	N of clinical trials	Type	Study phase	Applications
Alum	203	175 prophylactic 28 therapeutic	1 Pilot, 109 Phase I, 16 Phase I/II, 31 Phase II, 4 Phase II/III, II Phase III, 3 Phase IV 8 Phase I, 5 Phase I/II, 13 Phase II, 2 Phase III	Allergy, anthrax, botulism, candidiasis, <i>Campylobacter</i> , <i>Clostridium difficile</i> , dengue, encephalitis, <i>Helicobacter pylori</i> , hepatitis b, <i>Herpes simplex</i> , hookworm infection, human papillomavirus, influenza, leishmaniasis, malaria, <i>Meningococcus</i> , <i>Norovirus</i> , <i>Pneumococcus</i> , poliomyelitis, <i>Ross River virus</i> , SARS, schistosomiasis, shigellosis, <i>Staphylococcus</i> , <i>Streptococcus</i> , West Nile virus, yellow fever Cocaine dependence, colorectal cancer, diabetes, HDL, HIV, hypertension, malaria, melanoma, myasthenia gravis, nicotine dependence, prostate cancer, rhinconjunctivitis
Freund's incomplete adjuvant	190	9 prophylactic 181 therapeutic	4 Phase I, 1 Phase I/II, 2 Phase II, 2 Phase III 3 Pilot, 63 Phase I, 41 Phase I/II, 64 Phase II, 2 Phase II/III, 8 Phase III	Bladder cancer, carcinoma, influenza, malaria, melanoma Acute myeloid leukemia, adenocarcinoma, bladder cancer, bile duct cancer, brain cancer, breast cancer, carcinoma, chronic myeloid leukemia, colorectal cancer, esophageal cancer, gastric cancer, glioblastoma, HIV, HPV-induced cancer, kidney cancer, liver cancer, melanoma, multiple myeloma, multiple sclerosis, non-small-cell lung cancer, ovarian cancer, pancreatic cancer, prostate cancer, renal cell cancer
MF59	93	92 prophylactic 1 therapeutic	27 Phase I, 6 Phase I/II, 34 Phase II, 3 Phase II/III, 16 Phase III, 6 Phase IV 1 Phase I	<i>Cytomegalovirus</i> infections, influenza, HIV, respiratory syncytial virus HIV
Virosomes	23	23 prophylactic	8 Phase I, 1 Phase I/II, 1 Phase II, 9 Phase III, 4 Phase IV	Hepatitis A, Hepatitis C, influenza, malaria, vulvovaginal candidiasis
Virus-like particles	101	95 prophylactic 6 therapeutic	19 Phase I, 6 Phase I/II, 31 Phase II, 36 Phase III, 3 Phase IV 2 Phase I, 1 Phase I/II, 3 Phase II	Chikungunya, <i>Enterovirus 71</i> , HIV, human papillomavirus, influenza, malaria, <i>Norovirus</i> Hypertension, melanoma, respiratory syncytial virus
Poly(I:C)	16	1 prophylactic 15 therapeutic	1 Phase I/II 2 Pilot, 5 Phase I, 7 Phase I/II, 1 Phase II	Influenza Acute myeloid leukemia, allergy, breast cancer, glioblastoma, lymphoma, melanoma, non-small-cell lung cancer, ovarian cancer, prostate cancer
Poly-IC:LC	56	3 prophylactic 53 therapeutic	2 Phase I, 1 Phase II 6 Pilot, 19 Phase I, 17 Phase I/II, II Phase II	Colorectal cancer, HIV, melanoma Acute myeloid leukemia, astrocytoma, bladder cancer, breast cancer, colorectal cancer, epithelial ovarian cancer, glioblastoma, glioma, HIV, low grade B cell lymphoma, melanoma, myeloma, non-small-cell lung cancer, pancreatic adenocarcinoma, prostate cancer
Monophosphoryl lipid A	31	22 prophylactic 9 therapeutic	7 Phase I, 2 Phase I/II, 6 Phase II, 7 Phase III 2 Phase I, 1 Phase I/II, 5 Phase II, 1 Phase III	Hepatitis B, <i>Herpes simplex</i> , HIV, hookworm infections, malaria, <i>Norovirus</i> , visceral leishmaniasis Allergic rhinitis, cutaneous leishmaniasis, melanoma, type I hypersensitivity
Flagellin	6	6 prophylactic	4 Phase I, 1 Phase I/II, 1 Phase II	Diarrhea, influenza, plague

TABLE 2: Continued.

Adjuvant	N of clinical trials	Type	Study phase	Applications
Imiquimod	40	3 prophylactic	1 Phase II, 1 Phase II/III, 1 Phase III	Influenza, Hepatitis B, <i>Varicella zoster</i>
		37 therapeutic	2 Pilot, 20 Phase I, 2 Phase I/II, 9 Phase II, 2 Phase III, 2 Phase IV	Adenocarcinoma of the prostate, basal cell carcinoma, brain tumor, breast cancer, cervical cancer, ependymoma, gastric cancer, glioblastoma, glioma, human papillomavirus, melanoma, non-small-cell lung cancer, ovarian cancer, prostate cancer, sarcoma
Resiquimod	11	3 prophylactic	2 Phase I, 1 Phase I/II	Allergic rhinitis, Hepatitis B, influenza
		8 therapeutic	2 Pilot, 1 Phase I, 2 Phase I/II, 3 Phase II	Advanced malignances, bladder cancer, glioma, melanoma
CpG ODN	9	6 prophylactic	3 Phase I, 3 Phase I/II	Bacterial sepsis, HIV, hookworm infection, malaria
		3 therapeutic	1 Phase I, 1 Phase I/II, 1 Phase II	Allergic rhinitis, breast cancer, Hepatitis B, HIV
Muramyl dipeptide	1	1 prophylactic	1 Phase I	HIV
AS03	22	22 prophylactic	5 Phase I, 3 Phase I/II, 11 Phase II, 1 Phase III, 2 Phase IV	Dengue, influenza
		37 prophylactic	2 Phase I, 6 Phase II, 27 Phase III, 2 Phase IV	Cervical cancer, <i>Herpes simplex</i> , human papillomavirus
AS04	38	1 therapeutic	1 Phase II/III	Hepatitis B

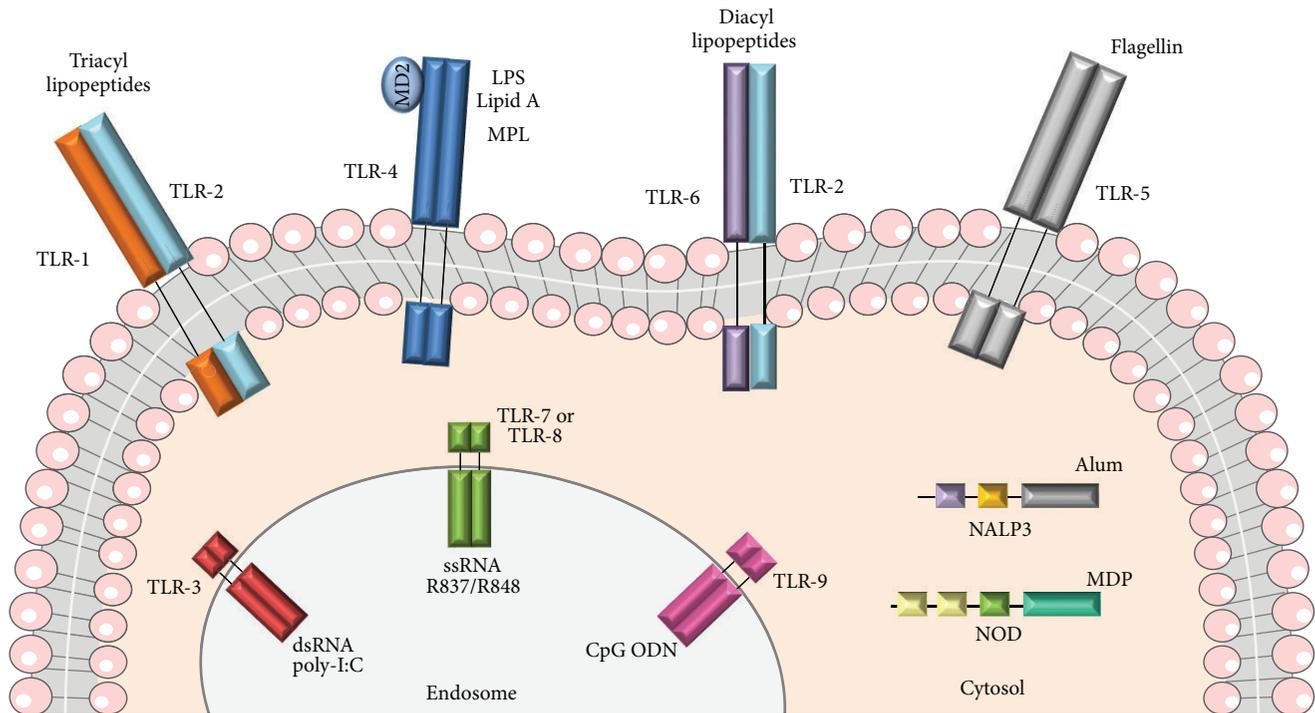


FIGURE 2: Adjuvants activate different immune innate receptors. TLRs (Toll-like receptors) and NLRs (NOD-like receptors).

produce IL-12 and type I IFN and upregulate MHC II expression [74, 75]. In response to IL-12, NK cells produce IFN $\gamma$  that in turn enhances T and B cell immunity. Type I IFN plays a critical role in the induction of Th1 responses and is also associated with cross-presentation [76]. Hence, poly(I:C) impacts APC maturation, antigen processing, and ultimately T and B cell immunity.

Poly(I:C) is the most TLR3 agonist tested as adjuvant against diseases including HIV [77, 78], dengue [79], malaria [80], and cancer [81, 82].

Poly-ICLC (Hiltonol<sup>®</sup>) is a poly(I:C) synthetic derivative stabilized with poly-L-lysine that is more resistant to RNAses [74, 83]. Several ongoing clinical trials (Table 2) are evaluating poly-ICLC for immunotherapy in patients with cancer [58]. More recently, poly-ICLC was also nasally delivered with a chimeric antibody containing HIV-p24 protein in mice and induced gastrointestinal immune responses [84].

**2.2.2. TLR4 Agonists.** Monophosphoryl lipid A (MPL) is the detoxified derivative of lipopolysaccharide (LPS) from Gram-negative bacteria (*Salmonella minnesota* R595). Removal of a phosphate residue from LPS renders MPL just 0.1% of the toxicity from the parental molecule. MPL mediates immune activation by interacting with TLR4 similarly to LPS [72]. MPL preferentially activates the TRIF signaling pathway [85] that triggers different cytokine production when compared to LPS that activates MyD88 and produces high amounts of TNF $\alpha$ . Indeed, MPL is able to induce IL-12 and IFN $\gamma$  production that promote Th1 responses.

MPL is approved for use in some countries as part of a vaccine against allergy (Pollinex Quattro<sup>®</sup>) [86] and in Canada for stage IV melanoma (Melacine<sup>®</sup>) [87]. Ongoing clinical trials evaluate MPL as a potential adjuvant for leishmaniasis, malaria, and *Herpes* antigens (Table 2).

**2.2.3. TLR5 Agonists.** Flagellin is the main component of bacterial flagella from both Gram-positive and Gram-negative bacteria and is recognized by the cell surface TLR5. Engagement of TLR5 induces TNF $\alpha$  production but flagellin, when administered together with a vaccine antigen of interest, is also able to induce high antibody titers and mixed Th1/Th2 responses [88, 89]. Flagellin can simultaneously target inflammasomes [90] through NLR4 phosphorylation [91, 92] and NAIP5 [93].

Flagellin can also be fused to the antigen of interest allowing its codelivery to the same APC. Influenza vaccines composed of fused flagellin-hemagglutinin (VAX128 and VAX125) and flagellin-matrix protein (VAX102) completed initial clinical trials [94, 95]. Results demonstrated that immunization with flagellin-fused proteins induced high antibody titers, seroconversion, and protection. Moreover, flagellin was also evaluated as a potent adjuvant to prevent rhinitis in mice [96].

**2.2.4. TLR7/8 Agonists.** Imiquimod (R837; 1-(2-methylpropyl)-1H-imidazo[4,5-c]quinolin-4-amine) and resiquimod (R848, 4-amino-2-(etoximetil)-a,a-dimethyl-1H-imidazo [4, 5-c]quinoline-1-ethanol) are imidazoquinolines with antiviral properties [97–99]. Imidazoquinolines mimic single

stranded RNAs (ssRNAs) that are recognized by TLR7/8 on endosomes triggering signaling through MyD88 [100–102]. Imiquimod is able to activate TLR7, while resiquimod activates TLR7 and TLR8. An important issue is the different TLR7 and TLR8 expression/function between human and mouse cells [103]. In mice, TLR7 is expressed by CD8<sup>-</sup> DC subsets but not by CD8<sup>+</sup> DCs [104]. Nevertheless, in both species TLR7 is expressed on plasmacytoid DCs (pDC), B cells, and neutrophils. In contrast, TLR8 is nonfunctional in mice whereas in humans it is expressed by myeloid DCs (mDC) and monocytes [105]. Activation of both DC subsets in humans (mDCs and pDCs) facilitates type I IFN and IL-12 production [106] and enhances expression of costimulatory molecules, inducing direct and cross-presentation to CD8<sup>+</sup> T cells [107], while it also induces NK cell activation [108]. Activation of Th1 cellular immune response can control viral replication, reactivation, and clearance [105]. Furthermore, resiquimod directly stimulates B cell proliferation by mimicking CD40 signal both in humans and in mice that ultimately stimulates antibody and cytokine production [109].

Imiquimod (Aldara) is approved for topical use in humans for treatment of actinic keratosis [110], basal cell carcinoma [111, 112], and genital warts caused by HPV 1, HPV 2, HPV 4, and HPV 7 [113, 114]. Resiquimod was tested in clinical trials to treat lesions caused by human *Herpes* virus (HSV) [115, 116]. Besides the use in therapy against established infections, these adjuvants are being evaluated for their ability to increase vaccine immunogenicity [78] and also in allergy and tumor therapy such as basocellular carcinoma and central nervous system tumors (Table 2) [117, 118].

Besides imiquimod and resiquimod, other TLR7/8 agonists have also been tested. Among them, we can cite the imidazoquinoline immune response modifier 3M-052 [119], the benzazepine TLR8 agonist, VTX-294 [120], and two benzonaphthyridines compounds SMIP.7-7 and SMIP.7-8 that bind to TLR7 [121].

**2.2.5. TLR9 Agonists.** CpG ODNs are 18–25 base synthetic oligodeoxynucleotides (ODN) composed of unmethylated CG motifs (cytosine phosphate guanidine) recognized by endosomal TLR9 [122–124]. Murine TLR9 is preferentially activated by GACGTT motif while the ideal sequence for human is GTCCGTT [125]. TLR9 engagement signals through MyD88, IRAK, and TRAF-6 that ultimately leads to upregulation of costimulatory molecules (CD40, CD80, and CD86) and proinflammatory cytokines (IL-6, IL-12, IL-18, and TNF $\alpha$ ) [125, 126].

Three different types of CpG ODNs have been identified: A, B, and C [127]. Type A CpG ODNs contain a central phosphodiester palindromic motif in a phosphorothioate backbone and induce type I IFN production by pDCs. B type CpG ODNs have an entire phosphorothioate backbone that protects from degradation by nucleases and stimulates proliferation, IL-6/IgM production by B cells, and IL-6/TNF $\alpha$  production by DCs [100, 126]. Type C CpG ODNs combine features of types A and B since they are composed of phosphorothioate backbone with palindromic motif and induce B cell responses as well as type I IFN production by pDCs

[128, 129]. In general, CpG ODNs increase antibody responses and polarize to Th1 profile.

One of the most promising clinical results showed that commercial Hepatitis B vaccine administered together with CpG induced higher protective antibody titers after fewer doses both in healthy and in hyporesponsive individuals [130, 131]. Moreover, CpG ODNs have also been used in combination with conventional treatments for cancer [132].

**2.2.6. NOD Agonists.** Muramyl dipeptide (N-acetylmuramyl-L-alanyl-D-isoglutamine) is a peptidoglycan biologically potent motif found on all bacteria cell walls. MDP was discovered in 1974 as the minimum component of mycobacteria's cell wall required for the efficacy of complete Freund's adjuvant [133].

MDP is able to activate NOD2 [134] leading to NF- $\kappa$ B transcription that results in the production of proinflammatory cytokines (TNF $\alpha$ , IL-1, IL-6, and IL-8) as well as Th2 cytokines, nitric oxide secretion, enhanced cytotoxicity, and upregulation of adhesion molecules (CD11a, CD11b, CD11c/CD18, CD54) [135]. Studies have focused on the use of MDP for solid tumor therapy based on its ability to stimulate cellular as well as the cytokine response, eliciting antibody production [136].

**2.3. Combination of Adjuvants.** A recent approach to optimize vaccine immune responses is the use of different adjuvant combinations that could trigger different signaling pathways [137]. Such observation comes from studies using effective live-attenuated vaccines such as yellow fever that induce activation of different PRRs [138].

Based on this observation, one strategy is to use different TLR agonists to trigger activation of different signaling pathways (e.g., MyD88 and TRIF). Previous work tested different TLR agonist combinations in human PBMCs and evaluated cytokine and chemokine production [139]. Combinations of TLR7+TLR9 agonists induced type I IFN whereas TLR4+TLR7/8 synergistically upregulated IFN $\gamma$  and IL2; TLR2+TLR7/8 synergistically upregulated IFN $\gamma$  and others. MF59 and Carbopol-971P in combination were able to increase specific anti-HIV antibody titers [140]. However, not all combinations increase the magnitude of immune responses. For example, mice immunized with a recombinant HIV gp140 together with MPL plus alum or MDP exert synergic effects on the magnitude and quality of humoral response. However, when the mixture contained MDP plus poly(I:C) or resiquimod, no impact on antibody titers was observed but a significant difference was observed in IgG subclasses [78]. Another study showed that immunization of mice with nanoparticles containing antigens plus TLR4 and TLR7 ligands induced synergistic increases in antigen-specific, neutralizing antibodies when compared to immunization with nanoparticles containing antigens plus a single TLR ligand [141]. DCs activation by different combinations of TLR ligands was also evaluated. Results showed that, in human DCs, agonists of TLR3 and TLR4 potentially acted in synergy with a TLR8 agonist and induced higher amounts of IL-12 and IL-23 than those induced by optimal concentrations

of single agonists. This synergism led to enhanced and sustained Th1-polarizing capacity [142].

**2.3.1. AS01 and AS02.** Adjuvant System 01 (AS01) and Adjuvant System 02 (AS02) were the first in this type to be developed and tested in the RTS,S (*Plasmodium falciparum* circumsporozoite protein) vaccine candidate against malaria [143]. They are composed of MPL and the saponin QS21, but AS01 contains a liposomal suspension while AS02 is an oil-in-water emulsion [144]. When the trial began, AS02 was primarily tested and showed protection against controlled human malaria infection (CHMI) by the bite of infected mosquitoes [143]. However, when AS01 was included a higher production of specific antibody and improved efficacy was observed when compared to AS02 [145, 146]. Several clinical trials are in progress with AS01 and AS02 as vaccine adjuvants against HIV, tuberculosis, and malaria.

**2.3.2. AS04.** AS04 is composed of a combination of MPL and aluminum salts. Currently, two adjuvant vaccines are licensed: against HPV (Cervarix) [147, 148] and HBV (Fendrix®) [149].

This adjuvant also leads to activation of NF- $\kappa$ B, production of proinflammatory cytokines and chemokines, and recruitment of monocytes and macrophages to the injection site, but specifically DCs. It is important to emphasize the need for AS04 and the antigen to be colocalized at the moment of antigen presentation on lymph nodes [144]. The advantage of AS04 for human vaccines is the induction of specific Th1 immune response and production of IL-2 and IFN $\gamma$ , a profile weakly induced when alum is used alone [88].

**2.4. Mucosal Adjuvants.** The first immunization through mucosal surface was accomplished with attenuated poliovirus in 1962. Thereafter, other mucosal vaccines based on *Salmonella typhi*, *Vibrio cholerae* [150], rotavirus [151], and influenza virus were developed [152]. Administration by mucosal route has some advantages as needle-free delivery, lower costs, few adverse effects, and induction of local mucosal immunity, an important feature when infection occurs at mucosal routes [150, 153].

The most promising adjuvants for mucosal immunization are bacterial toxins extracted from *Escherichia coli* (heat-labile enterotoxin, LT) and *Vibrio cholerae* (cholera toxin, CT), TLRs agonists [flagellin, poly(I:C), CpG ODNs], and novel small molecules ( $\alpha$ -galactosylceramide, chitosan, etc.). To avoid development of cholera and travellers' diarrhea symptoms, these toxins have been genetically modified to generate less toxic derivatives (LTK3, LTR-72, and CTB) [154, 155]. Alternative mucosal routes have been evaluated with LT mutants and CT, including nasal, intravaginal, and intrarectal. LTK3 and LTR-72 were shown to induce potent immune responses against influenza virus after oral immunization [156]. Oral immunization with LT was also efficient in protection against *H. pylori* infection in mice after challenge [157]. Studies that used intranasal delivery of LT as an adjuvant showed that immunization was able to induce strong immune response and protection against

*Herpes simplex virus* [158], *S. pneumonia* [159], and *B. pertussis* [160].

Mucosal adjuvants CT and LT amplify B and T responses and stimulate isotype switching to IgA and mixed Th1/Th2 profile [161]. Further studies also demonstrated their ability to increase antigen uptake/presentation and DCs maturation/activation due to antigen permeation across epithelial barriers [162].

Mice intranasally immunized with *Plasmodium vivax* merozoite surface protein 1 (MSP1<sub>19</sub>) in the presence of the adjuvants CT or LT presented high and long-lasting specific antibody titers. In the same study mice immunized with MSP1<sub>19</sub> fused to a T cell epitope (PADRE) in the presence of CpG ODN developed lower IgG titers when compared to mice that received CpG ODN plus CT [163]. In a recent study, an anti-HIV chimeric antibody ( $\alpha$ DEC205-p24) nasally delivered in combination with polyICLC induced polyfunctional immune responses within nasopharyngeal lymphoid sites and mucosal gastrointestinal tract [164].

Chitosan is a biopolymer based on glucosamine extracted from a crustacean shell and is a mucosal adjuvant commonly used for intranasal delivery. The adjuvant acts *in vitro* by the translocation of "tight junctions" that improve transepithelial antigen transport and reduces the mucociliary clearance rate that facilitates antigen phagocytosis [165]. A study using a nontoxic mutant (CRM197) of diphtheria toxin in combination with chitosan showed that intranasal immunization was able to increase Th2 responses and, after a boost with the conventional diphtheria toxoid vaccine, enhanced antigen-specific IFN $\gamma$  production [166]. Another study showed that intranasal administration of chitosan and CRM197 was as immunogenic as intramuscular immunization with the conventional diphtheria vaccine adsorbed to alum [167]. Furthermore, *H. pylori* vaccine with chitosan was used successfully in a therapeutic setting in mice with an equivalent performance as the traditional vaccine adjuvant, cholera toxin (CT). In addition, when infection was not fully eradicated, chitosan immunized mice presented lower bacteria density in the gastric mucosa when compared to CT groups [168].

### 3. Licensing

The introduction of an adjuvant in a new (or already licensed) vaccine formulation is still a challenge and may take several years. It is of utmost importance to test the compatibility of each component of the vaccine alone and in combination before any trials start [169]. Due to the urgent need to develop vaccines against infectious diseases, the Center for Biologics Evaluation and Research (CBER), a division of the US Food and Drug Administration, launched an important guide to facilitate the development of new formulations [170].

It is recommended that evaluation of safety/immunogenicity of a formulation begins with preclinical tests using an appropriate animal model (Figure 3). At this stage, the evaluation of adjuvant effect on the immune response is also recommended [171]. Of note, control groups composed of adjuvant and the antigen alone should also be included

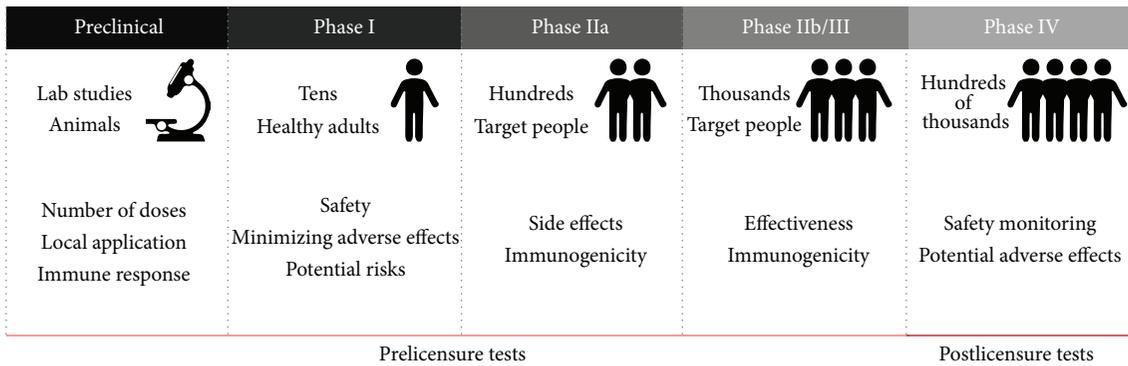


FIGURE 3: The different stages of vaccine development.

to provide evidence for adjuvant effect. The immunogenicity evaluation may include humoral (e.g., antibody titers, subclasses, avidity, and neutralization) and cellular (e.g., cytokine production, proliferation assays, and cell phenotyping) responses. If an animal model for the disease is available, initial protective efficacy information can be obtained [3].

After preclinical testing and GMP (good manufacturing practice) production of the vaccine formulation, human clinical trials begin. Phase I vaccine studies are conducted in healthy individuals ( $n < 100$ ) to evaluate safety—to minimize adverse events and potential risks—and the dosage. Safety concerns include, but are not limited to, pain, granuloma formation, fever, sterile abscess formation, nausea, headache, malaise, and other local or systemic events. Initial immunogenicity information can be obtained from Phase I.

Phase IIa trials are designed to evaluate immunogenicity, tolerability, and safety and typically involve hundreds of volunteers. When tests reach Phases IIb/III, an important goal is to ascertain the immunogenicity and efficacy in the vaccine target population (e.g., children). Another difference is based on the number of volunteers and the study duration; the more the people involved, the longer the trial duration (several years).

After the process that confirms safety and efficacy of the vaccine, it can be licensed and marketed. After that, the formulation undergoes a postmarket safety monitoring, Phase IV, to evaluate additional rare adverse reactions.

#### 4. Concluding Remarks

Adjuvants have been used to increase the immunogenicity of vaccines for almost a century. Until recently, adjuvant selection was empirical, but considerable advances in the field have allowed a rational/targeted use. This information together with an increasing understanding of the immune system will allow the development of effective vaccine formulations. Currently, only few adjuvant vaccines are licensed, but several ones are on clinical development and expected to reach approval in the near future. Finally, we believe that adjuvant selection could highly impact on rational vaccine design.

#### Competing Interests

The authors declare that they have no competing interests.

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## Research Article

# Neuroprotective Activity of (–)-Epigallocatechin Gallate against Lipopolysaccharide-Mediated Cytotoxicity

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Lipopolysaccharide- (LPS-) mediated systemic inflammation plays a critical role in neurodegenerative diseases. The present study was conducted to evaluate the protective effects of epigallocatechin gallate (EGCG), the major component in green tea, on LPS-mediated inflammation and neurotoxicity. LPS treatment of macrophages induced expression of proinflammatory cytokines (TNF- $\alpha$ , IL-1 $\beta$ , and IL-6). However, EGCG pretreatment of macrophages significantly inhibited LPS-mediated induction of these cytokines. In addition, EGCG significantly diminished LPS-induced inflammatory cytokines in the peripheral mononuclear blood cells (PBMCs). Supernatant from EGCG-pretreated and LPS-activated macrophage cultures was found to be less cytotoxic to neurons than that from non-EGCG-pretreated and LPS-activated macrophage cultures. Furthermore, EGCG treatment of neurons could inhibit LPS-induced production of reactive oxygen species (ROS). Thus EGCG represents a potent and useful neuroprotective agent for inflammation-mediated neurological disorders.

## 1. Introduction

Inflammation plays a critical role in the immunopathogenesis of neurodegenerative diseases such as Parkinson's disease, multiple sclerosis, Alzheimer's disease, and HIV-associated dementia (HAD). Activation of microglia, the intrinsic macrophages in the central nervous system (CNS) [1], is a characteristic feature of neurodegenerative diseases. Mounting evidence clearly indicates that macrophage/microglia activation contributes to inflammation and neuronal injury in the CNS [2, 3]. Lipopolysaccharide (LPS), a major element of Gram-negative bacteria, is a potent activator of immune cells, particularly macrophages and microglia, as it induces expression of proinflammatory cytokines such as TNF- $\alpha$ , IL-6, and IL-1 $\beta$  [4, 5]. These cytokines have direct or indirect neurotoxic effects on neuronal cells, causing neuronal injury. Microglial activation by LPS plays an important role in the progressive and selective loss of dopaminergic (DA) neurons

[6, 7]. Microglia-derived superoxide contributes to about 50% of LPS-induced DA neurotoxicity [8, 9].

Although microglia are vital in the inflammatory process in the CNS, they may have less chance to be activated during a peripheral bacterial infection, as LPS may not be able to enter the CNS due to the blood-brain barrier (BBB). However, monocytes/macrophages in peripheral systems can become activated by LPS, which results in overexpression of proinflammatory cytokines. These cytokines can penetrate BBB and induce an inflammatory environment in the CNS [10]. In addition, activated monocytes in HIV infection have the ability to migrate into the CNS, causing neuronal injury [11]. Furthermore, exposure of macrophages/microglia to invading pathogens leads to the production of ROS, which can benefit the clearance of pathogens but on the other hand cause irreparable damage to neurons [12].

Natural products and dietary components rich in polyphenols have been regarded as promising dietary agents

for the prevention and treatment of inflammation-related diseases [13]. (-)-Epigallocatechin gallate (EGCG) is the most abundant catechin in green tea, a beverage widely consumed worldwide. EGCG as a potent antioxidant has been shown to have both anti-inflammatory and antiatherogenic properties in experimental studies conducted *in vitro* and *in vivo* [14, 15]. EGCG was found to inhibit TNF- $\alpha$ -induced production of MCP-1/CCL2 from bovine coronary artery endothelial cells, providing direct vascular benefits in inflammatory cardiovascular diseases [16]. It has also been shown that EGCG attenuated the increase in malondialdehyde levels caused by cerebral ischemia and reduced the formation of postischemic brain edema and infarct volume [17]. The neuroprotective effect of EGCG against ischemia-induced brain damage was found, in part, due to the modulation of NOS isoforms and preservation of mitochondrial complex activity and integrity [18]. Thus, the *in vivo* neuroprotective effects of EGCG are not exclusively due to its antioxidant effects but involve more complex signal transduction mechanisms. In addition, the dose of EGCG is vital to be concerned in neuroprotective application, as EGCG presents a biphasic effect based on its concentration-dependent window of pharmacological action. EGCG can act as an antioxidant, reducing ROS at low concentrations [19, 20], and paradoxically may promote the production of ROS and decline of mitochondrial membrane potential and induce apoptosis at high concentrations [21]. In this study, we examined whether EGCG possesses the ability to protect primary human neurons from the macrophages-mediated inflammation and neurotoxicity.

## 2. Materials and Methods

**2.1. (-)-Epigallocatechin Gallate.** EGCG ( $\geq 95\%$ ) was purchased from Sigma-Aldrich, St. Louis, MO, USA (CAS# 989-51-5). EGCG stock solution was prepared in sterile double distilled water at 20 mM.

**2.2. Endotoxin-Induced Inflammatory Response and EGCG Treatment.** All animal experiments were conducted according to the guidelines for the care and use of laboratory animals and the protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Animal Biosafety Level III Laboratory at the Center for Animal Experiment. Sixteen adult male Sprague-Dawley rats weighing 200–300 g were obtained from the Center for Animal Experiment, Wuhan University. Briefly, rats were intraperitoneally injected with LPS (from *Escherichia coli*, 055:B5, Invivogen; 1 mg/kg;  $n = 4$ ) or EGCG (5 mg/kg;  $n = 4$ ) or EGCG (5 mg/kg) plus LPS (1 mg/kg;  $n = 4$ ) in 0.1 mL of endotoxin-free phosphate buffered saline (PBS) or 0.1 mL of PBS ( $n = 4$ ). After 24 h, the rats were anesthetized with ketamine and xylazine. Blood samples were collected by cardiac puncture into heparinized syringes. The peripheral blood mononuclear cells (PBMC) were isolated by Ficoll density gradient centrifugation. Following centrifugation (1500  $\times$ g, 30 min, room temperature), PBMC located at the interface were harvested and washed with PBS and lysed with Tri Reagent for RNA extraction.

**2.3. Monocyte-Derived Macrophage Cultures.** Monocytes were obtained from the Path BioResource of the University of Pennsylvania School of Medicine. Blood samples were screened for common blood-borne pathogens and certified to be pathogen-free. Monocytes were isolated by elutriation; the purity of isolated monocytes was higher than 95%. Freshly isolated monocytes were resuspended in DMEM supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL), streptomycin (100  $\mu$ g/mL), and 1% nonessential amino acids. Cells were cultured in 48-well plates (Corning CellBIND Surface, Corning Incorporated, Corning, NY) at  $2.5 \times 10^5$  cells per well. The medium was half-changed every 48 h. After culture for 7 days, monocytes differentiated into macrophages. Macrophages were incubated with different concentrations of EGCG (0, 10, 20, and 40  $\mu$ M) for 24 h prior to the treatment with LPS for additional 6 h after which the medium was replenished and cultured for additional 24 h. Supernatant collected from macrophage cultures was used to treat primary human neurons. The cytotoxicity of EGCG to macrophages was measured using a 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay as previously described [22].

**2.4. Primary Human Neuron Cultures.** Highly enriched neuronal cultures were prepared as described previously [23]. All of the experimental protocols were reviewed and approved by the Institutional Review Board of the University of Minnesota Medical School. Briefly, 11- to 19-week-old fetal brain tissues of aborted fetuses (3 donors) obtained from the Human Embryology Laboratory (University of Washington, Seattle, WA, USA) were dissociated and resuspended in neural basal medium containing B-27 serum-free supplement (contains antioxidants) plus penicillin (100 U/mL) and streptomycin (100  $\mu$ g/mL). Dispersed cells were plated onto collagen-coated plates ( $5 \times 10^5$  cells/well in 24-well plate) or chamber slides ( $4 \times 10^5$  cells/well in 4-well chambers). On day 12, these brain-cell cultures contained  $\sim 70$ – $80\%$  neurons (stained by anti-NeuN or anti-MAP2 antibodies), 15–25% astrocytes (stained by anti-GFAP antibody), and 3–7% microglial cells (stained by anti-CD68 antibody). For highly enriched neuronal cultures, cell cultures were treated with uridine (33.6  $\mu$ g/mL) and fluorodeoxyuridine (13.6  $\mu$ g/mL) on day 5, followed by replacement with neural basal medium with B-27 serum-free supplement (contains antioxidants) on day 6 and every 4 days thereafter. Highly purified neuronal cultures contained  $>95\%$  neurons, 2–3% astrocytes, and 1–2% microglial cells.

**2.5. Reverse Transcription and Quantitative Real-Time PCR.** Total RNA was extracted with Tri Reagent (Sigma-Aldrich) and quantitated by spectrophotometric analysis. Reverse transcription was performed using the AMV transcriptase and RNasin (Promega Co., Madison, WI, USA) according to the manufacturer's instruction. Quantitative real-time PCR (qRT-PCR) was performed with Brilliant SYBR Green Master Mix (Bio-Rad Laboratories, Hercules, CA, USA) described previously [24]. The oligonucleotide primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA, USA). The primers that we used for the

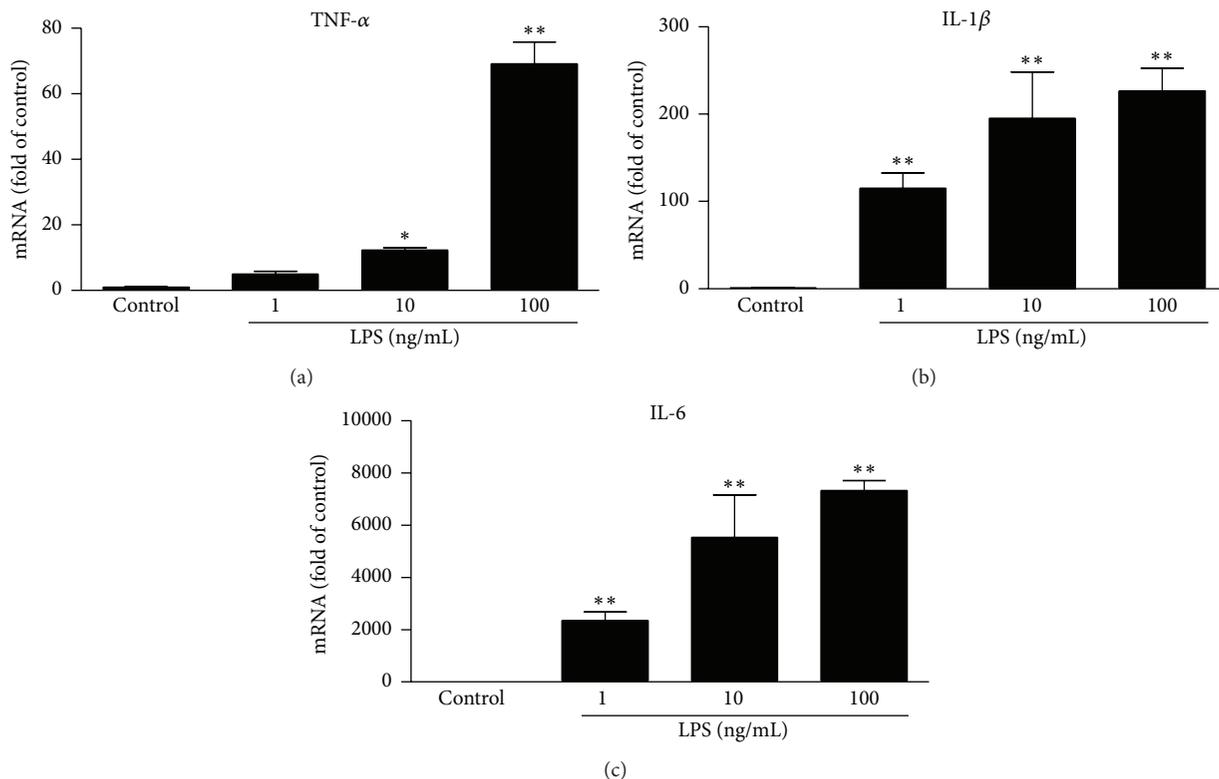


FIGURE 1: LPS induces the expression of inflammatory cytokines. Human macrophages derived from peripheral blood monocytes were treated with indicated concentrations of LPS for 24 h. Cellular RNA was extracted and subjected to quantitative real-time RT-PCR for TNF- $\alpha$  (a), IL-1 $\beta$  (b), and IL-6 (c). Data were expressed as mean  $\pm$  SD of three independent experiments. Data were expressed as mean  $\pm$  SD of three independent experiments. \* $P$  < 0.05; \*\* $P$  < 0.01.

PCR amplifications are listed as follows: glyceraldehyde 3-phosphate dehydrogenase (GAPDH): 5'-GGTGGTCTCCTCTGA CTTCAACA-3' (sense) and 5'-GTTGCTGTAGCCAAATTCGTTGT-3' (antisense); TNF- $\alpha$ : 5'-CGAGTGACAAGCCTGTAGC-3' (sense) and 5'-GGTGTGGGTGAGGAGC ACAT-3' (antisense); IL-1 $\beta$ : 5'-AAGCTGATGCCCTAAACAG-3' (sense) and 5'-AGGTGCATCGTGCACATAAG-3' (antisense); IL-6: 5'-AGGAGACTTGCC-TGGTGA AA-3' (sense) and 5'-CAGGGGTGGTTATTGCATCT-3' (antisense); iNOS: 5'-GCAGAATGTGACCATCATGG-3' (sense) and 5'-ACAACCTTGGTGTGAAGGC-3' (antisense). All values were calculated using the delta-delta cycle threshold method and expressed as the change relative to the expression of GAPDH.

**2.6. Immunofluorescence Staining and MAP-2 ELISA.** Neuronal cells were seeded on poly-L-lysine coated cover slips in 96-well plates and cultured for two weeks before treatment with LPS or supernatant from LPS-activated macrophage cultures. Cells were then washed with PBS three times and fixed in ice-cold methanol for 5 min. Nonspecific sites were blocked in Block A for 30 min. Cells were then incubated in mouse anti-MAP-2 antibody (1:100; Sigma-Aldrich, St. Louis, MO) for 1 h, followed by Alexa 488-conjugated anti-mouse IgG for 30 min. After Hoechst (2  $\mu$ g/mL) staining, the coverslips were mounted on glass slide and observed

under a fluorescence microscope (Olympus IX71). For MAP-2 ELISA, after block, cells were incubated with anti-MAP-2 antibody (1:1000) overnight at 4°C. After a wash with PBS, goat  $\alpha$ -mouse  $\beta$ -lactamase TEM-1 (Molecular Probes, Eugene, OR) conjugate (1:500; 2  $\mu$ g/mL) was added into each well and incubated for 30 min and then with fluorocillin green substrate (Invitrogen, Carlsbad, CA) solution in PBS (1  $\mu$ g/mL) for 1 h. Fluorescence was read at 485/527 nm on a SpectraMax® M3 Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA). The fluorescence of untreated neurons (control) was defined as 100%.

**2.7. Reactive Oxygen Species (ROS) Detection.** Macrophages were pretreated with or without EGCG for 1 h prior to LPS treatment for 24 h. Cells were then washed with serum-free medium and incubated in 10  $\mu$ M of 2',7'-dichlorofluorescein diacetate (DCFH<sub>2</sub>DA; Molecular Probes) at 37°C for 30 min [24]. After a counterstaining of nuclear with Hoechst 33342 (2  $\mu$ g/mL) for 5 min and wash, the ROS production was assessed using a fluorescence microscope (Olympus IX71) at 488/527 nm.

**2.8. Statistical Analysis.** Data are expressed as the mean  $\pm$  SD for at least three independent experiments. Statistical significance was analyzed using Student's *t*-test to compare the means of two groups. For comparison of means of

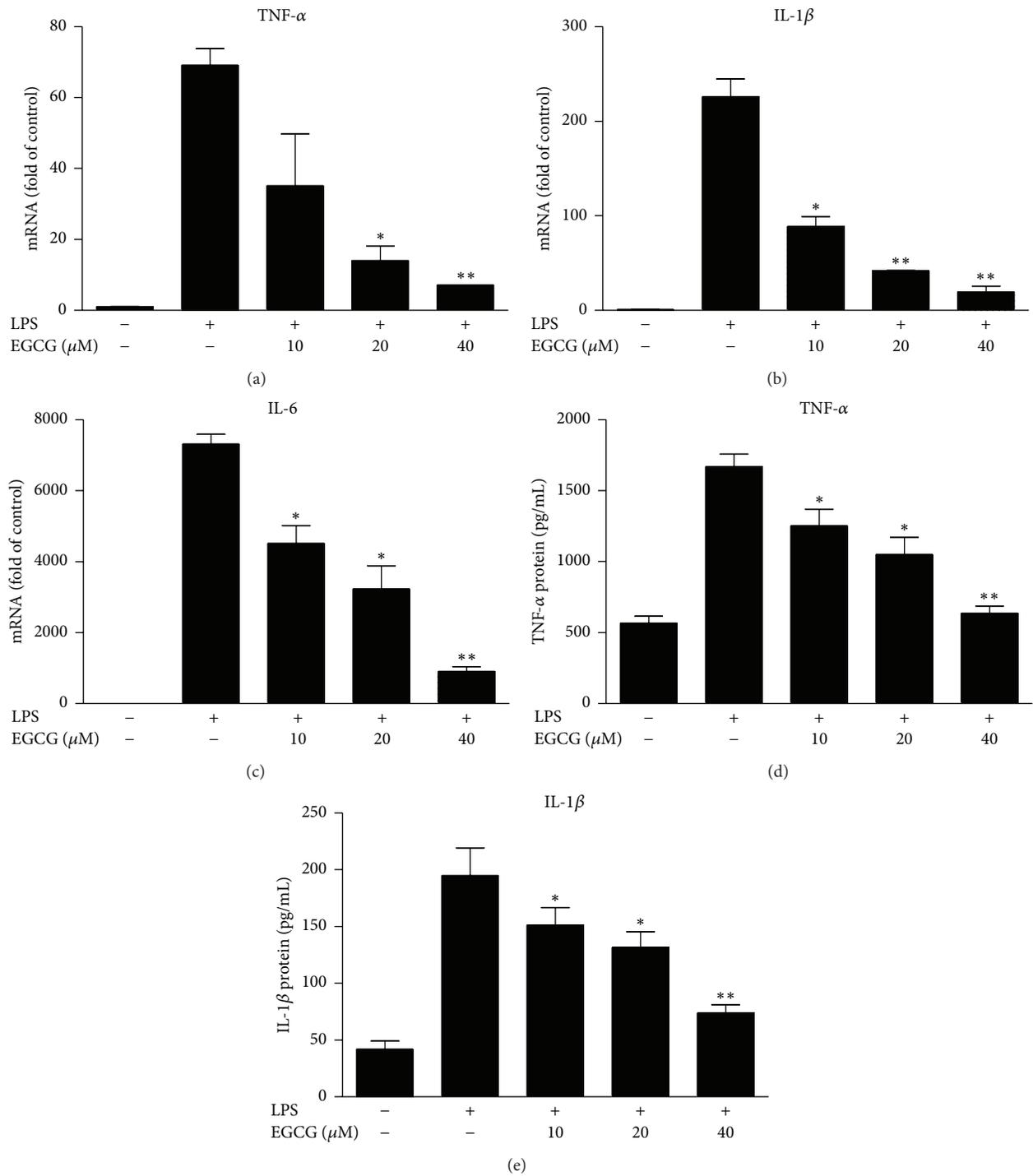


FIGURE 2: EGCG inhibits LPS-induced expression of inflammatory cytokines. Human macrophages derived from peripheral blood monocytes were treated with indicated concentrations of EGCG for 1 h prior to 100 ng/mL of LPS treatment for additional 24 h. RNA was extracted and subjected to quantitative real-time RT-PCR of TNF- $\alpha$ , IL-1 $\beta$ , and IL-6. Data were expressed as mean  $\pm$  SD of three independent experiments. \* $P < 0.05$ ; \*\* $P < 0.01$ , as compared with LPS treated.

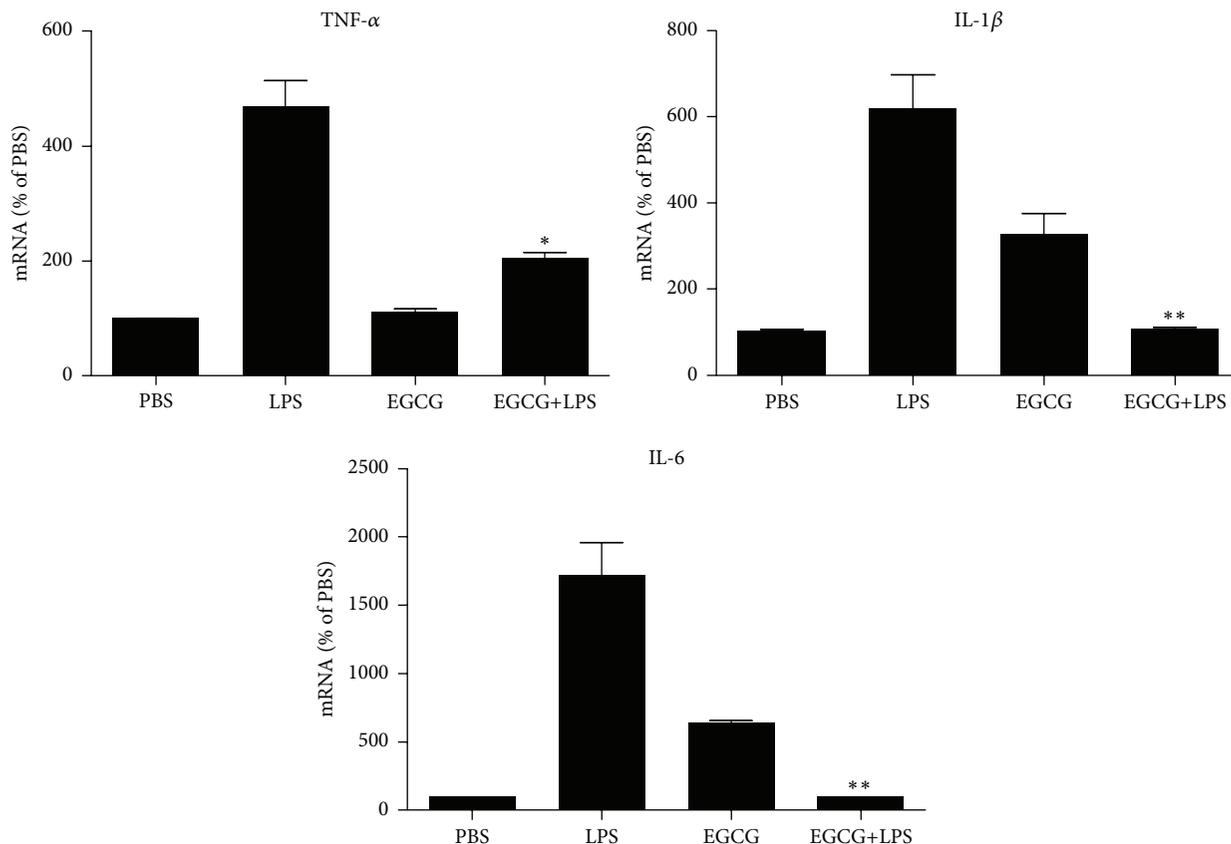


FIGURE 3: EGCG suppresses LPS-induced expression of inflammatory cytokines in rats. Sprague-Dawley rats were injected with PBS, LPS (1 mg/kg), EGCG (5 mg/kg), or LPS plus EGCG. Twenty-four hours posttreatment, rats ( $n = 4$ ) in each group were sacrificed 24 h after being anesthetized. PBMCs were isolated by Ficoll-plaque and lysed with Tri Reagent. RNA was extracted and subjected to quantitative real-time RT-PCR for TNF- $\alpha$ , IL-1 $\beta$ , and IL-6. Data were expressed as mean  $\pm$  SD of 4 animals in each group. \* $P < 0.05$ ; \*\* $P < 0.01$ , as compared with LPS treated.

multiple groups, one-way analysis of variance (ANOVA) was performed followed by post-Newman-Keuls test. Differences were considered to be statistically significant when the  $P$  value was less than 0.05.

### 3. Results

#### 3.1. EGCG Attenuates LPS-Induced Inflammatory Cytokines.

We first evaluated the *in vitro* effects of EGCG on LPS-induced inflammatory cytokines in primary human macrophages. As shown in Figure 1, LPS treatment of macrophages induced the expression of TNF- $\alpha$ , IL-1 $\beta$  (600-fold), and IL-6 (1700-fold). However, the expression of these cytokines were significantly reduced in macrophages pretreated with EGCG (Figure 2). This effect of EGCG was dose-dependent (Figure 2) without cytotoxicity (data not shown). We then examined the *in vivo* impact of EGCG on LPS-induced inflammatory cytokines in PBMCs of rats. As shown in Figure 3, LPS challenge of rats induced the expression of TNF- $\alpha$  (480-fold), IL-1 $\beta$  (600-fold), and IL-6 (1700-fold) in PBMCs. In contrast, EGCG administration significantly attenuated the induction of these cytokines by LPS (Figure 3).

#### 3.2. Effect of EGCG on LPS-Induced Neurotoxicity through Macrophages.

We next examined the protective effect of EGCG on LPS-induced neurotoxicity. Figure 4 shows that treatment of primary human neurons with supernatant from LPS-activated macrophage cultures significantly reduced the neuron numbers as identified by MAP-2 immunocytochemistry staining. However, EGCG pretreatment of macrophages remarkably inhibited LPS-induced neurotoxicity (Figure 4).

#### 3.3. EGCG Protects Neurons from LPS-Induced Neurotoxicity.

From the above we used macrophage cultures to mimic the microglia and we observed that supernatant from LPS-activated macrophages exerted neurotoxicity. Indeed, because it is difficult to obtain pure neuron population (even it can be >95%), there were microglia present in the neuronal cultures albeit at small numbers. We then examined whether LPS direct treatment of the neuronal cultures had neurotoxicity and whether EGCG could protect neurons in this context. When directly added to the neuronal cultures, LPS induced neurotoxicity as evidenced by the reduction of MAP-2 expression (Figure 5). Pretreatment of neurons with EGCG could protect neuronal cells from LPS-mediated cytotoxicity. However, the EGCG concentration (0.1  $\mu$ M) that

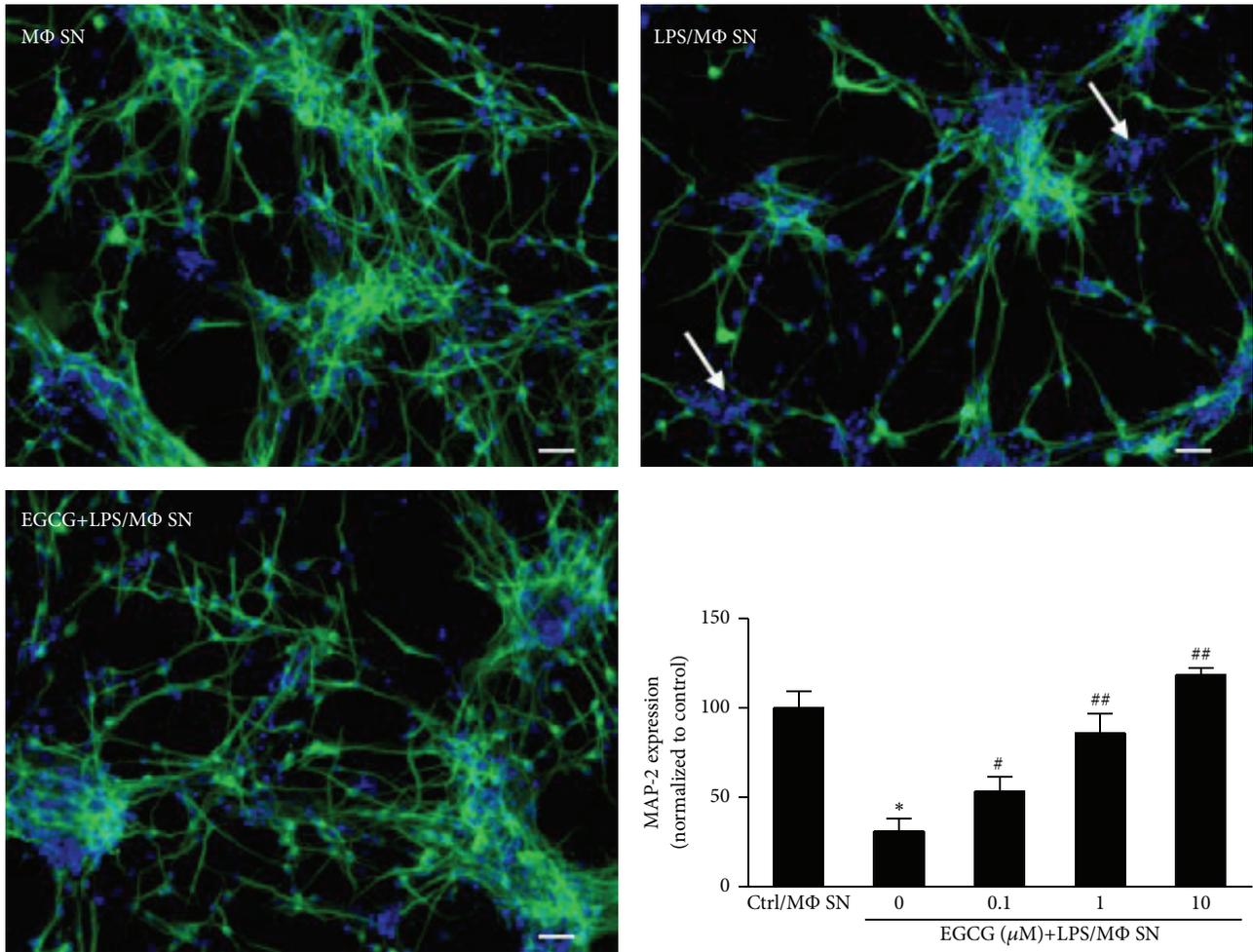


FIGURE 4: EGCG attenuates the neurocytotoxicity of supernatant from LPS-treated macrophage cultures to human primary neurons. Human macrophages derived from peripheral blood monocytes were treated with 100 ng/mL of LPS for 24 h. For EGCG group, macrophages were pretreated with 10  $\mu$ M of EGCG for 1 h. Culture supernatant from these macrophage cultures was used to treat human neurons (10%, v/v). The neurocytotoxicity was examined by a cell-based MAP-2 ELISA. Nuclei were counterstained with Hoechst. Data were expressed as mean  $\pm$  SD and representative data from three independent experiments was shown. Magnification:  $\times 100$ . \*  $P < 0.05$ , as compared with control; #  $P < 0.05$ ; ##  $P < 0.01$ , as compared with LPS treated. Scale bar = 50  $\mu$ m.

can protect neurons directly is much lower than the effective concentrations (10–40  $\mu$ M) in protecting macrophages from LPS-induced upregulation of cytokines and macrophage-mediated neurocytotoxicity.

**3.4. EGCG Inhibits LPS-Induced ROS Production in Neurons.** To investigate the mechanism(s) of EGCG against LPS-induced direct neurocytotoxicity, we examined the oxidative stress in the neuronal cultures. ROS has been reported as an important mediator for LPS-induced cytotoxicity. Figure 6(a) shows that LPS treatment of neurons directly induced ROS production and this effect was dose-dependent. EGCG pretreatment inhibited LPS-mediated induction of ROS (Figure 6(b)), as well as the upregulation of iNOS (Figure 6(c)), but the EGCG concentration (0.1  $\mu$ M) was much lower than that required to exhibit the anti-inflammatory effect in macrophages (10  $\mu$ M).

## 4. Discussion

It is well known that activated macrophages or microglia produce inflammatory mediators, which has a negative impact on the survival of neurons [1, 25, 26]. Overactivated microglia/macrophages are a chronic source of multiple neurotoxic factors, including TNF- $\alpha$ , NO, IL-1 $\beta$ , and ROS that can cause progressive neuron damage [26–28]. We found that culture supernatant from LPS-stimulated macrophages exerted neurotoxicity to primary human neurons as evidenced by the reduced expression of specific neuronal marker MAP-2. Systemic inflammatory response which resulted from microbial infection is partly mediated by various pathogen-associated molecular patterns (PAMPs), such as endotoxin [29]. Bacterial endotoxin challenge or exposure plays an important role in inflammation-related damages, including neurodegeneration [2, 30]. Although the production of proinflammatory cytokines (e.g., TNF- $\alpha$  and

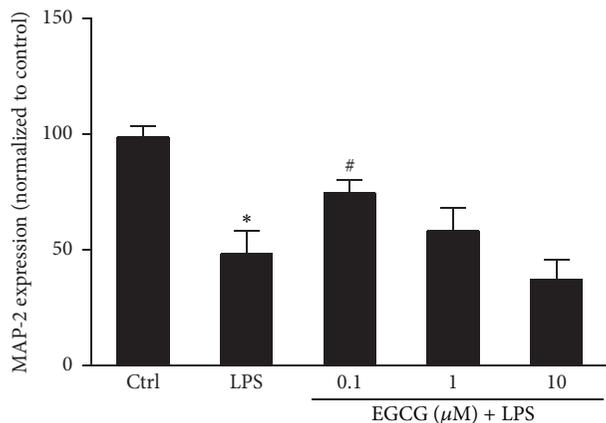


FIGURE 5: EGCG reduces neurocytotoxicity of LPS to neurons. Primary human neurons were treated with or without  $0.1 \mu\text{M}$  of EGCG for 1 h and then with  $100 \text{ ng/mL}$  of LPS for additional 48 h. Cells were fixed and stained with anti-MAP-2. Nuclei were counterstained with Hoechst. Data were expressed as mean  $\pm$  SD of three independent experiments and representative figures were shown. \* $P < 0.05$ , as compared with control; # $P < 0.05$ , as compared with LPS only.

IL-6) by macrophages/microglia is essential in early host defense against infection [31], excessive accumulation of these cytokines disrupts systemic or CNS homeostasis [32–35]. EGCG has been shown to inhibit the induction of TNF- $\alpha$  and IL-6 in murine peritoneal macrophages elicited by TLR2/4 signaling [4, 36]. Suppression of IFN- $\gamma$  and IL-6-induced STAT signaling by EGCG has also been reported in mouse splenic monocytes and PBMCs [37, 38]. In addition, our earlier *in vitro* study showed that EGCG pretreatment of human brain microvascular endothelial cells could inhibit LPS-induced expression of inflammatory cytokines [39]. We found that *in vivo* EGCG administration to rat significantly reduced LPS-induced expression of proinflammatory cytokines (TNF- $\alpha$ , IL-6, and IL-1 $\beta$ ) in PBMCs. The underlying mechanism(s) of the EGCG actions has largely been attributed to its suppression of NF- $\kappa\text{B}$  activation as well as the negative regulation of cytokine signaling [4, 38–41].

Green tea has been regarded as a nutrient component with possible beneficial effects on neurons although the cellular and molecular mechanism(s) remain unclear. EGCG is the main and most significantly bioactive polyphenol in green tea. We observed that EGCG inhibited the LPS-mediated induction of inflammatory cytokines and attenuated neurotoxicity by LPS-activated macrophages. In addition, EGCG at low dose ( $0.1 \mu\text{M}$ ) also exerted direct neuroprotective effect against LPS by mitigating the ROS production in neurons. These findings together with studies by others [39, 42, 43] support the notion that EGCG has potential for treating inflammation-induced neuronal injury. Several reports indicated that tea polyphenols can be attained in the brain and exert neuroprotective effect simply by drinking [44–46]. EGCG metabolite could be detected in the brain after oral administration of EGCG to rats [47, 48]. An early observation that there was a wide distribution of labelled

EGCG in mouse organs including brain suggests the ability of EGCG to penetrate the BBB [49]. A single, very high oral EGCG dose ( $500 \text{ mg/kg}$  body weight) to rats yielded EGCG concentrations of about  $0.5 \text{ nmol/g}$  in brain (measured by CL-HPLC) and 20-fold higher in plasma [50]. EGCG was also investigated as a therapeutics adjuvant in the combination therapy to treat multiple sclerosis in mice [51]. However, due to limited systemic absorption, the concentrations of EGCG or EGCG metabolite in the brain are much lower than those in plasma [49].

Interestingly, we revealed that EGCG, at a lower dose of  $0.1 \mu\text{M}$ , but not at higher concentrations (1 and  $10 \mu\text{M}$ ), protected neurons from LPS-induced direct neurotoxicity. This neuroprotective activity was concomitantly with the inhibition of ROS production by EGCG in LPS-treated neuronal cultures. Indeed, treatment of neurons with higher concentration ( $10 \mu\text{M}$ ) of EGCG increased ROS production (data now shown). This biphasic mode of antioxidant and prooxidant activities of EGCG has also been observed in other models [52, 53]. It has been proposed that EGCG exhibits prooxidant and proapoptotic activity at high concentrations, which are responsible for its anticancer cell death effect, while lower doses of EGCG exert neuroprotection against a wide spectrum of neurotoxic compounds [54, 55]. Kucera et al. showed that low doses ( $<10 \mu\text{M}$ ) of EGCG decreased ROS production whereas EGCG in concentrations of  $10 \mu\text{M}$  and higher induced increase in ROS formation with resultant cellular injury and a decrease in hepatocyte functions. It was revealed that EGCG at high doses led to an uncoupling of mitochondrial oxidative phosphorylation and to damage to the outer mitochondrial membrane [19]. The oxidant activity of EGCG has also been demonstrated in murine macrophages and human leukemic cell lines to increase  $\text{H}_2\text{O}_2$ -induced oxidative stress and DNA damage [56, 57]. Catechins, particularly EGCG ( $100 \mu\text{M}$ ), have been shown to increase the oxidative damage to isolated and cellular DNA after exposure to 8-oxo-7,8-dihydro-2'-deoxyguanosine [58, 59]. The prooxidant activity of EGCG was due to the generation of the hydroxyl radical and hydrogen peroxide in the presence of copper(II) and iron(III), suggesting that antioxidant mechanism of scavenging metals by catechins to stop the formation of free radicals may lead to prooxidant actions on DNA [60]. Excessive EGCG concentrations could also induce toxic levels of ROS *in vivo*. The prooxidative activities and dose-response relationship of EGCG have been implicated in the inhibition of lung cancer cell growth both *in vivo* and *in vitro* [21]. In our *in vivo* experiment, we noticed that EGCG treatment of rat also slightly induced the upregulation of IL-1 $\beta$  and IL-6, which might attribute to the prooxidant activity of EGCG. This concentration-dependent biphasic mode is common for some typical radical scavengers and antioxidants, such as ascorbic acid (vitamin C) [61].

In summary, we provide experimental evidence that EGCG attenuates LPS-induced inflammation and LPS-activated macrophage-mediated neurotoxicity at relative higher concentrations ( $10\text{--}40 \mu\text{M}$ ). EGCG at low dose ( $0.1 \mu\text{M}$ ), but not high concentrations used in macrophages, protects neurons from LPS-induced neurotoxicity and the

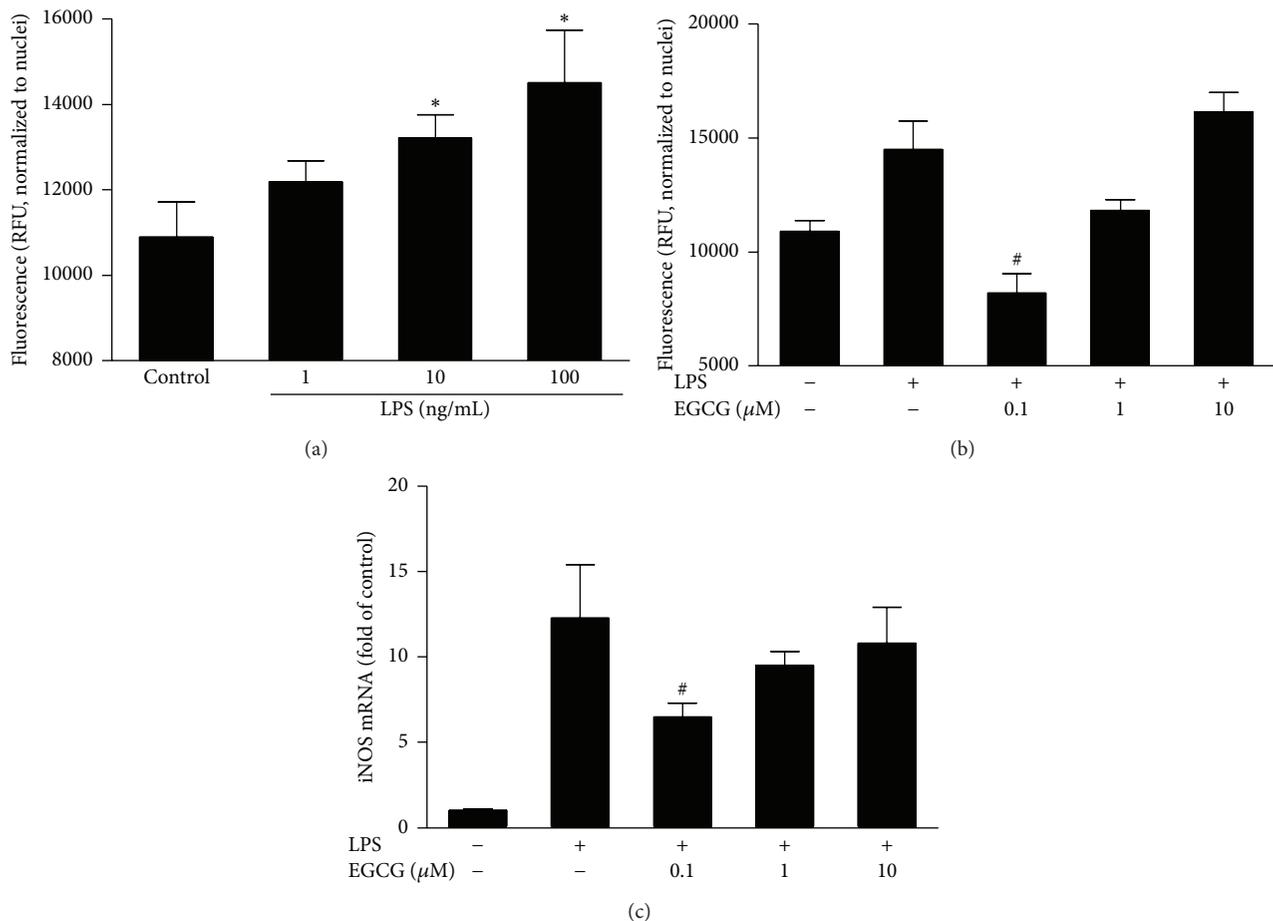


FIGURE 6: Effect of EGCG on LPS-induced production of ROS in neurons. Primary human neuronal cultures were treated with or without LPS at indicated concentrations for 48 h (a) or with indicated concentrations of EGCG for 1 h prior to 100 ng/mL of LPS treatment for additional 48 h (b). The ROS production was examined by labelling cells with a cell-permeable nonfluorescent probe 2',7'-dichlorofluorescein diacetate and the fluorescence was measured by a fluorescence microplate reader with excitation at 488 nm and emission at 527 nm. (c) The expression of iNOS in neurons treated with LPS in the presence of indicated concentration of EGCG. Data were expressed as mean  $\pm$  SD of three independent experiments. \* $P < 0.05$ , as compared with control; # $P < 0.05$ , as compared with LPS treated.

effect at least partially attributed to the antioxidant activity of EGCG at this concentration. This biphasic mode of action implicates that EGCG may be a good candidate for treatment of inflammation-associated neurodegenerative disorders given the limited availability of EGCG to the brain. Nevertheless, further studies with oral administration of EGCG to suitable animal model are needed.

## Competing Interests

The authors declare that they have no competing interests.

## Authors' Contributions

Jie-Liang Li and Wen-Zhe Ho conceived of the study and experiments and wrote the paper. Jie-Liang Li, Jin-Biao Liu, Yi-Zhong Wang, Li Zhou, Xu Wang, and Yu Zhou performed experiments. All authors have read and approved the final version of this paper.

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## Review Article

# Monocyte Activation in Immunopathology: Cellular Test for Development of Diagnostics and Therapy

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Several highly prevalent human diseases are associated with immunopathology. Alterations in the immune system are found in such life-threatening disorders as cancer and atherosclerosis. Monocyte activation followed by macrophage polarization is an important step in normal immune response to pathogens and other relevant stimuli. Depending on the nature of the activation signal, macrophages can acquire pro- or anti-inflammatory phenotypes that are characterized by the expression of distinct patterns of secreted cytokines and surface antigens. This process is disturbed in immunopathologies resulting in abnormal monocyte activation and/or bias of macrophage polarization towards one or the other phenotype. Such alterations could be used as important diagnostic markers and also as possible targets for the development of immunomodulating therapy. Recently developed cellular tests are designed to analyze the phenotype and activity of living cells circulating in patient's bloodstream. Monocyte/macrophage activation test is a successful example of cellular test relevant for atherosclerosis and oncopathology. This test demonstrated changes in macrophage activation in subclinical atherosclerosis and breast cancer and could also be used for screening a panel of natural agents with immunomodulatory activity. Further development of cellular tests will allow broadening the scope of their clinical implication. Such tests may become useful tools for drug research and therapy optimization.

## 1. Introduction

Immunopathology is associated with the most common life-threatening disorders, including atherosclerosis and related cardiovascular diseases, cancer, and chronic inflammation. A number of diseases, such as lupus erythematosus, rheumatoid arthritis, or HIV infections, are characterized by pronounced immunopathologies; others, such as atherosclerosis and cancer, by less obvious latent pathological changes in the immune system. Such changes may represent early events in the disease initiation and development and might therefore be especially interesting for timely diagnostics and for development of preventive treatment.

The role of the immune system dysfunction in cancer is currently well recognized [1]. Altered macrophage plasticity and polarization can contribute both to the malignancy

development and to the tumor vascularization [2]. In that regard, comprehensive analysis of the macrophage population diversity would be necessary for developing adequate therapeutic approaches and monitoring the therapy efficiency.

Recent studies have revealed many aspects of the complex and important role of macrophages in the pathogenesis of atherosclerosis [3]. Formation of the atherosclerotic plaque begins with monocyte activation and transformation into macrophages that reside in the subendothelial area of the blood vessel wall and accumulate lipids in their cytoplasm becoming foam cells. This lipid trapping is performed by means of uncontrolled phagocytosis. At the same time, certain types of macrophages are implicated in tissue repair, and these cells have been found in regressing plaques in mouse models [4, 5]. Therefore, different types of macrophages are

responsible for the plaque initiation, growth, and, eventually, regression [6–8]. Correspondingly, anti-inflammatory agents are considered as an important component of antiatherosclerotic therapy [9]. Here again, the analysis of macrophage phenotypic diversity could improve the understanding of the pathological process and assessment of the therapy efficiency.

According to current epidemiological data, atherosclerosis-related diseases and cancer are the two greatest contributors to the overall mortality in the developed countries [10, 11]. Given that these diseases are tightly associated with immunopathology, development of comprehensive diagnostic methods and therapeutic approaches to modulate the immune system appears to be of the greatest importance. However, the existing diagnostic methods are imperfect and their improvement remains challenging. Likewise, no drugs are available to date that allow targeted immune correction in atherosclerosis. It is clear that changes in cytokine expression and phenotypic features of macrophages may reflect the disease progression state. These features may therefore be used for monitoring the pathological process and treatment efficiency.

## 2. Cellular Tests for Diagnostics and Drug Research

In many pathological conditions, the analysis of different types of cells circulating in the bloodstream can provide valuable information about the disease progression. During the recent years, a number of cell types have been isolated and studied for possible application in diagnostics and drug development.

Circulating tumor cells (CTCs) can be extracted from patient's blood and used to analyze the expression of relevant genes and surface markers. For instance, successful isolation and molecular characterization have been described for metastatic breast cancer [12], metastatic colorectal cancer [13], and lung cancer [14]. This strategy is especially useful in cases of advanced metastatic cancer, where the patients could benefit from a personalized treatment. The analysis of CTCs has a great diagnostic potential but can also help in revealing the possible drug resistance of the tumor and designing the optimal therapy [15]. Many current studies are focused on the improvement of CTC-based analyses and their clinical implementation.

Peripheral blood mononuclear cells (PBMCs) are relatively easily obtainable cells that can be used for monitoring a wide spectrum of conditions and pathologies. The analysis of mRNA profiles of isolated PBMCs could be used for evaluation of metabolic changes [16]. PBMCs can also be kept in short-term culture and used for studying cytokine production induced by stimulation. For instance, changes in proinflammatory cytokine production by isolated PBMCs have been described in such conditions as allergies, alterations of immune response, and immunization [17–19]. Studies on PBMCs have demonstrated that vascular endothelial growth factor (VEGF) production was decreased in women with preeclampsia [20]. Isolated PBMCs can serve as a relevant system for testing various drugs, especially related to inflammation [21]. Recently, the potential of macrophage-based test

system for diagnostics and treatment of atherosclerosis has been explored [22].

Atherosclerosis is associated with life-threatening cardiovascular diseases [7]. Atherosclerosis progression is usually slow, and the disease often remains asymptomatic until the ischemia of organs and tissues becomes evident. This may happen due to the obstruction of a blood vessel with growing atherosclerotic plaque or the embolism caused by a thrombus formed on a destabilized plaque. Therefore, the first manifestations of the disease are often lethal [23, 24]. Diagnostic of preclinical (asymptomatic) atherosclerosis is therefore especially important. However, it is hindered by the absence of clinical symptoms and complaints and by the fact that the spectrum of risk factors is very wide, including genetic predisposition, lifestyle and diet patterns, chronic inflammation, and metabolic factors. Immunopathology is likely to be one of the mechanisms underlying atherosclerosis development starting from the early stages, and its assessment can therefore have an important diagnostic value.

## 3. Monocyte/Macrophage Diversity and Functions

Monocytes and macrophages are the key players in the innate immune system. These cells can eliminate pathogens by phagocytosis, release of reactive oxygen species, production of proinflammatory cytokines, and modulation of the T-cell immune response [25]. Macrophages are present in all organs and tissues and represent the first line of immune defence, responsible for removal of foreign agents and pathogens. They participate in all stages of the inflammatory process. The pool of macrophages remains constant in every tissue, and the cells are renewed from the population of circulating monocytes [26], although the results of recent studies suggest that these cells are also capable of self-renewal [27]. It has been known for a long time that changes in the phagocytic activity of macrophages might be dependent on changes in the peripheral blood monocyte population, and alterations of the monocyte pool lead to various pathological conditions [28]. Proliferation of promonocytes, which can be stimulated by systemic inflammatory stimuli, leads to the increase of the number of circulating monocytes [29, 30]. Monocytes and macrophages, together with their precursors and dendritic cells, form the mononuclear phagocyte system (MPS) [31], although the identity of the dendritic cells remains disputed [32, 33]. The development, maintenance, differentiation, and function of MPS are regulated mostly by colony-stimulating factor 1 (CSF-1) in homeostatic conditions [34] and by granulocyte-macrophage colony-stimulating factor (GM-CSF) during inflammation [35]. Inflammatory signals and various pathological conditions, including atherosclerosis development, stimulate the inactive circulating monocytes to become activated macrophages that can be distinguished by their phenotypic properties. Macrophages can acquire different functional phenotypes influenced by the surrounding microenvironment in a process known as macrophage polarization.

Studies of macrophage population revealed significant heterogeneity and plasticity of this cell type. Reaching consensus on macrophage classification was challenging due to the high variety of activation types, dependence of the results on the particular experimental setup, and differences of macrophage activation profiles between humans and animal models. Recently, a group of leading immunologists have summarized the current knowledge on the issue and drawn recommendations for conducting and reporting the experiments involving macrophage polarization [36]. Initially, two main classes of macrophages, M1 and M2, have been defined which could be obtained by activation of macrophages by proinflammatory interferon  $\gamma$  (IFN- $\gamma$ ) and lipopolysaccharide (LPS) or by interleukin-4 (IL-4), respectively [37–39]. A simplified scheme of macrophage polarization is presented in Figure 1.

M1 macrophages are characterized by the production of proinflammatory cytokines tumor necrosis factor- $\alpha$  (TNF $\alpha$ ), IL-1 $\beta$ , IL-6, IL-12, and proteolytic enzymes, as well as by the expression of Fc- $\gamma$  receptors on the cell surface [40, 41]. Polarization towards the proinflammatory phenotype can be induced *in vitro* by toll-like receptor (TLR) ligands, including TNF $\alpha$ , lipopolysaccharide (LPS), and interferon  $\gamma$  (IFN- $\gamma$ ) that might also play a role in the pathogenesis of atherosclerosis [42]. It has been demonstrated that M1 macrophages are present in the atherosclerotic plaques where they maintain the local inflammatory process and promote the extracellular matrix degradation contributing to the formation of unstable plaques that can induce thrombus formation and are therefore especially dangerous [43–45].

The subpopulation of M2 macrophages was further divided into several subtypes depending on the activation stimuli (Figure 1): M2a (activated by IL-4), M2b (activated by immune complexes), and M2c (activated by IL-10) [46]. Each of these subtypes is characterized by a distinct pattern of cytokine and surface marker expression which can also vary between the species. It has been proposed therefore to refer to different subtypes of macrophages indicating the activation type (e.g., M(IL-4) instead of M2a) [36]. M2a macrophages have strong anti-inflammatory properties and can be regarded as tissue-repairing cells [47]. They have poor phagocytic capacity and participate in the formation of extracellular matrix by stimulating production of collagen. They express IL-1 receptor antagonist (IL-1ra) and secrete CCL18, transforming growth factor  $\beta$  (TGF- $\beta$ ), and remodelling enzymes. M2b and M2c macrophages express different chemokine receptors, produce IL-10, and can modulate inflammation but do not synthesize the extracellular matrix and can therefore be regarded as regulatory macrophages [48]. Anti-inflammatory macrophages were also shown to express mannose receptor (CD206), stabilin-1, and decoy receptor IL-1RII on the surface [49–51]. M2 phenotype can also be induced by T regulatory cells [52]. However, the heterogeneity of this population requires further studies and standardization of the nomenclature. For the sake of simplicity, later in this work, we will refer to the IL-4-activated M2a macrophages as “M2 phenotype.”

The production and release of the proinflammatory cytokines, such as IL-1 $\beta$  and IL-18, are dependent on the

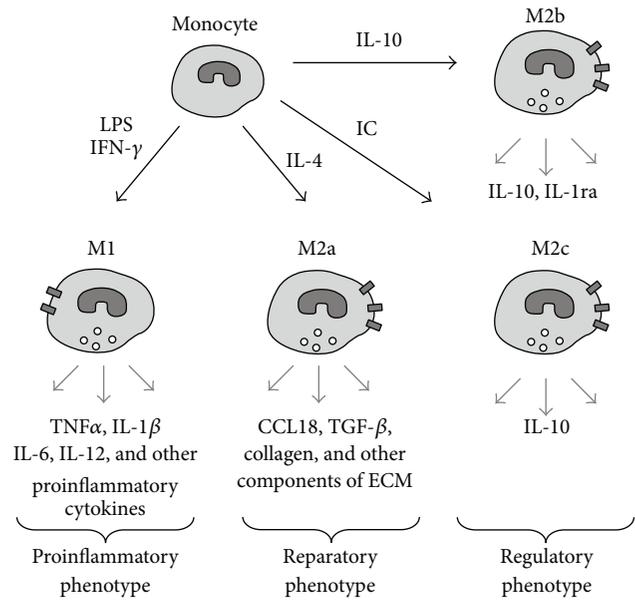


FIGURE 1: Simplified scheme of macrophage polarization. Activation of monocytes is followed by the polarization of macrophages to acquire proinflammatory phenotype (M1) or anti-inflammatory phenotypes (M2a–M2c) depending on the activation stimuli. Each phenotype is characterized by the secretion of a distinct pattern of pro- or anti-inflammatory cytokines and other molecules. For instance, M1 macrophages release TNF $\alpha$ , IL-1 $\beta$ , IL-6, IL-12, and other proinflammatory cytokines, whereas M2a macrophages produce CCL18, TGF- $\beta$ , collagen, and other extracellular matrix components. LPS: bacterial lipopolysaccharides; IC: immune complexes; IFN- $\gamma$ : interferon gamma; IL: interleukin; TNF $\alpha$ : tumor necrosis factor-alpha; TGF- $\beta$ : transforming growth factor beta; CCL18: CC chemokine ligand 18.

macrophage inflammasome status [53, 54]. Inflammasome is a caspase-activating complex formed by several proteins, including caspase-1, which is responsible for cytokine maturation. Active caspase-1 can also be released from the activated cells and may contribute to the damage of neighbouring cells [55]. The inflammasome activation has been reported in various pathological conditions and infections. It can be induced by danger- and pathogen-associated molecular patterns (DAMPs and PAMPs) [56–59]. Importantly, inflammasome is activated in atherosclerosis in response to cholesterol accumulation in the blood vessel wall and formation of cholesterol crystals in foam cells [60]. On the other hand, atherogenesis can be associated with ongoing infections with various pathogens, such as *Chlamydia pneumoniae* and *Helicobacter pylori*, which can induce the inflammasome activation [61–63]. There might exist other factors that contribute to the inflammasome activation and atherosclerosis progression, including the formation of uric acid crystals [64] and impaired autophagy [65]. Therefore, the inflammasome activation and release of proinflammatory cytokines and caspase-1 are relevant for atherosclerosis progression and can be regarded as important markers of the pathological process.

#### 4. Cellular Tests Based on Monocyte/Macrophage Phenotypic Changes in Atherosclerosis

Development of a reliable monocyte/macrophage-based functional test remains challenging due to several technical problems. Isolation of monocytes from blood can lead to their activation. A number of different isolation methods have been proposed during the recent years. The traditional method implies cell adhesion, which leads to monocytes activation and is therefore widely criticized. Another method is fluorescence-activated cell sorting (FACS), which is fast and accurate but requires labelling of cells with specific antibodies, which can also lead to activation. A pure fraction of monocytes/macrophages can be isolated using magnetic separation. In this method, unspecific activation can also occur due to possible phagocytosis of paramagnetic particles. So far, the only method that can extract nonactivated monocytes is elutriation [66, 67]. It requires, however, special equipment and is impossible to introduce into routine clinical practice. Because of these technical problems, recent studies focused on the identification of molecular markers that could substitute for functional tests in diagnostics of immunopathologies.

Studies of monocyte function include the assessment of their motility, adhesion, phagocytic activity, and low-density protein (LDL) uptake [68, 69]. Macrophages can take part in pathological processes via stimulation with circulating soluble activation factors, adhesion to the endothelium, and migration into the tissue where they meet local activation factors. The monocytes' response to these stimuli depends on their priming in circulation and therefore can have a diagnostic potential. The analysis of macrophage pro- and anti-inflammatory phenotypic classes can provide important information on the disease progression, as has been demonstrated for atherosclerosis [22].

A monocyte/macrophage-based assay has recently been designed to evaluate changes in monocyte response to pro- and anti-inflammatory stimuli and to reveal possible bias of the macrophage polarization towards M1 or M2 phenotype. In this method, a pure population of blood monocytes was isolated using magnetic separation [49, 70] (Figure 2). The analysis of macrophage activity was performed by stimulating the cells with LPS, proinflammatory stimulus IFN- $\gamma$ , and anti-inflammatory stimulus IL-4 [71]. Pro- and anti-inflammatory cytokine production measurement was used as readout. Proinflammatory activity of macrophages was assessed by the levels of secreted TNF $\alpha$  and IL-1 $\beta$  and anti-inflammatory activity by the levels of CCL18 production and IL-1ra expression. Inflammasome activation can also be assessed in this experimental system by measuring the IL-1 $\beta$  expression at mRNA level and comparing the results with the amount of mature IL-1 $\beta$  detected by ELISA. Another evidence of inflammasome activation that can be used as readout is the release of active caspase-1 [55]. Expression and release of the inflammasome-dependent cytokines TNF $\alpha$  and IL-8 should also be measured to provide the control of inflammasome activity. Further characterization of macrophages can be performed by analyzing such markers as

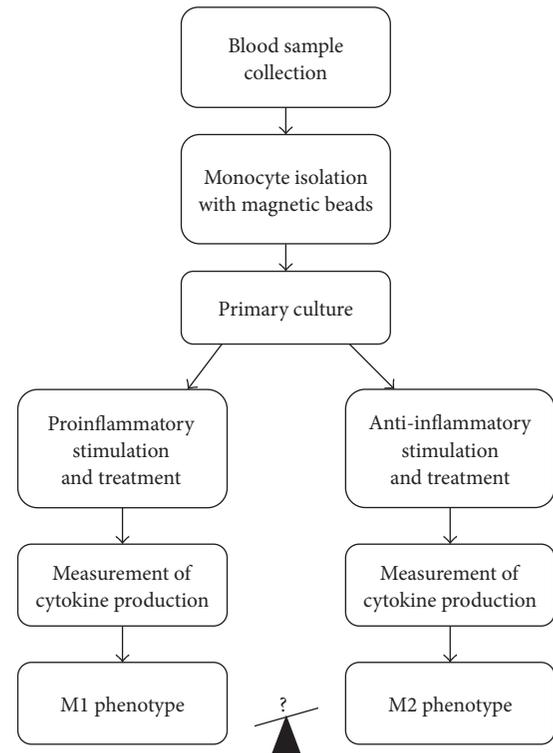


FIGURE 2: General design of the monocyte/macrophage activation assay.

MMR, CD163, TGF-RII, CSFR1, TNFR1, CD16, CD32, CD64, and stabilin-1, as well as the expression of TLR1, TLR2, and TLR4 at mRNA level and on the cell surface. Changes in the expression of these markers correlate with stimulation type and intensity. The described experimental system can provide important information on monocyte activation state and possible skew of the monocyte predisposition towards pro- or anti-inflammatory response. Apart from altered polarization towards one or the other phenotype, pathological conditions can be associated with other phenotypical alterations of monocytes/macrophage, including phagocytosis, migration, and proliferation. Alterations of macrophage phenotype and plasticity associated with atherosclerosis have recently been discussed in a comprehensive review [46].

The described method has been used to analyze activation of monocytes isolated from blood of healthy subjects ( $n = 19$ ), atherosclerosis patients ( $n = 22$ ), and breast cancer patients ( $n = 18$ ). The obtained results demonstrated that production of proinflammatory TNF $\alpha$  was significantly lower in atherosclerosis patients and significantly higher in cancer patients in comparison to healthy subjects. On the other hand, production of anti-inflammatory CCL18 was decreased both in atherosclerosis and in cancer patients [22].

To evaluate the diagnostic potential of macrophages' activation test in asymptomatic atherosclerosis, a study was performed on individuals with predisposition to atherosclerosis ( $n = 21$ , mean age  $63 \pm 9$  years) and subclinical atherosclerosis ( $n = 21$ , mean age  $62 \pm 7$  years) in comparison

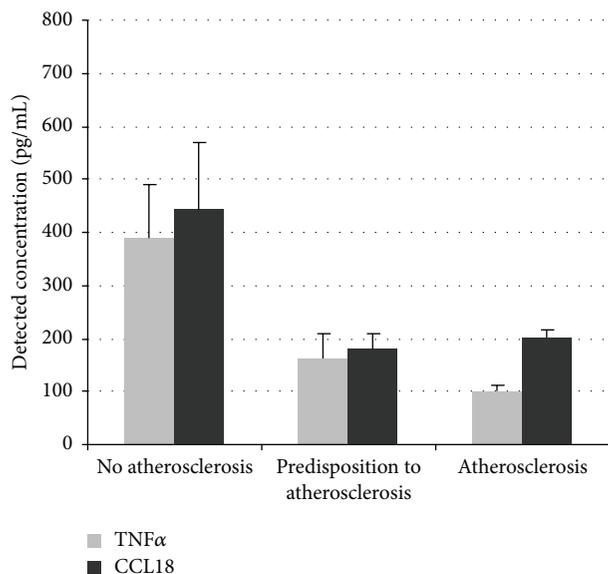


FIGURE 3: Study of monocyte/macrophage activation in subclinical and clinical atherosclerosis. Monocytes were isolated from the blood of subjects from 3 study groups ( $n = 20$  in each group). Cells were stimulated with IFN- $\gamma$  (100 ng/mL) or IL-4 (10 ng/mL). Secretion of TNF $\alpha$  and CCL18 was measured by ELISA.

to healthy subjects ( $n = 21$ , mean age  $60 \pm 9$  years). Predisposition to atherosclerosis and subclinical atherosclerosis were detected by measuring the age-adjusted carotid intima media thickness (cIMT). The analysis of TNF $\alpha$  and CCL18 production by stimulated macrophages revealed dramatic individual differences between the analyzed subjects that may reflect the individuals' predisposition to immunopathology. Macrophages from subjects with subclinical atherosclerosis were characterized by especially low degree of activation in response to stimuli [22] (Figure 3). Therefore, the ability of macrophages to polarize towards pro- and anti-inflammatory phenotypes was decreased at early stages of atherosclerosis development, although the causative significance of this observation remains unclear.

## 5. Application of Cellular Tests for Drug Development

Changes of the immune system occur early in many pathological processes, opening the intriguing possibility that patients may benefit from a preventive treatment targeting the underlying immunopathology. Imbalanced macrophage polarization is observed in such conditions as atherosclerosis and cancer. Enhanced monocyte activation may lead to macrophage polarization towards pro- or anti-inflammatory phenotype leading to chronic inflammation and atherosclerosis or to oncopathologies, respectively [22]. Therefore, macrophage depolarization might be exploited for the development of preventive treatment [72]. For instance, it has been demonstrated that depolarization of macrophages from the M2 phenotype was associated with tumor regression [73].

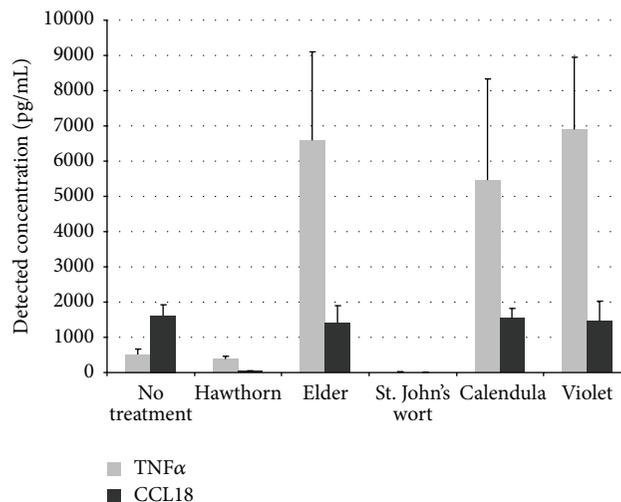


FIGURE 4: Modulation of macrophage polarization by natural agents. Macrophages were isolated from healthy subjects and brought into primary culture. Macrophages were incubated with extracts of various medicinal plants and stimulated with IFN- $\gamma$  (100 ng/mL) or IL-4 (10 ng/mL). Secretion of TNF $\alpha$  and CCL18 was measured by ELISA.

To explore the potential of the macrophage activation test for drug development, the macrophage depolarization effects of herbal extracts were studied on cells obtained from healthy subjects. Plant extracts with immune-modulating properties are widely used in traditional medicine, but their therapeutic potential for modern clinical practice remains to be investigated. The extracts of the following plants with known anti-inflammatory activity were included into the study: flowers of hawthorn (*Crataegus* sp.), elderberry (*Sambucus nigra*), and calendula (*Calendula officinalis*) and herbs of St. John's wort (*Hypericum perforatum*) and violet (*Viola* sp.). Cultured macrophages were exposed to pro- and anti-inflammatory stimuli (IFN- $\gamma$  and IL-4, resp.), and TNF $\alpha$  and CCL18 production was measured after 6 days. TNF $\alpha$  secretion by IFN- $\gamma$ -stimulated macrophages treated with elderberry, calendula, and violet extracts was 10–13-fold higher than that of untreated stimulated macrophages. On the other hand, hawthorn and St. John's wort extracts significantly inhibited TNF $\alpha$  secretion. Extracts of hawthorn and St. John's wort also suppressed the secretion of CCL18 by IL-4-stimulated macrophages (Figure 4). Therefore, St. John's wort and hawthorn extracts appear to be natural agents with immune-modulatory properties that could be used for macrophage depolarization. Importantly, natural agents are characterized by relatively good tolerance and minimal side effects and are therefore especially suitable for long-term therapy, which is necessary for successful immune correction.

One of the therapeutic strategies for treatment of atherosclerosis is the inhibition of intracellular cholesterol accumulation. It is well established that hypercholesterolemia is a potent risk factor for atherosclerosis development [74–76]. However, the accumulating evidence demonstrates that atherogenic potential depends not so much on the total level of cholesterol as on the nature of cholesterol-containing

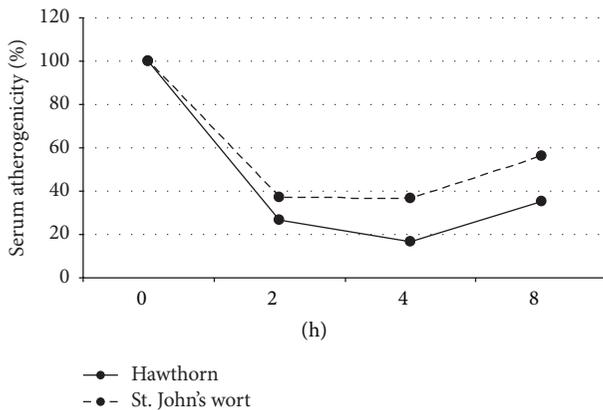


FIGURE 5: Study of serum atherogenicity in cellular assay. Four patients with atherosclerosis were given water extracts of 8 g of hawthorn berries or 3 g St. John's wort herb. Blood samples were collected before treatment and after 2, 4, and 8 hours and blood serum was added to cultured primary subendothelial intimal cells. Serum atherogenicity was measured as the increase of cholesterol content in cultured cells after 24 h.

lipoprotein particles that serve as a source of cholesterol storage in the arterial wall. Low-density lipoprotein (LDL) and especially its modifications, such as small dense, oxidized, desialylated, or electronegative LDL, play the key role in atherogenesis, and their levels positively correlate with the disease progression [77–79]. Moreover, modified LDL particles can provoke formation of autoantibodies that initiate the inflammatory response and form highly atherogenic immune complexes with LDL particles [80]. In that regard, LDL composition of blood plasma may be a decisive factor that triggers atherogenesis. The ability of blood serum to induce cholesterol accumulation is referred to as serum atherogenicity. It has been previously demonstrated that serum obtained from atherosclerosis patients caused cholesterol accumulation in cultured cells and therefore was highly atherogenic [81].

Primary culture of human aorta cells can be a useful system for testing various antiatherosclerotic substances. Using this system, atherogenic properties of patient's blood serum can be analyzed before and after drug administration to assess its therapeutic potential [82–84]. Antiatherogenic activity of hawthorn and St. John's wort extracts was tested using the described *ex vivo* model. Serum from study participants was added to cultured subendothelial intimal cells derived from uninvolved human aorta at concentration of 10%, and cholesterol accumulation was measured after 24 h using a previously established method [82]. It was demonstrated that blood serum atherogenicity decreased in study subjects treated with a single dose of hawthorn extract. The observed decrease was 73% and 83% after 2 and 4 hours, respectively, in comparison to the baseline (Figure 5). These results indicate that hawthorn extract may be regarded as a potent antiatherosclerotic agent. On the other hand, St. John's wort extract had no statistically significant effect on cellular cholesterol accumulation.

Taken together, the obtained results demonstrate that the combination of hawthorn and St. John's wort extracts appears promising for the development of antiatherosclerosis therapy. St. John's wort extract was a potent macrophage depolarizing agent, and hawthorn was demonstrated to reduce the serum atherogenicity. Further studies employing macrophage-based cellular test systems will allow identification of novel agents with therapeutic potential.

## 6. Conclusion

Monocyte/macrophage-based test system is a versatile tool to detect immunopathology, including increased monocyte activation and altered polarization of macrophages towards pro- or anti-inflammatory phenotypes. Alterations in monocyte activation and imbalance in macrophage polarization can be associated with a variety of pathological conditions, including different types of cancer and atherosclerosis. Therefore, the development of immunomodulating therapy might contribute significantly to the improvement of the existing treatment strategies. However, the complexity of the immunopathology requires flexible and reliable methods for diagnostics and monitoring of treatment efficiency. The monocyte/macrophage activation test is one of such methods. It was proven to be suitable for the analysis of immunopathology in subclinical atherosclerosis and breast cancer. This method can also be applied for studying numerous other pathologies, where monocyte/macrophage activation is implicated, including other types of cancer, chronic inflammation, and autoimmune disorders. Given the broad spectrum of cytokines that may be analyzed, the described method can be improved to perform a more detailed study of macrophage activation. The application of monocyte/macrophage activation test for drug research was illustrated by screening a series of medicinal plant extracts for antiatherosclerotic activity. Study of macrophage polarization revealed a potent immunomodulatory activity of hawthorn and St. John's wort extracts that might be beneficial for treatment and prevention of atherosclerosis. Together, these results demonstrate the possibilities of macrophage-based cellular tests for diagnostics and drug research in conditions associated with immunopathology.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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## Research Article

# Characterization of Adsorbents for Cytokine Removal from Blood in an *In Vitro* Model

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**Introduction.** Cytokines are basic targets that have to be removed effectively in order to improve the patient's health status in treating severe inflammation, sepsis, and septic shock. Although there are different adsorbents commercially available, the success of their clinical use is limited. Here, we tested different adsorbents for their effective removal of cytokines from plasma and the resulting effect on endothelial cell activation. **Methods.** The three polystyrene divinylbenzene (PS-DVB) based adsorbents Amberchrom CG161c and CG300m and a clinically approved haemoperfusion adsorbent (HAC) were studied with regard to cytokine removal in human blood. To induce cytokine release from leucocytes, human blood cells were stimulated with 1 ng/ml LPS for 4 hours. Plasma was separated and adsorption experiments in a dynamic model were performed. The effect of cytokine removal on endothelial cell activation was evaluated using a HUVEC-based cell culture model. The beneficial outcome was assessed by measuring ICAM-1, E-selectin, and secreted cytokines IL-8 and IL-6. Additionally the threshold concentration for HUVEC activation by TNF- $\alpha$  and IL-1 $\beta$  was determined using this cell culture model. **Results.** CG161c showed promising results in removing the investigated cytokines. Due to its pore size the adsorbent efficiently removed the key factor TNF- $\alpha$ , outperforming the commercially available adsorbents. The CG161c treatment reduced cytokine secretion and expression of cell adhesion molecules by HUVEC which underlines the importance of effective removal of TNF- $\alpha$  in inflammatory diseases. **Conclusion.** These results confirm the hypothesis that cytokine removal from the blood should approach physiological levels in order to reduce endothelial cell activation.

## 1. Introduction

Sepsis is a systemic inflammatory response syndrome (SIRS) that results from the body's innate immune response triggered by any of the several infectious stimuli. Lipopolysaccharides (endotoxins), peptidoglycan, flagellin, lipoteichoic acid from bacteria, mannan from fungi, and other antigens from infectious agents stimulate monocytes and macrophages to release tumour necrosis factor alpha (TNF- $\alpha$ ) as well as interleukins 1 and 6 (IL-1, IL-6) into the circulation [1–4]. These again activate additional proinflammatory pathways within endothelial cells and leukocytes. A very high and uncontrolled release of proinflammatory cytokines also stimulates leukocytes to release anti-inflammatory mediators and transforming growth factor-beta, which inhibit the synthesis of proinflammatory cytokines and exert direct

anti-inflammatory effects on monocytes, macrophages, and endothelial cells [5]. In many cases progress (the further course) of the disease will lead either to an unbalanced coexistence of pro- and anti-inflammatory mediators (mixed antagonistic response syndrome) or to an excess of anti-inflammatory cytokines which end up in immunosuppression. This so-called sepsis-induced “immunoparalysis” is characterized by restricted innate and adaptive immune responses, including enhanced apoptosis and dysfunction of lymphocytes and impaired phagocyte functions [6]. A sensitive balance between proinflammatory and anti-inflammatory response is necessary for cytokine release to achieve homeostasis. Attempts were made to restore the cytokine imbalance by using anticytokine monoclonal antibodies. These attempts, where particular cytokines were blocked, yielded no clinically detectable benefits but

indicated that the modulation of several cytokines at the same time to reach rather physiological blood levels may help to achieve homeostasis [7]. Consequently, extracorporeal blood purification (EBP) techniques were applied to modulate pro- and anti-inflammatory cytokines of sepsis patients. Currently, there are four main techniques in clinical use for cytokine removal: high-flux hemofiltration, high cutoff membranes, adsorption techniques, and combined plasma filtration adsorption [7]. A hemoperfusion cartridge that is used for cytokine removal in intensive care medicine is the Cytosorb cartridge, which is filled with 300 mL hemadsorption beads [8]. Cytosorb hemadsorption beads are porous polystyrene-divinylbenzene (PS-DVB) particles coated with biocompatible polyvinylpyrrolidone exhibiting 450  $\mu\text{m}$  average particle diameter and 0.8–5 nm pores [9, 10]. Another device for cytokine removal is the Coupled Plasma Filtration Adsorption (CPFA). CPFA is an extracorporeal therapy that was developed and patented by Bellco for the treatment of patients with multiorgan failure or sepsis. CPFA combines plasma sorption and hemofiltration for cytokine elimination in patients' blood. The unspecific removal of inflammatory mediators is achieved by an Amberchrom adsorbent [11]. This hydrophobic polystyrene resin with an average pore size of 30 nm has a high affinity and capacity for many cytokines and mediators [12]. Both adsorbents were clinically tested and capable of decreasing proinflammatory cytokines significantly, but a reduction of mortality in patients with septic shock was not observed [13, 14]. Probably the removal rate of cytokines was not sufficient to reach homeostasis. In a previous study conducted by our group, the optimal pore size for cytokine removal was investigated [15] and revealed that the Amberchrom CG161c, a neutral PS-DVB based adsorbent with 15 nm pores, shows promising results for cytokine removal from human plasma. The aim of this study was to compare, by *in vitro* experiments using human plasma, the capability of cytokine removal between the new CG161c adsorbent and the two PS-DVB based cytokine adsorbents available for clinical use. Furthermore, the consequence of the level of cytokine removal achieved by each adsorbent on endothelial cell activation was tested using human umbilical vein endothelial cells (HUVECs).

## 2. Materials and Methods

**2.1. Materials.** The clinically approved hemoperfusion adsorbent for cytokine removal (HAC) was obtained from Euromed (Euromed GmbH, Vienna, Austria) and the two Amberchrom adsorbents CG300m and CG161c were provided by Dow Chemical (Philadelphia, PA, USA). Tetrahydrofuran, toluene, and polystyrene standards for inverse size exclusion chromatography (iSEC) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and ethanol was obtained from VWR (Vienna, Austria). Blood bags were ordered from the Red Cross (Vienna, Austria) and the Bio-Plex cytokine array was purchased from Biorad (Biorad, Vienna, Austria). Recombinant TNF- $\alpha$  and IL-1 $\beta$  were obtained from R&D Systems (Minneapolis, MN). Hank's Balanced Salt Solution, cell culture medium M199, penicillin, streptomycin, fetal bovine serum (FBS), endothelial

cell growth supplement (ECGS), and HEPES buffer were purchased from Sigma-Aldrich (St. Louis, MO, USA).

### 2.2. Adsorbent Characterization

**2.2.1. SEM.** The structural characteristics and accessible pore size of each adsorbent were determined by scanning electron microscopy (SEM) and inverse size exclusion chromatography. The adsorbent particles were washed with pure ethanol and dried at 100°C for 12 hours. The particles were then sputtered with gold (Q150R ES, QUORUM) and imaged by SEM (TM-1000, Table Microscope, Hitachi).

**2.2.2. Particle Size.** Particle size distributions of the microspheres were determined by laser-light scattering (Mastersizer 2000, Malvern Instruments, Malvern, UK). Approximately 500  $\mu\text{L}$  of microspheres suspension was dispersed in 100 mL distilled water and sonicated to avoid agglomeration of particles during measurements. The particle size distribution results are volume based.

**2.2.3. Pore Size.** Inverse size exclusion chromatography was used to determine the accessible pore size and intraparticle porosity of each adsorbent based on the retention of toluene and polystyrene standards with molecular masses between 0.5 and 1,000 kDa. For this purpose, each adsorbent was flow packed in 0.46  $\times$  15 cm HPLC columns from Grace Davison Discovery Sciences (GRACE). A Waters HPLC System (Milford, USA) with a Waters 2487 UV detector was used to determine the retention volume of individual standards after injection of 20  $\mu\text{L}$  samples containing 10 mg/mL polystyrene at a flow rate of 0.5 mL/min. The retention volume of each polystyrene standard was experimentally determined and the SEC distribution coefficient has been calculated according to the following:

$$K_d = \frac{V_R - V_0}{V_T - V_0}, \quad (1)$$

where  $V_R$  is the retention volume,  $V_0$  is the interparticle void volume, and  $V_T$  is the total mobile phase volume. The mobile phase was represented by Tetrahydrofuran. Toluene was used as a small molecule tracer and acetonitrile only for washing.  $K_d$  values range between 0, for a compound that is excluded completely corresponding to polystyrene with a molecular mass of 1,000 kDa, and 1, for compounds able to access and permeate the total pore volume represented by toluene with a molecular mass of 92 Da. Since  $(V_T - V_0)$  represents the intraparticle mobile phase volume,  $K_d$  represents the extent of permeation for molecules into the pore volume of the stationary phase. The following correlation was used in order to interrelate the molecular mass  $M_W$  of a polystyrene sample and the size of the pores ( $\Phi$ ) from which it is excluded:

$$M_W = 2.25 \times \Phi^{1.7}, \quad (2)$$

where the pore size diameter is given in  $\text{\AA}$  [16, 17].

The adsorbent porosity  $\varepsilon_P$  was calculated from the following [18, 19]:

$$\varepsilon_P = \frac{V_T - V_0}{V_B - V_0}, \quad (3)$$

where  $V_B$  is the column bed volume.

The pore volume ( $V_P$ ) of the adsorbent materials was calculated according to

$$V_P = V_T - V_0. \quad (4)$$

**2.3. An In Vitro Sepsis Model.** The three PS-DVB based adsorbents Amberchrom CG161c, Amberchrom CG300m, and HAC were studied in a dynamic model with regard to cytokine removal in human plasma. Furthermore, the effect of cytokine removal on endothelial cell activation was evaluated using human umbilical vein endothelial cells (HUVECs). This model comprises three steps: whole blood stimulation, the adsorption study in a dynamic model, and the cell culture model (see Figure 1).

**2.3.1. Whole Blood Stimulation.** Blood bags containing between 400 and 500 mL fresh donated blood were ordered from the Red Cross (Vienna, Austria). The overproduction of cytokines by leucocytes was induced by stimulating human blood with 1 ng/mL LPS from *E. coli* (Sigma, St. Louis, MO, USA) at 37°C for 4 hours. The plasma, including the inflammatory mediators, was separated by centrifugation at 3000 ×g for 10 min and then stored at -80°C until adsorption experiments were performed in a dynamic model.

**2.3.2. Adsorption Studies in a Dynamic Model.** The dynamic model consists of a commercially available 5 mL Rezorian cartridge (Sigma, St. Louis, MO, USA) packed with 5 mL of adsorbent material. The bead volume of the cartridge was downscaled (approximately 60x) in comparison to the 300 mL cartridge which is normally used clinically for the HAC device. The recirculation reservoir volume, 60 mL, and flow rates, 1 mL/min (55 cm/h), used in the experiments were also scaled down from clinical hemadsorption, 100 to 300 mL/min (212–635 cm/h), and a total blood volume of 4 to 6 liters in the average adult, using this factor (see Figure 1). A circuit with an empty cartridge acted as a control. The experiment was carried out for 6 hours at 37°C, and samples were taken hourly and stored at -80°C until cytokine quantification using the Bio-Plex cytokine array and the cell culture model for endothelial cell activation were performed. In order to ensure the plasma stability during the experiment, albumin, total protein, antithrombin III, protein C, and fibrinogen were measured at the beginning and at the end of the experiment using a Hitachi/Roche 902 automated analyser with the according test kits (Roche, Penzberg, Germany).

### 2.3.3. Endothelial Cell Activation

**(1) Cell Culture.** The effect of cytokine removal on endothelial cell activation was evaluated using a cell culture model with

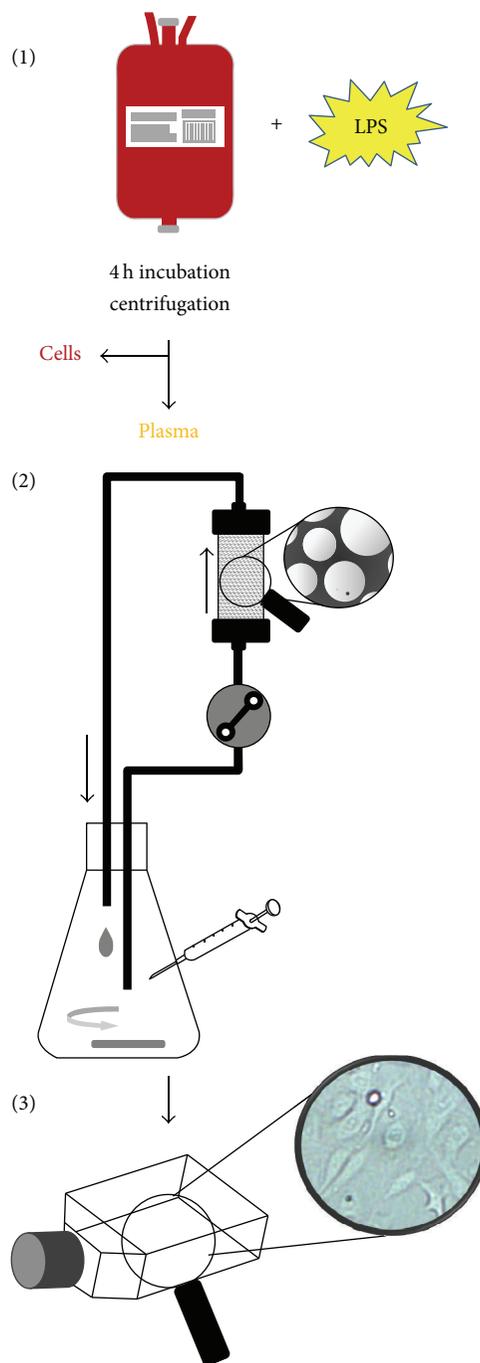


FIGURE 1: Schematic procedure of the experiments. The experiments were conducted in three parts: (1) blood stimulation and centrifugation, (2) adsorption experiments by a dynamic model, and (3) cell culture model with HUVEC.

HUVEC. The beneficial outcome was assessed by measuring the adhesion molecules ICAM-1 and E-selectin and an array of secreted cytokines after incubation of HUVEC with 10% of plasma from the adsorption experiments. Primary HUVECs were isolated from umbilical cord veins provided by the local hospital (LKH-Krems, Austria) after informed

consent of the donors and stored at 4°C in sterile HBSS. HUVECs were isolated according to Marin et al. with minor changes [20]. Cannulated umbilical veins were perfused with M199 containing 0.02 M HEPES and 100 mM penicillin-streptomycin (M199/HEPES/PS) at 37°C to remove the blood. The veins were filled with dispase (BD Biosciences Europe, Vienna, Austria) and incubated at 37°C for 15 min. After incubation, the dispase solution containing the HUVEC was collected by perfusion of the cord with basal medium (M199/HEPES/PS). The cells were collected by centrifugation at 500 ×g for 5 min and resuspended in growth medium (M199/HEPES/PS containing 20% FBS, 15 IU/mL heparin, and 10 mg/mL ECGS) and transferred to a 75 cm<sup>2</sup> cell culture flask. One day after isolation, cells were washed with basal medium and supplied with fresh growth medium. Isolated HUVECs were used between passages 4 and 7 for the assays.

(2) *Stimulation of HUVEC.* HUVECs ( $8.5 \times 10^5$ ) were seeded in 25 cm<sup>2</sup> cell culture flasks with 5 mL growth medium and incubated for 24 hours at 37°C and 5% CO<sub>2</sub>. The cell activation tests were performed after cell vitality and near confluency were confirmed by microscopy, as follows: plasma samples from the adsorption experiments were thawed and diluted 1:10 with 5 mL of basal medium. The HUVEC monolayer was washed once with basal medium and the sample medium was added to the corresponding cell culture flask. The cells were incubated with the sample medium and control medium (basal medium including 10% native plasma) for 16 hours at 37°C and 5% CO<sub>2</sub> atmosphere. After incubation the supernatants were centrifuged for 10 min at 1000 g, aliquoted, and stored at -80°C until cytokine analysis by the Bio-Plex cytokine array. The cells were washed with 3 mL of ice-cold PBS and detached with 1.5 mL 0.02% EDTA per flask. After addition of 3 mL PBS, cells were pelleted at 500 ×g for 5 min and used for flow cytometry analysis.

(3) *Flow Cytometry Analysis.* The detached cells were counted and aliquots of  $2.5 \times 10^5$  cells per sample were prepared in FACS tubes. The cells were washed with ice-cold PBS and stained by incubation with FITC-conjugated anti-CD31, PE-conjugated anti-ICAM-1, PE-Cy5-conjugated anti-E-selectin (BD, Franklin Lakes, NJ, USA), or the corresponding control antibodies for 30 min on ice in the dark. All antibodies were from the IgG isotype. After two further washing steps with PBS, cells were analysed on a flow cytometer (Cytomics FC 500 MPL, Beckman Coulter, CA, US), using the FlowJo 7.6.5 software (Tree Star Inc., Ashland, OR, USA).

*2.4. TNF- $\alpha$  and IL-1 $\beta$  Dependent Activation of HUVEC in the Cell Culture Model.* To evaluate the level, to which the cytokines have to be lowered by any extracorporeal treatment, for preventing or reducing the endothelial cell activation, a separate experiment was performed. Heparinized (5 IU/mL) human plasma with different recombinant TNF- $\alpha$  and IL-1 $\beta$  concentrations (0, 50, 100, 500, 1000, 5000, and 10000 pg/mL) was used in our cell culture model to determine their threshold level for endothelial cells activation. The HUVECs were cultivated with sample medium (as described above) including 10% of spiked plasma which leads to a tenfold

dilution of the spiked recombinant cytokines. After 16 hours of incubation, the supernatants were aspirated, centrifuged for 10 min at 1000 ×g, aliquoted, and stored at -80°C until IL-8 and IL-6 were quantified by the Bio-Plex cytokine array. To verify the expression of the adhesion molecules ICAM-1 and E-selectin, the HUVECs were washed and analysed by flow cytometry as described above.

### 3. Results and Discussion

#### 3.1. Adsorbent Characterization

*3.1.1. SEM, Particle Size, and Inverse Size Exclusion.* SEM of the manually cracked particles illustrates that the outer thin shell of the adsorbent particles acts as a molecular sieve for entering the inner surface, which is the adsorbent surface for the target molecules (see Figure 2 and Table 1). The  $K_d$  values obtained for toluol and polystyrene probes from the iSEC experiments are shown in Table 2. Complete molecular exclusion is achieved when the  $K_d$  value reaches zero at a certain molecular weight. For the largest pore size, an acceptable  $K_d$  was defined with a value of 0.1, which means that molecules with a  $K_d$  of 0.1 are allowed to pass through the outer pore shell and reach the inner adsorbent surface. As shown in Table 2,  $K_d$  approaches 0.1 at a pore size between 10.0 and 16.2 nm for CG161c, 20.6 and 26.0 nm for CG300m, and 7.6 and 10.0 nm for HAC. The porosities  $\epsilon_p$  (see Table 2) of the three tested adsorbent particles were similar and always above 80%. HAC was found to have the highest porosity at 86.6% followed by CG161c, 86.2%, and CG300m, 82.3%.

*3.2. Adsorption Studies in Dynamic Model.* The ability of the adsorbents to remove cytokine was investigated in dynamic model experiments using inflammatory mediator rich human plasma obtained after whole blood stimulation. The concentrations of the following cytokines were measured hourly over a 6-hour period: TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-8, and IL-10. These cytokines are considered to be key factors as well as markers in inflammation [21–23]. However, there are many other cytokines and mediators involved in this complex and dynamic process. The interleukins were efficiently removed by both CG161c and CG300m at comparable levels. HAC performed consistently worse than the other adsorbents for all tested cytokines. Sufficient TNF- $\alpha$  removal could only be observed in case of CG161c (Figure 3 and Table 3) with a removal rate of  $94.3 \pm 0.23\%$ . The two commercially available cytokine adsorbents offered limited removal of TNF- $\alpha$ :  $63.5 \pm 0.5\%$  for CG300m and  $53.4 \pm 6.8\%$  for HAC. The molecular mass of TNF- $\alpha$  ranges from 17 to 51 kDa depending on oligomerization, that is, monomer, dimer, or trimer. The homotrimer is the most active form of TNF- $\alpha$ , which is the largest cytokine with respect to the crystal structure and viscosity radius [15]. Because of the large size of the trimer, the removal of TNF- $\alpha$  from the bloodstream represents a considerable significant challenge. The mechanism of adsorption of the three adsorbents under investigation is the same. The target molecules have to enter the pores of the outer surface to reach the inner surface composed of PS-DVB copolymer, where they will be adsorbed. The particle size, the pore size

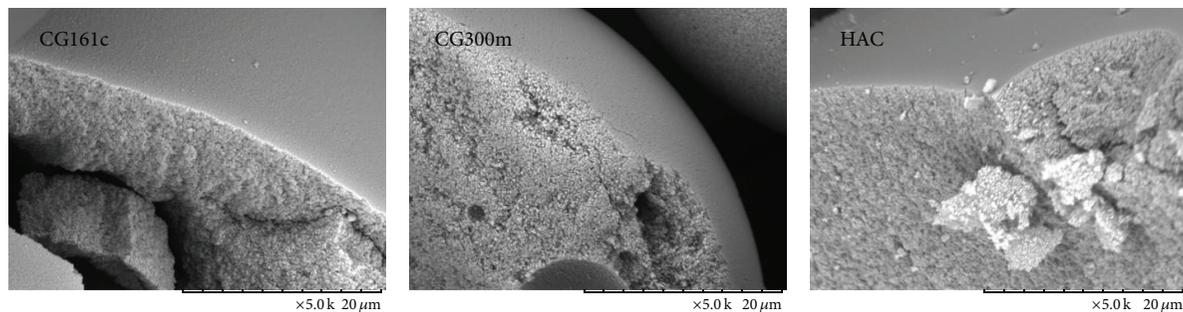


FIGURE 2: Imaging and particle size of the used adsorbents. SEM images at 5000x magnification and particle size distribution using laser-light scattering of the three tested adsorbents.

TABLE 1

Adsorbent	$D(0.1)$ [ $\mu\text{m}$ ]	$D(0.5)$ [ $\mu\text{m}$ ]	$D(0.9)$ [ $\mu\text{m}$ ]	Vol. weighted mean $D$ [ $\mu\text{m}$ ]
CG300m	60	82	112	84
CG161c	86	117	158	120
HAC	363	492	656	504

(determined by iSEC), and the blood compatible outer PVP layer in case of HAC are the only differentiation factors suggesting that the reduced adsorption is primarily due to the different pore sizes of the adsorbents. When the pores are too small, TNF- $\alpha$  cannot enter the adsorbent beads to be immobilized at the inner surface. Contrariwise, if the pore size is too large, bound TNF- $\alpha$  may be replaced by high molecular weight plasma proteins and plasma lipids due to their high binding affinity via hydrophobic interactions according to the Vroman effect [24]. There was no significant change in the parameters (albumin, total protein, antithrombin III, protein C, and fibrinogen) which were observed in order to ensure plasma stability during the experiment (data not shown).

**3.3. Cell Culture Model.** The endothelium takes part in the regulation of numerous physiological functions and lies at the interface of circulating blood and the vessel wall. Under physiological conditions, it is responsible for anticoagulant and antiadhesive properties and it regulates vasomotor tone and vascular homeostasis. Endothelial dysfunction has been associated with many pathophysiological processes, such as inflammation and oxidative stresses. Endothelial cells are precociously exposed to circulating signalling molecules and physical stresses, like in sepsis and septic shock [25]. It is well known that sepsis in humans is associated with activation of the endothelium as evidenced by increased levels of expressed ICAM-1 and E-selectin and secreted cytokines/chemokines such as IL-6 and IL-8. To test whether cytokine removal has a positive effect on endothelial cell activation, the treated plasma derived from the adsorbent experiments was used to stimulate HUVEC. The results of the flow cytometry analysis indicate that the CG161c adsorbent is most effective at reducing the expression of cell adhesion molecules by HUVEC.

TABLE 2: Summary of inverse size exclusion chromatography. The pore size of the adsorbents was determined by SEC using polystyrene standards.  $R_S$ : stokes radius;  $K_d$ : SEC distribution coefficient.

$M_r$	$R_S$ [nm]	$K_d$		
		CG161c	CG300m	HAC
92	0.18	1.00	1.00	1.00
570	0.51	0.82	0.85	0.56
1920	1.05	0.67	0.76	0.29
3460	1.48	0.59	0.70	0.21
9630	2.71	0.41	0.60	0.11
17300	3.82	0.29	0.53	0.10
27500	5.01	0.15	0.44	0.08
62300	8.11	0.02	0.26	0.04
96000	10.46	0.00	0.13	0.03
139000	13.00	0.00	0.07	0.02
319000	21.19	0.00	0.01	0.01
524000	28.37	0.00	0.00	0.00
925000	39.63	0.00	0.00	0.00
		CG161c	CG300m	HAC
$V_T$ (Toluol) [mL]		2.30	2.25	2.31
$V_0$ (PS 1000 kDa) [mL]		1.06	1.14	1.13
$V_B$ (column) [mL]		1.792	1.792	1.792
$V_p$ [mL]		1.23	1.11	1.18
$\epsilon_p$		86.2	82.3	86.6
* iSEC pore radius $r$ [nm]		$5.0 < r < 8.1$	$10.3 < r < 13.0$	$3.8 < r < 5.0$
+ Pore radius [nm]		7.5	15	2.5

\*  $K_d > 0.1$ .

+ Manufacturer data.

The expression of the adhesion molecule E-selectin could effectively be suppressed by all three adsorbent treatments. A marked difference, however, was observed in case of ICAM-1

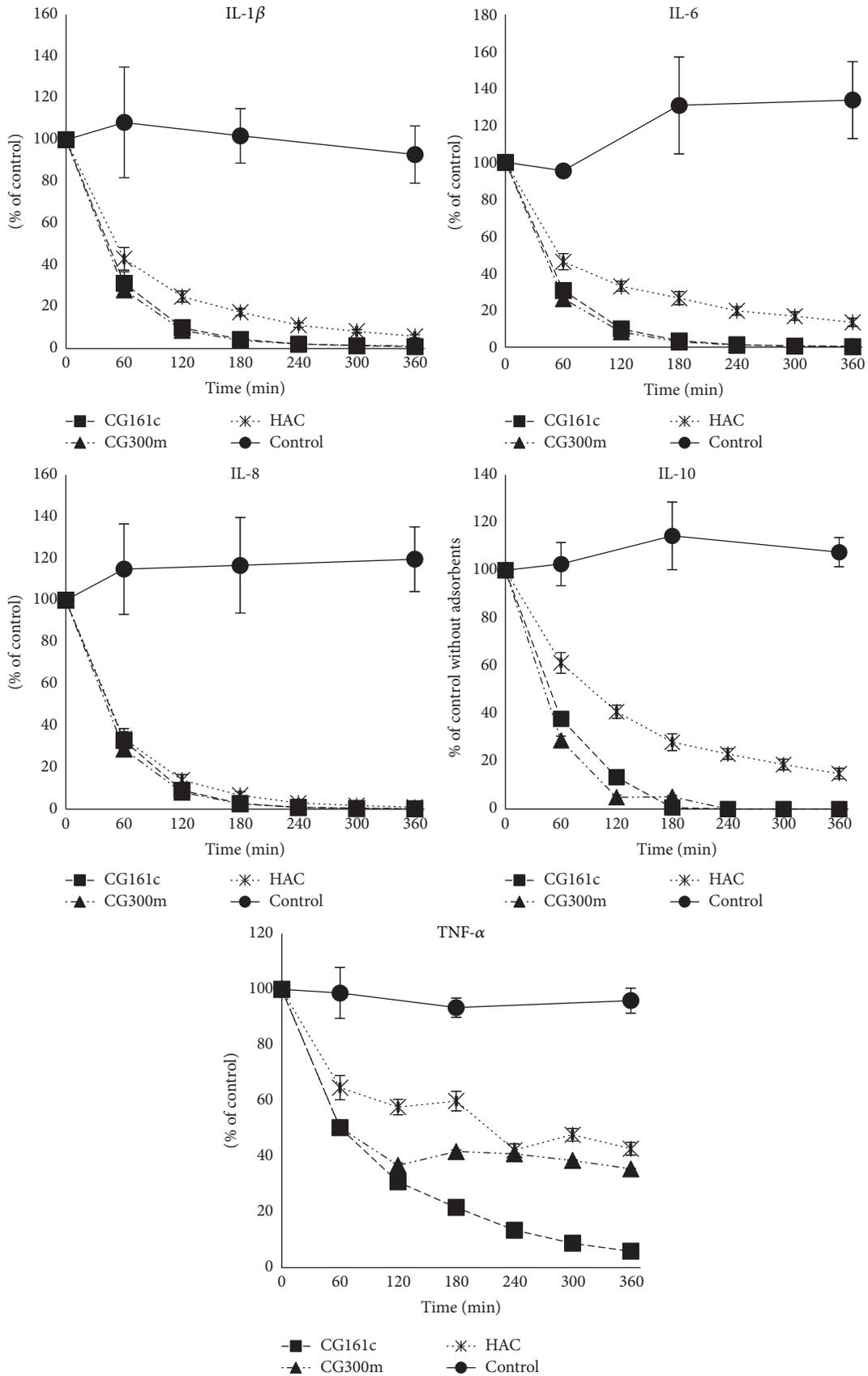


FIGURE 3: Cytokine removal in the dynamic model. Cytokine levels (TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-8, and IL-10) in the plasma pool during 6 hours of treatment with the three tested adsorbents in the dynamic model. The results are shown in mean  $\pm$  SD.

TABLE 3: Cytokine levels of treated plasma. Mean cytokine concentration  $\pm$  SD ( $n = 3$ ) in plasma after 6 hours of treatment with different adsorbents in the dynamic model. Plasma from LPS treated blood circulating through an empty cartridge acted as control.

	TNF- $\alpha$ [pg/mL]	IL-1 $\beta$ [pg/mL]	IL-6 [pg/mL]	IL-8 [pg/mL]	IL-10 [pg/mL]
Control	3102 $\pm$ 533	830 $\pm$ 190	24273 $\pm$ 13446	4837 $\pm$ 2300	51 $\pm$ 9
CG161c	177 $\pm$ 7	7 $\pm$ 1	59 $\pm$ 18	8 $\pm$ 7	<2
CG300m	1131 $\pm$ 16	10 $\pm$ 1	65 $\pm$ 3	10 $\pm$ 6	<2
HAC	1445 $\pm$ 212	45 $\pm$ 3	2587 $\pm$ 1254	60 $\pm$ 52	7 $\pm$ 4

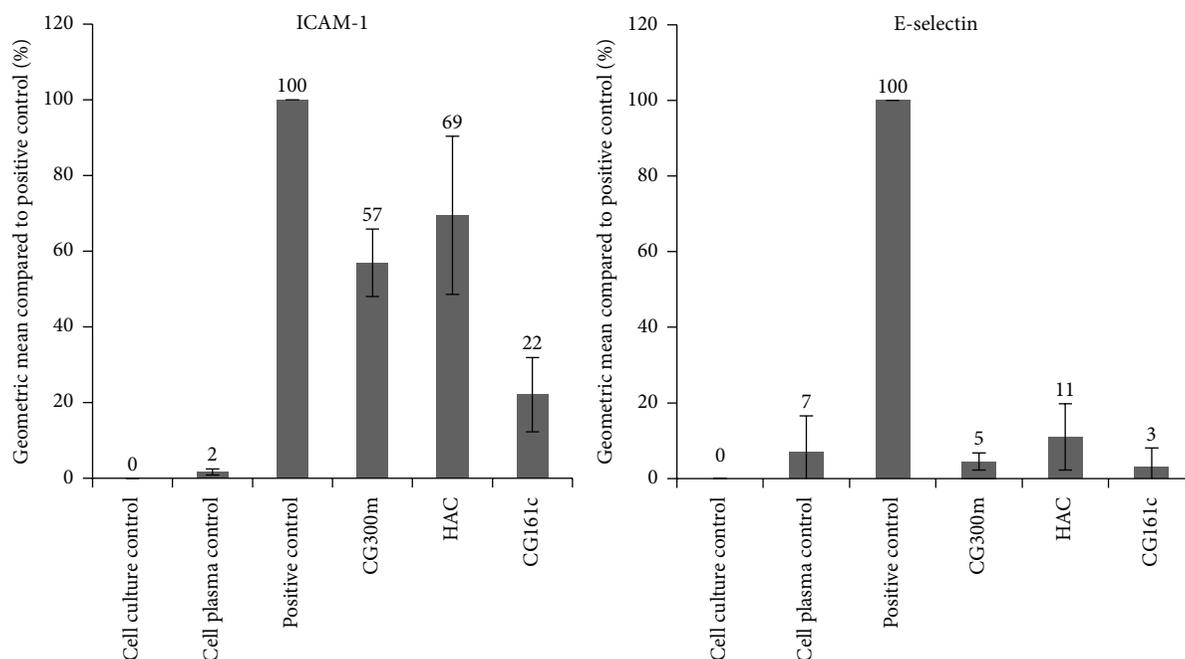


FIGURE 4: ICAM-1 and E-selectin expression by HUVEC. The effect of cytokine removal on endothelial cell activation was assessed by measuring the expressed adhesion molecules ICAM-1 and E-selectin after incubation of HUVECs with 10% of sample plasma from the adsorption experiments. The results are shown in mean  $\pm$  SD.

with a highly reduced expression to  $22 \pm 10\%$  for CG161c and a moderate beneficial effect for CG300m ( $57 \pm 9\%$ ) as well as for HAC ( $69 \pm 21\%$ ) compared to the untreated cytokine rich plasma. It is well documented that ICAM-1 expression in vascular endothelium can be induced by IL-1 and TNF- $\alpha$  [26]. This fact should be considered when the results of TNF- $\alpha$  removal (Figure 3) and the resulting ICAM-1 expression (Figure 4) are compared. It confirms that the lower the TNF- $\alpha$  content in plasma, the lower the ICAM-1 expression on HUVEC's surface. Thus, only an effective removal of cytokines to a physiological concentration in plasma, which are usually below 100 pg/mL [27], can significantly reduce endothelial cell activation.

A similar effect was observed when the secreted cytokines of HUVEC were determined. The plasma treated with the CG161c adsorbent elicits the lowest IL-6 and IL-8 levels in cell culture but also the other two tested adsorbents provoke a high reduction in cytokine release compared to untreated plasma (Figure 5 and Table 4). Makó et al. reported that the expression of E-selectin, IL-6, and IL-8 was induced most efficiently by IL-1 $\beta$ , while that of LPS and TNF- $\alpha$  was less

TABLE 4: IL-6 and IL-8 secretion by HUVECs. Mean IL-6 and IL-8 secretion  $\pm$  standard deviation ( $n = 3$ ) of HUVECs after a 16-hour treatment with cell media containing 10% of plasma from the different adsorption studies in the dynamic model. Untreated plasma from LPS stimulated blood acts as positive control and cell plasma control denotes plasma from blood without LPS stimulation.

	IL-8 [pg/mL]	IL-6 [pg/mL]
Cell plasma control	326 $\pm$ 15	231 $\pm$ 187
Positive control	6249 $\pm$ 858	4056 $\pm$ 2124
CG300m	657 $\pm$ 72	141 $\pm$ 23
HAC	600 $\pm$ 53	143 $\pm$ 14
CG161c	130 $\pm$ 15	66 $\pm$ 6

efficient, and ICAM-1 expression was not different between stimuli [28]. Our findings are in agreement with those of Makó et al. (see Figure 6), because IL-1 $\beta$  was removed very efficiently by all tested adsorbents; also the ICAM-1 expression as well as IL-6 and IL-8 secretion from the HUVEC was reduced.

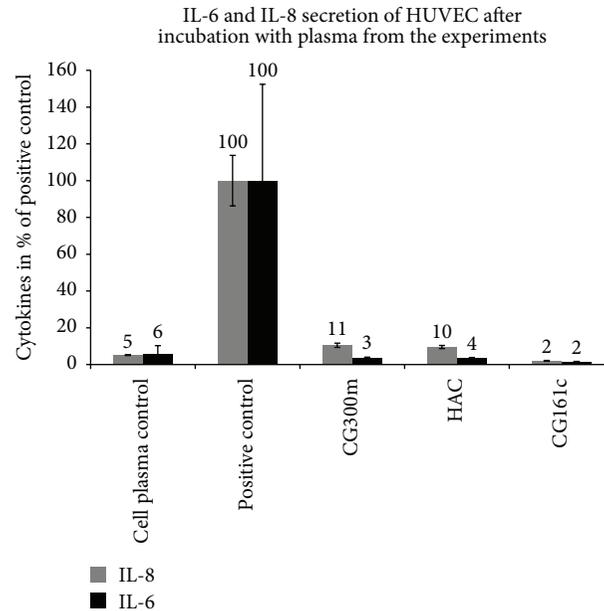


FIGURE 5: IL-6 and IL-8 secretion by HUVEC. The effect of cytokine removal on endothelial cell activation regarding IL-8 and IL-6 secretion after 16 h incubation of HUVEC with 10% of sample plasma from the adsorption experiments. Untreated plasma (empty cartridge without adsorbent) acts as positive control. The results are shown in mean  $\pm$  SD.

**3.4. TNF- $\alpha$  and IL-1 $\beta$  Dependent Activation of HUVEC in the Cell Culture Model.** Endothelial cells are activated primarily by the two cytokines TNF- $\alpha$  and IL-1 $\beta$  [28, 29]. In our cell culture model the threshold concentrations of IL-1 $\beta$  and TNF- $\alpha$  for HUVEC activation were between 10 and 50 pg/mL (see Figure 6) regarding IL-8 and IL-6 secretion as well as ICAM-1 expression (Figures 6 and 7). E-selectin expression was induced by low IL-1 $\beta$  concentration (50 pg/mL) in contrast to TNF- $\alpha$  which activates the expression of E-selectin not below 500 pg/mL. Thus the three tested adsorbents which removed IL-1 $\beta$  very efficiently also were able to reduce the expression of E-selectin in contrast to ICAM-1 expression which was only suppressed by the CG161c treatment because of efficient TNF- $\alpha$  removal. These results confirm the assumption that it is not sufficient merely to reduce the cytokine levels, for example, by EBP. The cytokine levels have to be reduced to physiological levels in order to prevent endothelial cell activation. Based on their molecular size, especially trimeric TNF- $\alpha$  with 52 kDa, the cytokines are not able to rapidly cross the usually applied dialysis membranes. Consequently, an effective removal of a wide array of cytokines from the plasma cannot be achieved using only membrane based technologies like high-volume hemofiltration and high-cutoff hemodialysis or hemofiltration. This can only be realised by using adsorption techniques or by a combination of adsorption and membrane technologies.

#### 4. Conclusion

Cytokines are considered to be targets that have to be modulated in order to improve the patient's health in case of severe inflammation, sepsis, and septic shock. Although there are different adsorbents commercially available, their clinical

utility is limited [30]. In order to suppress systemic effects in these disease patterns, effective removal of cytokines below a critical threshold is necessary. The three PS-DVB based adsorbents Amberchrom CG161c, Amberchrom CG300m, and HAC were studied with regard to cytokine removal capacity from human plasma. The new PS-DVB based cytokine adsorbent CG161c exhibited promising results in terms of all tested cytokines. Especially in case of removing the key factor TNF- $\alpha$ , it outperforms commercially available adsorbents such as HAC or CG300m due to its optimized pore size. With respect to endothelial cell activation, the CG161c treatment highly reduced cytokine secretion and expression of cell adhesion molecules in HUVEC, which emphasizes the importance of the effective removal of TNF- $\alpha$  in inflammatory diseases when using a cytokine adsorbent. A successful sepsis treatment strategy regarding effective cytokine modulation may use a combination of membrane and adsorption based technique. A promising adsorbent for such a blood purification device could be the CG161c adsorbent. However, the findings here are based on *in vitro* studies and are not yet confirmed by clinical data.

#### Key Messages

- (i) The Amberchrom adsorbent CG161c is promising for cytokine removal from human plasma compared to other tested cytokine adsorbents.
- (ii) The threshold concentrations of TNF- $\alpha$  and IL-1 $\beta$  for HUVEC stimulation are below 50 pg/mL.
- (iii) Cytokines circulating in the blood should be modulated to physiological levels during treatment of sepsis in order to reduce endothelial cell activation.

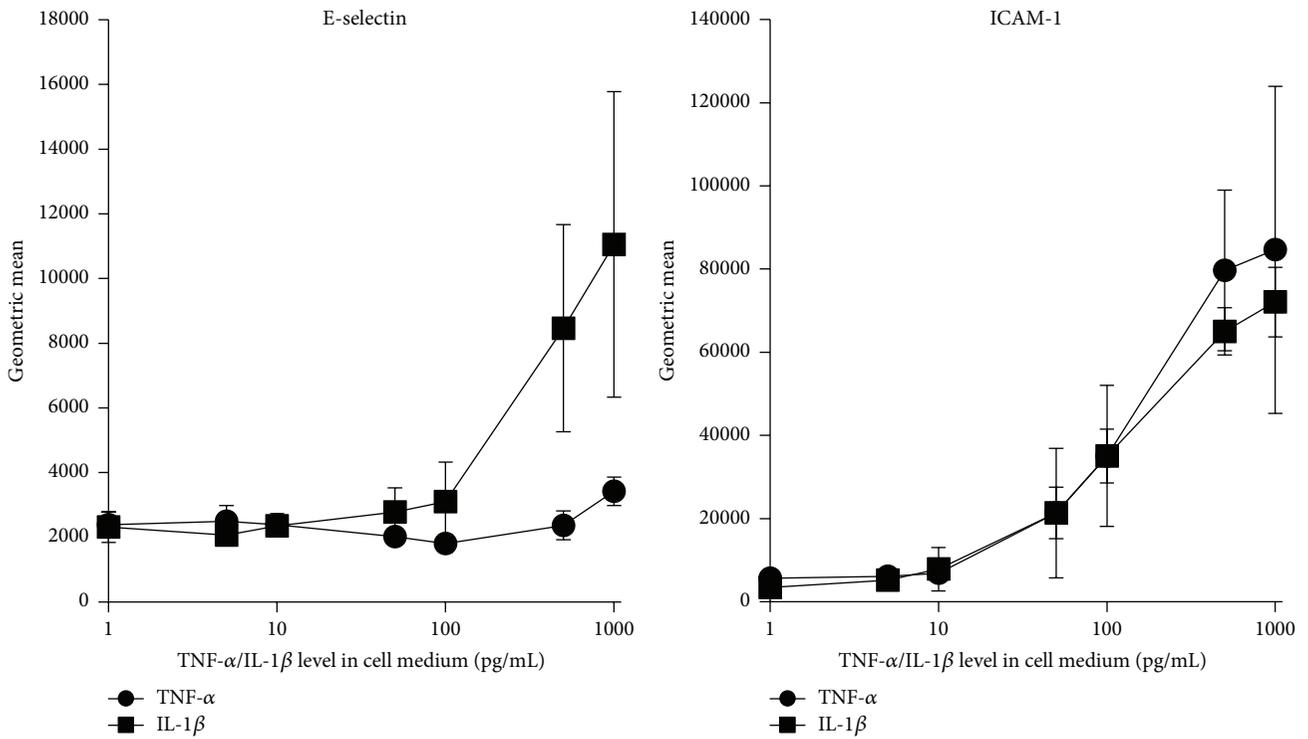


FIGURE 6: E-selectin and ICAM-1 expression of HUVEC as a function of TNF- $\alpha$  or IL-1 $\beta$  level. ICAM-1 and E-selectin expression by HUVEC after 16-hour incubation in cell media with 10% of plasma spiked with increasing amounts of TNF- $\alpha$  or IL-1 $\beta$  (mean  $\pm$  SD,  $n = 3$ ).

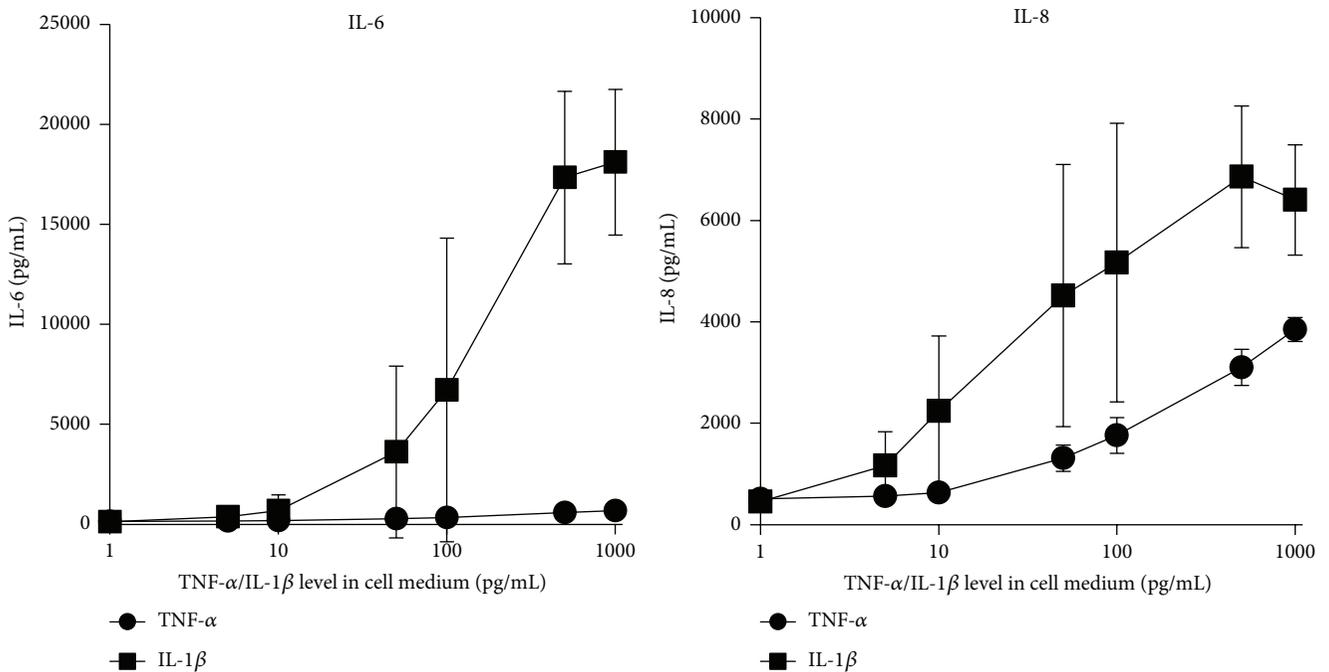


FIGURE 7: TNF- $\alpha$  or IL-1 $\beta$  dose-dependent production of IL-6 and IL-8 by HUVEC. IL-6 and IL-8 secretion by HUVEC after 16-hour incubation in cell media with 10% of plasma spiked with increasing amounts of TNF- $\alpha$  or IL-1 $\beta$  (mean  $\pm$  SD,  $n = 3$ ).

## Conflict of Interests

This study has received research funding from Fresenius Medical Care (Bad Homburg, Germany).

## Authors' Contribution

Stephan Harm, Franz Gabor, and Jens Hartmann participated in research design, writing of the paper, performance of the research, and data analysis. The authors contributed to the critical review and revision of the paper and approved it for publication.

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