Clinical and Developmental Immunology

Immunobiology and Pharmacologic Manipulation of Dendritic and Regulatory Cells

Guest Editors: Mohamad Mohty, Arnon Nagler, Nicolaus Kröger, and Beatrice Gaugler

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Editorial Board

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Editorial

Immunobiology and Pharmacologic Manipulation of Dendritic and Regulatory Cells

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Antigen presenting cells (APCs), especially dendritic cells (DCs), play a major role in the hierarchy of the induction of immune reactions. DCs are widely accepted as the most potent APCs capable of inducing protective adaptive immune responses in addition to tolerance to self-antigens. The role of DCs is currently being investigated in the context of many disease and therapeutic settings. In response to a variety of microbial and endogenous stimuli, resting DCs in peripheral tissues undergo a complex maturation process that might involve the regulation of genes that control distinct DC functions. The different functional properties of DCs are also linked to the existence of several subpopulations in humans and animals that differ in response to stimuli. In this special issue, several articles addressed some recent findings highlighting the interactions between key immune effectors both in vitro and in vivo and in different disease settings.

V. R. Yanofsky et al. provided an in-depth analysis of DC biology, with a particular focus on skin DCs and their role in cutaneous carcinoma. C. Y. J. Chung et al. provided an overview of the role of DCs in the immunopathogenesis of autoimmunity, as well as recent concepts of DC-based therapeutic opportunities in autoimmune diseases. On the other hand, P. Rúiz et al. described the different treatments and some of the novel immunotherapeutic strategies undertaken to induce transplantation tolerance in general. More specifically, M. Michael et al. discussed the current knowledge of Treg biology and its potential for cell-based immunotherapy in allogeneic stem cell transplantation. The review by A. K. Gloudemans et al. summarized the latest literature on the role of mucosal IgA in protection against allergic airway disease, the mechanisms described to induce secretory IgA, and the role of (mucosal) DCs in this process.

D.-Y. Chen et al. examined the effect of dextromethorphan (DXM), a common cough suppressant, on the activation and function of DCs. Interestingly, DXM decreased the LPS-induced surface expression of CD80, CD83, and HLA-DR and the secretion of IL-6 and IL-12 in human monocyte-derived DCs. These findings provide a new insight into the impact of DXM treatment on DCs and suggest that DXM has the potential to be used in treating DC-related acute and chronic diseases. Similarly, L. Adalid-Peralta et al. showed that cysticerci may modulate DCs to favor a suppressive environment, which may help parasite establishment, minimizing the excessive inflammation, which may lead to tissue damage. In the same line, R. N. Ramos et al. described that cytokines such as IL-10 and TGF-beta, as well as cell surface molecules like PD-L1 and ICOS, seem to be significantly involved in the redirection of DCs towards tolerance induction, and tumor cells may modulate distinct DC subpopulations through the involvement of these molecules. Another work from X. Gu et al. suggested that regulation of B7-H1 expression on hepatic stellate cells by IFN-γ represents an important

mechanism that regulates immune responses in the liver favoring tolerogenicity rather than immunogenicity. Finally, A. Brosbøl-Ravnborg et al. described how vitamin D3 and TLR agonists acted in synergy to alter secretion of cytokines from human DCs in a direction that may provide an anti-inflammatory environment.

From a therapeutic perspective, C. Penna et al. elegantly showed that a combined treatment, including dexamethasone preconditioning followed by an inoculation of short-term LPS-stimulated type II collagen-loaded DCs, provides an improved strategy for attenuating arthritis severity. On the other hand, data from S. O. Åkefeldt et al. suggested that targeting MCL1 and BCL2A1 in infiltrating DC may affect the clinical outcomes in rheumatoid arthritis (RA) and Langerhans cell histiocytosis (LCH). P. Cordiali-Fei et al. showed that in patients with limited systemic sclerosis, Treg cells were inversely correlated to disease duration, suggesting that their levels may represent a marker of disease progression. Interestingly, C. Doñas et al. showed that Trichostatin A (TSA) could potentially be used to enhance the differentiation and suppressive function of CD4+Foxp3+ Treg cells.

Finally, L. de la Cruz-Merino et al. reviewed some important aspects about the role of tumor-infiltrating lymphocytes (TIL) and their subtypes, tumor-associated macrophages (TAM), and myeloid-derived suppressive cells (MDSC) that will eventually be incorporated into diagnostic and therapeutic algorithms of breast cancer. The work from W. Maes et al. discussed based on an animal model the value of local elimination of Tregs within the tumor microenvironment which might represent an important tool from both fundamental and clinical perspectives.

All in all, articles published in this special issue clearly show that DCs and Tregs are currently being considered as attractive targets towards manipulation of the immune system for therapeutic purposes in different human disease settings.

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Review Article

New Insights into the Role of the Immune Microenvironment in Breast Carcinoma

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Recently, immune edition has been recognized as a new hallmark of cancer. In this respect, some clinical trials in breast cancer have reported imppressive outcomes related to laboratory immune findings, especially in the neoadjuvant and metastatic setting. Infiltration by tumor infiltrating lymphocytes (TIL) and their subtypes, tumor-associated macrophages (TAM) and myeloid-derived suppressive cells (MDSC) seem bona fide prognostic and even predictive biomarkers, that will eventually be incorporated into diagnostic and therapeutic algorithms of breast cancer. In addition, the complex interaction of costimulatory and coinhibitory molecules on the immune synapse and the different signals that they may exert represent another exciting field to explore. In this review we try to summarize and elucidate these new concepts and knowledge from a translational perspective focusing on breast cancer, paying special attention to those aspects that might have more significance in clinical practice and could be useful to design successful therapeutic strategies in the future.

1. Introduction

Neoplasms represent a wide group of heterogeneous diseases with several different alterations at genomic and proteomic levels, which finally confer them the acquisition of the neoplastic phenotype. Every human carcinoma induces an immune response in its microenvironment. Generally, this immune reaction is considered ineffective to destroy cancer cells; however, in the last years evidence has emerged demonstrating the importance of tumor lymphocyte infiltration in the clinical evolution of many cancer types. Importantly, this immune "awakening" against tumors may be induced by some new and classical antineoplastic strategies. Hence, we will analyze this new knowledge from a clinical point of view focusing on breast cancer, giving eventual clues to overcome and break immune tolerance in this disease.

2. Clinical Consequences of Immune-Related Events in Breast Carcinoma

In the last few years, some translational studies in patients with breast carcinoma have suggested that infiltration by

tumor infiltrating lymphocytes (TIL) and regulatory T (Treg) cells might have a great significance in the final clinical outcomes.

In the neoadjuvant setting Demaria et al. found a change in the frequency of TIL after treatment with paclitaxel. Importantly, response was correlated with TIL density suggesting that apoptosis induced by taxanes is a powerful immunogenic stimulus [1].

Recently, Denkert et al. investigated the hypothesis that the presence of an intense lymphocytic infiltrate might predict the response to neoadjuvant chemotherapy in breast cancer [2]. They examined pretherapeutic core biopsies of 1058 patients enrolled in the GeparDuo and GeparTrio studies. Results of these analyses showed that the presence of intratumoral lymphocytes and lymphocyte-predominant breast cancers were associated with a 31 and 41% pathological complete response (pCR) rates, respectively [2]. On the opposite, pCR rates were only 2% in patients without any lymphocytic infiltration. In a multivariate analysis, intratumoral lymphocytes, age, and estrogen receptor status were the only independent predictive parameters for pCR. Several other studies have reported consistent data in the same direction [3]. These results should not be overlooked as they confirm a strong association between lymphocytic infiltrate and chemotherapy response in a large set of more than 1000 samples of breast cancer.

Despite the impressive results achieved in the study conducted by Denkert, TIL subtypes were not specifically analyzed. On the contrary, Ladoire et al. reported another interesting study conducted on 56 patients with operable breast carcinoma and treated with preoperative chemotherapy [4]. Overall, the histological analyses of surgical specimens revealed a pathologic complete response in 21.4% of the cases. pCR was achieved in 40% of the tumors overexpressing HER2 and only in 11% of the patients with HER2-negative tumors. Results of T-cell infiltrates analyses were of great interest at this point. After neoadjuvant chemotherapy CD3 and CD8 infiltrates remain stable, whereas FOXP3+ Treg cell numbers significantly decreased in surgical specimens. Importantly, pCR patients had a significantly lower number of FOXP3 cells than nonresponders [4]. Accordingly with this study, Perez et al. found that advanced breast cancer patients with HER2+ tumors exhibited an overall significantly increased frequency of circulating Treg, and, among them, therapeutic intervention with trastuzumab led to an overall reduction to normal levels in the frequency of Treg [5]. Remarkably, a good clinical response to trastuzumab therapy was associated with a significant reduction in Treg frequency, whereas disease recurrence correlated with a significant increase in the percentage of circulating Treg [5].

Recently Mahmoud et al. analyzed the influence of density of CD8+ cytotoxic lymphocytes on prognosis in a large series of 1334 patients with primary invasive breast carcinomas. CD8+ T cells were counted in three locations per tumor (intratumoral compartment, adjacent, and distant stroma), and the total number was determined by the sum of the counts of these three compartments. Higher total CD8+ lymphocyte counts were independently associated with longer breast cancer specific survival after multivariate analysis (Hazard Ratio, 0.55; 95% CI, 0.39-0.78; $P = 0.001$) in a model that included the standard prognostic and predictive factors [6].

Taking into account the above mentioned studies (Table 1), it is tempting to speculate that TIL infiltration may represent not only a prognostic but rather a predictive factor of clinical response in breast cancer, although prospective validation studies are needed to confirm this hypothesis.

3. Regulatory T Lymphocytes and Host Antitumor Response

Tumor infiltration by TIL is well recognized as a good prognosis factor in multiple solid human neoplasms [7–10]. TIL are considered to be a manifestation of the host antitumor reaction. However, there is growing evidence that the specific type of immune cells rather than their quantity governs the host-versus-tumor immune response.

The majority of TIL in solid tumors is CD3+ T-cell phenotype. CD3 can be divided into CD4+ helper cells, including Th1 and Th2 subtypes, based on their cytokine profile, CD4+ regulatory T cells (Treg), and CD8+ cytotoxic effector cells. Treg are thymus-derived CD4+CD25+ T lymphocytes that constitutively express cell surface cytotoxic T lymphocyte antigen-4 (CTLA-4) and secrete immunosuppressive cytokines such as TGF-beta and IL-10 [11]. Treg may also be induced in the periphery from naïve T cells under certain conditions, like stimulation with TGF-β [12].

Treg represent roughly 10% of CD4 T cells and specifically express the forkhead box P3 transcription factor (FOXP3) [13, 14]. Recent studies have shown that Treg play an essential role in sustaining self-tolerance by expressing a wide variety of pathological immune responses against self, nonself, and tumor antigens [11]. Although the exact mechanisms of Treg suppression remain unknown, this effect seems to be largely dependent on the expression of the transcription factor FOXP3 which controls some genes encoding proteins like CD25, GITR, CTLA-4, and others, capable of mediating Treg suppressive functions [15, 16]. In addition, FOXP3 inhibits production of effector cytokines like interleukin-2 (IL-2) after T-cell receptor (TCR) stimulation of T cells [17]. Other mechanisms of immunosuppression are direct cell-to-cell contact with antigen presenting cells (APC) via transforming growth factor β (TGF-β) or CTLA-4 and secretion of immunosuppressive cytokines such as interleukin-10 (IL-10), TGF-β, and others [18, 19].

Specifically in breast carcinoma, the number of FOXP3 CD4+CD25+ Treg and decreased ratios of CD8 T cells/FOXP3 Treg are correlated with a poor prognosis [20]. Bates et al., after analyzing 222 breast carcinoma specimens, observed that elevated numbers of Treg confer a significantly shorter overall and recurrence-free survival and that Treg quantity correlates significantly with more aggressive breast cancer features (high tumor grade, node positive disease) [20]. Although FOXP3 expression was thought to be restricted to Treg, recently it has been elucidated that this transcription factor is also present in various types of tumor cells, including breast cancer [21–23].

However, clinical implications of FOXP3 expression in breast cancer cells are contradictory. Balsari et al. reported that accumulation of FOXP3 Treg predicts a striking reduction of patient survival [21]. On the contrary, Ladoire et al., in a large prospective cohort of node positive breast cancer included in the PACS01 trial, have recently reported better outcomes for those patients that express FOXP3 in breast cancer cells (37% of the entire population) but only in the group treated without taxanes [22]. The same group has published data of a retrospective study restricted to a HER2-positive population treated with neoadjuvant chemotherapy [23]. Again in this case FOXP3 expression in tumor cells correlates with better relapse free and overall survival. In line with these results, FOXP3 has been recently demonstrated to be a tumor suppressor gene that acts as a transcriptional repressor of some breast cancer oncogenes [23].

As mentioned before, immune function is generally compromised in cancer patients, which have lower absolute numbers of peripheral blood lymphocytes but increased numbers of functionally suppressive CD4+CD25+ Treg and dysfunctional dendritic cells (DC) in peripheral blood and tumor microenvironment [24]. In addition, higher numbers of Treg in blood from patients with breast cancer have been reported in relation to normal donors [25].

4. Mechanisms of Immune Tolerance: Effects of Costimulatory and Coinhibitory Molecules on the Immune Synapse

The immune synapse is a region of physical contact between the T cell and the antigen presenting cell (APC), and it represents one of the major determinants of the immune response against tumoral antigens [26]. Two main signals are required for an effective T-cell activation. The first signal is provided by the recognition of cognate antigen bound major histocompatibility complex (MHC) by the T-cell receptor (TCR) [27]. Additional costimulatory signals are provided by engagement of coreceptors. The canonical coreceptor CD28 binds to members of the B7 family present on APC. However, soon after T-cell priming, other negative regulatory molecules are induced on T-cells leading to downregulation of the T cell response. Some of the main costimulatory and coinhibitory molecules that act as immune checkpoints on the immune synapse are resumed in the following lines.

(a) CD40: CD40 is a member of the tumor necrosis factor receptor family expressed on macrophages, dendritic cells, endothelial and B cells, and fibroblasts [28]. Binding of CD40 with its CD40 ligand (CD40L) or CD154 acts on APC and T cells mediating both cellular and humoral responses. Specifically on APC, CD40 plays a central role in priming and expansion of antigen-specific CD4 T cells by regulating the expression of costimulatory molecules on APC such as CD80 and CD86 (B7.1 and B7.2) and by production of cytokines such as IL-12, IL-8, or TNF-α [28, 29].

(b) Cytotoxic T lymphocyte antigen-4 (CTLA-4): CTLA-4 acts as a key negative regulator of CD28 dependent T-cell activation to limit self-damage [30]. CTLA-4 is produced and mobilized from the internal side of the cell membrane, to the immune synapses 2 to 3 days after T-cell activation has taken place. There, it is bound to either one of the costimulatory molecules, CD80 and CD86. CTLA-4 expression turns the activated T cell to an inhibitory T cell [31]. A delay in CTLA-4 expression favours T-cell activation and could be a pathway to improve or expand the immune response against tumors (Figure 1). Recently, an interesting study analyzed the effect of CTLA-4 in breast carcinoma [32]. CTLA-4 expression was detected in breast tissue and blood of breast cancer patients and normal donors. Patients showed strong expression of CTLA-4 in tumor cells of all specimens. By contrast, weakly positive or negative expression of CTLA-4 was found in normal breast tissue. In addition, patients with higher mRNA level of CTLA-4 had breast cancer with worse features, and spontaneous expression of CD3+CTLA-4+ on peripheral blood of patients with tumors was also significantly higher than that of the controls [32].

(c) Programmed death 1 (PD-1): PD-1 is expressed on activated T and B cells, natural killer, dendritic cells, and activated monocytes [33]. PD-1 plays a major role in maintenance of T-cell tolerance limiting effector T-cell responses. There are two ligands of PD-1, PD-L1 and PD-L2 (or B7-H1 and B7-H2), although PD-L1 is considered the most important one [34]. PD-L1 is aberrantly expressed in some tumors including breast cancer, and thus it can induce immune suppression through signaling PD-1 [35]. In breast cancer PD-L1 expression (in tumor tissue and TIL) has been shown to be correlated with worsen clinicopathological data like larger tumor size, histologic grade III tumors, or negative hormone receptors [36].

(d) OX-40: OX-40 is a member of the tumor necrosis factor (TNF) superfamily that needs T-cell activation to be expressed [37]. OX-40 is present in CD4+ and CD8+ T cells, whereas its ligand OX40L is expressed on activated APC, B cells, and macrophages [38]. Binding of OX40 to the OX40L enhances proliferation and survival of T cells leading to a larger expansion of effector T and a larger pool of memory T cells [37]. In addition, CD40 signaling increases cytokine secretion by CD4+ T cells and enhances the development of Th1 and Th2 cells [39].

The immune synapse is to be considered altogether as a complex battlefield where many different molecules and cells interact. It seems crucial to understand in depth the mechanisms that may trigger a successful immune response or on the contrary lead to immunotolerance at this level, in order to find out emerging therapeutic tools targeting the immune synapse.

Table 1: Studies correlating immunobiomarkers with clinical results.

Study	*N* (patients)	Immune biomarker	Results
Balsari et al. [21]	DCIS: 62 Invasive: 257 Normal breast: 10	FOXP3	High FOXP3 in invasive and in situ breast carcinoma than in normal breast High FOXP3 shorter PFS and OS Negative correlation between FOXP3 and ER
Ladoire et al. [4]	56	CD3 CD8 FOXP3	Poor prognostic factors (RE−, high-tumor grade and nodal involvement) correlate with higher number of FOXP3 before chemotherapy >pCR to neoadjuvant chemotherapy correlates with absence of FOXP3 cells and presence of high number of CD8 T cell
Bates et al. [20]	183 + 214	FOXP3	FOXP3 expression in tumor associated with worse overall survival FOXP3 prognostic factor for distant metastases free survival
Demaria et al. [1]	25	TIL	Development of TIL after treatment correlates with clinical response to neoadjuvant chemotherapy
Denkert et al. [2]	1058 (2 cohort)	TIL	High TILs: pCR rates 42 and 40% versus 3 and 7%
Perez et al. [5]	24 normal breast 74 breast cancer (28 HER−; 46 HER+)	Tregs	Treg frecuency in HER2+ was significantly increased. Trastuzumab therapy: decreased Treg frecuency/objective clinical response
Mahmoud et al. [6]	1334	CD8+ T	TIL CD8+ density associated with improved clinical outcome

PFS: progression free survival; OS: overall survival; ER: estrogen receptor; and pCR: pathologic complete response.

5. Tumor-Associated Macrophages, Myeloid-Derived Suppressor Cells, and Related Cytokines

Chronic inflammation in some tissues correlates with higher risk of developing cancer [40]. Within the tumoral microenvironment, tumor-associated macrophages (TAM) and myeloid-derived suppressive cells (MDSC) seem to play a critical role in the progression of tumoral development through nonimmune (mostly proangiogenic) and immune mechanisms [41].

TAMs are a heterogeneous population of cells depending on oxygen availability and phases of tumor development [42]. In early stages, tumors are generally infiltrated by type 1 macrophages (M1) that release proinflammatory cytokines and chemokines promoting Th17 cell differentiation from naïve CD4+ T cells [43]. On the other hand, in advanced stages, TAMs polarize to a type 2 macrophage (M2) related cell that releases cytokines such as transforming growth factors β1 (TGF β1) and IL-10, which induce Th2 differentiation and recruitment, favouring Treg development and thus promoting tumor development through inhibition of anticancer immune responses [44].

In breast cancer, a sample of 128 matching invasive (88% stages I-II) and ductal carcinomas in situ specimens, along with normal breast tissues, was analyzed regarding macrophage phenotype [45]. Increased M2-TAM was significatively associated with more aggressive histopathological features (high tumor grade), increased microvessel density, and decreased overall survival, whereas M1-TAM phenotype was not associated with a worse overall survival. Furthermore, M1-TAM tumors were predominantly low grade [45].

MDSC represent a heterogeneous population of immature myeloid cells in different stages of myeloid cell differentiation [46]. MDSC within the tumor microenvironment exert a variety of immune suppressive functions by perturbing both innate and adaptive immune responses. These effects are largely dependent on cytokines (TGF-β, IL-10, and IL-6) and cellular factors observed in the surroundings of the tumors [47]. Recently, Cole et al. demonstrated that circulating MDSC in metastatic breast cancer significantly correlate with overall survival, observing worse outcomes in patients with high percentages of MDSC (OS, 6.9 versus 19.6 months; $P = 0.05$) [48].

These data suggest that MDSC might be a good biomarker and even a therapeutic target in breast cancer.

As previously cited, cytokines are molecules of critical importance in the tumor microenvironment that modulate the activity of immune cells and may induce different effects during tumor progression. Among immunosuppressive cytokines the role of TGF-β and IL-10 merits special consideration. Functions of both cytokines are intriguing and complex as long as they seem to play initially and antitumor effect preventing angiogenesis and inflammation by inhibiting macrophage activation [43]. Nevertheless, in established tumors, their effects are mostly protumorigenic, encouraging cell survival, and suppressing effector T cells [43]. Specifically, TGF-β plays a central role in the generation and function of CD4+CD25+ Treg and suppression of IFN-γ production by

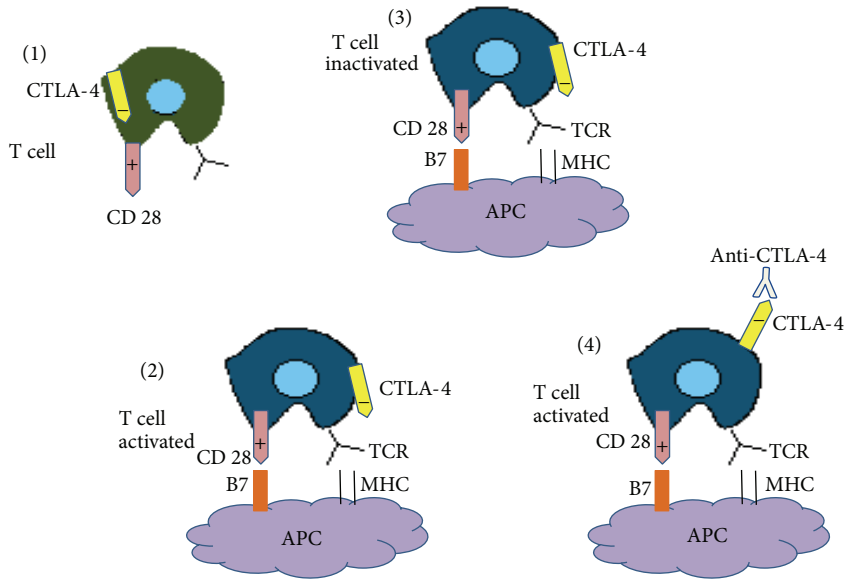

Figure 1: Role of CTLA-4 in T-cell activation. (1) CTLA-4 is a negative regulator of T-cell activation. (2) Conventional T cells are activated by engagement of MHC and B7. (3) Upon activation, T-cells express CTLA-4. Binding of CTLA-4 with B7 inhibit T cell activation. (4) Blockade of CTLA-4 produces the liberation of CD28 that engages with B7 activating T cells. APC: antigen presenting cell; MHC: major histocompatibility complex; and TCR: T-cell receptor.

Th1 and CD8+ T cells, finally impeding a successful immune response and favouring tumor progression [19, 49, 50].

However, there are also cytokines in the microenvironment like GM-CSF or IL-2 that exert costimulatory effects [31]. GM-CSF has pleiotropic properties, including the mobilisation, differentiation, and function of dendritic cells [51], and it has also been studied in the clinical setting in many cancer types showing some promising results [52–54]. In this sense, Honkoop et al. reported an interesting study in locally advanced breast carcinoma treated with chemotherapy and GM-CSF [54]. The authors reported a positive correlation between the number of cycles received with GM-CSF and overall survival (OS) and disease-free survival (DFS) [54]. One of the hypothesis to explain these results relies in the large number of overexpressed tumoral antigens (Her-2/neu, CEA, MUC-1, etc.) in breast cancer released after CT, which represent an excellent target for an immune environment boosted by GM-CSF [55].

6. Strategies to Overcome Immunotolerance in Breast Cancer

6.1. Depletion of Treg. Depletion of FOXP3 Treg can enhance antitumor immunity, and thus different strategies are being pursued to attenuate the suppressive function of Treg [11].

Metronomic chemotherapy consists in administration of low doses of chemotherapeuticals with the aim of reducing tumor angiogenesis. Recently, it has been elucidated that low doses of oral metronomic cyclophosphamide in advanced cancer patients induce a profound and selective reduction of circulating regulatory T cells, associated with a suppression of their inhibitory functions on conventional T and NK cells, therefore, leading to reduction of tumor-induced immune tolerance and a better disease control [56, 57].

Treg are highly IL-2 dependent for their survival. IL-2 neutralization with specific antibodies may substantially reduce the number of Treg [58]. Denileukin diftitox (Ontak), a recombinant fusion protein consisting of IL-2 and diphtheria toxin, may deplete Treg and so reduce immune suppression boosting antitumor immunity [58, 59]. CD25 is the IL-2 receptor α chain, so denileukin diftitox binds to the IL-2 receptor and inhibits protein translation following internalization, leading to apoptosis [59]. Curiel demonstrated in a phase 0/1 trial that Ontak at 9 or 12 μg/kg decreased the number of blood Treg and the suppression mediated by the CD4+CD25+ blood T-cell population in patients with advanced stage epithelial carcinomas, including cases of breast carcinoma [60].

6.2. Immune Synapses as a Therapeutic Target. As mentioned before, the immune synapses are virtual spaces where the complex controls and checkpoints that modulate the interaction of effector cells with their targets take place. Many of the molecules and cells that compose the immune synapses are attractive targets to be exploited clinically, and among them CTLA-4 is probably the most widely studied molecule.

In preclinical studies with knockout mice it has been reported that CTLA-4 deficiency in CD4+CD25+ Treg impairs its suppressive function in tumor immunity [61, 62]. In addition, exclusive blockade of CTLA-4 signal in either CD4+CD25+ Treg or nonTreg T cell in mice leads not only to attenuation of Treg suppression but to augmentate effector T-cell activity [61].

A recent study demonstrated that antibodies against CTLA-4 (anti-CTLA-4) induce proliferation of TCR stimulated T effector cells and abrogate Treg suppressive activity by enhancing IL-2 and IFNγ release in response to polyclonal or tumor antigen stimulation [63]. Curiously, anti-CTLA-4 does not reduce the amount of Treg, suggesting that it mediates immune responses by direct activation of T effector cells and not by depleting Treg [63].

There are 2 CTLA-4 blocking antibodies for use in humans [64]. Recently, ipilimumab (Bristol-Myers Squibb, Princeton, JC, USA) has demonstrated significant benefits in overall survival in randomized phase III studies in the first or second line treatment of metastatic melanoma [65, 66], gaining FDA approval.

Clinical research of anti-CTLA-4 in other solid neoplasms like breast carcinoma is scarce until now [67]. However a phase I study in advanced breast carcinoma with the combination of exemestane and tremelimumab has been recently reported, demonstrating that the combination is well tolerated and associated with an increased expression of inducible costimulator (ICOS+) in peripheral T CD4+ and CD8+ cells, which likely signals immune activation secondary to CTLA-4 blockade [68].

A better understanding of the mechanism of action of anti-CTLA-4, along with its use in the context of combinatorial strategies, may enable us to explore the eventual efficacy of these molecules in nonmelanoma populations [67, 69]. Combination of CTLA4 and PD-1 blockade with anti-PD-1:B7-H1 monoclonal antibodies increases effector T-cell infiltration into B16 melanoma in mice, resulting in an elevated effector to Treg cell ratio within the tumor [70]. Phase I studies in humans with single agent anti-PD-1 in refractory solid tumors have been performed with promising results [71].

As previously cited, CD40 is another molecule that plays an essential role in the immune synapses [35]. Several agonistic antibodies against CD40 are under clinical research, and preliminary data in murine models suggest strong immune effects resulting in CD4 T-cell priming and cytotoxic T-cell responses [72, 73]. Interestingly, a clinical study in 21 patients with advanced pancreatic carcinoma has been recently reported testing the combination of gemcitabine with CD40 agonist CP-870.893 [74]. Metabolic evaluation by PET assessment revealed an impressive response rate of 88% after two cycles, with a median progression free and overall survival of 5.6 and 7.4 months, respectively [74]. These interesting results deserve confirmation in phase II and III studies. Again, preclinical data have revealed that anti-CTLA-4 and CD40 are more effective when combined that either therapy alone [75].

Finally, another immunogenic molecule is OX40. It is elucidated that signaling through OX40 and OX40L enhances antitumor immunity [37]. In rodents, Murata et al. demonstrated that combination of a GM-CSF secreting tumor cell vaccine with anti-OX40 antibody induced a potent CD8+ T-cell response, leading to eradication of established breast carcinomas [76]. This effect seems related to the prolonged expansion and survival of tumor specific T cells. Another interesting strategy relies in the combination of OX40 therapy with radiotherapy (RT) and/or chemotherapy. CT and RT imply an enhanced expression of tumoral antigens with an increase in tumor antigen-specific cytotoxicity and OX40 expression. Clinical trials testing this hypothesis are ongoing [77, 78].

Although early in their clinical development, it is tempting to speculate that the universal mechanism of action of anti-CTLA-4, anti-PD-1, and CD40 or OX-40 agonists, among other molecules, may not only be restricted to melanoma patients but rather may be useful in a wide range of other oncologic diseases. Preclinical data support clinical research in this field especially in the context of combinatorial strategies. Likewise clinicians must be aware that conventional response criteria seem no longer valid in this context, and new guidelines for the evaluation of immune-related responses must be considered [79].

6.3. Impact of Chemotherapy (CT) in Breast Cancer Microenvironment. Chemotherapy remains the therapeutical modality of choice for the systemic treatment of many breast carcinomas, especially in the neoadjuvant and metastatic setting. Impact of conventional chemotherapy on the relationship between the tumor and the immune system is extremely important (Table 1). Some groups argue that cell death induced by chemotherapy implies a variety of immune reactions that mediate a sort of vaccination effect via release of an "antigenic milieu" that, in turn, may represent the major determinants of the therapeutical success of the chemotherapy in oncological diseases [80].

Cytotoxic drugs destroy tumor cells by apoptosis [57], and recent studies suggest that some chemotherapeutics may induce tumoral destruction improving cancer cell recognition by the immune system [81, 82]. Some preclinical studies support the idea that immune stimulation might be mediated by chemotherapy in murine cancer models treated with gemcitabine and doxorubicin [83, 84]. The explanation to this selective immune activation is an increased CD8 T-lymphocyte expansion and an increased density of TIL mediated by an effective MHC class I cross-presentation of tumor antigens released and phagocytosed [85].

There is now clear evidence supporting the fact that drugs like anthracyclines, cyclophosphamide, or gemcitabine may promote apoptosis in cancer cells with immunogenic effects through several mechanisms [80, 85, 86] (Figure 2). This sort of immunogenic tumor cell death is characterized by a temporal sequence of events including early translocation of calreticulin (CRT) to the cell surface and thereafter interaction of CRT with multiple receptors on DC with apoptotic bodies phagocytosis, release and exposure of heat shock proteins, and late release of HMGB1 [85]. HMGB1 is able to bind to the TLR4 receptor on DC, which allows tumor-derived antigens to be processed and presented along with MHC and costimulatory molecules on the surface of DC [58, 62]. These mechanisms altogether serve to trigger DC-mediated specific antitumor response, which may be enhanced by the use of costimulatory molecules [31].

In addition, other more general effects of chemotherapy on the surrounding stroma are postulated like secondary

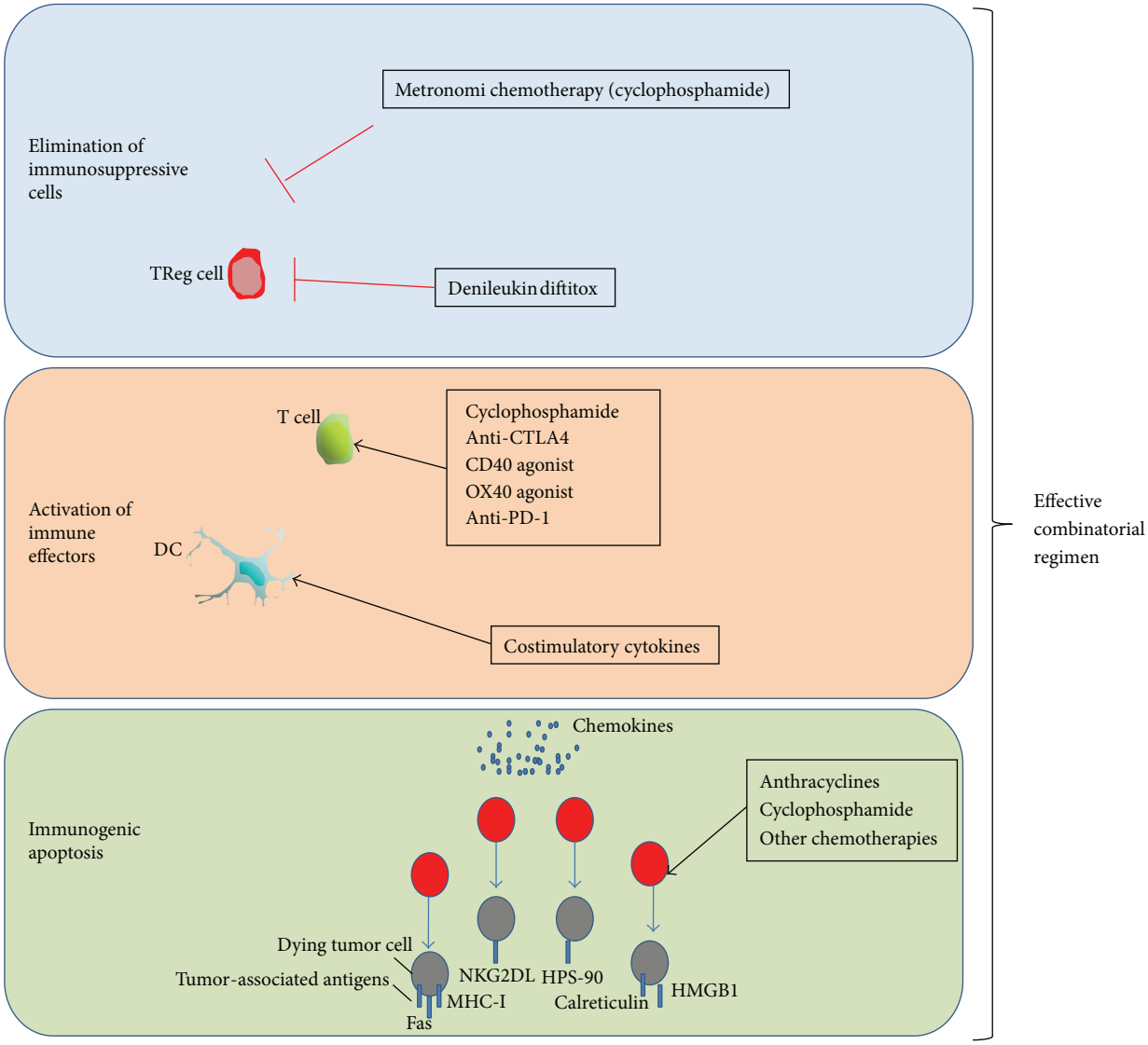

Figure 2: Mechanisms of action of conventional antineoplastic agents and new immunostimulatory drugs.

necrosis or eradication of tumor cells [87]. Furthermore, gemcitabine has demonstrated the ability to restore immune surveillance by reducing MDSC levels in murine models, which represents another interesting field to explore among the immune effects of chemotherapy over microenvironment [88].

In conclusion, emerging evidence led Lake and Robinson to announce a paradigm shift in the way of understanding the effects of CT on the surrounding stroma [87, 89]. These new concepts may serve to consider chemotherapeutics as less empirical and more specific drugs, thus may help to customize treatments taking into account its potential effects on the microenvironment.

7. Conclusions

Available data support the hypothesis of an immune-mediated antitumor activity in breast carcinoma, and several lines of research are ongoing [90]. It is critical to understand what happens in the tumoral microenvironment in order to design biological agents that may modulate the immune response towards cancer cell destruction (Figure 2). Combination strategies of chemoimmunotherapy will eventually synergize and obtain meaningful clinical results.

Finally, the fact that probably the most successful strategy in oncology in the last decade has been the combination of chemotherapy and passive immunotherapy merits special consideration. Rituximab and trastuzumab monoclonal antibodies (MoAb) have obtained impressive results when administered to the right populations [91, 92]. Interestingly enough, the powerful effect of these MoAb is enhanced when combined with chemotherapy, as a new evidence of the priming of the APC tumor antigen presentation and T-cell activation. Moreover, preclinical data exploring the effects of a HER2/neu peptide vaccine combined with trastuzumab have demonstrated synergistic immune enhancement [93].

In our opinion there is large amount of available data which provides sufficient evidence to consider the host immune reaction as one of the main determinants of the clinical evolution in breast cancer. Importantly, this immune response is capable of being modulated in clinical practice, so new therapeutical strategies based on chemoimmunotherapeutic approaches might be worthy of consideration in the coming future to rise another step in the global battle against breast cancer.

Conflict of Interests

The authors declare no conflict of interests.

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Research Article

Dexamethasone Preconditioning Improves the Response of Collagen-Induced Arthritis to Treatment with Short-Term Lipopolysaccharide-Stimulated Collagen-Loaded Dendritic Cells

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Background. Pharmacologically modulated dendritic cells (DCs) have been shown to restore tolerance in type II collagen-(CII-) induced arthritis (CIA). We examined the effect of dexamethasone (DXM) administration as a preconditioning agent, followed by an injection of lipopolysaccharide-(LPS-) stimulated and CII-loaded DCs on the CIA course. *Methods*. After CIA induction, mice pretreated with DXM were injected with 4-hour LPS-stimulated DCs loaded with CII (DXM/4hLPS/CII/DCs). *Results*. Mice injected with DXM/4hLPS/CII/DCs displayed significantly less severe clinical disease compared to animals receiving 4hLPS/CII/DCs alone or those in which only DXM was administered. Cytokine profile evaluation showed that CD4+ T cells from DXM/4hLPS/CII/DCs and 4hLPS/CII/DCs groups release higher IL-10 levels than those from mice receiving DXM alone or CIA mice. CD4+ T cells from all DC-treated groups showed less IL-17 release when compared to the CIA group. On the contrary, CD4+ T cells from DXM/4hLPS/CII/DCs and 4hLPS/CII/DCs groups released higher IFN-γ levels than those from CIA group. *Conclusion*. A combined treatment, including DXM preconditioning followed by an inoculation of short-term LPS-stimulated CII-loaded DCs, provides an improved strategy for attenuating CIA severity. Our results suggest that this benefit is driven by a modulation in the cytokine profile secreted by CD4+ T cells.

1. Introduction

Current evidence indicates that dendritic cells (DCs) functions are related to the stage of maturation and associated cytokine production profile. Unlike mature DCs, immature and semimature DCs exhibit a reduced costimulatory capacity and show a distinctive IL-10 high/IL-12 low cytokine profile, which might endow them with tolerogenic functions [1]. In order to induce and maintain a tolerogenic phenotype on *in vitro* generated DCs for therapeutic purposes, several strategies have been used, including modulation with agents such as IL-10, TNF, neuropeptides, lipopolysaccharide (LPS), dexamethasone (DXM), and vitamin D3 plus LPS, among others [2–7]. Our group has demonstrated that short-term LPS stimulation can induce IL-10-producing tolerogenic DCs that, when administered to mice with established collagen-induced arthritis (CIA), can interfere with the disease course [6, 8].

A major challenge that therapeutic administration of tolerogenic DCs has to face is to avoid the conversion of DCs into immunogenic antigen-presenting cells as they encounter a proinflammatory environment. This goal could be achieved if a transient immunosuppression is induced in the recipient just before receiving an injection with tolerogenic DCs. Data from animal models of autoimmunity and patients affected by autoimmune diseases has shown that nonmyeloablative immunosuppression followed by hematopoietic stem cell transplantation led to a reset of the dysregulated immune system of the recipient, which in some cases, reach complete remission of the disease [9].

In the present study, we demonstrate that the outcome of CIA mice receiving short-term LPS-stimulated collagen-loaded DCs inoculation can be improved by a previous DXM administration. In addition, we prove that CD4+ T cells from mice receiving the combined treatment produced high levels of IL-10 and IFN-γ, while they were low IL-17 producers.

2. Materials and Methods

2.1. Bone Marrow-Derived DCs. Bone marrow cells from DBA1/lacJ mice were differentiated into DCs as described by Salazar et al., 2008 [6]. After 6 days of culture, bone marrow-derived DCs were purified by positive selection using magnetic beads coupled to anti-CD11c antibodies (Miltenyi Biotec, Germany) and then stimulated with 1 μg/mL LPS (*Escherichia coli*, Sigma-Aldrich, USA) for the last 4 hours (4hLPS/CII/DCs) and loaded for 24 hours with bovine type II collagen (CII) (Chondrex, Redmond, WA, USA), or left unloaded (4hLPS/DCs). LPS-untreated DCs loaded with CII (0hLPS/CII/DCs) or unloaded (0hLPS/DCs) or treated for 24 hours with LPS (24hLPS/DCs) were used as controls.

2.2. Characterization of DCs Phenotype and Cytokine Secretion Profile. The following antibodies were purchased from eBioscience (San Diego, CA, USA): fluorescein isothiocyanate-(FITC-) labeled anti-CD11c, phycoerythrin-(PE-) labeled anti-CD86, anti-MHC class II, and anti-CD40. Stained DCs were acquired in a FACSCalibur flow cytometer (BD Bioscience, San Diego, CA, USA) and data was analyzed using the WinMDI 2.9 software. IL-10 and IL-12 production was measured in supernatants from differentially treated DCs by ELISA (Bender MedSystems, Austria).

2.3. CIA Induction and Clinical Evaluation. CIA induction and clinical evaluation with the Joint Score and the Swollen Joint Severity Score were performed as described by Salazar et al., 2008 [6]. Protocols were approved by the Bioethics Committee of Universidad de Chile.

2.4. DXM Administration. DXM (Sigma-Aldrich, USA) was intraperitoneally administered in different doses (0.5, 1.0, and 2.0 mg/kg). The drug was given daily for 6 days, starting at day 29 until day 34 after disease induction.

2.5. DCs Inoculation. Differentially treated DCs (5×10^5) were intraperitoneally administered to 8–10 mice per group by a single injection at day 35 after CIA induction.

2.6. CD4+ T Cells Cytokines Production Assessment. Spleens were obtained from different groups of mice at day 47 after first CII inoculation. CD4+ T cells from spleens were purified by negative selection using magnetic beads (Miltenyi Biotec). Isolated CD4+ T cells were incubated in the presence of 5 μg/mL concanavalin A at 37°C for 72 hours, and supernatants were collected and frozen at −85°C. IL-10, IFN-γ, and IL-17 were quantified by ELISA (Bender MedSystems).

2.7. Statistical Analysis. Comparisons between different groups of DCs or CD4+ T cells were performed with a one-way ANOVA test, corrected with Bonferroni's post-test. Two-way ANOVA test, corrected with Bonferroni's post-test, was applied for comparisons between clinical scores of different groups of mice. $P < 0.05$ was considered statistically significant. For statistical analyses and graphics, the software GraphPad Prism 4 was used.

3. Results

3.1. DXM Dosing for CIA Inhibition. In order to define the dose of DXM capable of providing the most intensive anti-inflammatory effect on CIA mice, three groups of animals were treated with 0.5, 1.0, and 2.0 mg/kg DXM, respectively, for six consecutive days, starting at day 29 after the first CII inoculation. As depicted in Figure 1, all DXM doses were able to interfere with the onset and the course of CIA. However, from day 39 and 46, respectively, both Joint Score and Swollen Joint Severity Score increased irrespectively of the DXM dose administered, reaching values that did not differ statistically from those observed in untreated CIA animals. According to the Joint Score, mice that received DXM at 2.0 mg/kg, were the only group to show significant lower mean scores compared with CIA mice up to day 39 (Figure 1). For this reason, we used this dose in subsequent experiments.

3.2. Effect of DXM Pretreatment Followed by Short-Term LPS-Stimulated DCs Inoculation on Established CIA. To assess whether the transient anti-inflammatory status achieved by DXM conditioning could enhance the tolerogenic effect of short-term LPS-stimulated DCs, mice with CIA were treated with 2.0 mg/kg DXM for six days as described above, and at day 35, animals were split into three study groups, which received the following intraperitoneal injections: saline buffer (DXM group), DCs loaded with CII and stimulated for 4 hours with LPS (DXM/4hLPS/CII/DCs group), or unloaded DCs stimulated for 4 hours with LPS (DXM/4hLPS/DCs group). Also, animals with CIA injected at day 35 only with 4hLPS/CII/DCs or with vehicle were used as controls.

Previous to inoculation, DCs were phenotypically characterized by cell surface markers expression (MHC class II, CD86, and CD40) (Figure 2(a)) and functionally by evaluating their IL-10 and IL-12 production (Figure 2(b)). As depicted in Figure 2(a), 4hLPS/DCs and 4hLPS/CII/DCs displayed lower CD40 and CD86 expressions than 24hLPS/DCs ($P < 0.001$) and higher than 0hLPS/DCs or 0hLPS/CII/DCs ($P < 0.001$ and $P < 0.01$ for CD40 and CD86, resp.). As shown in Figure 2(b), we detected that both 4hLPS/DCs and 4hLPS/CII/DCs showed a higher IL-10 production than 24hLPS/DCs ($P < 0.001$) and 0hLPS/DCs ($P < 0.01$), while they produced lesser IL-12 than 24hLPS/DCs ($P < 0.001$). These data suggest that both phenotypic and functional features of 4hLPS/DCs are consistent with those displayed by tolerogenic DCs, as reported previously [6], and that these features are not affected by antigen loading.

Once 4hLPS/DCs and 4hLPS/CII/DCs were characterized, they were administered to CIA mice, which had previously received the DXM pretreatment. We compared the

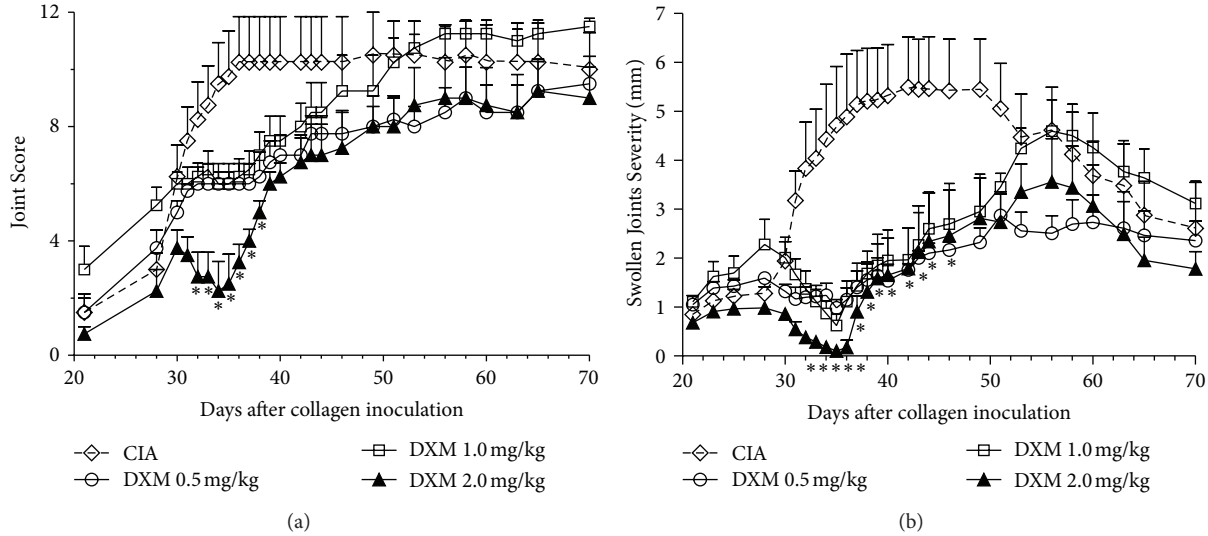

Figure 1: Dexamethasone (DXM) dosing for collagen-induced arthritis (CIA) inhibition. (a) Different doses of DXM (0.5, 1.0, and 2.0 mg/kg) were intraperitoneally administered to DBA1/lacJ mice from days 29 to day 34 after CIA induction. The mean Joint Score and the Swollen Joint Severity Score were determined until day 70. Data from a representative experiment of three experiments performed with 8–10 mice per group. $^{*}P < 0.001$, in DXM 2.0 mg/kg versus CIA group.

severity curves from day 44 onwards to avoid the effect of DXM conditioning on its self. As depicted in Figure 2(c), according to the Joint Score, the DXM/4hLPS/CII/DCs group displayed a significantly less severe clinical disease from day 44 up to day 70, when compared to CIA mice ($P < 0.001$). In contrast, this effect was not observed in the 4hLPS/CII/DCs or DXM groups. This observation was also valid for the Swollen Joint Severity Score, although in this case both groups, DXM/4hLPS/CII/DCs and 4hLPS/CII/DCs, exhibit significantly lower scores than the CIA group (Figure 2(c)). Interestingly, the mice group receiving DXM/4hLPS/DCs showed a more severe clinical disease than mice from the DXM/4hLPS/CII/DCs or 4hLPS/CII/DCs groups, highlighting the antigen dependence of the tolerogenic effect.

3.3. Cytokine Production by CD4+ T Cells from CIA Mice Receiving DXM Preconditioning Followed by a Short-Term LPS-Stimulated DCs Injection. In order to evaluate whether the administration of 4hLPS/CII/DCs after preconditioning with DXM could modify the profile of cytokines secreted by CD4+ T cells in CIA mice, we assessed the secretion of IL-10, IL-17, and IFN-γ by stimulated splenic CD4+ T cells obtained at day 47 after CIA induction. We detected that CD4+ T cells from mice treated either with DXM/4hLPS/CII/DCs or 4hLPS/CII/DCs secrete higher IL-10 levels than those from the DXM group or from the CIA group ($P < 0.001$ for each comparison) (Figure 3). Interestingly, CD4+ T cells from all treated groups released significantly lower IL-17 levels when compared to those produced by CD4+ T cells from the CIA group ($P < 0.05$ for each comparison) (Figure 3). Furthermore, the evaluation of IFN-γ showed that CD4+ T cells from the 4hLPS/CII/DCs and DXM/4hLPS/CII/DCs groups released higher levels than those from the DXM group or the CIA group ($P < 0.001$ for each comparison) (Figure 3).

4. Discussion

Corticosteroids are potent anti-inflammatory drugs that are widely used in the treatment of rheumatoid arthritis (RA), having a beneficial role in both short-term and long-term management of the disease. In short-term use, corticosteroids are more effective anti-inflammatory agents than nonsteroidal anti-inflammatory drugs, and their long-term use has been shown to stop progression of bone erosions caused by RA, similar to other disease-modifying antirheumatic drugs [10].

Anti-inflammatory effects of corticosteroids have been associated with a strong inhibition of IL-2 by T cells, as well as signal transduction through the IL-2 receptor [11]. In addition, mice receiving DXM exhibit an expansion of regulatory T cells [12], and human CD4+ T cells treated *in vitro* with DXM increased the expression of regulatory T cell-associated transcription factor FoxP3 and the anti-inflammatory cytokine IL-10 [13], although it has been proposed that this induction is not correlated with an increased suppressive function [14].

Corticosteroids can affect other immune cells. For instance, it has been shown that DXM can induce an enhancement in human B cells ability to secrete IL-10 [15]. Also, it has been demonstrated that corticosteroids downregulate the production of IL-1β, IL-6, and TNF by monocytes and macrophages [16]. Studies in monocyte-derived DCs in humans and in bone marrow-derived DCs from mice have shown that hydrocortisone or DXM added *in vitro* strongly inhibit the production of IL-12p70, TNF, and IL-6 [17, 18]. Furthermore, hydrocortisone-treated monocyte-derived DCs induced less IFN-γ production and higher levels of IL-10 secretion by stimulated CD4+ T cells [17].

On the other hand, tolerogenic DCs have been used to restore tolerance in experimental autoimmune

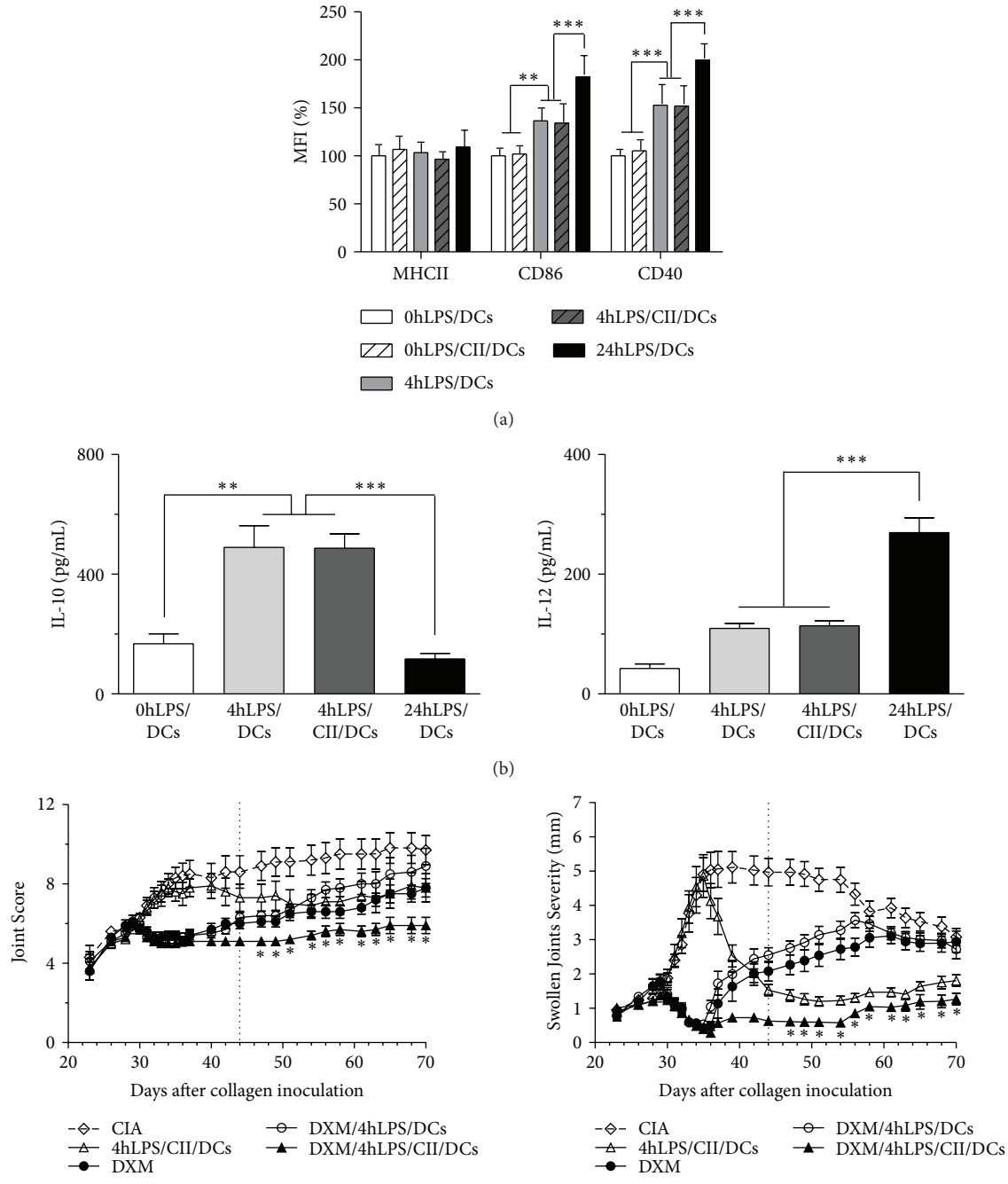

Figure 2: Dexamethasone (DXM) preconditioning improves the effect of 4-hour lipopolysaccharide-(LPS-) stimulated dendritic cells (DCs) in modulating active CIA. (a) Isolated CD11c+ DCs were stimulated with LPS for 4 hours (4hLPS/CII/DCs) and loaded for 24 hours with bovine type II collagen (CII) or left unloaded (4hLPS/DCs). LPS-untreated DCs loaded with CII (0hLPS/CII/DCs) or unloaded (0hLPS/DCs), or treated for 24 hours with LPS (24hLPS/DCs) were used as controls. The expression of major histocompatibility complex (MHC) class II and costimulatory molecules (CD86 and CD40) was analyzed by flow cytometry. Values are expressed as percentage of increase in mean fluorescence intensity (MFI) related to 0hLPS/DCs. Data from a representative experiment of three experiments performed are shown. $^{**}P < 0.01$ and $^{***}P < 0.001$. (b) Cytokine production by differentially stimulated DCs was assessed by ELISA. Bars represent the mean of three experiments performed in duplicate. $^{**}P < 0.01$ and $^{***}P < 0.001$. (c) Mice with active CIA received 2.0 mg/kg DXM from days 29 to day 34 after CIA induction (DXM group). Then, mice were inoculated intraperitoneally at day 35 with 5×10^5 DCs as follows: 4-hour LPS-stimulated DCs (DXM/4hLPS/DCs) and 4-hour LPS-stimulated DCs loaded with CII (DXM/4hLPS/CII/DCs). The 4hLPS/CII/DCs group received 4-hour LPS-stimulated DCs loaded with CII, but without DXM preconditioning. The CIA control group corresponds to mice that did not receive any treatment. The two-tailed ANOVA test and Bonferroni's post-test were applied when comparing Joint Score and Swollen Joints Severity Score curves from day 44 to day 70. Data from a representative experiment of three experiments performed with 8–10 mice per group are shown. $^{*}P < 0.001$ in DXM/4hLPS/CII/DCs versus CIA group.

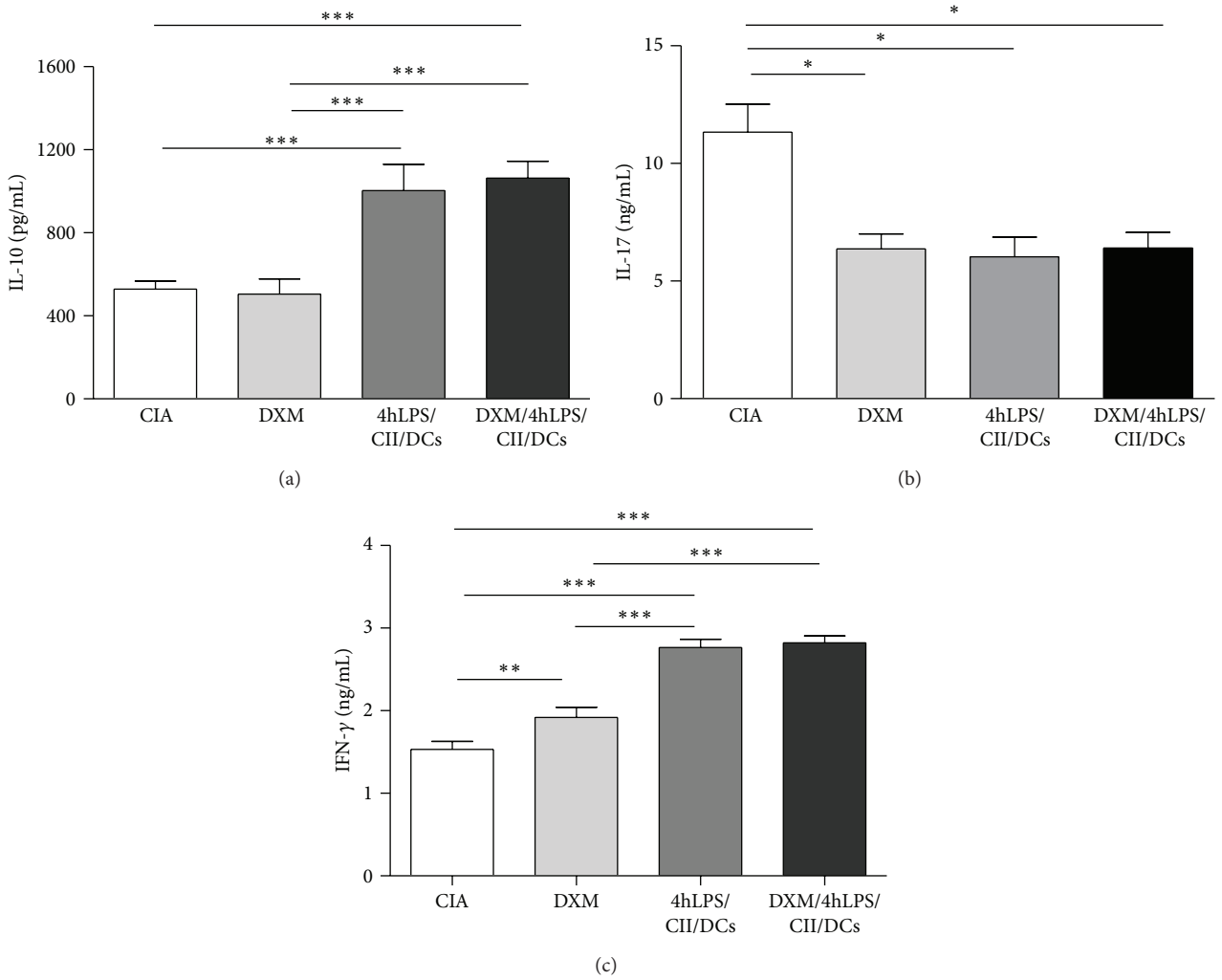

Figure 3: Cytokine production by CD4+ T cells from mice with active collagen-induced arthritis (CIA), treated with an inoculation of 4-hour lipopolysaccharide-(LPS-) stimulated dendritic cells (DCs) and dexamethasone (DXM) preconditioning. Mice received 2.0 mg/kg DXM from day 29 to day 34 after CIA induction (DXM group). Another group of mice were additionally injected at day 35 with 5×10^5 4-hour LPS-stimulated DCs loaded with type II collagen (CII) (DXM/4hLPS/CII/DCs group). The 4hLPS/CII/DCs group received 4-hour LPS-stimulated DCs loaded with CII, but without DXM preconditioning. The CIA control group corresponds to mice that did not receive any treatment. CD4+ T cells purified from mouse spleens at day 47 after CIA induction were stimulated with concanavalin A for 72 hours, and IL-10, IL-17, and IFN-γ levels in supernatants were quantified by ELISA. Bars represent the mean of three experiments.

encephalomyelitis and CIA by preventive injections of TNF-maturated DCs [5, 7] or by therapeutic injection of LPS-stimulated DCs in mice with established CIA [6, 8]. Among others, studies performed by van Duivenvoorde et al., 2007, also demonstrate that a preventive inoculation with DXM-modulated DCs is able to inhibit the induction of CIA [7].

Based on the successful approach of nonmyeloablative conditioning previous to stem cell transplantation for the treatment of severe systemic autoimmune diseases, including experimental arthritis [19], we have hypothesized that the corticosteroid preconditioning of mice with established CIA may significantly reduce the inflammatory status, thereby strengthening the regulatory activity of tolerogenic 4hLPS/CII/DCs. Our results indicate that the conditioning with DXM was able to interfere with disease onset and progression during a short period. However, this quenching of the inflammation could have facilitated that antigen-pulsed tolerogenic DCs display a higher effectiveness in controlling CIA progression than that observed when tolerogenic DCs were inoculated without DXM conditioning.

Treatment with 4hLPS/CII/DCs alone or in combination with DXM significantly reduced the deleterious Th17 immune response, as we have previously reported [8]. In addition, both schemes were able to expand IL-10-producing CD4+ T cell subsets, which could be responsible for interfering with disease progression. Interestingly, the conditioning with DXM alone also inhibited the IL-17 production, providing a low inflammation environment, which could allow IL-10-producing CD4+ T cells to be generated by the effect of

tolerogenic 4hLPS/CII/DCs, and to participate by inhibiting the autoimmune-mediated process in a synergic way.

Remarkably, IFN-γ levels produced by CD4+ T cells increased significantly after 4hLPS/CII/DCs inoculation with or without DXM conditioning. As reported by van Duivenvoorde et al., 2007, and by our group [7, 8], tolerogenic DCs are able to induce a high percentage of IFN-γ-producing T cells in CIA mice. Our finding is in agreement with the protective role attributed to IFN-γ, according to the new paradigm accepted for CIA pathogenesis [20]; however, the present work was not intended to prove this issue.

Although the use of LPS with therapeutic purposes is inadmissible, it has been demonstrated that LPS activation is essential for inducing migratory and antigen-presenting activity in tolerogenic DCs [21]. The lack of proinflammatory stimulation does not allow DCs to upregulate polysialic acid production, a requirement to express high levels of the chemokine receptor CCR7, and they may therefore not be able to migrate to secondary lymphoid tissue [22]. Therefore, in order to improve the CCR7-mediated migratory capacity of tolerogenic DCs, without compromising their tolerogenic function, more investigation on the potential of safe LPS-derivatives or other pro-inflammatory reagents is required.

In conclusion, our findings support the use of DXM conditioning as an intervention that improves the effect of antigen-loaded short-term LPS-stimulated DCs as treatment for an animal model of RA. In addition, we demonstrated that CD4+ T cells from mice receiving the combined treatment produced high levels of IL-10 and IFN-γ, while they were low IL-17 producers. Nevertheless, regarding human application of this therapeutic strategy for RA, the antigenic proteins to be used for loading tolerogenic DCs is an important issue that remains unsolved and might be the target of intense research in the area for the future years.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

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Clinical Study

Assessment of T Regulatory Cells and Expanded Profiling of Autoantibodies May Offer Novel Biomarkers for the Clinical Management of Systemic Sclerosis and Undifferentiated Connective Tissue Disease

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In order to identify disease biomarkers for the clinical and therapeutic management of autoimmune diseases such as systemic sclerosis (SSc) and undifferentiated connective tissue disease (UCTD), we have explored the setting of peripheral T regulatory (T reg) cells and assessed an expanded profile of autoantibodies in patients with SSc, including either limited (lcSSc) or diffuse (dcSSc) disease, and in patients presenting with clinical signs and symptoms of UCTD. A large panel of serum antibodies directed towards nuclear, nucleolar, and cytoplasmic antigens, including well-recognized molecules as well as less frequently tested antigens, was assessed in order to determine whether different antibody profiles might be associated with distinct clinical settings. Beside the well-recognized association between lcSSc and anti-centromeric or dcSSC and anti-topoisomerase-I antibodies, we found a significative association between dcSSc and anti-SRP or anti-PL-7/12 antibodies. In addition, two distinct groups emerged on the basis of anti-RNP or anti-PM-Scl 75/100 antibody production among UCTD patients. The levels of T reg cells were significantly lower in patients with SSc as compared to patients with UCTD or to healthy controls; in patients with lcSSc, T reg cells were inversely correlated to disease duration, suggesting that their levels may represent a marker of disease progression.

1. Introduction

Systemic sclerosis is an autoimmune systemic disease characterized by diffuse fibrosis and vasculopathy. The diffuse alteration of small blood vessel leads to tissue ischemia and fibroblast stimulation, which result in accumulation of collagen in the skin and internal organs [1]. Patients with SSc can be classified into distinct clinical categories, characterized by different outcomes and life expectancy [2]. According to the extent of skin involvement, patients are classified as limited cutaneous scleroderma (lcSSc) and diffuse cutaneous scleroderma (dcSSc) [3]. In lcSSc, fibrosis is mainly restricted to the hands, arms, and face. Raynaud's phenomenon is generally present for several years before fibrosis appears and pulmonary hypertension represents a frequent clinical complication. In dcSSc, which represents a rapidly progressing disease, a large area of the skin is affected by fibrosis which extends to one or more internal organs. Autoantibodies characteristically targeting nuclear antigens are recognised as one of the hallmarks of SSc and their presence is considered a key feature for the diagnosis. In addition, the presence of different types of antinuclear antibodies (ANAs) appears to

be associated with distinct outcomes of the disease including clinical severity [4]. Although the current criteria of the American College of Rheumatology for SSc staging do not include the presence of ANAs [5, 6], their detection might offer an additional tool for the clinical management of the disease, since they might help distinguish patients with an early SSc from those presenting an undifferentiated connective tissue disease (UCTD). According to the more recently proposed criteria [7], UCTD is characterized by a persistent oligosymptomatic condition (at least 3 years) which might evolve into aggressive autoimmune diseases as SSc, systemic lupus erythematosus, primary Sjögren's syndrome, mixed connective tissue disease (MCTD), systemic vasculitis, polydermatomyositis (PM/DM), and rheumatoid arthritis (RA) [8]. The laboratory determination of the autoantibody profile represents a useful tool for both diagnosis and characterization of distinct clinical manifestations of autoimmune diseases; however, their presence or titer tends to persist during the course of the disease, even following therapeutic interventions [4]. Indeed, both in SSC and UCTD the role of autoantibodies in inducing the disease is, as yet, unclear [9]. However, some authors have reported a favorable outcome in SSc patients who lose anti-topo I antibody during the disease course [10], and previous studies have shown a marked reduction of organ inflammation following the suppression of autoantibody production both in human [11] and experimental lupus [12], strongly, though indirectly, suggesting that antibodies reacting with self-components can trigger a chronic, site-specific, inflammation, which, in turn, can be responsible for organ damage. In this view, accumulating evidence has pointed at the pivotal role played by T reg cells in autoimmune diseases, since these cells are key for the regulation, including the initiation as well as the termination, of the adaptive immune response [13]. Previous studies suggested that T reg cells may play a role either in controlling autoantibody production [14] or in limiting autoantibody-induced inflammation through IL-10 production [15, 16] or downregulation of costimulatory molecules on APCs [17]. In order to identify "disease biomarkers" useful for the clinical and therapeutic management of autoimmune disorders, in the present study we assessed an extended panel of nuclear, nucleolar, and cytoplasmic autoantigens, including those associated with SSc (Topoisomerase-I, Cenp-A/B, RNAP III, Th/To, Fibrillarin, PDGFR) as well as dermatomyositis (Mi-2, Jo-1, PL-7, PL-12, EJ, OJ, SRP) or other overlapping syndromes (PM-Scl 75 e PM Scl 100, Ku, Ro-52, NOR 90) [18] facing the determination of the regulatory T cell levels in patients with different clinical forms of SSc and in subjects presenting with a UCTD.

2. Patients and Methods

2.1. Study Design. A cross-sectional study was performed on consecutive patients with diagnosed or suspected SSc incoming the autoimmune outpatients' clinical department (San Gallicano Dermatology Institute) between January 2011 and December 2012. The diagnosis of cSSc was based on the presence of the Raynaud phenomenon associated to abnormal nailfold capillary examination and dermal skin thickness evaluated by clinical palpation of 17 areas of the body [19]. In limited disease (lcSSc) cutaneous involvement was distal to the elbows, knees, and clavicles; in diffuse disease (dcSSc) cutaneous involvement was present also in arms, chest, abdomen, back, or thighs according to the classification criteria established by Carwile LeRoy et al. [3, 20]. Blood samples were collected upon informed consent at the time of the first medical examination and consisted of 3 mL EDTA for flow cytometry analysis and 3 mL for serological assays.

2.2. Patients. The diagnosis of UCTD was based on clinical and serological manifestations suggesting a systemic connective autoimmune disease (arthralgias, Raynaud's disease, ANA reactivity different from antitopoisomerase I and anticentromere, disease duration) [7]. Forty-eight patients were examined in the study: 11 had dcSSc, 14 had lcSSc, and 23 were diagnosed as UCTD. Forty-five of them were women and 3 men. The median age and disease duration from diagnosis to blood sampling as well as a description of the clinical symptoms are summarized in Table 1. All patients were under therapy with 0,5–2 ng of iloprost/kg/min. The control group was composed by 15 healthcare workers, 12F/3 M, median age 53 yrs (range 36–64). All subjects were enrolled upon signature of written informed consent.

2.3. Autoantibodies. An immunofluorescence assay (IFA) (Kallestad HEp-2 cell line substrate, Bio-Rad Laboratories, Redmond, WA) was used to detect SSc-associated autoantibodies. An IFA titre greater than 1 : 80 dilution was considered positive and the specific fluorescence pattern was recorded. Sera were further analyzed by the Bioplex 2200 ANA screen system (Bio-Rad Laboratories, Hercules, CA), a fully automated system based on multiplexed bead technology, which allows the simultaneous detection of different autoantibodies directed toward a panel of autoantigens including dsDNA, chromatin, centromere-B, Scl-70, RNP (68 kDa, RNP-A), SSA (52 and 68 kDa), SSB, Sm, Sm/RNP, P ribosomal, and Jo-1. The sera were further tested by two distinct immunoblot assays (Euroline Systemic Sclerosis Nucleoli profile and Euroline Myositis profile, Euroimmun, Germany, resp.) against purified (Scl-70) or recombinant nuclear (CENP A and CENP B, SSA 52, Ku, Mi-2), nucleolar (Th/To, RNAP III, Fibrillarin, NOR 90, PM-Scl 75, PM-Scl 100), and cytoplasmic antigens (Jo-1, SRP, PL-7, PL-12, EJ, OJ), following the manufacturer's instructions.

2.4. Immunophenotyping. Peripheral blood lymphocyte subsets were examined by flow cytometry. Absolute lymphocyte counts were determined by BD FACSCanto II flow cytometer using BD FACSCanto clinical software v2.4 (BD Biosciences) after incubation of 50 uL of whole blood with 20 uL of a mix of monoclonal antibodies (anti-CD45/CD3/CD4/CD8/CD19/CD16-CD56, BD Multitest 6-color TBNK reagent; BD Trucount tubes). T regulatory cells were identified through surface expression of CD4/CD25 antigens and intracellular expression of FoxP3 (Human T reg Flow Kit, Biolegend, San Diego, CA).

Table 1: Clinical and demographic characteristics of patients.

	lcSSc (14)	dcSSc (11)	UCTD (23)
Sex F/M	14/0	8/3	23/0
Age range (median yrs)	41–66 (56)	35–80 (68)	18–73 (51)
Disease duration range (median yrs)	7–20 (12)	6–30 (24)	3–20 (8)
Raynaud's phenomenon P/N	14/0	11/0	23/0
Fingertip ulcers P/N	14/0	11/0	14/9
Pulmonary arterial hypertension P/N	0/14	7/4	2/21
Pulmunary fibrosis P/N	0/14	7/4	0/23
Therapy (iloprost)	Yes	Yes	Yes

2.5. Statistical Analysis. The results (i.e., different responses toward distinct antigens) were compared by contingency tables and X^2 values using the GraphPad Prism5 software (GraphPad Software, Inc., San Diego, CA, USA). The relationship between clinical parameters and serological profiles was assessed by Multiple Correspondence Analysis of data using the SPSS software (SPSS Statistics for Windows, Version 19.0. IBM Corp. Armonk, NY). Results of lymphocyte subsets, including T regulatory cells, were compared among the different subject groups by nonparametric test using the GraphPad Prism5 software (GraphPad Software, Inc., San Diego, CA, USA).

3. Results

3.1. Autoantibody Profiles. The frequency of antinuclear antibodies and the distribution of the different fluorescence patterns are shown in Table 2. All patients with lcSSc had antinuclear antibodies as determined by IFA. These were mainly directed against centromeric antigens (78,6%). Patients with dcSSc had antibodies against both nuclear and nucleolar antigens in 54,5% of cases while UCTD patients had speckled antinuclear (65,2%) or antinucleolar (21.7%) responses. The antibody profiles determined by Bioplex and immunoblot are summarized in Table 3. None of the collected sera had antibodies against fibrillarin, OJ, or EJ as well as Th/To antigens. As expected, a highly significant association was found between production of anti-Cenp A/B antibodies and lcSSc as well as between anti Scl-70 and dcSSc. A significantly high frequency of patients with dcSSc had also antibodies against PL-7/12 or SRP antigens (27.3% and 36.4%, resp.). Among UCTD patients two subgroups could be distinguished on the basis of anti-PM-Scl (26.0%) or anti-RNP (30.4%) antibodies. Multiple correspondence analysis (MCA), a descriptive and exploratory technique designed to analyze simple two-way and multiway data, was used to evaluate the possible association among the production of specific antibodies and the clinical outcome. These data, which are shown in Figure 1,

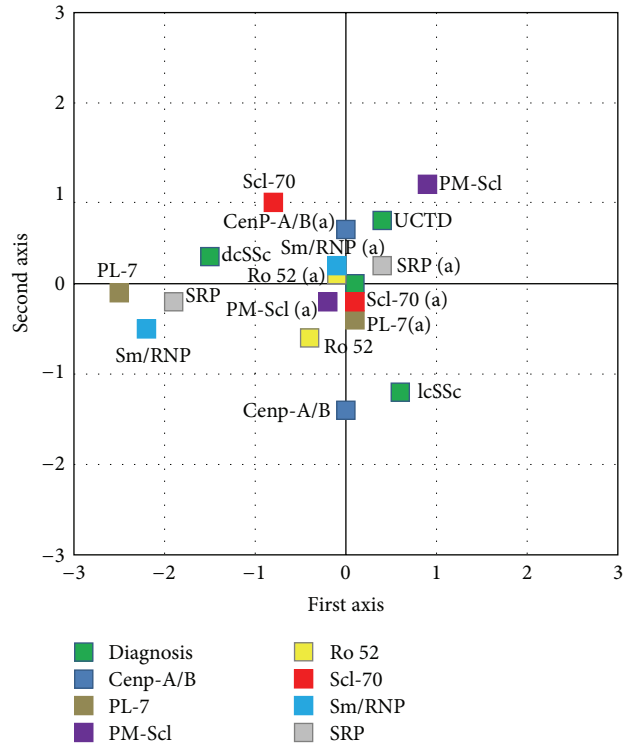

Figure 1: Multiple correspondence analysis showing the association between clinical and serological variables.

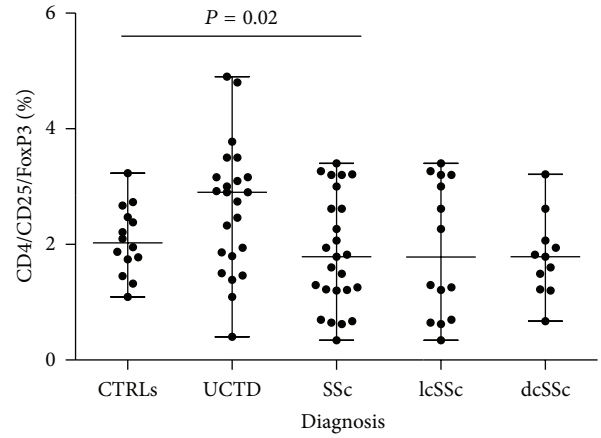

Figure 2: Levels of T reg cells (CD4+/CD25+/FoxP3+) in controls and patients with UCTD or cSSc are significantly different.

further confirmed the recognized association between lcSSc and anti-Cenp A/B as well as between dcSSc and anti-Scl70 antibodies. Interestingly, anti-PM/Scl responses were mainly associated to patients with UCTD.

3.2. Lymphocyte Subsets. The analysis of lymphocyte subsets is described in Table 4. The results showed that patients with SSc, either affected by lcSSc or dcSSc, have higher T cell counts and significantly lower frequencies of T reg cells (also depicted in Figure 2) as compared to UCTD

Table 2: Frequency of anti-nuclear antibodies and fluoroscopic patterns.

Anti-nuclear antibodies (ANA)	Diagnosis lcSSc (14)	dcSSc (11)	UCTD (23)	P
P/N	14 (100%)	8 (73%)	20 (87%)	
Titer (range)	1 : 5120 (1 : 640–1 : 5120)	1 : 5120 (1 : 80–1 : 5120)	1 : 320 (1 : 80–1 : 1280)	
Centromere	**11 (78.6%)**	1 (12.5%)	0	<0.0001
Nucleolar	3 (21.4%)	0	5 (25.0%)	n.s.
Homogeneous and nucleolar	0	**6 (75.0%)**	0	<0.0001
Speckled	0	0	**15 (75.0%)**	<0.0001
Mitotic Spindle	0	1 (12.5%)	0	n.s.

Table 3: Frequency of reactivity to nuclear, nucleolar, or cytoplasmic antigens.

Antibody reactivity	Diagnosis lcSSc (14)	dcSSc (11)	UCTD (23)	P
	P/N	P/N	P/N	
ANA (I.F.A.)	14/0	11/0	18/5	0.02
Cenp-A/B	11/3	3/8	0/23	<0.0001
Scl-70	0/14	6/5	0/23	<0.0001
Ro-52	2/12	3/8	1/22	
RNP	0/14	0/11	7/16	0.011
Mi-2	1/13	1/10	0/23	
PM/Scl	1/13	1/10	5/18	
NOR	2/12	1/10	2/21	
Ku	1/13	2/9	3/20	
RP	1/13	0/11	1/22	
PL-7-12	0/14	3/8	0/23	0.005
SRP	0/14	4/7	1/22	0.005
Jo-1	0/14	0/11	0/23	
≥2 antigens	4/7	8/6	3/20	0.017

patients or to healthy controls. To further explore the setting of T reg cells during the progression of the disease, the relationship between T reg cell levels and disease duration was examined (Figure 3). The results showed a significant correlation between disease duration and reduced T reg cell percentages (Figure 3(a)), although a strong statistical significance was found only in the group of patients with lcSSc (Figure 3(c)).

4. Discussion and Conclusions

In the present paper,we have assessed an expanded profile of autoantibodies and determined the levels of peripheral T reg cells in patients with SSc, including either limited or diffuse disease, and in patients presenting with clinical signs and symptoms of UCTD, in order to identify disease biomarkers useful for the clinical and therapeutic management. The results indicate that testing an extended antibody profile may provide possible advantages for the clinical classification of patients with SSc and UCTD, while assessing the levels of T reg lymphocytes might help monitoring disease progression. In fact, a multiparametric profiling of antibodies directed towards nuclear, nucleolar, and cytoplasmic antigens, including well-recognized molecules as well as less frequently studied antigens helped at identifying distinctively different clinical outcomes. The use of an extended panel of antigens, mainly related to PM or DM, has been previously proposed in order to identify SSc patients with overlapping syndromes [21]. None of our patients had clinical evidence of an overlapping syndrome (i.e., PM/SSc). However, anti-SRP and anti-PL7/12 antibodies proved useful to identify the most severe clinical forms of SSc, which, in turn, presented with the lowest levels of T reg cells. As previously described from different authors, we also found an association between anticentromeric antigens and lcSSc or antitopoisomerase-I and dcSSc [21]. In fact, according to the analysis of results from EULAR study, ACA and antitopoisomerasi are independent predictors of disease presentation/organ involvement [22]. However, in this latter clinical setting, corresponding to the most severe of the autoimmune disorders included in this study, we found also a significatively higher prevalence of antisignal recognition particle (SRP) and/or anti-PL-7/12 antibodies as compared to the lcSSc or the UCTD syndromes. SRP is a ribonuclear protein that regulates protein traslocation across the endoplasmic reticulum membrane during protein synthesis. Anti-SRP antibodies were initially found in patients with PM [20], and they have been subsequently recognized as myositis-specific antibodies [23]. However, the presence of anti-SRP antibodies has been occasionally reported in patients with other immunologic disorders, including SSc, however in the absence of an evident myopathy [24]. Nevertheless, skeletal muscle involvement represents a well-recognized characteristic of SSc [25–28]. Anti-PL-7, threonyl-tRNA synthetase, and anti-PL-12, alanyl-tRNA synthetase, antibodies have been previously implicated in the pathogenesis of the antisynthetase syndrome, a disease characterized by varying degrees of interstitial lung disease, myositis, arthropathy, fever, Raynaud's phenomenon, and mechanic's hands [29]. A recent analysis of the clinical profile of patients with connective tissue disease, anti-PL-12 autoantibodies suggest a strong association with idiopathic lung fibrosis rather than myositis or arthritis [30]. In the

Table 4: Peripheral lymphocyte subsets including T reg cells in patients and controls.

Diagnosis	Lymphocyte subsets						
	CD3+ n/mmc median value range	CD3+CD4+ n/mmc median value range	CD3+CD8+ n/mmc median value range	CD19+ n/mmc median value range	CD16+CD56+ n/mmc median value range	CD4/CD8 ratio median value range	CD4/CD25/FoxP3 % of CD4+ cells median value range
lcSSc	1538* 1004–2666	1196* 683–1939	351 221–727	250 99–763	162 54–475	3.05* 1.88–4.53	1.67* 1.21–3.27
dcSSc	1704* 952–3426	1317* 592–2637	506 170–938	159 45–1031	158 90–752	2.99 1.11–7.1	1.85* 1.22–3.21
UCTD	1308 490–2506	967 282–1830	437 143–694	181 36–866	186 25–397	2.30 0.56–5.11	2.90 0.4–4.90
CTRLS	1160 1300–1680	840 738–1040	504 272–760	231 100–310	294 100–810	2.10 1.2–2.9	2.10 1.1–3.2
P	<0.0001	0.0003	0.0800	0.0440	0.0860	0.0007	0.0030

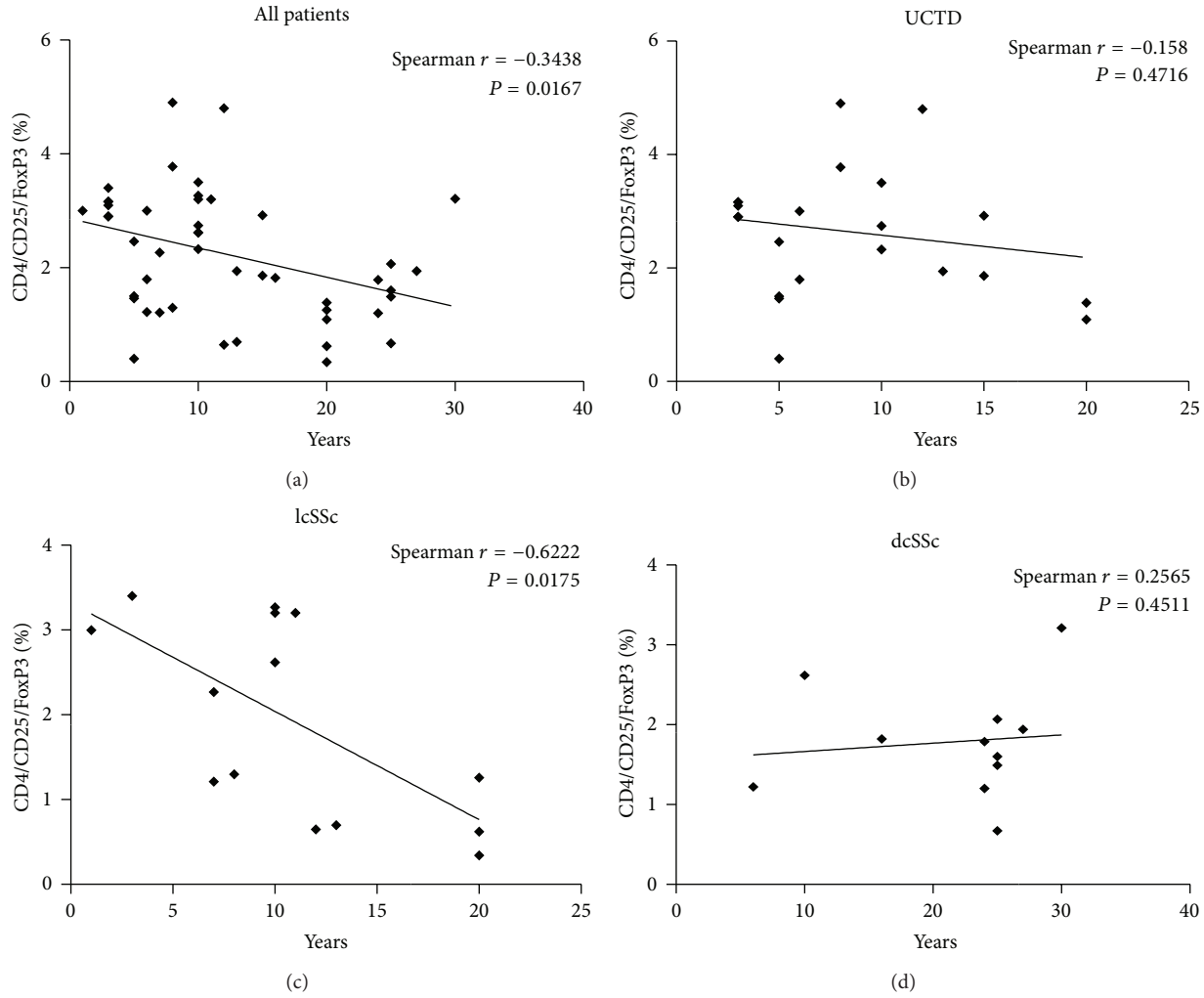

Figure 3: Correlation of T reg cell levels and disease duration in all patients (a), patients with UCTD (b), patients with SSc, limited (c), or diffuse (d) disease.

group of UCTD patients examined in the present study, two main autoantibodies were present: anti-RNP or anti-PM/Scl antibodies. Anti-RNP have been associated with the mixed connective tissue disease (MCTD) while anti-PM-Scl antibodies have been described in SSc patients with milder symptoms, eventually susceptible to evolve to SSc within 5–15 years from the onset of clinical symptoms [31]. However, these patients presented a stable clinical outcome characterized by the presence of typical RP/SSc-like capillaroscopic findings during a period of several years of followup. A clinical and laboratory followup over a more extended period could be necessary to establish or exclude the eventual evolution towards a definite connective disease.

The assessment of T reg lymphocytes may represent an important parameter to measure the immune dysregulation underlying autoimmune disorders. In fact, by either initiating or terminating the adaptive immune response [13], T reg cells may play a central role in the pathogenesis of autoimmunity. It has been shown that an increased expression of genes associated with SSc susceptibility and/or disease manifestations plays a major role also in the regulation of the immune system [32]. Furthermore, an inadequate number of T reg lymphocytes can lead to autoimmunity in humans, as it is clearly shown in patients with immune dysregulation, polyendocrinopathy, enteropathy, and X-linked (IPEX) syndrome, who completely lack T reg cells as results of a mutation in FoxP3 [33]. Indeed, recent studies aimed at assessing the number of T reg cells, pointed at a decrease of T reg frequencies in individuals with immunologic disorders such as rheumatic diseases, including scleroderma [34–36]. However, authors which previously afforded the analysis of T cell reg in SSc patients, underlined an alteration of T cell homeostasis in these patients with an increase of peripheral CD4 T cells and of T reg cells, correlated to disease severity [37, 38] or irrespective of disease phenotype but associated to an impaired regulatory function [39]. In the present study, the results of the analysis of lymphocyte subsets confirmed an increase of CD4 T cells, but in association with a decrease of the frequency of CD4 T reg lymphocytes in SSc patients. In fact, decreased percentages of T reg lymphocytes were found either in the limited or the diffuse disease, although in the dcSSc, which arise as severe clinical condition, their levels do not vary with disease duration, and in the lcSSc, which can be milder at the onset but advance with time, T reg levels decrease according to disease duration, suggesting a relationship between the setting of T reg cells and the progression of the disease. Of note, these results did not depend upon therapeutic intervention since all the patients examined in this study underwent the same therapeutic protocol (iloprost 0,5–2,0 ng/kg/min). This notion also suggests that such therapeutic intervention, though effective at ameliorating some clinical signs and symptoms, exerts a limited impact on regulatory immune circuitries, which appears to play a key pathogenic role and to be chronically and progressively altered in autoimmune disorders. This further emphasizes the need of novel therapeutic strategies capable of targeting regulatory cells/signals involved in the generation and termination of the adaptive immune response. In fact, although numerous studies have focused on the pathogenic mechanism of immune activation and tissue fibrosis in SSc [40] and potential therapeutic targets have been identified [41], efficacious treatment strategy for this disease has not been established yet.

Taken together, our data indicate the following: (i) dcSSc, which corresponds to a severe autoimmune disorder and is associated to the presence of several class of autoantibodies, presents with very low levels of T regs, which do not appear to depend upon disease duration; (ii) lcSSc, which is characterized by high titers of anti-centromere antibodies from the earlier clinical stages and generally starts with mild symptoms which progress during time, presents levels of T reg which are progressively lower in association with disease duration; (iii) UCTD, which is characterized by an undifferentiated onset, which could remain clinically undetermined for an indefinite time or may even show a clinical remission [8], presents with a reduced spectrum and low levels of autoantibodies and shows levels of T reg which are comparable to healthy controls. These observations support the notion that T reg lymphocytes may play a central role in the pathogenesis of SSc and that the determination of the levels of peripheral T reg cells may represent a useful tool in the diagnosis, prognosis, and monitoring of patients presenting with clinical signs and symptoms of autoimmune inflammatory diseases and might prove a key disease biomarker for the clinical and therapeutic management of major autoimmune disorders.

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Research Article

Dextromethorphan Inhibits Activations and Functions in Dendritic Cells

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Dendritic cells (DCs) play an important role in connecting innate and adaptive immunity. Thus, DCs have been regarded as a major target for the development of immunomodulators. In this study, we examined the effect of dextromethorphan (DXM), a common cough suppressant with a high safety profile, on the activation and function of DCs. In the presence of DXM, the LPS-induced expression of the costimulatory molecules in murine bone marrow-derived dendritic cells (BMDCs) was significantly suppressed. In addition, DXM treatment reduced the production of reactive oxygen species (ROS), proinflammatory cytokines, and chemokines in maturing BMDCs that were activated by LPS. Therefore, DXM abrogated the ability of LPS-stimulated DCs to induce Ag-specific T-cell activation, as determined by their decreased proliferation and IFN-γ secretion in mixed leukocyte cultures. Moreover, the inhibition of LPS-induced MAPK activation and NF-κB translocation may contribute to the suppressive effect of DXM on BMDCs. Remarkably, DXM decreased the LPS-induced surface expression of CD80, CD83, and HLA-DR and the secretion of IL-6 and IL-12 in human monocyte-derived dendritic cells (MDDCs). These findings provide a new insight into the impact of DXM treatment on DCs and suggest that DXM has the potential to be used in treating DC-related acute and chronic diseases.

1. Introduction

Dendritic cells (DCs), a highly specialized type of bone marrow-derived leukocytes that are important for the initiation of T-cell responses, link innate and adaptive immunity. They are present in different stages of maturation in the circulation as well as in lymphoid and nonlymphoid organs. DCs reside in an immature form in nonlymphoid tissues, where they act as sentinels [1–3]. After they capture and process antigens in peripheral nonlymphoid tissues, DCs migrate through afferent lymph to the T-cell-dependent areas of secondary lymphoid organs (e.g., lymph nodes), where they activate naive T-cell responses and undergo phenotypic and functional changes (i.e., maturation). The immunostimulatory properties of mature DCs include increased surface expression of major histocompatibility complexes (MHCs) with Ag-peptides and costimulatory molecules (e.g., CD40, CD80), increased secretion of cytokines and chemokines,

and reduced Ag uptake [4, 5]. While mature DCs can potently initiate primary T-cell-mediated immune responses, immature DCs stimulate T-cell responses only weakly or may even promote the generation of regulatory T (Treg) cells [6].

Because pharmacological modulation of DC activation prevents the development of several T-cell-mediated diseases [7], DCs may represent a new therapeutic approach for treating harmful immune responses such as hypersensitivity reactions and autoimmunity [8, 9]. Notably, the clinical efficacy of corticosteroids and other antirheumatic drugs, such as gold sodium thiomalate, leflunomide, mycophenolic acid, and valproic acid, may be due to their significant disruption of DC function [10–15].

Dextromethorphan (d-3-methoxy-17-methylmorphinan, abbreviated DXM), a dextrorotatory morphinan, is widely and clinically used as an antitussive. There is an increasing evidence that DXM has anti-inflammatory and immunomodulatory effects. DXM protects mice against lipopolysaccharide/GalN-induced endotoxemia and liver damage; the mechanism of protection may involve faster TNF-α clearance, decreased superoxide production, and decreased expression of genes associated with inflammation and hepatocellular death [16]. In addition, DXM prevents moderate experimental autoimmune encephalomyelitis by inhibiting the NOX2-mediated production of ROS and decreasing the infiltration of monocytes and lymphocytes into the spinal cord [17]. DXM reduces Group A Streptococcal (GAS)-induced systemic inflammatory responses and organ injury in mice [18]. Furthermore, DXM reduces cytokine and superoxide production in macrophages by inhibiting NAPDH oxidase, resulting in decreased atherosclerosis and neointima in mice [19]. DXM attenuates oxidative stress and inflammation markers in habitual smokers [20]. Because the cellular targets of DXM in the immune system have yet to be studied, the role of DXM in the cellular maturation and immunoregulatory activity of DCs is an open question.

In this study, we examined the potential effects of DXM on the maturation and functional properties of DCs. We found that DXM inhibited the LPS-induced functional maturation of murine BMDCs and human MDDCs. In addition, DXM downregulated the LPS-induced MAPK signaling pathways (ERK1/2, JNK, and p38 MAPK), IκB expression, and NF-κBp65 nuclear translocation. Taken together, these results suggest that DXM manipulates the immunostimulatory properties of DC and may have important applications against harmful immune responses such as chronic inflammation, autoimmunity, and transplantation.

2. Material and Methods

2.1. Mice and Preparation of Bone Marrow-Derived Murine DCs. Five- to eight-week-old specific pathogen-free female C57BL/6 (H-2^b) mice were purchased from the National Laboratory Animal Center (Taipei, Taiwan) or the National Cheng-Kung University (Tainan, Taiwan). OT-I TCR transgenic mice were purchased from Jackson Lab (Bar Harbor, ME, USA), and OT-II TCR transgenic mice were provided by Dr. Clifford Lowell (UCSF, San Francisco, CA, USA). All mice were housed in the barrier facility at Taichung Veterans General Hospital (Taichung, Taiwan) in accordance with the Institutional Animal Care and Use Committee guidelines for animal experimentation. Murine bone marrow-derived DCs were generated as previously described [21]. Briefly, femurs and tibias were aseptically removed from mice. After the surrounding muscle tissue was removed, the bones were placed in a 10 mm dish with 70% alcohol for 1 min, washed twice with phosphate-buffered saline (PBS), and transferred into a fresh dish with RPMI 1640 medium. Both ends of the bones were cut with scissors, and the marrow was flushed with RPMI 1640 using a syringe and a 25-gauge needle. The red cells were lysed with ammonium chloride. Bone marrow cells ($5–7 \times 10^5$ cells/mL) were suspended in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum, 2 mm L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 5×10^5 M 2-ME, 10 mM HEPES (pH 7.4), 20 ng/mL recombinant murine granulocyte macrophage colony-stimulating factor (PeproTech), and 20 ng/mL recombinant murine IL-4 (PeproTech). Cells were placed in 6-well plates. The culture medium was changed every 2 days, and nonadherent or loosely adherent cells were harvested on day 7 and used as immature DCs. More than 80% of the cells expressed CD11c, as determined using flow cytometry. $CD11c^+$ DCs were further selected from BM cells with CD11c (N418) microbeads (Miltenyi Biotec), according to the manufacturer's instructions, and these cells were used for the experiments. The purity of the $CD11c^+$ cells was >90% (data not shown).

2.2. Generation of Human Monocyte-Derived DCs. DCs were prepared from peripheral blood monocytes (PBMCs) by standard procedures. Briefly, peripheral blood was collected from healthy volunteer donors, and PBMCs were isolated from peripheral blood buffy coats by magnetic cell sorting with anti-CD14 MicroBeads, per the manufacturer's protocol (Miltenyi Biotec). The purity of the CD14+ fraction was always >90%, as assessed using flow cytometry. Purified monocytes were seeded in 6-well plates and cultured in complete medium (RPMI 1640 (Gibco) containing 10% FBS (Gibco)), recombinant human 80 ng mL^{-1} GM-CSF (PeproTech), and 100 ng mL^{-1} IL-4 (PeproTech) to generate immature DCs. Every two days, fresh medium containing GM-CSF and IL-4 was added to the cells. After 7 days of culture, nonadherent or loosely adherent cells were harvested, washed once with PBS, and used for the experiments.

2.3. Flow Cytometry Analysis. The expression of cell surface molecules was quantified by flow cytometry as follows. DXM hydrobromide hydrate was purchased from Sigma-Aldrich, and a 12.5 mM stock solution was made with PBS. Aliquots of 2×10^5 immature BMDCs or MDDCs were cultured in the presence or absence of DXM for 1 h and then stimulated with 100 ng/mL *Escherichia coli* serotype O26:B6 LPS (Sigma) or 100 ng/mL LPS plus 10 ng/mL IFN-γ (PeproTech) for 18 h. The control group was treated with PBS alone. After incubation, DCs were harvested and stained with

the following antibodies for 45 min on ice (1 μg/mL diluted in PBS/1.0% FCS (v/v)): FITC-conjugated anti-human CD1a$^+$ or anti-murine CD11c$^+$; phycoerythrin (PE)-conjugated anti-human CD80$^+$, anti-human CD83$^+$, anti-murine CD40, anti-murine CD80, anti-murine CD86, anti-murine MHC class I, anti-murine MHC class II or isotype-matched control mAbs (all of the above from Biolegends); or PE-conjugated anti-human HLA-DR (BD Pharmingen). After washing with PBS, the cells were analyzed in a FACSCalibur flow cytometer (BD Biosciences), and the data were analyzed using WINMDI software (Scripps, La Jolla, CA, USA).

2.4. Cytokine Assay. Supernatants were collected from DCs (1×10^6/mL) propagated in the presence or absence of DXM for 1 h. The cells were then stimulated with 100 ng/mL LPS or 100 ng/mL LPS plus 10 ng/mL IFN-γ or other TLR ligands, including Pam3CSK4 (5 μg/mL, TLR1/TLR2), PolyI:C (250 μg/mL, TLR3), flagellin (500 ng/mL, TLR5), and CpG ODN 1826 (200 nM TLR-9) (all from InvivoGen) for 18 h (6 h for TNF-alpha and RANTES). The control group was treated with PBS alone. After incubation, cytokine and chemokine production in DC supernatants was determined using sandwich ELISA assays, according to the manufacturer's specifications (PeproTech).

2.5. Measurement of Reactive Oxygen Species (ROS). ROS generation was measured after staining the cells with the oxidative sensitive dye 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (DCFDA, Molecular Probes). For this assay, DC cells at a density of 3×10^5 cells/mL were cultured in the presence or absence of DXM (50 μM) for 1 h followed by stimulation with 100 ng/mL LPS. The control group was treated with PBS alone. After LPS stimulation for 6 h, the medium was removed, and culture medium containing 5 μM DCFDA was added under low-light conditions. The cells were incubated for 30 min at 37°C, and the amount of ROS was analyzed by flow cytometry as described above.

2.6. DXM Cytotoxicity Assay. To determine cell viability and apoptosis, murine BMDCs and human MDDCs (2×10^5 cells/mL) were cultured in the presence or absence of DXM for 1 h and stimulated with 100 ng/mL LPS for 18 h. The control group was treated with PBS alone. Cell viability was determined using the CCK-8 colorimetric assay according to the manufacturer's instructions (Sigma). To detect apoptosis, cell death was measured by flow cytometry using a phycoerythrin-conjugated Annexin V detection kit-I (BioVision) per the manufacturer's protocol. Flow cytometry was performed as described above by gating on 2×10^4 CD11c$^+$ DC cells per sample.

2.7. OVA-Specific T-Cell Activation. The protocol was modified from our previous report [22]. Briefly, purified DCs were pulsed with 2 μg/mL $OVA_{257-264}$ ($OVAP_1$) or $OVA_{323-339}$ ($OVAP_2$) (synthesized by Echo Chemical Co., Taiwan) and incubated with LPS (100 ng/mL), DXM (50 μM), or LPS plus DXM for 18 h. After incubation, the cells were harvested and washed with PBS. $OVAP_1$ specific CD8$^+$ T cells and $OVAP_2$ specific CD4$^+$ T cells were positively enriched from the spleens of OT-1 and OT-2 mice using the EasySep Murine CD8a or CD4 positive selection kits, respectively, according to the manufacturer's protocols (stem cells). The cells were more than 90% pure, as determined by flow cytometry with FITC-conjugated anti-CD4 and CD8 mAbs (Biolegends). Purified T cells (2×10^5) and different treated DCs were added at various DC : T-cell ratios to 96-well round-bottom plates. After 3 days, T-cell proliferation was measured. [^{3}H] thymidine (1 μCi; GE Healthcare) was added to the culture, and after an overnight incubation period, the incorporated [^{3}H] thymidine was quantified by liquid scintillation counting (β-Counter; Beckman). In addition, supernatants from the DC—OT-I/OT-II cultures were collected after 3 days, and their IFN-γ levels were measured using an ELISA kit (eBioscience).

2.8. Preparation of Nuclear Extracts and Western Blot Analysis. Briefly, purified DCs were cultured in the presence or absence of 50 μM DXM for 1 h and stimulated with LPS (100 ng/mL). Whole-cell lysates were prepared at the indicated time points, as described previously [23]. Nuclear extracts were prepared using the NE-PER nuclear and cytoplasmic Extraction system (Pierce), per the manufacturer's instructions. All of the steps in the preparations included the protease inhibitors leupeptin (Sigma-Aldrich) and aprotinin (Sigma-Aldrich) at 10 μg/mL. Protein concentrations were determined using a BCA protein assay kit (Pierce). Protein extracts were boiled, resolved by SDS-PAGE and electrotransferred to nitrocellulose membranes. After blocking in 10% milk in TBS, the membranes were incubated with antibodies for phospho-p38 (Thr180/Tyr182), p38, phospho-p42/44 (Thr202/Tyr204, 20G11), total p42/44 (137F5), phosphor-JNK (81E11), JNK, anti-IκB (56G8), anti- NF-κB p65 (C22B4) (all purchased from cell signaling) or anti-Lamin B (M-20), anti-IDO Ab (mIDO-48) were purchased from Santa Cruz Biotechnology. The membranes were then washed, incubated with horseradish peroxidase-labeled secondary Abs (Jackson ImmunoResearch, West Grove, PA, USA), developed with enhanced chemoluminescence (Amersham), and analyzed with the LAS3000 system (Fujifilm, Tokyo, Japan). Densitometric analysis was performed with ImageJ software (National Institute of Health, Bethesda, MD, USA).

2.9. Statistical Analysis. The results are expressed as the mean ± SD. Statistical analyses were performed by one-way ANOVA, followed by Tukey's post-hoc test (Graphpad Prism 4.0, GraphPad Software). P values < 0.05 were considered statistically significant.

3. Results

3.1. DXM Affects the Expression of Cell Surface Molecules in LPS-Stimulated Murine BMDCs. In the first series of experiments, we investigated the effects of DXM on the maturation of immature DCs. Immature BMDCs were cultured in the presence of DXM (12.5, 25, 50, and 100 μM) and then exposed

to bacterial LPS which is a strong inducer of DC maturation. In general, DC maturation is accompanied by the enhanced expression of surface molecules, including costimulatory molecules and major histocompatibility complex molecules (MHC) that mediate adhesion with T cells by stabilizing the DC/T cells contact zone. Consistent with previously published data, the LPS stimulation of BMDCs resulted in the significant upregulation of costimulatory molecules (CD80, CD86 and CD40) and major histocompatibility complex molecules (MHC class II, MHC class I) within 18 h. While the DXM inhibition of LPS-induced maturation was dose-dependent, the expression of CD80, CD86, CD40, MHC class I, and MHC class II was significantly lower in the presence of DXM than in untreated mature BMDCs cells (Figure 1). These effects were not due to an increase in the number of dead cells (as determined by CCK-8 or flow cytometry with Annexin V); there was no marked difference in the percentage of dead cells in cultures containing 100 μM DXM or PBS-treated controls (Supplemental Figure 1(a) available online at http://dx.doi.org/10.1155/2013/125643). These observations suggested that DXM impaired LPS-induced DC phenotypic maturation.

3.2. DXM Modulates Cytokine, Chemokine, and ROS Production in LPS-Stimulated BMDCs. Mature DCs secrete cytokines and chemokines that modulate inflammatory responses and adaptive immunity [24]. We examined whether DXM altered cytokine and chemokine secretion in LPS-stimulated BMDCs. First, we examined changes in the BMDC TNF-alpha production, which is a hallmark of DC activation. TNF-alpha was quantified using ELISA for supernatants that were collected from LPS-triggered BMDCs propagated in the presence or absence of DXM. Figure 2 shows that unstimulated immature BMDCs did not produce detectable levels of TNF-alpha. As expected, BMDCs started producing a large amount of TNF-alpha after stimulation with LPS, but DXM pretreatment led to dose-dependent significant decreases in TNF-alpha production. The secretion of other proinflammatory cytokines (e.g., IL-6, IL-12) and chemokines (e.g., MCP-1, MIP-1 alpha, and RANTES) was also inhibited by DXM. IL-12 production is an important marker for DC maturation and can be used to select Th1-dominant adjuvants (Figure 2). Additionally, increased levels of reactive oxygen species (ROS) are involved in the activation of DCs by different stimuli, and antioxidants inhibit DC activation [25]. To assess the potential intracellular mechanisms for DXM inhibition of DC maturation, we analyzed ROS levels in BMDCs pretreated with DXM and matured with LPS, which is known to increase ROS in DCs [25]. As expected, ROS levels were increased following treatment with LPS (Figure 3). However, treatment with DXM reduced LPS-induced ROS in BMDCs. These results further suggest that DXM attenuates the maturation and immunostimulatory activity of DCs activated by LPS.

3.3. DXM Inhibits the Ability of LPS-Stimulated BMDCs to Stimulate OVA-Specific T-Cell Proliferation. Because the critical function of mature DCs is to activate T-cell proliferation, we determined whether DXM-treated BMDCs could induce antigen-specific $CD4^+$ and $CD8^+$ T-cell responses. $OVA_{257-264}$ ($OVAP_1$) or $OVA_{323-339}$ ($OVAP_2$) peptide-loaded immature BMDCs were preincubated in the presence or absence of DXM, stimulated with LPS, and tested for their ability to stimulate allogeneic OVA-specific $CD4^+$ OT-II or $CD8^+$ OT-I T cells. T-cell proliferation was measured by [^{3}H] thymidine incorporation. Coculture with LPS-stimulated BMDCs effectively enhanced $CD4^+$ OT-II and $CD8^+$ OT-I T-cell proliferative responses, but this proliferation was reduced by DXM (Figure 4). Because IFN-γ is produced by activated T cells, IFN-γ in the culture supernatants was measured using ELISA. As shown in Figure 5, DXM treatment reduced the IFN-γ produced by activated $CD4^+$ and $CD8^+$ T cells. Thus, DXM attenuated the ability of DCs to activate Ag-specific T-cell immune responses.

3.4. DXM Suppressed MAPK and NF-κB Pathways in LPS-Stimulated BMDCs. The activation of MAPKs and NF-κB is crucial for DC maturation and the inflammatory response [26]. The LPS stimulation of TLR-4 signaling activates MAPKs and NF-κB signal pathways, resulting in DC maturation [27, 28]. To explore the molecular mechanisms of the DXM inhibitory effect, we determined whether MAPKs and NF-κB activation were altered by DXM in LPS-stimulated BMDCs. DXM treatment blocked the phosphorylation of MAPKs ERK, p38, and JNK but did not affect the level of unphosphorylated proteins (Figure 5(a)). To determine whether DXM decreased NF-κB activation, the expression of Iκb protein and nuclear translocation of NF-κB p65 were measured. Iκb is known to be an inhibitor of NF-κb and can form a complex with the NF-κb, thereby preventing nuclear translocation of NF-κb. Under partial external stimulus such as LPS, Iκb undergoes phosphorylation and degradation, thereby unlocking NF-κb and resulting in the nuclear translocation of NF-κb and the activation of related signaling pathways. As shown in Figure 5, in LPS-stimulated BMDCs, DXM treatment prevented downregulation of Iκbα protein (Figure 5(a)) and decreased NF-κB p65 nuclear localization (Figure 5(b)). These results suggest that DXM inhibits LPS-induced DC activation, possibly by disrupting the MAPK and NF-κB pathways.

3.5. DXM Affects the Expression of Surface Markers and Cytokine Secretion in Human Monocyte-Derived DCs (MDDCs). In addition to murine BMDCs, we examined whether DXM regulates LPS-induced surface molecule expression and cytokine production in human MDDCs. MDDCs were cultured in the presence or absence of DXM for 1 h and then stimulated with LPS (100 ng/mL) plus IFN-γ (10 ng/mL). Immature MDDCs stimulated with LPS plus IFN-γ released IL-6 and IL-12. The release of these cytokines was suppressed by incubation with DXM (Figure 6(a)). We also analyzed the effect of DXM on the expression of DC surface activation markers. The LPS stimulation of MDDCs resulted in the upregulation of CD80, CD83, and HLA-DR; however, this upregulation was significantly inhibited by

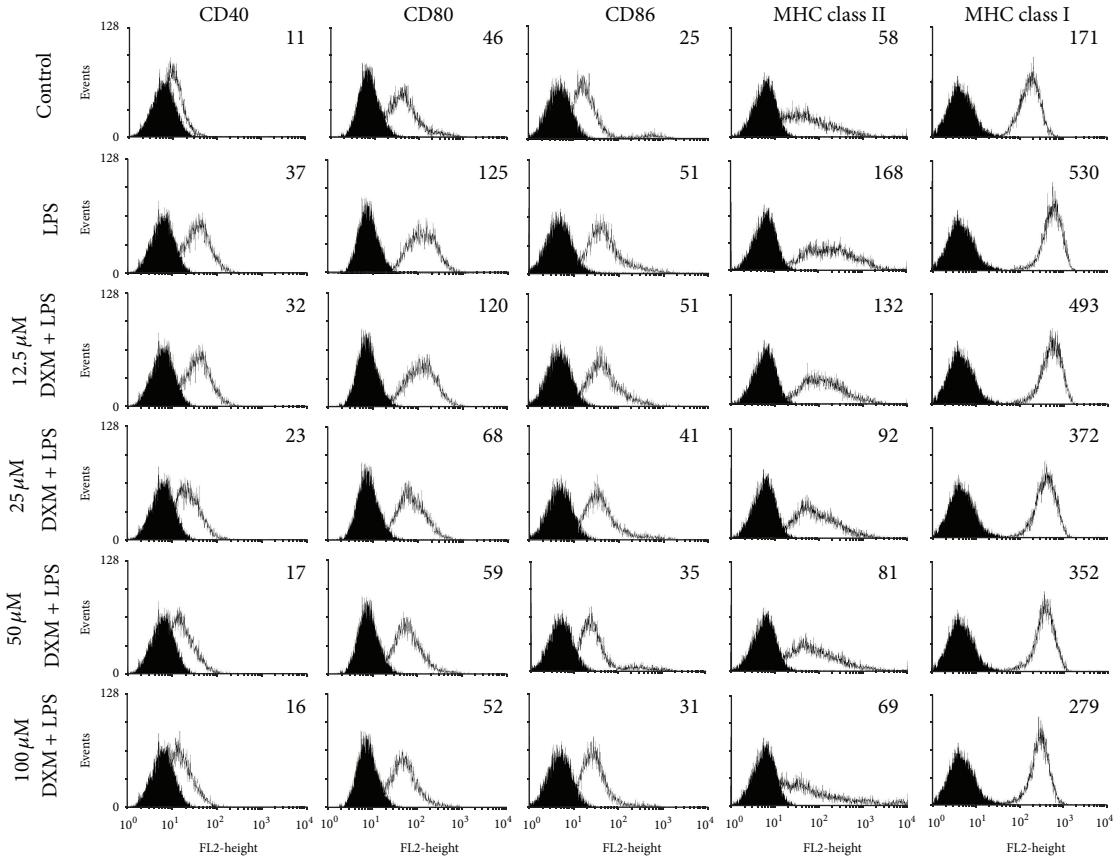

Figure 1: DXM reduces the expression of immunomodulatory cell surface markers in LPS-induced BMDCs. Immature BMDCs were stimulated with 100 ng/mL LPS with or without DXM for 18 h. Control groups were treated with PBS alone. After incubation, the expression of the surface markers CD40, CD80, CD86, MHC class I and MHC class II was analyzed by flow cytometry with fluorescently labeled Abs. The gray-filled area represents staining with an isotype-matched control Ab. The geometric mean fluorescence intensity (GMFI) of LPS or LPS+DXM is indicated. All data are representative of three independent experiments showing similar results.

DXM (Figure 6(b)). Also, these inhibited effects were not due to cytotoxicity of DXM, because there were no marked difference in the cell viability and percentage of Annexin V^+/dead cells in cultures containing DXM or PBS-treated controls (Supplemental Figure 1(b)).

4. Discussion

Because DCs can initiate primary T-cell responses, they form a crucial interface between innate and adaptive immunity. Potential interference with this essential cell type might affect the pharmacological profile of an immunosuppressive drug [10–14]. In this study, we examined the activity of DXM, a widely used antitussive, on the immune function of DCs. We showed that DXM interfered with DC maturation, as measured using costimulatory molecules, cytokine, reactive oxygen species (ROS), and stimulation of allogeneic T cells. This is the first study to report that DXM has an immunomodulatory effect on DCs.

The NF-κB signaling pathway is critical for DC maturation and cytokine production [28]. The NF-κB signaling pathway includes several important molecules such as NF-κB, IκB, and IκB kinase [29]. DXM inhibits LPS-induced IκBα degradation and the nuclear translocation of p65 in human endothelial cells [30]. MAPK signaling pathways have also received attention as molecular targets for DC therapies [26–28, 31, 32]. The minimal MAPK cascade consists of a three kinase core where an MAP3 K (MAP2 K kinase) activates a MAP2 K (MAPK kinase) that activates an MAPK (ERK, JNK, p38), resulting in the activation of NF-κB pathways that contribute to cell growth, survival, and antiapoptosis [33]. In this study, we showed that DXM decreased NF-κB and MAPK (ERK, p38, JNK) activation in LPS-treated BMDCs (Figure 5), and this inhibitory effect was associated with DC maturation.

Reactive oxygen species (ROS) are also known to have important signaling properties, including activation of NF-κB and MAPK signaling in many cell types [34]. ROS are also known to influence the production and secretion of cytokines; after exposure to ROS, DCs more efficiently present antigens [25]. Previous studies have shown that DXM has antioxidant properties in many cell types [16, 19, 35–37]. In this study, we investigated whether DXM could affect ROS formation during the process of LPS-stimulated dendritic cell maturation. Our results confirmed that DXM inhibited

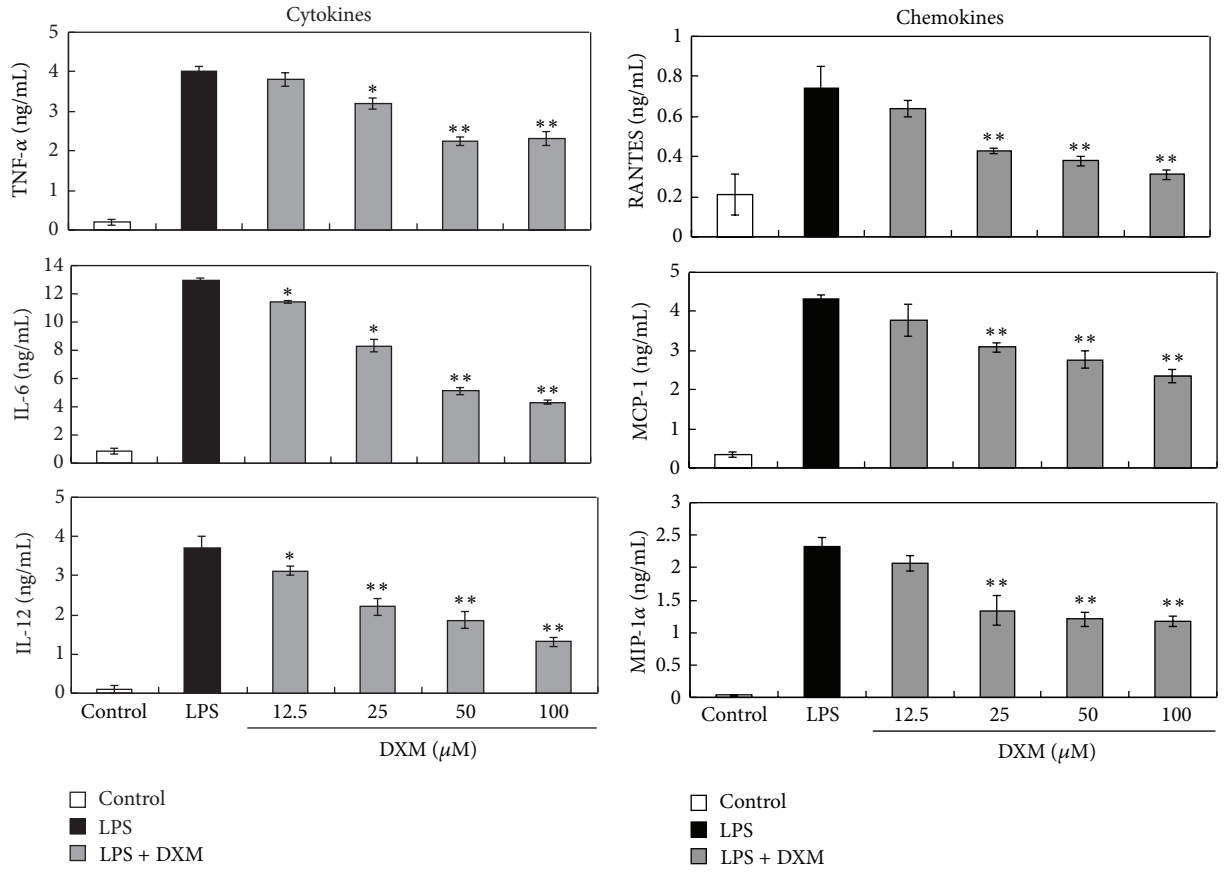

Figure 2: DXM impaired the release of cytokines and chemokines from LPS-stimulated BMDCs. Immature BMDCs were stimulated with 100 ng/mL LPS with or without DXM. The control group was treated with PBS alone. Culture supernatants were collected after 18 h (4 h for TNF-alpha and RANTES), and cytokines and chemokines were quantified by ELISA. Data are presented as the means ± SD of samples from three wells. Significant differences between DXM-treated and untreated LPS-activated BMDCs are shown with asterisks ($^{*}P < 0.05$, $^{**}P < 0.01$). All data are representative of three independent experiments.

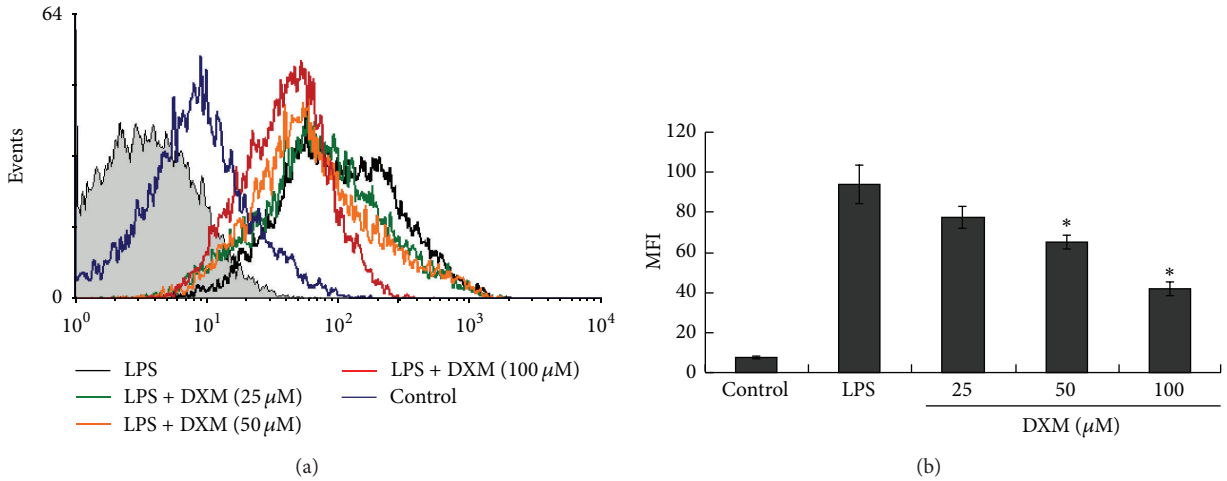

Figure 3: ROS production in LPS-stimulated BMDCs was impaired by DXM. Immature BMDCs were stimulated with 100 ng/mL LPS with or without DXM for 18 h. The control group was treated with PBS alone. After incubation, the cells were harvested, stained with DCFDA, and analyzed by flow cytometry. The mean fluorescence intensities for ROS generation were tabulated. The data are represented as the mean ± SD in triplicate tests. All data are representative of three independent experiments. Significant differences between DXM-treated and untreated LPS-activated BMDCs are shown with asterisks ($^{*}P < 0.05$).

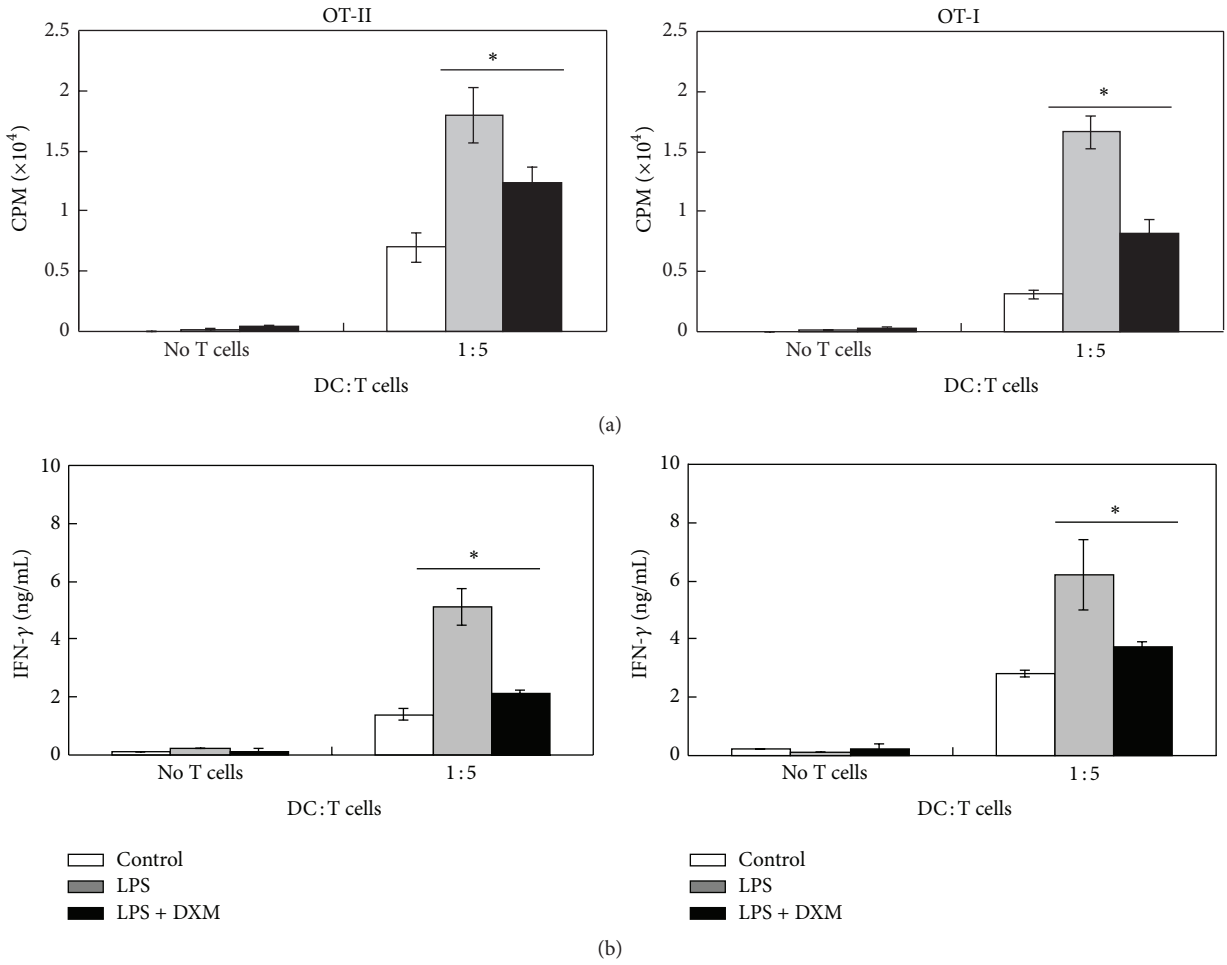

Figure 4: DXM inhibits Ag-specific T-cell activation by LPS-stimulated BMDCs. (a) Either OT-I $CD8^+$ T cells or OT-II $CD4^+$ T cells were cocultured with BMDCs pulsed with OVA peptide and treated with PBS, LPS (100 ng/mL) + PBS, or LPS + DXM (50 μM) at the indicated ratio of DC : T cells for 3 days. The cells were exposed to [^{3}H]-thymidine for 18 h before cell-associated radioactivity was determined. (b) Supernatants were collected from cultures after 4 days. IFN-γ production was measured by ELISA. The data shown are the mean ± SD of samples of three wells. Significant differences between DXM-treated and untreated LPS-activated BMDCs are shown with asterisks ($^*P < 0.05$). All data are representative of three independent experiments.

ROS production in LPS-stimulated BMDCs. Although the underlying mechanism remains unclear, suppressed ROS production due to the inhibition of NOX2, iNOS, or NADPH oxidase expression and activity is possible [16, 19, 35, 38]. NOX2- and NADPH oxidase-deleted dendritic cells cannot be induced to mature [39, 40]. Further investigation of the influence and possible mechanism of action of DXM on NOX2 and NADPH oxidase in dendritic cells is necessary.

Because activated DCs regulate T-cell responses, the type of cytokines that they release may determine whether $CD4^+$ T cells mature into Th1, Th2, Th17, or Treg cells [41]. IL-12 drives T helper type 1 (Th1) responses, whereas IL-4 promotes Th2-type responses [42]. We observed that DXM significantly inhibited LPS-induced IL-12 production in murine and human DCs (Figures 2 and 6). In addition, we showed that LPS-stimulated OVA peptide-pulsed BMDCs skewed naive OT-II T cells toward IFN-γ-producing T cells, but OT-II T cells stimulated with OVA-pulsed BMDCs exposed to DXM produced lower levels of IFN-γ (Figure 4). Because IFN-γ is a major product of Th1 cells [43], these results suggest that DXM may be effective in several Th1-dominant chronic inflammatory diseases, such as multiple sclerosis (MS), diabetes, and rheumatoid arthritis (RA) [44].

The present study used the TLR-4 ligand LPS to stimulate DC maturation. LPS induces strong Th1-like responses but not Th2 immune responses [45]. We did not observe IL-4 expression in DCs after LPS stimulation (data not shown). However, we cannot exclude the possibility that DXM affects Th2 responses. Therefore, substances capable of stimulating Th2 immune responses, such as dust mite allergens [46], should be used in future investigations of the effects of DXM on DC-mediated Th2 responses.

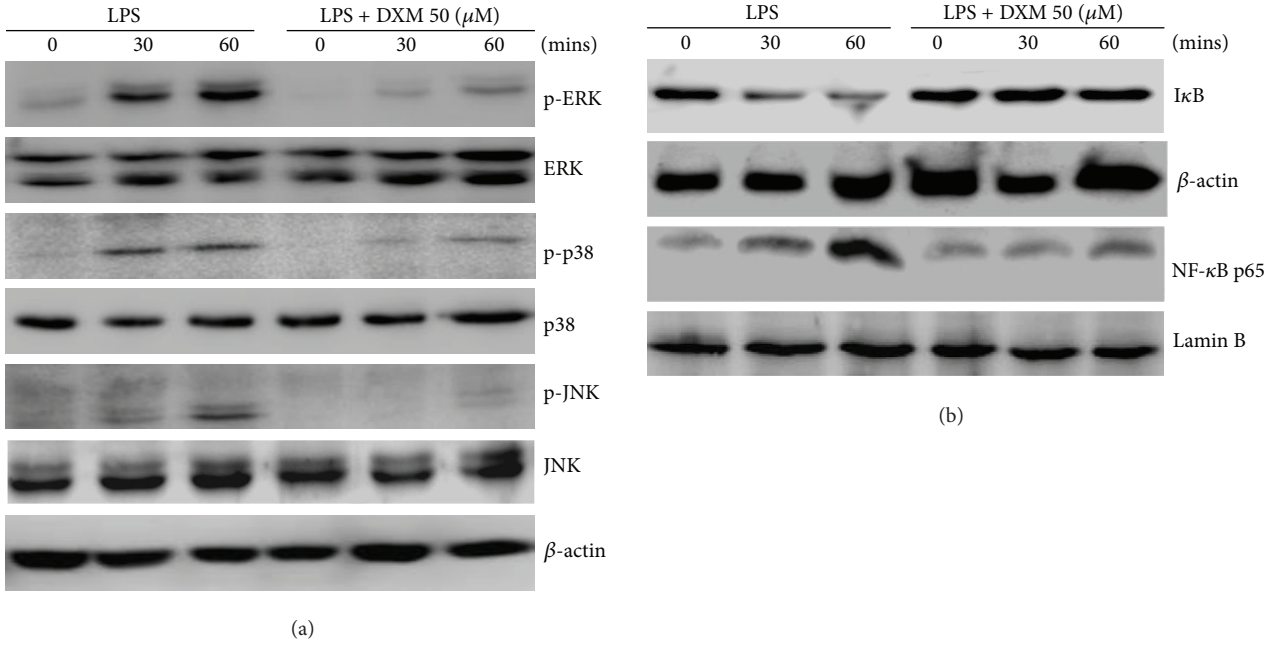

Figure 5: DXM inhibition of MAPK and NF-κB activation in BMDCs. Immature BMDCs were stimulated with 100 ng/mL LPS with or without DXM and lysed at the indicated time points. (a) The ERK, JNK, and p38 MAPK (native and phosphorylated) in whole cell lystes. (b) IκBα in whole-cell lystes and NF-κB p65 in nuclear extracts as mentioned were determined by Western blot with antibodies. β-actin and Lamin B loading controls are also shown to demonstrate relatively equal protein load across all lanes. The data are representative of three independent experiments showing similar results.

We also found that DXM suppressed TNF-alpha expression when it was given before or after LPS stimulation (Supplemental Figure 2), implying that the anti-inflammatory and immunomodulatory effects of DXM could be used for prevention or treatment purposes. Although LPS was the main stimulus used for DC maturation in this study, we also tested whether DXM could modulate the activation of immature BMDCs by other TLR ligands and applied Pam3CSK4, PolyI:C, flagellin, and CpG ODN ligands for TLR1/TLR2, TLR3, TLR5, and TLR9, respectively. The presence of each substance resulted in the release of the proinflammatory cytokine TNF-alpha. This release was completely inhibited by 50 μM of DXM (Supplemental Figure 3). Although the mechanism of DXM interference with DC activation after TLR ligand stimulation is not entirely clear, we suggest that it may be related to the inhibitory effect of DXM on MAPK and NF-κB activation. NF-κB is required for DCs to secrete inflammatory cytokines after they are stimulated with various TLR ligands [29]. Further investigation of the effect of DXM on DC maturation through other non-TLR pathways such asflt3 or c-kit ligands [47] or GM-CSF, IL-1β, and IL-7 (FKGm17) cytokine stimuli is necessary.

Increased IDO expression in DCs can cause T-cell apoptosis via tryptophan starvation [48]. IDO expression in DCs may be related to the differentiation of Treg cells [49, 50]. We found that DXM at 50 μM did not induce IDO expression or alter LPS-induced IDO expression (Supplemental Figure 4(a)). Previous studies reported that IL-10 inhibits effector T-cell responses and may induce Tr1 regulatory T-cell differentiation [51, 52]. In this study, ELISA indicated that no significant alteration in IL-10 expression was found in BMDCs treated with or without LPS (Supplemental Figure 4(b)). Based on these results, we suggest that the T-cell-inhibitory effect of DXM might occur via the suppression of surface costimulatory receptor expression and cytokine release. Further analysis of more immunomodulatory factors, such as high levels of PD-L1 (programmed death-1 ligand), retinoic acid (RA), TGF-beta, or other factors capable of activating Treg differentiation or activation is required [53, 54].

The clinical dose of DXM for adult human is 60–120 mg/kg/day, and the peak concentration of DXM is about 8–16 μM in serum after administration [55]. Another report describes that the maximum concentration of DXM is about 0.8–9.64 mg/kg (as high as 2 μM) in the serum of neurosurgery patients; however, the concentration of DXM in brain can be 68-fold higher than that in serum [56]. In our in vitro study, we found that 12.5 μM–50 μM DXM can attenuate the LPS-induced murine and human DC activation, which dosage is possible in physiological condition, suggesting that DXM may have a potential to modulate DC function in vivo.

5. Conclusion

In summary, we provided evidence for a novel cellular target of DXM in alloimmune responses in addition to its well-known T-cell inhibitory capacity. Because of its potent effects

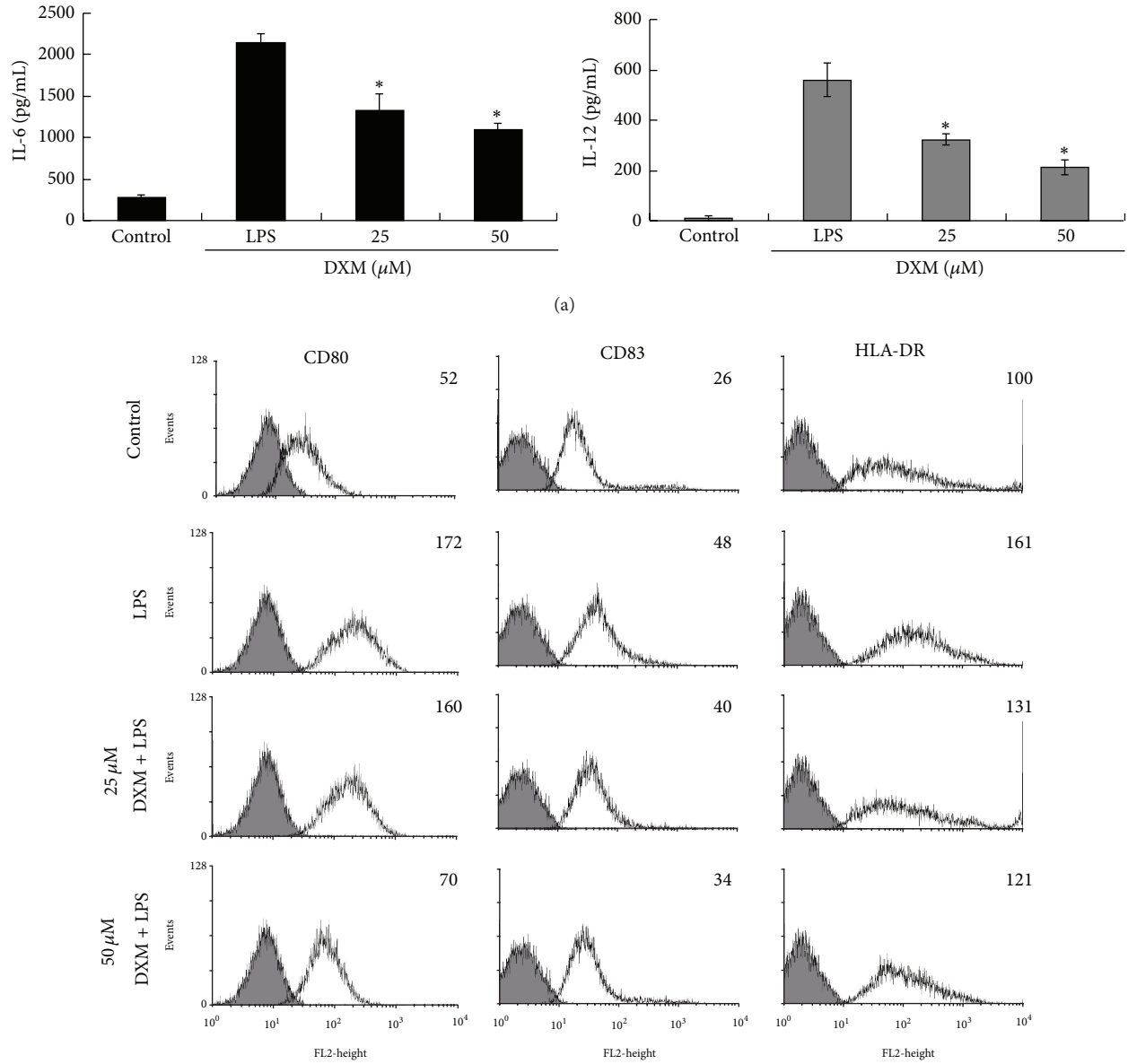

Figure 6: DXM inhibited human MDDC activation. Immature MDDCs were treated with LPS (100 ng/mL) + IFN-γ (10 ng/mL), LPS (100 ng/mL) + IFN-γ (10 ng/mL) + DXM (25, 50 μM) for 18 h. The control group was treated with PBS alone. (a) Supernatants were collected 18 h later (TNF, 6 h), and TNF-alpha, IL-6, and IL-12 production was measured by ELISA. Data are presented as the mean ± SD of samples from three wells. Significant differences between DXM-treated and untreated LPS + IFN-γ-activated DCs are shown with asterisks ($^*P < 0.05$). (b) The expression of CD80, CD83, and HLA was determined by flow cytometry. All data were gated on CD1a$^+$ cells. The gray-filled area represents staining with an isotype-matched control Ab. The change of geometric mean fluorescence intensity in the LPS + IFN-γ or LPS + IFN-γ + DXM samples is indicated. All data are representative of five independent experiments with cells from individual donors.

on DCs, DXM may potentially prevent or treat DC-associated chronic or acute immune diseases, such as MS, diabetes, and RA [44]. Because DCs are important for the eradication of tumors and pathogens [57–59], future clinical studies should identify the risks associated with long-term DXM use.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contribution

Der-Yuan Chen and Pei-Sang Song contributed equally to this work.

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Clinical Study

Cysticerci Drive Dendritic Cells to Promote *In Vitro* and *In Vivo* Tregs Differentiation

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Regulatory T cells (Tregs) play a crucial role in immune homeostasis. Treg induction is a strategy that parasites have evolved to modulate the host's inflammatory environment, facilitating their establishment and permanence. In human *Taenia solium* neurocysticercosis (NC), the concurrence of increased peripheral and central Treg levels and their capacity to inhibit T cell activation and proliferation support their role in controlling neuroinflammation. This study is aimed at identifing possible mechanisms of Treg induction in human NC. Monocyte-derived dendritic cells (DC) from healthy human donors, cocultivated with autologous $CD4^+$ naïve cells either in the presence or absence of cysticerci, promoted $CD25^{high}$ Foxp3+ Treg differentiation. An increased Treg induction was observed when cysticerci were present. Moreover, an augmentation of suppressive-related molecules (SLAMF1, B7-H1, and CD205) was found in parasite-induced DC differentiation. Increased Tregs and a higher *in vivo* DC expression of the regulatory molecules SLAMF1 and CD205 in NC patients were also found. SLAMF1 gene was downregulated in NC patients with extraparenchymal cysticerci, exhibiting higher inflammation levels than patients with parenchymal parasites. Our findings suggest that cysticerci may modulate DC to favor a suppressive environment, which may help parasite establishment, minimizing the excessive inflammation, which may lead to tissue damage.

1. Introduction

Natural (thymic) and inducible regulatory T cells (Tregs) play a pivotal role in maintaining the immune system homeostasis. While natural Tregs are produced in the thymus at any time, inducible Tregs acquire a regulatory function in the context of a given infection or a neoplastic process [1]. A variety of inducible Treg subpopulations mediating their immune suppressive effects by different mechanisms have been reported [2–4]. Disregarding their effectiveness in controlling inflammation, it is conceivable that Tregs could promote a more permissive environment for parasite establishment [5, 6]. The increased Treg levels found in many different protozoa and cestode infections are consistent with this possibility [7, 8].

The role of Tregs in neurocysticercosis (NC), a parasitic disease caused by the establishment of *Taenia solium* metacestode in the central nervous system (CNS), begins to be explored. Increased central and peripheral Treg levels were observed in severe NC patients. These Tregs seem to

participate in the control of the inflammatory response, since a negative correlation between the percentage of peripheral Tregs and activated CD8+ and $CD4^+$ T cells, along with a depressed T cell proliferative response, was observed [9]. However, the mechanisms underlying Treg induction in NC are still unknown.

Dendritic cells play a prominent role in Treg induction in the beginning of the immune response to pathogens, either by promoting the conversion of naïve T cells to Treg subpopulations or by expanding the population of preexisting Treg cells [10]. Mature DCs direct conventional $CD4^+$ cells to become either specific T helper or T regulatory cell subsets, depending on the affinity of their TCR to the antigen, the strength of the costimulatory signals provided by antigen-presenting cells (APCs), and the cytokine milieu. In addition, the absence of inflammation arrests dendritic cells into an immature or semimature state (iDC), which promotes T cell tolerance by conversion of naïve T cells into Tregs [11]. iDCs are characterized by expressing MHC II, by a high phagocytosis capacity, and by low CD80/CD86 expression. iDCs also produce IL-10, but neither IL-12 nor TNFα [10, 12]. Moreover, it has been proposed that preexisting Tregs can educate iDCs to become tolerogenic, promoting Treg generation [10, 13]. Helminths, as well as other pathogens, may modulate the immune tolerance properties of DCs [14–17].

This study was designed to evaluate the capacity of cysticerci to modulate dendritic cells, driving them to Treg induction, both *in vitro* and *in vivo*.

2. Materials and Methods

2.1. Parasites. *Taenia solium* metacestodes were obtained from naturally infected pigs coming from villages of Guerrero, an endemic region in Mexico. Pigs were euthanized according to ethical animal handling regulations in Mexico. Cysticerci were individually harvested from muscle tissue and maintained in RPMI 1640 medium containing 10% fetal calf serum and 1% antibiotics (Invitrogen, NY, USA). Cysticerci of *Taenia crassiceps ORF* strain were also used in this study to evaluate their potential to induce iDCs, and therefore Tregs. *T. crassiceps* cysticerci were obtained from infected BALB/cAnN female mice. Parasites were harvested from the peritoneal cavity of stock female mice after 10 weeks of infection.

2.2. Cell Purification and DC Generation in the Presence of Live Cysticerci. Human cells were isolated from buffy coats from blood of healthy donors (Banco de Sangre del Centro Médico Nacional Siglo XXI, México, DF).

A RosetteSep Human $CD4^+$ T Cell Enrichment Cocktail kit was used for $CD4^+$ T cell purification (StemCell, Vancouver, Canada). Purified $CD4^+$ T lymphocytes including >90% of CD3+ $CD4^+$ cells were kept frozen at –80°C until used.

Monocyte-derived DCs (MDDCs) were isolated from mononuclear cells from healthy donors using a RosetteSep Human Monocyte Enrichment Cocktail kit (StemCell, Vancouver, Canada). MDDCs were generated as previously reported [18] with only minor modifications. Briefly, monocytes were plated in Petri dishes (P100) at 5×10^5 cells/mL in 10 mL of RPMI 1640 medium (Invitrogen, NY, USA) containing 10% human AB serum (HSAB) and 1% antibiotics, 20 ng/mL IL-4 (eBiosciences CA, USA), and 100 ng/mL granulocyte-macrophage colony-stimulating factor (eBiosciences, San Diego, CA, USA) for 8 days. One half of complete medium, including cytokines, was replaced at day 4.

To evaluate the role of cysticerci during DC differentiation, monocytes were cultured as described above, either with or without vesicular *Taenia crassiceps* (40 cysts) or *Taenia solium* (5 cysts) cysticerci per dish. The effect of both cestodes on DC differentiation was tested considering that murine cysticerci may eventually provide a controlled parasite source of purified immune-modulatory components. Dexamethasone at 10^{-7} M was used as a positive control.

2.3. In Vitro Treg Induction with Cysticercal Antigens. Parasite-induced MDDCs were seeded at 2.5×10^4 cell/mL in 1 mL RPMI 1640 medium (Invitrogen, NY, USA) containing 10% human AB serum and 1% antibiotics (Invitrogen, NY, USA). MDDCs were maintained in culture for 24 h. During this period, dexamethasone at 10^{-7} M, which maintains a tolerogenic DC phenotype, was used as control [19]. Parasite-induced DCs were pulsed using 3 μg/mL of total antigens from *T. crassiceps* cysticerci. *T. crassiceps* antigens were employed considering that *T. solium* and *T. crassiceps* cysticerci share in common more than 98% of the expressed antigens [20]. 1.25×10^5 autologous $CD4^+$ T lymphocytes were cocultured for 7 days with MDDCs in RPMI 1640 medium (Invitrogen, NY, USA) containing 10% human AB serum and 1% antibiotics (Invitrogen, NY, USA). Then, the phenotype of regulatory T cells was evaluated by flow cytometry as described below. Supernatants of these cultures were used to evaluate cytokine profile.

2.4. Cell Phenotype. The following combination of antibodies was used to characterize the phenotype of MDDCs: (a) MHC-II FITC (mouse IgG2a k), SLAMF1 PE (mouse IgG1 k), CD11c PerCP-eFluor 710 (mouse IgG1 k), and B7/H1 APC (mouse IgG1 k); (b) CD83 FITC, CD80 PE, CD11c PerCP-eFluor 710 (mouse IgG1 k), and CD86 APC (mouse IgG1 k); (c) CD205 FITC (mouse IgG2b k), CD40 PE (mouse IgG1 k), CD11c PerCP-eFluor 710 (mouse IgG1 k), and ILT3 APC (mouse IgG1 k). Inducible regulatory T cell phenotype was assessed using CD127 FITC (mouse IgG1 k), Foxp3 PE (Rat IgG2a k), CD4 PerCp (mouse IgG1 k), and CD25 APC (mouse IgG1 k). Regulatory T cells were intracellularly stained for Foxp3 using the eBiosciences kit (most antibodies used for cell staining were purchased from eBiosciences, CA, USA). All antibodies were titrated for optimal detection of positive populations prior to its use, considering the manufacturer's recommended concentrations.

For cytometry analyses, cells were first gated according to lymphocyte forward and side light-scattering properties. Treg numbers were defined as the fraction of Foxp3+ cells among $CD4^+$ $CD25^{high}$ cells.

2.5. Cytokines. Cytokine levels in supernatants were measured using the cytometric bead array Cytokine Kit II (BD Biosciences Pharmingen, CA, USA), according to the manufacturer's instructions, in an FACSCalibur cytometer. Data were analyzed with the BD Cytometric Bead Array software (BD Biosciences Pharmingen, CA, USA). Flow cytometer was calibrated using BD FACSComp (BD Biosciences Pharmingen, CA, USA) and BD CaliBRITE beads (BD Biosciences Pharmingen, CA, USA). Assay sensitivities were set as follows: IL-2 (2.6 pg/mL), IL-6 (3.0 pg/mL), IL-10 (2.8 pg/mL), TNF-α (2.8 pg/mL), and IFN-γ (7.1 pg/mL). TGF-β were measured using the human/mouse TGF-beta 1 ELISA Ready-SET-Go (eBiosciences, CA, USA). ELISA was performed according to the manufacturer's instructions; detection limit was 60 pg/mL. All samples were run in duplicate.

2.6. DC and Treg Phenotype in NC Patients. Blood samples from 13 patients who attended at the Instituto Nacional de Neurología y Neurocirugía and Centro Médico Nacional Siglo XXI in Mexico City with confirmed NC diagnosis were included in this study. All samples were taken before any treatment was administered. Eight male (age mean 56.25 ± 3.8 years) and five female (age mean 37 ± 1.6 years) patients were included in this study.

In all cases, NC was diagnosed based on clinical manifestations (seizures, focal deficit, and intracranial hypertension) and radiological studies such as MRI and computed tomography (CT).

Vesicular parasites were observed in most cases (12/13 patients). A solitary cysticercus was seen in 9 patients, and multiple cysticerci in 2 patients; the rest of the patients had calcified and colloidal forms. In seven patients, parasite was located at the subarachnoid space of the base (SAB), while the rest of the patients showed parasites at the cerebral parenchyma (P). Patients with SAB cysticerci exhibited inflammatory traits in cerebral spinal fluid (glucose 30.7 ± 28 mg/dL, proteins 421 ± 407 mg/dL, and 70.7 ± 96.9 cell/mm^3). In contrast, patients with parenchymal cysticerci showed no inflammation signs. Blood samples from 5 healthy subjects were included as controls.

2.7. Microarrays. DNA microarrays were used to compare the gene expression profile of mononuclear peripheral cells (PBMCs) from a cohort of 8 NC patients, 40 ± 14 years old. PBMC from all patients were obtained 8 days after cysticidal treatment with albendazole, 30 mg per kg, and concomitant corticosteroid administration. These 8 (4 females and 4 males) patients showed cysticerci in different locations. Cysticerci were established in the subarachnoid basal space or in the ventricles (SAB/IV) in 5 patients and were established in the cerebral parenchyma (P) in 3 patients. When required for patients' followup, CSF was obtained by lumbar puncture at the National Institute of Neurology and Neurosurgery of Mexico City. A mean of 74 cell/mm^3 (range 1–261) was found in SAB/IV-NC patients, and a mean of 6.6 cell/mm^3 (range 0–18) cells was found in P-NC patients. PBMCs were isolated and then cultured by 72 h with *T. solium* cysticercal antigens, obtained as previously described [21].

Recovered cells were placed in TRIzol (Gibco, NY, USA) for RNA purification using the RNeasy mini kit (QIAGEN, TX, USA) following the manufacturer's instructions. The amount of obtained RNA was estimated in an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Deutschland). Comparisons were done with respect to parasite location (SAB/IV-NC versus P-NC) using the R-language platform, under the terms of the Free Software Foundations GNU General License (see http://cran.r-project.org/). Data were standardized and normalized. All genes were analyzed using the Reactome software (see http://www.reactome.org/) to find a possible biological pathway involved.

2.8. Ethical Considerations. The present study fulfilled all regulations for research with human subjects as required by the Mexican law and international regulations. It also complied with all ethical aspects considered in the General Rules of Health for Clinical Investigation. Ethics Committee at Instituto Nacional de Neurología y Neurocirugía, México approved the protocol. Written informed consent was obtained from all participants. Patients were informed that samples obtained would be used for this work.

2.9. Statistical Analysis. Data were processed in InStat (GraphPad Software Inc., CA, USA). Variables were described using mean ± SD. Differences between groups were calculated with Students' t-test. P values less than 0.05 were considered significant.

3. Results

3.1. Effect of Cysticerci on Dendritic Cell Phenotype. The semimature DC phenotype is associated with tolerance and Treg induction [12]. To evaluate the effect of cysticerci on dendritic cell differentiation, the phenotype of DC generated from CD14+ monocytes either in the presence or absence of vesicular cysticerci after 8-day culture was studied. Dexamethasone (10^{-7} M) was employed as a positive control (Figure 1). The expression of CD83+, as well as the costimulatory (CD80, CD86, CD40, HLA-DR) and regulatory molecules (SLAMF1, B7-H1, CD205, and ILT3) on CD11c+, was measured. As shown in Figure 1, a higher expression of SLAMF1, B7-H1, and CD205 was observed in cells differentiated in the presence of cysticerci. The expression of HLA-DR, CD80, CD83, CD86, CD40, and ILT3 molecules did not differ between control and parasite-driven differentiated DC cells. On the other hand, CD80, CD40, and SLAMF1 were diminished in dexamethasone-treated cells with respect to control (Figure 1). HLA-DR and ILT3 expression did not differ from control at any tested condition (data not shown). Altogether, these results indicate that parasite promotes the expression of molecules related to a DC tolerogenic phenotype.

3.2. Taenia Solium and T. crassiceps Cysticerci Promote In Vitro Regulatory T Cells Differentiation. We investigated whether

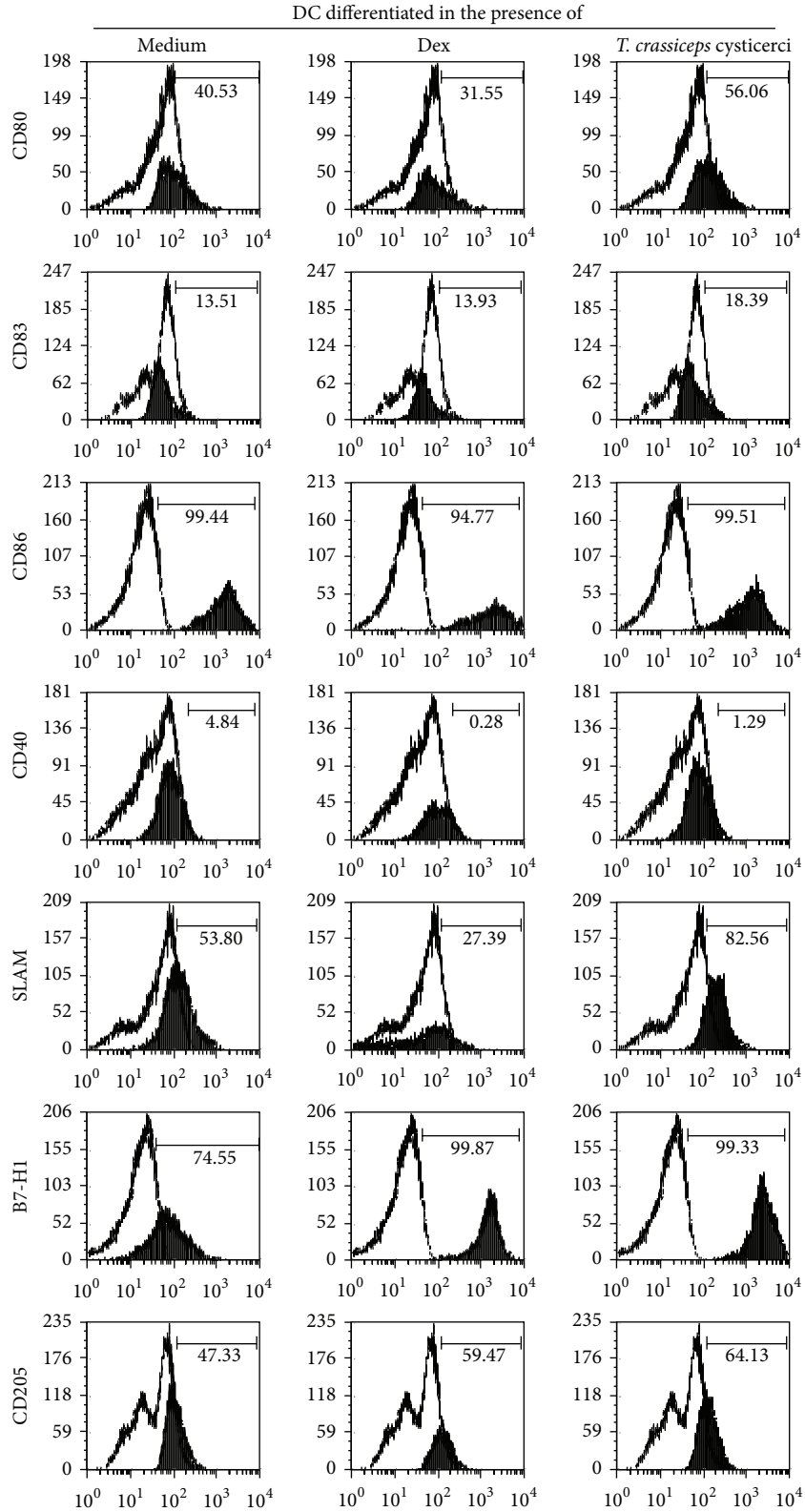

Figure 1: Parasite effect on DC differentiation and activation. Monocytes were differentiated to DC in presence of IL-4 and GM-CSF, plus medium or dexamethasone or *Taenia crassiceps* cysticerci. On day 8, the DC phenotype was studied. Representative data of three independent experiments are shown. Expression of CD80, CD83, CD86, CD40, SLAMF1, B7-H1, and CD205 gated on CD11c+ cells was analyzed by FACS. The isotype controls are shown in white histograms.

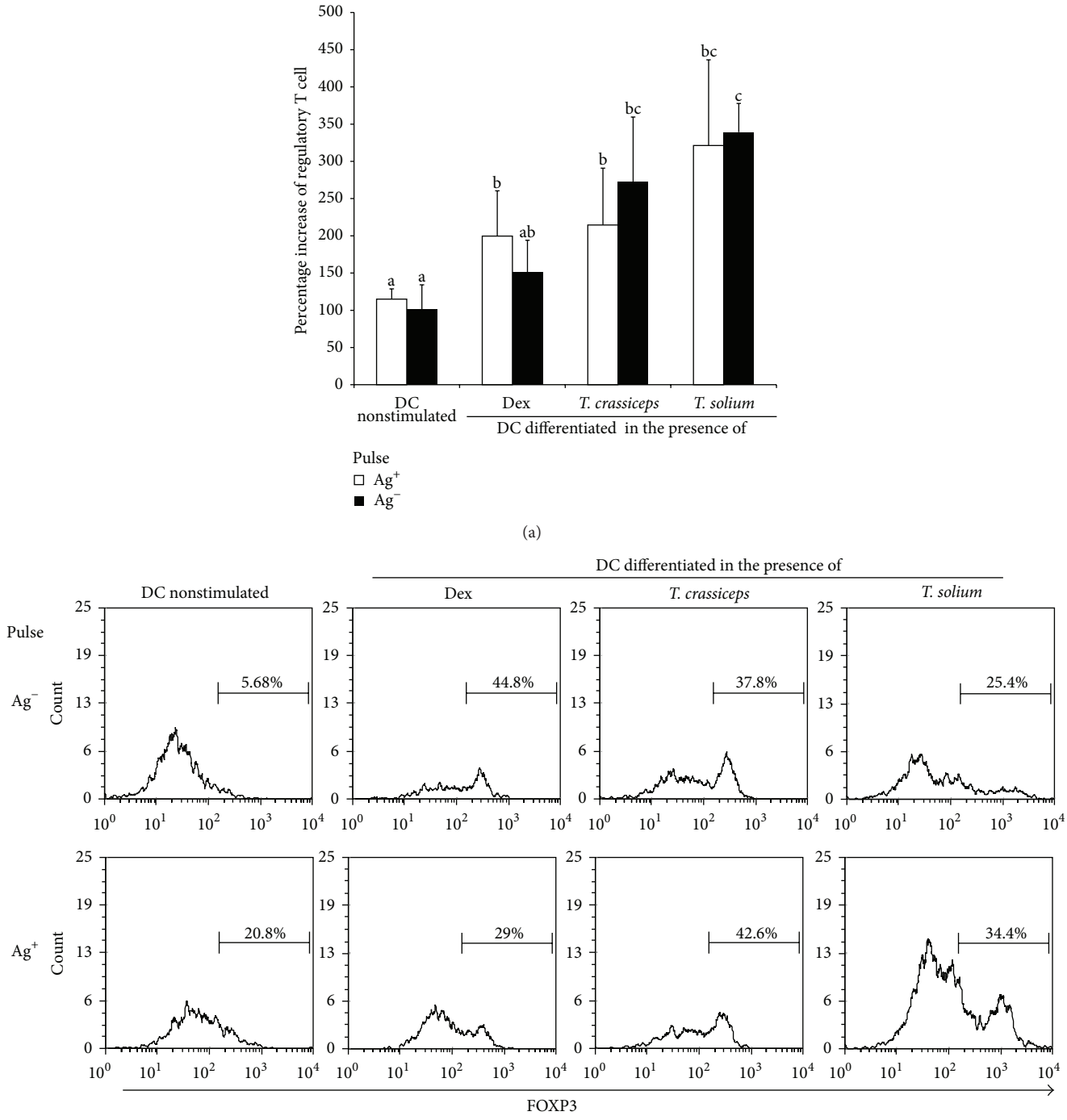

Figure 2: *In vitro* differentiation of dendritic cells in the presence of *Taenia solium* or *T. crassiceps* cysticerci promotes Treg induction. (a) Increased percentage in Treg cell induction. Percentage was calculated as follows: (percentage of Tregs induced/percentage of basal Tregs) × 100. Different letters indicate significant differences between groups at $P < 0.05$. (b) Representative histograms showing Foxp3 induction within $CD4^+$ $CD25^{high}$ cells. Data are representative of three independent experiments.

differentiation *in vitro* in the presence of live cysticerci stimulated the differentiation to $CD4^+$ $CD25^{high}$ Foxp3+ Tregs. Human DCs, either differentiated in the presence or absence of cysticerci, were pulsed or not with cysticercal proteins and used to stimulate autologous $CD4^+$ T lymphocytes. Seven days later, the expression of $CD25^{high}$ and Foxp3 within $CD4^+$ T lymphocytes was measured. As a positive control of Treg induction, differentiated DCs were treated with dexamethasone (10^{-7} M). As shown in Figure 2, cocultivation with cysticerci significantly increased the percentage of $CD4^+$

Table 1: Levels of cytokines in supernatants from *in vitro* DC differentiation and *in vitro* regulatory T cell induction.

	IFN-γ	TNF-α	IL-10	IL-6	IL-2	TGF-β
DC nonstimulated	0.39 ± 0.88	3.98 ± 3.82	3.17 ± 1.79	>5000	0.34 ± 0.77	406.50 ± 191.69
[†]Control medium 10% HSAB	3.55 ± 0.51	5.78 ± 1.79	5.12 ± 1.50	>5000	1.59 ± 1.41	ND
[b]DC differentiation in the presence of						
Dexamethasone	0	3.12 ± 0.47	30.84 ± 5.82[a]	>5000	0	545.17 ± 289.37
Taenia crassiceps	0.78 ± 1.75	2.98 ± 1.75	3.77 ± 1.76	>5000	0.72 ± 0.98	341.50 ± 238.19
Taenia solium	0	4.58 ± 4.44	7.31 ± 4.88	>5000	0	436.00 ± 243.95
Treg induction from naïve $CD4^+$ cells						
DC nonstimulated, nonpulsed (Ag^-)	0.76 ± 1.31	1.21 ± 1.07	0.99 ± 0.93	2.58 ± 0.70	0.45 ± 0.78	124.60 ± 5.35
DC nonstimulated pulsed (Ag^+)	0.78 ± 1.75	2.06 ± 0.88	1.22 ± 1.12	2.78 ± 0.60	0.43 ± 0.95	93.07 ± 16.98
Dexamethasone nonpulsed (Ag^-)	0.63 ± 1.26	1.68 ± 0.34	1.01 ± 1.18	2.97 ± 2.02	0.39 ± 0.78	95.49 ± 19.66
Dexamethasone pulsed (Ag^+)	1.00 ± 1.74	2.49 ± 0.36	2.32 ± 1.36	2.78 ± 1.10	1.38 ± 1.27	98.63 ± 26.39
T. crassiceps nonpulsed (Ag^-)	1.23 ± 2.13	2.09 ± 0.79	1.02 ± 1.76	8.15 ± 1.26	1.31 ± 1.21	99.22 ± 31.64
T. crassiceps pulsed (Ag^+)	1.26 ± 1.81	2.18 ± 0.38	1.44 ± 0.81	29.34 ± 46.75	0.43 ± 0.95	119.80 ± 42.56
T. solium nonpulsed (Ag^-)	4.50 ± 2.61	2.35 ± 0.68	1.48 ± 2.10	56.99 ± 5.21	12.14 ± 11.79	111.00 ± 3.54
T. solium pulsed (Ag^+)	1.73 ± 2.45	2.28 ± 0.68	1.95 ± 0.09	11.68 ± 6.02	6.61 ± 6.16	78.50 ± 21.21
Control T cell	1.54 ± 1.41	1.90 ± 0.48	1.77 ± 0.99	2.01 ± 0.45	0.88 ± 1.20	101.00 ± 16.58

[a]Significantly different ($P < 0.05$) compared to nonstimulated DC. [b]Human AB sera from healthy donors were used in DC differentiation experiments.
[†]Levels of cytokines in the control medium supplemented with 10% HSAB are shown.

lymphocytes coexpressing the $CD25^{high}$ and Foxp3 fraction with respect to non-stimulated DC, from 115 ± 13.7 to 214.6 ± 76.3 when cocultured with *T. crassiceps* ($P = 0.04$) and to 321.2 ± 115 ($P = 0.05$) when cocultured with *T. solium* cysticerci. When DCs were pulsed with parasite antigens, Treg cells were also significantly increased, from 101.2 ± 33 to 273 ± 86.5 ($P = 0.01$) with *T. crassiceps*-differentiated DC cells, and to 339.3 ± 38.6 ($P = 0.0002$) with *T. solium*-differentiated DC cells (Figure 2).

3.3. Cytokine Profile. The levels of induced cytokines were measured in supernatants from *in vitro* DC differentiation after 8 days of culture and from *in vitro* Treg induction after 7 days of culture. During DC differentiation, only IL-10 was significantly increased when cells were differentiated in presence of dexamethasone (Table 1). No difference was observed in the other tested conditions.

3.4. Dendritic Cells and Regulatory T Cells in NC Patients. The *in vivo* effect of parasite products on the phenotype of peripheral DC from NC patients was studied. The percentage of DC and the expression of regulatory (SLAMF1, CD205, and ILT3) and costimulatory molecules (HLA-DR, CD86, and CD40) in CD11c cells in 13 NC patients and 5 healthy subjects was measured. As Table 2 shows, SLAMF1, and CD205 are significantly increased in DC from NC patients from 3.8 to 9.7 and from 4.3 to 21.5, respectively ($P < 0.05$). Moreover, peripheral Tregs are also increased in NC patients from 4.35 to 14.22.

3.5. SLAMF1 Is Downregulated in PBMC from Patients with SAB/IV Parasites. As Table 3 shows, only four immune-related genes of the 32,322 included in the array were found downregulated in severe SAB/IV-NC patients with respect to P-NC patients. Among them figures the SLAMF1 gene, whose expressed protein was also found in dendritic cells from non-treated NC patients (Table 2). The other downregulated genes found in these patients were MTOR, NFKB2, and IL12RB2 (Table 3). In these severe SAB/IV-NC patients, TGB2 and IL24 genes were found to be up-regulated.

Table 2: Phenotype of peripheral dendritic and regulatory T cells in NC patients and healthy subjects.

Phenotype	NC patients	Healthy subjects	P
Dendritic cells			
$SLAM^+CD11C^+$	9.65 ± 5.66	3.80 ± 1.88	0.045
$CD205^+CD11C^+$	21.55 ± 13.64	4.27 ± 3.12	0.014
$ILT3^+CD11C^+$	35.01 ± 27.61	31.48 ± 28.70	0.813
$HLA\text{-}DR^+CD11C^+$	71.47 ± 19.87	69.05 ± 12.96	0.805
$CD86^+CD11C^+$	39.70 ± 25.87	54.21 ±12.45	0.250
$CD40^+CD11C^+$	7.92 ± 7.98	2.81 ± 1.49	0.182
T cells			
$CD4^+CD25^{High}$ $FoxP3^+CD127^{-/low}$	14.22 ± 9.08	4.35 ± 4.26	0.0194
$CD4^+$ T lymphocytes	30.85 ± 11.49	43.59 ± 5.60	0.07

4. Discussion

Tregs play a pivotal role in modulating the host environment, so parasites may find more appropriate conditions for their establishment and development [7]. It is also possible that Tregs may favor the parasite persistence even when a specific treatment is used to promote the destruction of the parasite [22]. During *Taenia solium* infection, an increase in the levels of regulatory T cells in blood and CSF in NC patients

Table 3: Genes differentially expressed in NC patients with respect to the parasite location after 8 days of treatment.

	NC caused by cysticerci localized in the subarachnoid base or intraventricular versus parenchymal		
	Genes	Log	*B*
Downregulated	MTOR	−0.69	0.95
	NFKB2	−0.93	0.78
	SLAMF1	−1.05	2.18
	IL12RB2	−0.81	1.19
Upregulated	IL24	0.82	0.96
	TGFB2	1.06	2.13

The log fold change (Log FC) describes how much a quantity changes from an initial to a final value in a determinate gene. *B* value represents the possibility that the gene is differentially expressed.

has been observed, probably promoted by the parasite for controlling the central inflammatory environment and thus favoring its survival [9]. Increased Tregs in NC patients may result from expanded natural Tregs or may be induced by the parasite. The latter possibility was explored herein. On the other hand, DCs may drive T cell differentiation to regulatory or effector cells, depending on their activation status. In this study, *in vivo* and *in vitro* lines of evidence point to the effect of cysticercal components on the activation of dendritic cells and their impact on promoting T cell differentiation to $CD25^{high}$ Foxp3+ CD4 T cells. Indeed, co-cultivation of dendritic cells with either *T. solium* or *T. crassiceps* cysticerci promotes a status that favors the differentiation of peripheral T cells to $CD4^+$ Tregs. Comparable results were found when peripheral cells from non-treated NC patients were studied.

The similar effects induced by the presence both of *T. solium* or *T. crassiceps* cysticerci merit some comments. It is well known that both cestodes share multiple antigens [23], a fact that has been exploited to use the murine cysticercal antigens for diagnosis [24, 25].

Parasite-differentiated dendritic cells show no difference with respect to control in the maturation marker (CD83), neither in the costimulatory molecules HLA-DR, CD80, CD86, or CD40; these findings are compatible with an immature dendritic phenotype. In contrast, an overexpression of SLAMF1, B7-H1, and CD205 was observed. The latter two molecules are related to a tolerogenic DC phenotype, as reported in many studies [12, 26]. It is important to note that the expression of SLAMF1, B7-H1, and CD205 was found accompanied by Treg induction in this study, as well. However, their participation in Treg induction remains to be elucidated. This may be particularly relevant since no increase in the two main regulatory cytokines, IL10 and TGFβ, was observed in the supernatants recovered during *in vitro* Treg induction (Table 1). An increased expression of SLAMF1 and CD205 in dendritic cells and in $CD25^{high}$ Foxp3+ CD4 T cells was observed in NC patients. Both *in vitro* and *in vivo* findings reinforce the relevance of SLAMF1 and CD205 dendritic cells, defining their critical role in T cell immunity regulation [27, 28].

It is also worth noticing the downexpression of SLAMF1 in the group of 8 SAB/IV-NC patients with respect to those patients harboring cysticerci in the parenchyma. This apparent discordance with the *in vitro* results from non-treated NC patients can be traced to differences promoted by *in vivo* or *in vitro* conditions, which may differentially modulate the expression of the SLAM-associated protein (SAP) in lymphocytes. Indeed, the expression of this adaptor SAP protein promotes the inflammatory response, while its absence promotes a regulatory environment [28]. SLAM-family receptors presenting cells ligands carry out important immunomodulatory functions: by one side, they regulate lymphocyte interactions and adhesion [28, 29] as well as a tolerogenic immune profile [11, 12]. Although there is no previous information about the functions of SLAM receptors during NC, the results shown in this study point to their possible participation in modulating the inflammatory response promoted by cysticidal and/or corticosteroid treatments. Changes found in MTOR, IL12RB2, NFKB2, and TGF-β genes match with an immunoregulatory environment. MTOR [30], IL12RB2 [31], and NFKB2 [32, 33] genes, coding for proteins that promote an inflammatory environment, are found downregulated, whilst TGF-β, which promotes a regulatory immune response [7], was up-regulated.

5. Conclusions

Overall, the results shown in this study reinforce our previous findings on the relevance of Tregs in controlling the extent of the inflammatory response in NC patients, and added *in vivo* and *in vitro* lines of evidence that cysticerci may drive a particular dendritic cell phenotype that induces regulatory T cells, even though the mechanisms that underlined this phenomenon remain to be elucidated. Additionally, while the clinical relevance of the promotion of this regulatory environment needs to be evaluated, it is feasible to propose that it could favor parasite survival.

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Review Article

Targeting BCL2 Family in Human Myeloid Dendritic Cells: A Challenge to Cure Diseases with Chronic Inflammations Associated with Bone Loss

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Rheumatoid arthritis (RA) and Langerhans cell histiocytosis (LCH) are common and rare diseases, respectively. They associate myeloid cell recruitment and survival in inflammatory conditions with tissue destruction and bone resorption. Manipulating dendritic cell (DC), and, especially, regulating their half-life and fusion, is a challenge. Indeed, these myeloid cells display pathogenic roles in both diseases and may be an important source of precursors for differentiation of osteoclasts, the bone-resorbing multinucleated giant cells. We have recently documented that the proinflammatory cytokine IL-17A regulates long-term survival of DC by inducing BCL2A1 expression, in addition to the constitutive MCL1 expression. We summarize bibliography of the BCL2 family members and their therapeutic targeting, with a special emphasis on MCL1 and BCL2A1, discussing their potential impact on RA and LCH. Our recent knowledge in the survival pathway, which is activated to perform DC fusion in the presence of IL-17A, suggests that targeting MCL1 and BCL2A1 in infiltrating DC may affect the clinical outcomes in RA and LCH. The development of new therapies, interfering with MCL1 and BCL2A1 expression, to target long-term surviving inflammatory DC should be translated into preclinical studies with the aim to increase the well-being of patients with RA and LCH.

1. Introduction

Myeloid dendritic cells (DCs) can be derived from monocytes in the presence of GM-CSF and IL-4, both *in vitro* and *in vivo* [1]. Therefore, DC and monocytes are closely related. They share (Figure 1) the ability to phagocytose extracellular bacteria, to synthesize the apoptotic factor TRAIL in response to interferons (IFN) in the context of viral infections [2, 3], and to present antigens to T cells. They also undergo cell fusion in the presence of M-CSF and RANKL, thus forming osteoclasts (OCs), the bone-resorbing multinucleated giant cells (MGCs) [4, 5]. By contrast, two functional properties discriminate DC from monocytes. First, DCs initiate adaptive immune responses versus tolerance, as demonstrated in mouse models of DC short-term ablation, *in vivo* [6]. Second, DCs undergo cell fusion in the presence of the pro-inflammatory cytokine

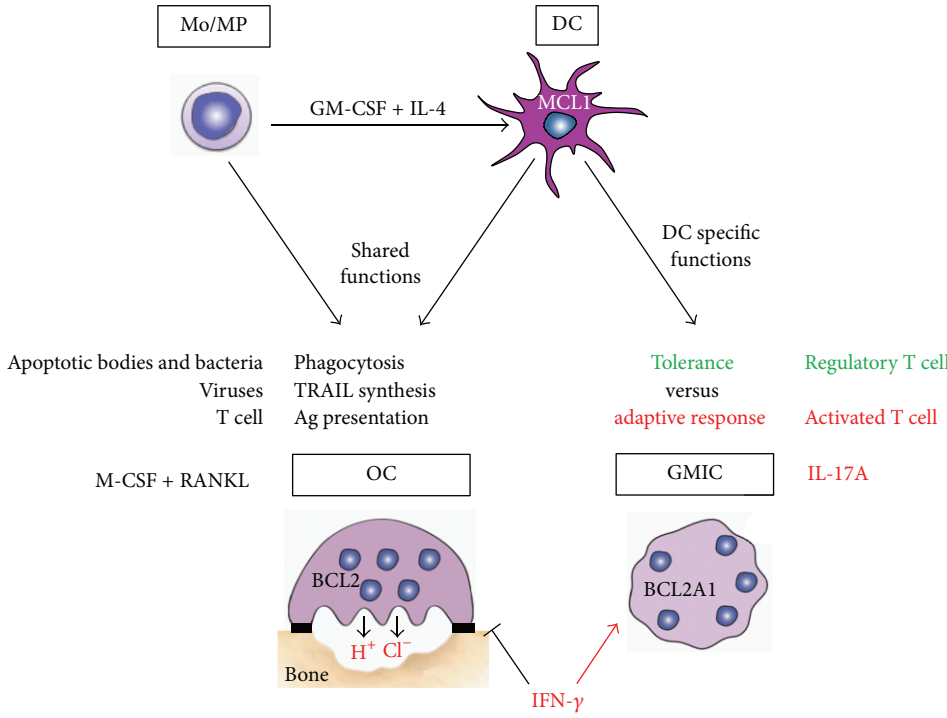


Figure 1: Dendritic cell functions compared to monocytes. Monocyte-derived DCs share four functions with monocytes: phagocytosis, TRAIL synthesis, Ag presentation, and differentiation into OC in the presence of M-CSF and RANKL. Conversely, initiation of tolerance, adaptive response, and IL-17A-dependent differentiation of GMIC are DC-specific functions. IFN-γ differentially regulates MGC formation by inhibiting OC and stimulating GMIC formation.

IL-17A, a mechanism highly potentiated by IFN-γ [7, 8]. To discriminate OC from the IL-17A-dependent MGC, these latter will be called giant myeloid inflammatory cells (GMICs) in this review. Exacerbation of these fusion pathways may be involved in two diseases of unknown etiology: rheumatoid arthritis (RA) and a rare disease called Langerhans cell histiocytosis (LCH). In both diseases, bone loss is observed, and the three cytokines M-CSF, RANKL, and IL-17A have been detected [7, 9–11]. While untreated immature DCs have a short two-day lifespan, both OC and GMIC survive more than two weeks, thus demonstrating that survival pathways are activated along the DC fusion process, *in vitro*. In 2008, it was shown that B-cell lymphoma 2 (BCL2) is critical for OC survival as demonstrated by the increased bone mass of $BCL2^{-/-}$ mice [12]. We have recently documented that an unexpected member of the BCL2 family, *BCL2A1,* is involved in the survival of GMIC [13]. In the light of this last finding, we reviewed the available knowledge to investigate whether targeting members of the BCL2 family in DC may open novel treatment opportunities in chronic inflammatory diseases associated with bone loss. We first focus on the two BCL2 members expressed by DC, MCL1, and BCL2A1. Then, we review the therapeutic compounds able to target MCL1 or BCL2A1. Finally, we discuss challenges and opportunities to target MCL1 and BCL2A1 in RA and LCH, in the aim to block formation of OC and GMIC from DC.

2. Bcl2 Family and the Focus on MCL1 and BCL2A1 Expressed by Myeloid Dendritic Cells

Apoptosis is initiated by extrinsic and intrinsic pathways, depending on the stimulus, as death receptor ligation on the cell surface (Fas, TNFRSF1, TNFRSF10, etc.) and intracellular stress (UV, cytokine withdrawal, cytotoxic drugs, etc.), respectively [14]. Ligation of death receptors causes formation of a death-inducing signaling complex (DISC) that triggers caspases and then apoptosis, independently of mitochondria. Such mechanism can be inhibited by CFLAR (or viral homologs), previously named FLICE-inhibitory proteins (FLIP). Interestingly, myeloid DCs expressing Fas on their membrane were not sensitive to Fas-mediated apoptosis unless sensitized by cycloheximide [15]. This resistance is attributed to the expression of CFLAR by myeloid DC. By contrast, immature (but not mature) human myeloid DCs are very sensitive to UVB irradiation [16], a process involving intracellular oxidative stress and caspase activation through the intrinsic pathway of apoptosis.

The mitochondria-dependent intrinsic pathway involves the BCL2 members, whose main functions include embryogenesis control, tissue homeostasis, leukocyte development, and defense against pathogens. The BCL2 family displays three groups of proteins, sharing sequence homology in

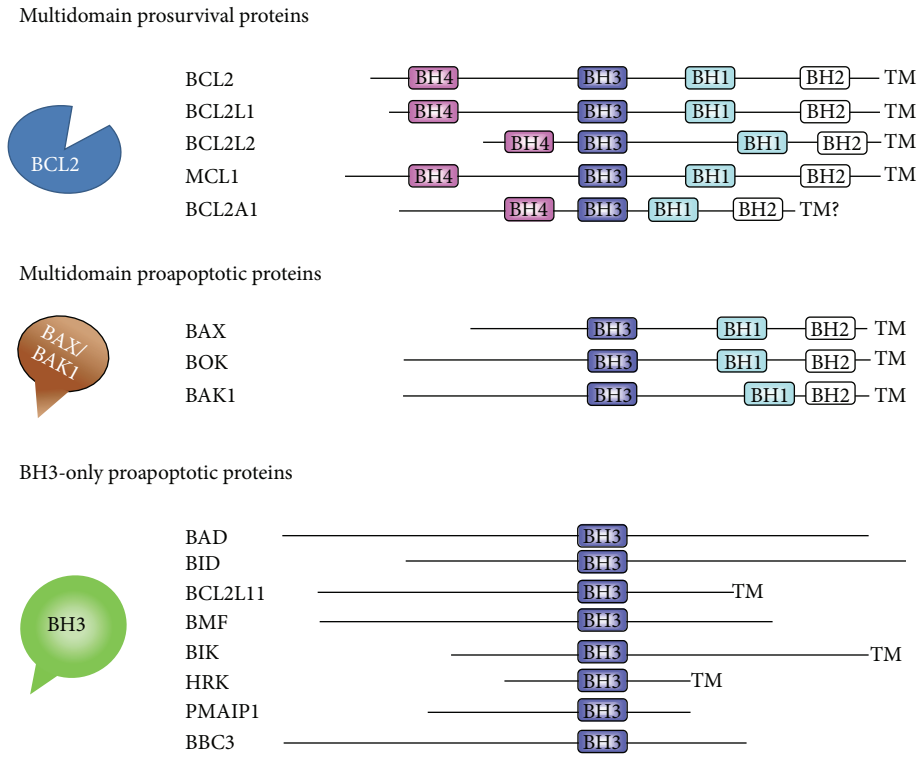


FIGURE 2: Classification of the BCL2 family into three subfamilies of BCL2-related proteins. The four BCL2 homology (BH) domains are the most highly conserved among BCL2 family. The prosurvival proteins contain four α-helix BH domains (BH1-4). BH1-3 domains of prosurvival proteins form a hydrophobic cleft that binds proapoptotic proteins through their hydrophobic BH3 domains.The multidomain proapoptotic proteins have BH1-3 domains, while the proapoptotic BH3-only proteins share only the BH3 domain with the other BCL2-related proteins. Most members have a carboxy-terminal hydrophobic transmembrane (TM) domain, with the exceptions of many of the BH3-only proteins and probably BCL2A1.

their BCL2 homology (BH) domains (Figure 2, Table 1). They include the prosurvival proteins (such as BCL2, MCL1, and BCL2A1) and the proapoptotic proteins, comprising the multidomain proteins (such as BAX and BAK1) and the BH3-only proteins (including BAD, BID, and BIM) [17]. How these three groups integrate cell signaling into the decision to live or die is not completely understood and the mechanism remains controversial [18]. A set of interactions between BCL2 members ultimately controls the release of cytochrome c from mitochondria, the caspase activation, and then apoptosis. In living normal cells, proapoptotic proteins are sequestered by prosurvival members, thus inhibiting the release of cytochrome c from mitochondria (Figure 3(a)). However, there are some reports indicating that the mitochondria are not essential for initiation of cell death induced from intrinsic pathway and that apoptosis can also occur in the absence of caspase activation [19]. After an intracellular apoptotic stimulus, the BH3-only proteins activate the multidomain proapoptotic proteins, thereby triggering cytochrome c release and apoptosis (Figure 3(b)). Impaired apoptosis associated with an enhanced expression of prosurvival BCL2 proteins is a hallmark of human cancers (Figure 3(c)) and is frequently associated with resistance to therapy [18, 20, 21]. In addition, upregulation of prosurvival BCL2 proteins has also been observed in chronic inflammatory disorders.

Compared to lymphocytes, relatively little knowledge is available on the regulation of myeloid cell survival by the BCL2 family members. Among the prosurvival proteins, both MCL1 and BCL2A1 appear to have physiologically important roles in regulating myeloid cell survival. MCL1 is expressed at steady state in neutrophils, monocytes, and DC, but not in macrophages, unless they are activated [21, 22]. MCL1 provides short-term enhancement of myeloid cell survival during the critical transition differentiation process [22]. In normal or tumoral myeloid cells, the cytokines GM-CSF, IFN-α, IL-3, IL-5, IL-6, IL-15, or IL-22 upregulate MCL1 expression [22]. Upon exposure to pro-inflammatory cytokines, MCL1 is upregulated in granulocytes, monocytes, and macrophages and associated with BCL2A1 induction [19]. During chronic inflammatory diseases, apoptosis of neutrophils is significantly delayed due to upregulation of MCL1 [23]. Interestingly, BCL2A1 is not expressed in myeloid cells at steady state; yet, inflammatory stimuli including bacterial endotoxin like lipopolysaccharide, G-CSF, GM-CSF, IL-1β, TNF-α, IFN-γ, IL-8, and IL-17A induce it, thus extending the survival of neutrophils, granulocytes, mast cells, macrophages, and, as we recently documented, DC [13, 19, 24–26]. BCL2A1, an NF-κB target gene expressed in activated myeloid cells, supports key function in inflammation. In chronic inflammatory disorders, regulation of MCL1

Table 1: Approved Hugo gene nomenclature of the BCL2 family.

Approved symbol	Approved name	Activity	Location	Synonym
BCL2	B-cell CLL/lymphoma 2	Prosurvival	18q21.3	Bcl-2, PPP1R50
BCL2L1	BCL2-like 1	Prosurvival	20q11.21	Bcl-X, bcl-xL, bcl-xS, BCL2L, BCLX, PPP1R52
BCL2L2	BCL2-like 2	Prosurvival	14q11.2-q12	BCL-W, KIAA0271, PPP1R51
MCL1	Myeloid cell leukemia sequence 1 (BCL2-related)	Prosurvival	1q21	BCL2L3, Mcl-1
BCL2A1	BCL2-related protein A1	Prosurvival	15q24.3	ACC-1, ACC-2, BCL2L5, BFL1, GRS
BAX	BCL2-associated X protein	Proapoptotic multidomain	19q13.3-q13.4	BCL2L4
BOK	BCL2-related ovarian killer	Proapoptotic multidomain	2q37.3	BCL2L9, BOKL, MGC4631
BAK1	BCL2-antagonist/killer 1	Proapoptotic multidomain	6p21.31	BAK, BCL2L7
BAD	BCL2-associated agonist of cell death	Proapoptotic BH3-only	11q13.1	BBC2, BCL2L8
BID	BH3 interacting domain death agonist	Proapoptotic BH3-only	22q11.2	—
BCL2L11	BCL2-like 11 (apoptosis facilitator)	Proapoptotic BH3-only	2q13	BIM, BimEL, BimL, BimS, BOD
BMF	BCL2 modifying factor	Proapoptotic BH3-only	15q14	FLJ00065
BIK	BCL2-interacting killer (apoptosis-inducing)	Proapoptotic BH3-only	22q13.31	NBK
HRK	Harakiri, BCL2 interacting protein	Proapoptotic BH3-only	12q24.2	DP5
PMAIP1	Phorbol-12-myristate-13-acetate-induced protein 1	Proapoptotic BH3-only	18q21.32	APR, NOXA
BBC3	BCL2 binding component 3	Proapoptotic BH3-only	19q13.3-q13.4	JFY1, PUMA

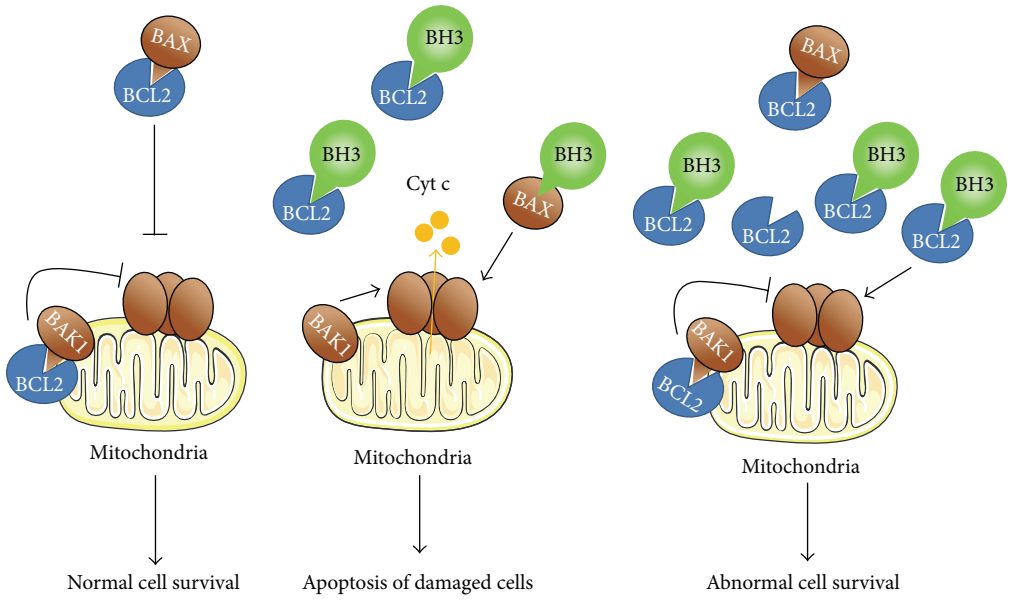
and BCL2A1 gene expressions results in recruitment and stabilization of myeloid cells of the immune system [27, 28]. We propose that, in healthy DC, while MCL1 expression provides short-term (two days) survival, additional BCL2A1 expression switches the DC phenotype and allow long-term survival. In this context, BCL2A1 induction operates downstream of NF-κB activation in IL-17A-stimulated DC [13] and may be very important in sustaining chronic T-cell activation in IL-17A-related diseases. This new concept places IL-17A-stimulated MCL1$^+$ BCL2A1$^+$ DC in the sunlight and makes MCL1 and BCL2A1 novel attractive therapeutic targets in chronic inflammatory diseases.

3. Targeting MCL1 and BCL2A1 by Chemotherapeutic Compounds

To overcome BCL2-family-mediated resistance to chemotherapy, different strategies have been tried, including their targeting by antisense oligonucleotides peptide inhibitors and small molecules inhibitors (SMIs) [29, 30]. Most widely used so far is the SMI ABT-737 (and its orally active follow-up ABT-263) which mimics the BH3-only proteins and binds with high-affinity BCL2, BCL2L1, and BCL2L2, inducing apoptosis in a BAX- and BAK-dependent way [31]. However, it binds only weakly to MCL1 and BCL2A1, and resistance to ABT-737 has been associated with high expression of MCL1 and BCL2A1 [32, 33].

Expression of both MCL1 and BCL2A1 has, in several hematological malignancies, been associated with chemoresistance or poor prognosis [34, 35]; thus, new drugs targeting these proteins must be developed. Some of the SMIs under development, including Obatoclax and Sabutoclax, have been shown to better target MCL1 or MCL1 and BCL2A1, respectively [36, 37]. Sorafenib, developed as a BRAF inhibitor, reduces MCL1 translation leading to increased apoptosis in leukemia cells [38] while Flavopiridol, a cyclin-dependent kinase inhibitor, suppresses MCL1 and has been used to treat patients with high-risk chronic lymphocytic leukemia (CLL) [39, 40].

We recently showed that monocyte-derived DCs, treated with IL-17A and IFN-γ that mimic chronic inflammation conditions, develop resistance to apoptosis. This resistance is associated with a robust coexpression of MCL1 and BCL2A1 and is dependent on IL-17A that induces BCL2A1 in MCL1$^+$ DC [13]. IL-17A- and IFN-γ-treated DCs were resistant to a variety of chemotherapeutic drugs. However, they were highly sensitive to the antimicrotubule drugs vinblastine and, to a lesser extent, vincristine and cytarabine. We showed



(a) At steady state or response to activation signals: BCR, TCR, CD40, proinflammatory cytokines, and so on

(b) Response to intracellular damage signals: growth factor deprivation, stress, UV, viruses, and so on

(c) In cancers or chronic inflammatory disorders: oncogenes, viruses, proinflammatory cytokines, and so on

Figure 3: Model of survival control by the BCL2 family in physiological and inflammatory conditions. (a) In a physiological context or following an immune response, constitutive or inducible prosurvival proteins (here BCL2) bind multidomain proapoptotic proteins (BAX, BAK) that become unable to oligomerize, thereby resulting in normal cell survival at steady or activated state. (b) In response to intracellular damage, activator BH3-only proteins are induced or activated. They inhibit the prosurvival proteins and activate the effector multidomain proapoptotic proteins, which in turn are homooligomerize, triggering cytochrome c release (Cyt c) and apoptosis. Potentially abnormal cells are then eliminated. (c) In a pathological context, such as cancers and chronic inflammatory diseases, prosurvival proteins are upregulated, inhibiting the multidomain and BH3-only proapoptotic proteins. As a result, the membrane of mitochondria remains intact and abnormal cells survive.

that exposure to vinblastine or cytarabine decreased MCL1 expression. Antimicrotubuli agents are widely used in various cancers, including hematological malignancies [41]. They induce mitotic arrest and trigger apoptosis through mechanisms which are not fully clear. However, Wertz et al. recently showed that vincristine-induced apoptosis is mediated by the molecular partnership between the ubiquitin-ligase FBW7 and MCL1, once it has been phosphorylated, downstream of vincristine treatment [42]. Ubiquitination of phospho-MCL1 by FBW7 led to the destruction of MCL1 by the proteasome. Our recent studies documented MCL1 degradation by vinblastine. We also confirmed that adding vinblastine to GMIC led to disorganization of the microtubule network and cell death.

IL-17A- and IFN-γ-treated DCs also underwent apoptosis upon addition of antibodies neutralizing IL-17A, which selectively reduced BCL2A1 expression. Our interpretation is that the long-term DC survival is dependent on both MCL1 and BCL2A1 expressions. In the future, it would be interesting to evaluate the targeting of both MCL1 and BCL2A1 in chronic IL-17A-related inflammatory diseases, using either Sabutoclax or the combination of toxic compounds targeting MCL1 (such as vinblastin, vincristine, cytarabine, or Obatoclax) with antibodies neutralizing IL-17A, the pro-inflammatory cytokine that induces BCL2A1 in human DC.

4. Role of Dendritic Cells and Regulatory T Cells in Rheumatoid Arthritis

RA is a chronic inflammatory disease of the synovium, a delicate membrane that lines the nonweight-bearing surfaces of the joint. In the absence of disease, synoviocytes produce factors that provide nutrition and lubrication for the surrounding cartilage tissue; few cellular infiltrates are seen in the synovium. In RA, the synovium is infiltrated by chronic inflammatory cells, such as macrophages, DC, neutrophils, T cells, and B cells. The resident fibroblasts adopt a quasi-malignant phenotype with upregulation of oncogenes, inhibition of apoptosis, and secretion of cytokines, chemokines, and enzymes that reinforce the inflammation and catalyse joint destruction. The resulting pannus acquires the ability to invade and destroy adjacent articular cartilage. Activation of OC in periarticular bone leads to resorption and erosion, a radiologically detectable hallmark of the disease. Similar processes affect the synovium that lines the tendon sheaths, resulting in tendon weakness and rupture, which are responsible for the characteristic deformities of RA. This is a potentially devastating disease that affects the whole individual, reducing the social contribution, destroying the quality of life, and ultimately shortening the patient's lifespan. Pro-inflammatory cytokines are amongst the most

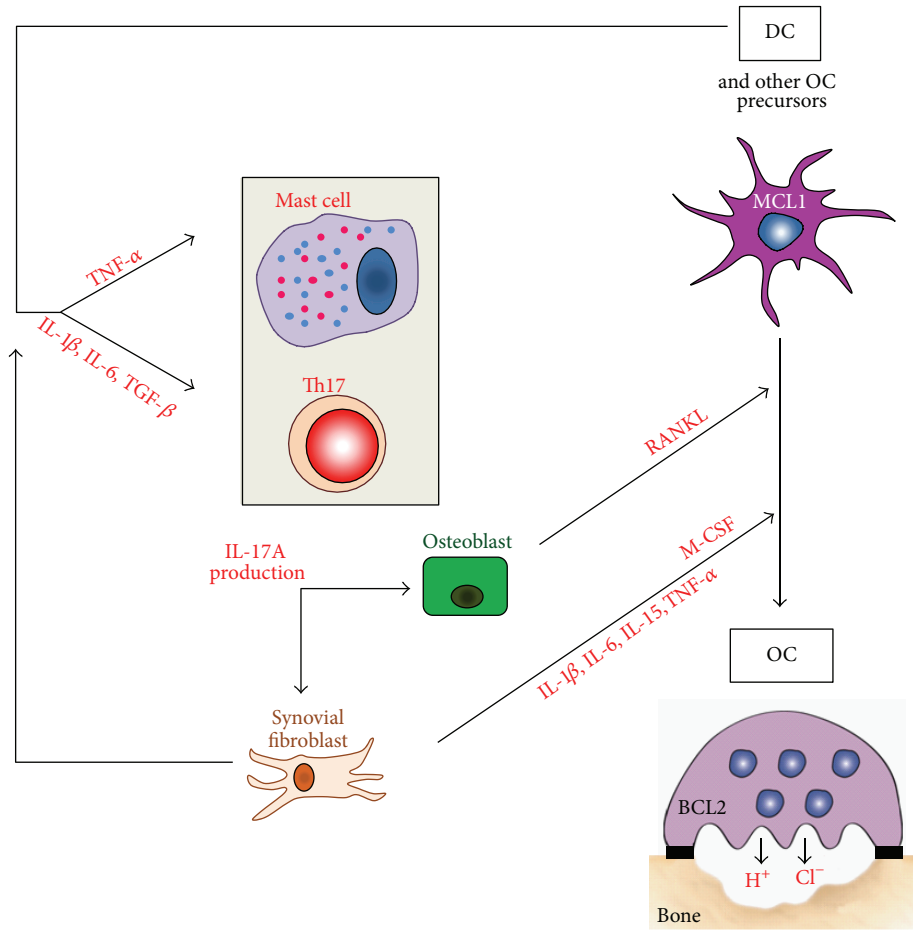


Figure 4: Pro-inflammatory cytokines drive bone resorption in rheumatoid arthritis. Cytokines are amongst the most important mechanisms driving bone resorption associated to inflammation mediated by M-CSF, RANKL, TNF-α, IL-1-β, IL-6, and finally IL-17A. In RA, IL-17A is mainly produced by Th17 and mastocytes, further amplifying inflammation by enhancing pro-inflammatory cytokine production of synovial fibroblast. IL-17A also increases bone resorption by inducing RANKL production by osteoblasts and M-CSF production by synovial fibroblasts; M-CSF and RANKL are the two cytokines required to differentiate OC from different cell sources: DC, monocytes, macrophages, or bone-marrow progenitors.

important mechanisms driving this process. In particular, M-CSF, RANKL, TNF-α, IL-1, and IL-17A play dominant roles in the pathogenesis of arthritis-associated bone loss (Figure 4). A common first line of treatment is methotrexate monotherapy, while nonresponders are treated with agents neutralizing TNF-α activity. Recently, in a phase I clinical study, biotherapy involving neutralization of IL-17A reduced signs and symptoms of RA with no strong adverse effects [43].

DCs are key players in RA. Rheumatoid synovium is characterized by accumulation of immature and mature DC subsets perivascularly, in close association with T cells and B-cell follicles [44–46]. Synovial fluid contains significant numbers of myeloid DC compared to blood, suggesting a role for these antigen presenting cells in disease perpetuation [47, 48]. DC may contribute to ongoing inflammation through presentation of autoantigens, as suggested by animal models of autoimmune arthritis [49] or secretion of crucial pro-inflammatory mediators or differentiation into OC [4]. Whether regulatory T-cell (T_{REG}) defects are present in patients with RA is not clear. The number of $CD4^+CD25^{high}$ T_{REG} in the peripheral blood of patients with RA was found to be higher than in healthy individuals in one study, but not in others [50]. DCs show evidence of activation *in vivo*: upregulation of MHC, costimulatory molecules, and expression of NF-κB, RANKL, and RANK [51]. The killing of activated DC and the investigation of tolerance induction by shaping DC plasticity towards tolerogenic DC may possibly give rise to a withdrawal of therapy.

5. Targeting MCL1 and BCL2A1 in Rheumatoid Arthritis

MCL1 is critical for the survival of macrophages in the joints of patients with RA, thus representing a potential therapeutic target in this disease [52]. In a mouse study, BIM-BH3 mimetic therapy reduced arthritis through the activation of myeloid cell apoptosis, thus demonstrating that BH3 mimetic therapy may have a significant potential for the treatment of RA [53]. More recently, Oliveira et al. evaluated gene

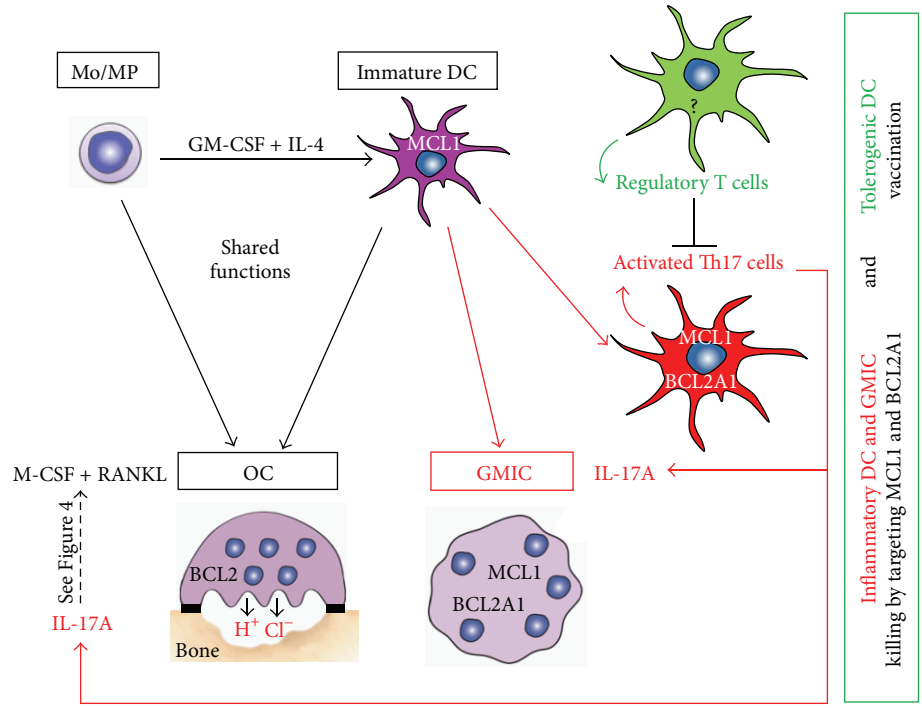


Figure 5: Model for the therapeutical management of diseases associated with IL-17A-dependent chronic inflammation with bone loss. In the context of IL-17A-driven inflammation, IL-17A amplifies OC formation and consequently bone resorption (see details in Figure 4). In addition, IL-17A induces BCL2A1 expression in MCL1$^+$ monocyte-derived DC, DC clustering, and fusion, leading to a mixed culture, containing both mononuclear and multinuclear (GMIC) inflammatory DC. These inflammatory DC and GMIC express MCL1 and BCL2A1 contrary to OC whose survival is under the control of BCL2. Tolerogenic DCs activate regulatory T cells able to control activated Th17 cells. The question mark indicates that the status of BCL2 family in tolerogenic DC is unknown and should be studied to validate this model. In order to cure diseases with IL-17A-dependent chronic inflammation associated with bone loss, we suggest killing inflammatory DC and GMIC by targeting both MCL1 and BCL2A1. In parallel performing therapeutic autologous vaccination with tolerogenic DC may help breaking IL-17A-dependent chronic inflammation to restore normal bone physiology.

expression profiles of (i) RA patient responders and nonresponders to methotrexate and, in the case of nonresponders, (ii) the responders and nonresponders to methotrexate combined with anti-TNF-α biotherapy [54]. They identified nine genes in methotrexate nonresponders and three genes in methotrexate plus anti-TNF-α nonresponders. Two genes were common in both lists: CCL4 and BCL2A1. This is a strong argument to further evaluate the role of BCL2A1 in RA and, in particular, a potentially overlooked role of long-term surviving IL-17A-stimulated MCL1$^+$ BCL2A1$^+$-activated DCs. Segura et al. have just characterized that inflammatory DCs, found in human inflammatory fluids, represent a distinct human DC subset, sharing gene signatures with *in vitro* monocyte-derived DC and involved in the induction and maintenance of Th17 cell responses [55]. If the survival pathway of these inflammatory DC is different from that of tolerogenic DC (Figure 5), it would be possible, on the one hand, to vaccinate with autologous DC exhibiting potent tolerogenic functions and, on the other hand, to induce apoptosis of inflammatory DC, in order to reinstate immune tolerance [56] and to abrogate IL-17A-dependent DC-driven inflammation.

6. Role of Dendritic Cells and Regulatory T Cells in Langerhans Cell Histiocytosis

LCH is a rare disease which belongs to the histiocytic disorders characterized by tissue damage induced by infiltrating cells (histiocytes), derived from the monocytic lineage [57]. LCH occurs predominantly in children but can occur at any age. Clinical manifestations can vary from a single self-resolving lesion to a severe life-threatening systemic form. Multiple organs may be affected by this disease including bone (80% of the patients), skin, lymph nodes, endocrine glands, and the central nervous system. LCH lesions are heterogeneous and form aggressive granulomas containing CD1a$^+$ CD207$^{+/-}$ cells (presumed to be pathogenic LCH cells) admixed with macrophages, T cells, eosinophils, and MGC [58]. Killing the lesional tissue-aggressive LCH cells is difficult but may be achieved in most patients by chemotherapy regimens containing the combination of prednisone and vinblastine or, in salvage settings, cladribine and cytarabine.

The exact origin of pathogenic LCH cells is unclear. Based on many common features, it was proposed that they arise from epidermal CD207$^+$ Langerhans cells. However, in 2008,

we proposed that pathogenic DC may derive from monocytes rather than belonging to the Langerhans cell lineage [7]. This is, in keeping with data from a recent gene expression profile study of human cells isolated from LCH granulomas, also suggesting that LCH lesions originate from accumulation of immature myeloid DC rather than epidermal Langerhans cells [59]. Finally, LCH DCs exhibit a unique transcription profile that separates them from all previously known DCs based on their expression of both Notch ligand and its receptor [60].

The etiology of LCH remains controversial between an inflammatory disorder, a neoplasm, or even both since induction of long-term DC survival by inflammation may license accumulation of mutations; this might provide to LCH DC a more apoptotic-resistant behavior. Senechal et al. found that less than 2% of cells were proliferating within lesions and propose that pathogenic DC accumulation is mainly the consequence of increased survival rather than proliferation [61]. This was associated with a local and systemic expansion of $CD25^{high}$ $FoxP3^{high}$ T_{REG} possibly impairing the resolution of LCH granulomas [61]. Altogether, these data suggest that immunological mechanisms play the major role in the development of LCH. Furthermore, evidence of concordance for LCH in monozygous twins supports the concept of a genetic predisposition to develop LCH, possibly affecting the immune system regulation [62]. However, Badalian-Very and colleagues found that 57% (35 of 61) of examined LCH specimens display the oncogenic BRAF V600E mutation [63]. These findings were also confirmed by additional independent studies [64, 65]. BRAF is a pivotal protein kinase of the RAS-RAF-MAPK signaling pathway which regulates cell survival and proliferation. In pathological LCH cells, constitutive activity of the mutant BRAF V600E protein may lead to a deregulated signaling through this pathway, thereby resulting in increased cell survival [66].

7. Targeting MCL1 and BCL2A1 in Langerhans Cell Histiocytosis

In LCH lesions, apoptotic pathways have been shown to be active alongside prosurvival pathways [67–69], and the expansion or healing of a granuloma is likely the sum of these apparently conflicting activities. Concerning the BCL2 family members, BCL2 expression was documented in LCH DC in two separate studies using immunohistochemistry and *in situ* hybridization [68, 69]. However, BCL2 was not found to be elevated in $CD207^+$ cells from LCH lesions analyzed by transcriptome analysis by Allen et al. [59], who instead showed upregulation of BCL2L1 and BAX. Similar data on BCL2L1 had been previously described in pulmonary LCH [70]. Whether upregulation of survival molecules in LCH is due to exogenous stimuli, such as cytokines, or intrinsic mutations such as BRAF V600E, in the RAS-RAF-MAPK signaling cascade [63] in a majority of samples from LCH biopsies, is still not clarified. So far, no other cancerogenic mutations have been found in LCH and the BRAF V600E mutation by itself is not sufficient for tumor development [71]. Southern blot analysis performed by the Savell team showed no evidence for gene rearrangement of the BCL2 gene [68]. An abundant number of cytokines have been described in LCH lesions, many with the potential to affect cell survival [72, 73]. Considering the presence also of IL-17A in LCH and the therapeutic efficacy of vinblastine, targeting MCL1, it would be interesting to study the role of MCL1 and BCL2A1 in LCH and to correlate their expression to disease progress and drug resistance. Depending on these future studies, targeting MCL1 and BCL2A1 in LCH may be of importance, at least to prevent the intense bone resorption occurring in 80% of the patients with LCH.

8. Exploiting DC Surface Molecules to Specifically Target BCL2A1-Expressing DC

There are a variety of *in vivo* DC-targeting strategies used in different contexts including autoimmune disease therapies [74], vaccine-induced immunity [75], and cancer therapy [76]. Specific targeting of long-term survival $BCL2A1^+$ DCs may form a promising therapeutic avenue in inflammatory conditions. Two different strategies can be suggested: inhibition of the intracytoplasmic activity of BCL2A1 or prevention of IL-17A signal transduction in DCs. In the former, a BCL2A1 inhibitory peptide (that should be developed) may be delivered directly to DCs by using a fusion protein built with this peptide and GM-CSF, whose receptor is expressed by $BCL2A1^+$ DCs. More specifically, it can be loaded into biodegradable nanoparticles attached to monoclonal antibodies that recognize specific DC surface receptor(s). Since human inflammatory DCs appear most likely to be derived from monocytes *in vivo* [55], a potential target surface receptor is CD209/DC-SIGN, expressed by these cells in tissues and absent on the surface of other DC subpopulations. Specific inhibition of IL-17A signal transduction in DCs may be achieved through an approach which combines anti-DC-SIGN and anti-IL-17A antibodies. This may result in the neutralization of IL-17A and the subsequently induced BCL2A1 expression in DCs.

9. Conclusion

DCs are critical regulators of immune responses not only at initiation, but also, as recently demonstrated, for the maintenance of chronic inflammation, especially the IL-17A-driven chronic inflammation [55]. We demonstrated that IL-17A activates long-term survival pathway by inducing BCL2A1 in DC, thus providing a molecular basis for DC maintenance in IL-17A-mediated chronic inflammation. Cytokines and BCL2-related survival pathway may interplay to determine not only myeloid cell accumulation and inflammatory DC maintenance, but also their fusion and final differentiation into either GMIC or OC, the bone-resorbing giant cells (Figure 5). This may affect the clinical course and final long-term outcomes of patients. However, fundamental research is required to solve the question mark: a better understanding of the disease-related alterations in BCL2-related survival pathways and functions of DC might offer the opportunity

to design and fine-tune approaches aimed at killing inflammatory DC, while therapeutic vaccination may reintroduce tolerogenic DC. Since chemotherapy remains, at present, the standard of care for LCH, introduction of immunemodulation is highly warranted. Currently available data suggest that manipulation of the BCL2 family (with the decrease of both MCL1 and BCL2A1) in DC, associated with a therapeutic vaccination with autologous tolerogenic DC, might represent a suitable treatment in rheumatoid arthritis and Langerhans cell histiocytosis, possibly leading to a cure.

Abbreviations

BCL2: B-cell lymphoma 2
BCL2A1: BCL2-related protein A1
DC: Dendritic cell
GMIC: Giant myeloid inflammatory cells
IFN: Interferon
LCH: Langerhans cell histiocytosis
MCL1: Myeloid cell leukemia sequence 1
MGC: Multinucleated giant cells
OC: Osteoclast
RA: Rheumatoid arthritis
SMI: Small molecules inhibitors
T_{REG}: Regulatory T cell.

Conflict of Interests

The authors declare no conflict of interests.

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Review Article

What Are the Molecules Involved in Regulatory T-Cells Induction by Dendritic Cells in Cancer?

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Dendritic cells (DCs) are essential for the maintenance of homeostasis in the organism, and they do that by modulating lymphocyte priming, expansion, and response patterns according to signals they receive from the environment. The induction of suppressive lymphocytes by DCs is essential to hinder the development of autoimmune diseases but can be reverted against homeostasis when in the context of neoplasia. In this setting, the induction of suppressive or regulatory T cells contributes to the establishment of a state of tolerance towards the tumor, allowing it to grow unchecked by an otherwise functional immune system. Besides affecting its local environment, tumor also has been described as potent sources of anti-inflammatory/suppressive factors, which may act systemically, generating defects in the differentiation and maturation of immune cells, far beyond the immediate vicinity of the tumor mass. Cytokines, as IL-10 and TGF-beta, as well as cell surface molecules like PD-L1 and ICOS seem to be significantly involved in the redirection of DCs towards tolerance induction, and recent data suggest that tumor cells may, indeed, modulate distinct DCs subpopulations through the involvement of these molecules. It is to be expected that the identification of such molecules should provide molecular targets for more effective immunotherapeutic approaches to cancer.

1. Background

Regulatory T cells (Tregs) are crucial to the maintenance of tolerance to autoantigens [1]. The failure of Treg function or their depletion has been implicated in the development of many autoimmune diseases in humans and in mouse models [2]. However, Treg-mediated suppressive activity can also contribute to the immune escape of pathogens or tumors [3, 4]. Nowadays, regulatory T cells (Tregs) are considered one of the major obstacles to the success of immunotherapeutic approaches to cancer [5–8]. Several studies have described the direct association between Treg increase and tumor development, implicating this phenomenon as one of the most important escape mechanisms in different tumor types [7, 9, 10]. Many evidences have demonstrated that Treg accumulation is not restricted to the tumor site but is observed in the peripheral blood as well, from patients with distinct malignant tumors, including pancreas and breast [11], lung [12], and ovarian cancer [4, 12]. Indeed, elimination of Tregs in mouse tumor models can improve antitumor immune responses and survival [9, 13].

Dendritic cells (DCs) are believed to act as sensors of the homeostatic equilibrium of their environment, where they capture antigens to present to T lymphocytes. Thus, depending on the status of the tissue, they might induce immunity or tolerance to the antigens they present. Indeed, many *in vitro* studies have demonstrated that DCs are essential for regulatory T-cells induction [14, 15], apparently depending on various distinct mechanisms [16], but also, frequently, on external sources of cytokines, among which TGF-beta seems to play a predominant role [17]. Not surprisingly, therefore, during tumor development the balancing role of DCs in the T helper versus Treg stimulation seems to be deeply modified [8, 18].

However, despite all the accumulated data, the precise role of DCs in the imbalance between T helper and Tregs

in cancer is still unclear. Do the observed biases of DC function in tumor bearers reflect a previous disturbance in their immune homeostasis or are these deviations of DC function the cause of the other immunological abnormalities? How significant is the contribution of these DC deficits to the escape of tumors from the body's control? Though the answer to these questions is not available yet, the increasing knowledge and characterization of DC behavior in the presence of tumors allows us to predict that it will be, and, furthermore, that, once reached, it will provide us with powerful tools for the clinical management of cancer. With these goals in view, we discuss, here, the impact of tumor presence in the membrane phenotype and function of DCs and their bias to induce/expand regulatory T cells.

2. The Tumor Microenvironment: A Tolerogenic Milieu

Several studies have described the potential impact of tumor-derived products in the suppression of immunity. Signals derived from tumors not only act directly upon immune effector cells but also induce the conversion and/or the recruitment of cells with suppressive functions to their microenvironment [19]. In consequence, tumors are typically characterized by the presence of higher concentrations of anti-inflammatory molecules, such as TGF-beta, IL-10, and prostaglandin E2 [20–23], increased amounts of angiogenic factors, as the vascular endothelial growth factor (VEGF) [24], and augmented CCL22 chemokine gradient [25] in addition to the local expression of immune-inhibitory molecules, including CTLA4 and PD-1/PD-L1 [26, 27]. Altogether, these constitute, nowadays, the most highly sought targets to achieve the breakdown of tumor-associated microenvironment-induced tolerance. Still, in order to obtain an immune recovery in face of tumors, we still need to identify the source of the tolerogenic signals. Though tumors cells may produce such mediators, also tumor-infiltrating leukocytes may be their source, and, indeed, the study of such populations has revealed that regulatory $Foxp3^+$ T cells (Tregs) [28], anti-inflammatory M2-macrophages [29], plasmacytoid dendritic cells (pDCs) [30], and immature myeloid DCs [31] accumulate in human neoplastic tissues and patients' blood [4] and have been associated with poor prognosis for the patients specific cancer types.

As mentioned, the presence of tolerance-inducing conditions seems not to be restricted to the tumor microenvironment. Several studies have demonstrated the increase of anti-inflammatory cytokines and the higher frequency of suppressive cells in the bloodstream and lymph nodes from cancer patients. The detection of higher amounts of cytokines like TGF-beta [32], M-CSF [33], and IL-6 [34, 35] in patients' serum could suggest that the tumor presence affects cells in distant organs, thus resulting in systemic alterations which could allow tumors not only to grow locally unchecked but also to metastasize without an effective immune barrier. In agreement with that are: the higher frequency of myeloid-derived suppressor cells (MDSCs) (a group of immature but potent suppressor cells capable of down-regulating anti-tumor immunity) found in cancer patients' circulation [36]; the decreased frequency of circulating and tumor-infiltrating myeloid DCs [37, 38]; and the CD4 lymphopenia observed in cancer patients [39–41]; all three important alterations of immune homeostasis in cancer patients that, consequently, hamper the effectiveness of their treatment.

3. DCs: Targets to the Tumor Tolerogenic Milieu

Dendritic cells (DCs) are the best adapted professional antigen-presenting cells (APCs) able to initiate, coordinate, and regulate the adaptive immune responses by inducing naive T-cells differentiation into diverse T helper lymphocyte subtypes [42–46]. Generally, at homeostasis condition, tissue-resting DCs are in immature status (lower MHC class II and costimulatory molecules expression) and strategically located to sense and acquire antigenic products from the environment. Using nonspecific receptors, immature DCs can recognize pathogens or danger-associated molecular patterns (as known, PAMPs and DAMPs, resp.) and migrate to lymphoid organs, at the same time as they increase their expression of MHC, CD80, CD86, and CD40 surface molecules and become ready to activate naïve T lymphocytes [44]. DCs are also crucial for the induction/maintenance of T-cell tolerance to antigens acquired in "healthy" tissues, thus performing an essential role in the prevention of autoimmunity [47].

It is also evident that the term DC is applied to several distinct subpopulations, classified, still incompletely, in relation to their tissue localization, migratory ability, surface markers' expression, and the profile of soluble factors they release. Though still uncertain, it is becoming increasingly clear that any classification of DCs will be insufficient to accommodate all the plasticity of these cells. Therefore, a better approach to the problem would be to describe, as well as possible, the DCs found in a certain condition, and from that, to correlate their phenotype in that specific situation with the known functions of these cells. This has been done in relation to DCs within tumors and has shown that tumors modify significantly the phenotype of DCs within their microenvironment [8, 22]. Various observations point to a mainly functional deficit of these cells in immune stimulation, due to a decreased frequency of mature, functionally competent DCs within tumors [31] and in peripheral blood [48]. Actually, we have already shown an altered expression of CD86 in Mo-DCs from advanced cancer patients, which was, apparently, corrected by an immunotherapeutic approach [49]. Importantly, the presence of pDCs in tumor sites has been also related to poor prognosis in cancer patients [30], and their functional investigation revealed a considerable low to absent IFN-alpha production in breast and ovarian cancer [50, 51]. The tumor-associated stroma and cancer cells *per se* can generate signals that drive DC to a tolerogenic pathway, characterized, mainly, by a poor upregulation of MHC class II and costimulatory molecules and absent or low production of proinflammatory cytokines [52], thus favoring tumor

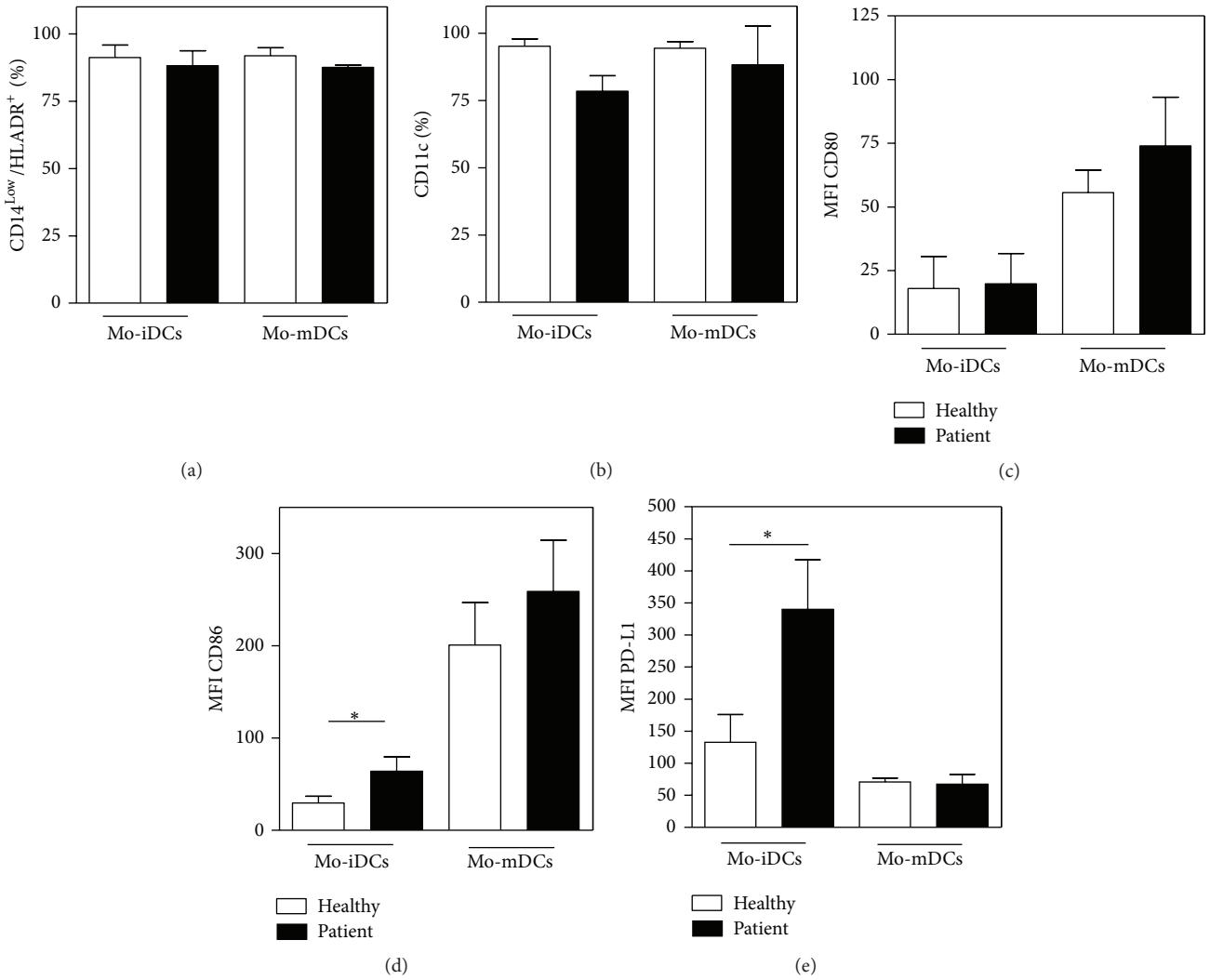

Figure 1: Patients' immature Mo-DCs express higher levels of PD-L1. Blood monocytes from control, and breast cancer patient subjects were cultured in the presence of IL-4 and GM-CSF for seven days and, subsequently, characterized. Flow cytometry analysis showing grouped frequency of $CD14^{Low}/HLADR^{+}$ (a) and $CD11c^{+}$ (b) cells and Mean Intensity Fluorescence (MFI) values of $CD14^{Low}/HLADR^{+}$ gated cells to CD80 (c), CD86 (d), and PD-L1 (e) molecules in Mo-iDCs from healthy donors and breast cancer patients ($^{*}P < 0.05$, two-tailed unpaired t-test; healthy $n = 5$; patients $n = 9$). (Mature Mo-DCs were activated by TNF-alfa for 48 hours.)

evasion from the immune system. Interestingly, in tumor-bearing mouse, the presence of DCs is also crucial for cancer vascularization, and when DCs are depleted, the elimination of malignant cells can be enhanced [53, 54]. Additionally, another elegant study showed that human myeloid DCs expressing OX40L stimulate Th2 immunity *in vitro,* under the influence of thymic stromal lymphopoietin (TSLP) derived from breast tumor cells [55]. Such findings may explain the bias towards a Th2 inflammatory tumor microenvironment found in breast cancer.

Since it became possible to achieve DC differentiation from human blood monocytes (Mo-DCs) [56], the immunostimulatory potential of these cells could be harnessed for cancer immunotherapy [57–60]. On the other hand, *in vitro* findings, describe that tumor cells present during human monocyte differentiation cause alteration in their molecular expression and unsuccessful DC differentiation, even under exogenous cytokine addition [61–63]. In addition, we have shown that breast cancer patients' monocyte-derived DCs are phenotypic altered and biased to induce Tregs [64], even though differentiated without the presence of tumor cells in the culture.

Immature Mo-DCs from patients express higher levels of CD86 and PD-L1 membrane molecules after 7 days in the presence of IL-4 and GM-CSF (Figure 1). Though the expression of CD86 could be interpreted as an enhanced costimulatory ability, the same cannot be implied for PD-L1. PD-L1, also known as B7-H1, has been described as an inhibitory molecule in T lymphocyte activation [65, 66] and also related to T effector to Treg conversion [67] and the induction of T cell anergy by Mo-DCs [68]. Furthermore, its expression has been described as enhanced in monocytes from peritumoral stroma in hepatocellular carcinoma [26] and in lung cancer infiltrating DCs [69].

4. Regulatory T-Cells Induction by Tumor-Affected DCs

The induction and expansion of Tregs by DCs are generally related to their role in the maintenance of tolerance to self [16]. Several studies have been developed, trying to identify the signals that drive DCs into that function and, thus, eventually allow the use of such educated DCs to control unwanted immune responses, like those against transplanted tissues or in autoimmune diseases [70]. Actually, the acquisition of the ability to promote Tregs is an integral part of the physiologic function of DCs, as can be noted, for instance, in the presence of apoptotic cells [71, 72]. In this search, anti-inflammatory cytokines as IL-10 [73, 74], TGF-beta [75], and vitamin D3 addition [76, 77] have been shown to affect mouse and human DCs, causing them to stimulate regulatory or suppressive T lymphocytes [78]. Intriguingly, even inflammatory cytokines, as TNF-alpha, have been associated with tolerogenic DC induction in autoimmune disorders like the murine Experimental Autoimmune Encephalomyelitis (EAE) [79]. Paradoxically, the same functional status of DCs, which is the still unreached aim of research in autoimmunity and transplantation studies, is the natural status of DCs in cancer, which is, again, beyond our powers of effective modulation. Tumor cells are associated with lower activation of immune cells and hinder APC activation [32, 80, 81] and, also can attract regulatory T cells to their microenvironment [4, 11, 12], all phenomena which would be more than welcome in the aforementioned autoimmune and transplant recipients. Regarding APCs, *in vitro* studies showed Treg induction by human Mo-DCs stimulated by pancreatic or lung tumor cells [61, 62], the ability of human intratumoral pDCs, to expand Tregs *ex vivo* in breast cancer [50] and to induce suppressive activity by T cells in prostate cancer [82]. These findings show that tumor cells are able to promote Tregs induction by DCs in patients, and also to affect DCs from healthy donors, causing them to stimulate Tregs. Finally, our group has demonstrated that this effect of tumors upon DCs does not depend on the continuous presence of neoplastic cells, since Mo-DCs from breast cancer patients even when differentiated *in vitro* and, therefore, away from the direct tumor influence, are poor T-cell stimulators and biased to induce $CD4^+CD25^+Foxp3^+$ regulatory T cells when cocultured with naïve $CD4^+CD45RA^+$ lymphocytes (Figure 2). It should be noted that this bias was present, regardless of the maturation stimulus used to activate the patients' Mo-DCs [64]. Taken together, these data indicate that during tumor development a systemic tolerogenic status of DCs is favored, enhancing their ability to expand/recruit Tregs and whose specific mechanisms are still largely undetermined.

5. Potential Mechanisms of Tumor-Affected DCs in the Induction of Tregs

Cytokines, as TGF-beta and IL-10 in addition to IL-2, are currently used to expand effectively murine and human Tregs *in vitro*. Interestingly, the same cytokines can also induce DCs to stimulate Tregs *in vitro* [17, 74, 75]. This may suggest that the major signals responsible for the generation and expansion of regulatory T cells *in vitro* and in vivo are already known. However, few data are available in regards to the mechanism of tumor-conditioned DCs in Tregs induction. Recent findings have demonstrated that infiltrating pDCs from ovarian [83] and breast tumor [84] can express high levels of ICOS-L, a phenomenon that could explain their ability to stimulate $Foxp3^+$ Tregs *in vitro*. Our own data also have shown that the Tregs induction by Mo-DCs from cancer patients could be partially reversed by blocking of TGF-beta *in vitro*, and not by LPS, proinflammatory cocktail, or sCD40L activation [64]. TGF-beta is a multifunctional cytokine that regulates T-cell growth and development [85], inhibits IL-2 production, and has potent antiproliferative effects on $CD4^+$ T cells [86], principally by inducing regulatory T cells [87]. However, since blocking of this cytokine was not enough to abolish the Treg-induction bias of the patients' Mo-DCs, it is likely that the TGF-beta signal may act together with other factors. Among the candidates for this cosignaling it is interesting to note that patients' Mo-DCs expressed higher levels of surface CD86 and PD-L1 (Figure 1), both molecules that have been also implicated in the balance of Tregs stimulation [88–91]. Thus, the TGF-beta signal may actuate together with surface molecules signals to "complement" the patients' Mo-DCs signalization in the induction/expansion of Tregs, as we showed here that DC-T cell contact is essential in that phenomenon (Figure 3).

6. Concluding Remarks

Tregs are recognized as central in the maintenance of tolerance to self [1] but may be also involved in the failure of the immune system to eliminate or control infections [3], tumors [13] and to respond to therapeutic vaccination [92]. Nowadays, it is also broadly accepted that DCs may play a crucial role in tolerance by the induction of Tregs at peripheral tissues and organs [16]. On the other hand, it is also known that tumor cells can alter profoundly the ability of DCs to instruct the immune system to generate adaptive antitumor responses [22], thus deviating the response to tolerance. The physiological DC ability to induce Treg activation depends on various cytokines and costimulatory molecules, but the exact balance between these, particularly, in DCs from cancer patients, is still unclear. CD86 and CD80 bind to both stimulatory (CD28) and inhibitory (CTLA-4) receptors on T cells, with different affinities [93]. In human DCs, the induction and upregulation of CD86 was shown to influence significantly T-cell activation [94], while studies in knockout mice have indicated that DCs ability to generate/expand Treg subsets can be related to the balance of CD80 and CD86 [89, 95].

Confirming the significant role of CTLA-4 signaling in the immunosuppression of cancer patients, the blockage of this molecule in clinical settings by monoclonal antibodies has been able to improve significantly the survival of metastatic melanoma patients [96, 97]. Additionally, PD-L1, ICOS-L, and TGF-beta seem to emerge as good candidates for the *in vitro* manipulation of DC phenotype/function for immunotherapeutic approaches. More recently, clinical trials targeting the PD-1/PD-L1 axis with anti-PD1 monoclonal

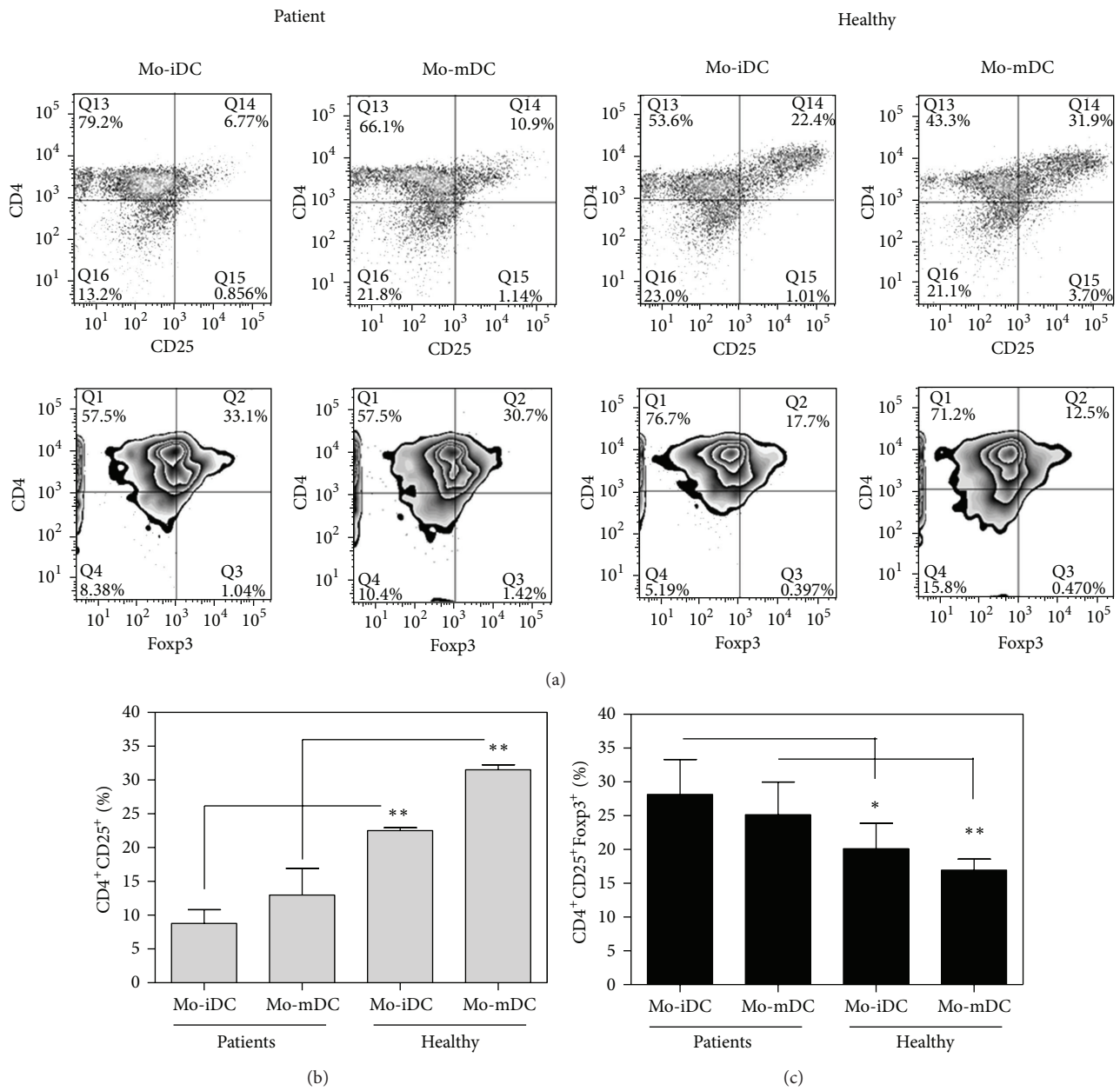

Figure 2: Patients' Mo-DCs fail to activate $CD4^+$ lymphocytes and induce higher Foxp3 expression even after maturation. Mo-DCs from controls and breast cancer patients were cocultured with allogeneic $CD4^+CD45RA^+$ cells for five days. At the end of culture the phenotype of lymphocytes was evaluated by flow cytometry. (a) Representative experiments of CD25 and Foxp3 expression in $CD4^+$ lymphocytes stimulated by immature DCs (Mo-iDCs) or mature DCs (Mo-mDCs) from healthy donors or breast cancer patients. Average frequency of $CD25^+$ cells (b) and $CD4^+CD25^+Foxp3^+$ cells (c) after $CD4^+CD45RA^+$ lymphocytes' coculture with Mo-DCs ($^*P < 0.05$; $^{**}P < 0.01$, two-tailed unpaired t-test; $n = 4$). (Mature Mo-DCs were activated by TNF-alfa for 48 hours.)

antibodies revealed their safety [98] and achieved promising results, with tumor regressions in patients with advanced cancer [99, 100], thus indicating another possible pathway to be explored in the clinic.

Nevertheless, these data are still sparse and much needs to be determined before an effective manipulation of DC phenotype and function is achieved. In order to accomplish this, however, studies addressing the intracellular signaling pathways in tumor-affected DCs are urgently needed and may shed light on the precise mechanisms of their response to tumors as well as provide molecular targets for their effective manipulation.

Abbreviations

APCs: Antigen-presenting cells
DCs: Dendritic cells
GM-CSF: Granulocyte macrophage colony stimulating factor

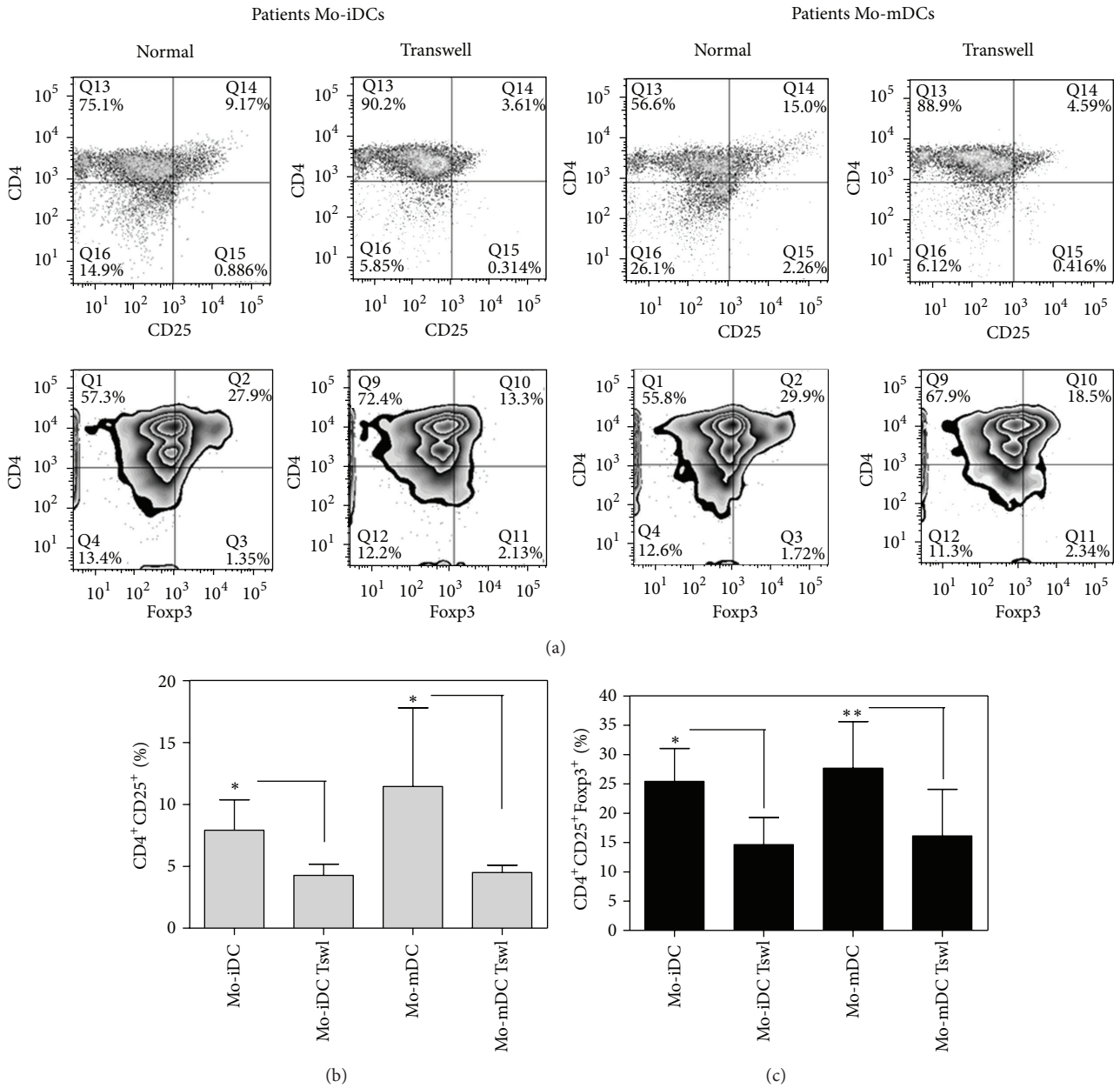

Figure 3: Patients' Mo-DCs induce expansion of regulatory T lymphocytes with the cooperation of contact molecules. Mo-DCs from breast cancer patients were cocultured with allogeneic $CD4^+CD45RA^+$ cells for five days in a transwell system or not. At the end of culture the phenotype of lymphocytes was evaluated by flow cytometry. (a) Representative experiments of CD25 and Foxp3 expression in $CD4^+$ lymphocytes stimulated by immature DCs (Mo-iDCs) or mature DCs (Mo-mDCs) from breast cancer patients in normal or transwell condition. Average frequency of $CD25^+$ cells (b) and $CD4^+CD25^+Foxp3^+$ cells (c) after $CD4^+CD45RA^+$ lymphocytes' coculture with patients' Mo-DCs ($^*P < 0.05$; $^{**}P < 0.01$, paired t-test; $n = 4$). (Tswl: transwell system; mature Mo-DCs were activated by TNF-alfa for 48 hours.)

IFN-gamma: Interferon-gamma
MFI: Median fluorescence intensity
MHC: Major histocompatibility complex
Mo-DCs: Monocyte-derived dendritic cells
Mo-iDCs: Monocyte-derived immature dendritic cells
Mo-mDCs: Monocyte-derived mature dendritic cells
PBMCs: Peripheral blood mononuclear cells
pDCs: Plasmacytoid dendritic cells
TGF-beta: Transforming growth factor-beta
TNF-alpha: Tumor necrosis factor-alpha
Tregs: Regulatory T cells
TSLP: Thymic stromal lymphopoietin
Tswl: Transwell coculture system.

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Review Article

Dendritic Cells: Cellular Mediators for Immunological Tolerance

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In general, immunological tolerance is acquired upon treatment with non-specific immunosuppressive drugs. This indiscriminate immunosuppression of the patient often causes serious side-effects, such as opportunistic infectious diseases. Therefore, the need for antigen-specific modulation of pathogenic immune responses is of crucial importance in the treatment of inflammatory diseases. In this perspective, dendritic cells (DCs) can have an important immune-regulatory function, besides their notorious antigen-presenting capacity. DCs appear to be essential for both central and peripheral tolerance. In the thymus, DCs are involved in clonal deletion of autoreactive immature T cells by presenting self-antigens. Additionally, tolerance is achieved by their interactions with T cells in the periphery and subsequent induction of T cell anergy, T cell deletion, and induction of regulatory T cells (Treg). Various studies have described, modulation of DC characteristics with the purpose to induce antigen-specific tolerance in autoimmune diseases, graft-versus-host-disease (GVHD), and transplantations. Promising results in animal models have prompted researchers to initiate first-in-men clinical trials. The purpose of current review is to provide an overview of the role of DCs in the immunopathogenesis of autoimmunity, as well as recent concepts of dendritic cell-based therapeutic opportunities in autoimmune diseases.

1. Introduction

Dendritic cells (DCs) are widely recognized as the most professional antigen-presenting cells (APCs). Moreover, they are indispensable in the regulation of the delicate balance between immunity and tolerance [1–3]. By interacting with other cells of the immune system through cell-cell contact or the production of cytokines, DCs induce an appropriate answer to a specific antigen. DCs can also prevent (auto)immunity by inducing apoptosis of autoreactive T cells in the thymus on the one hand (i.e., central tolerance), and by induction of anergy, deletion, or tolerance through cooperation with regulatory T cells (Treg) in the periphery on the other hand (i.e., peripheral tolerance). Consequently, it has been hypothesized that defects in the number, phenotype, and/or function of DCs cause the development of autoimmune diseases. Furthermore, DC-based antigen-specific modulation of the unwanted responses is evaluated for therapeutic approaches in recent years and may have several advantages in contrast to standard treatments which can induce a variety of complications and have serious side-effects. Indeed, considering the key role of DCs in the induction and activation of both effector T cells and Treg, DCs can be used to suppress or redirect immune responses in an antigen-specific manner. Recent investigations have shown promising results for the role of DCs as cellular treatment of autoimmune diseases and in preventing transplant rejections. Here, we discuss the role of DCs in the immunopathogenesis

of autoimmunity, especially with regard to mechanisms underlying T cell tolerance, and recent concepts of DC-based therapeutic opportunities in autoimmune diseases.

2. Dendritic Cells: Key Regulators of Immunity and Tolerance

2.1. DC Subsets and Differentiation Stages. DCs originate from CD34+ hematopoietic progenitor cells in the bone marrow and are generally classified in two groups: myeloid or classical DCs (cDCs) and plasmacytoid DCs (pDCs) [1, 4]. pDCs are characterized by expression of CD123 and a high production of type I interferon (IFN). Whereas pDCs differentiate from lymphoid progenitor cells in lymphoid organs, cDCs are derived from myeloid progenitor cells in the bone marrow and differentiate into immature DCs (iDCs) with different features. (i) Langerhans cells are characterized by expression of CD11c and CD1a. Once they enter the blood circulation, they migrate to the epidermis. (ii) Interstitial DCs are CD11c+CD1a− and are found in the interstitium of various organs including the lungs, the gastrointestinal tract, afferent lymphatic vessels, and the dermis. (iii) During physiological stress, monocyte-derived DCs can originate from CD14+ monocytes under the influence of a combination of stimuli, such as granulocyte-macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor-α (TNF-α), and interleukin (IL)-4.

The widespread distribution of DCs underlines their sentinel function. Indeed, DCs are most concentrated in places of the body where invasion of pathogens is most likely. Additionally, they are also present in organs such as the heart and kidneys and lymphoid structures, including the spleen, lymph nodes, and the thymus. Where present, iDCs take up both foreign as well as self-proteins and structures and process them intracellularly to antigens that are subsequently presented in the context of major histocompatibility (MHC) class I and II molecules on the cell's surface. Once DCs capture these antigens in the presence of so-called "danger signals," DCs undergo a complex maturation process. For this, DCs are equipped with pathogen-recognition receptors (PRRs) which detect foreign antigens (i.e., pathogen-associated molecular patterns, PAMPs) thereby activating specific signalling pathways to drive biological and immunological responses. These stimuli can be bacterial products, such as lipopolysaccharide (LPS), or viral products, including double-stranded RNA, but also proinflammatory cytokines like TNF-α [1, 5]. Upon maturation, DCs efficiently present the antigen/MHC complex in combination with co-stimulatory molecules, have changed their pattern of cytokine production [6], and will migrate to the lymph nodes where they eventually activate T cells [1, 7].

2.2. The Immunological Synapse. DCs bridge innate and adaptive immunity, integrate a variety of stimuli, and establish protective immunity. For this, efficient communication between DCs and T cells is warranted and must take place in the presence of at least 3 signals. First, the presented antigen/MHC complex must bind with the T cell receptor (TCR) of T cells (i.e., "signal 1"). Second, costimulation is obligatory for T cell activation (i.e., "signal 2"). For instance, binding of CD80/86 molecules on DCs with CD28 present on the cell membrane of T cells results in T cell stimulation. For a long time, it was believed that antigen recognition in the absence of co-stimulatory factors results in T cell anergy [5]. However, to date a variety of co-stimulatory pathways have been identified and are currently classified based on their impact on primed T cells [8]. Indeed, pathways delivering activatory signals to T cells are termed co-stimulatory pathways, whereas pathways delivering tolerogenic signals to T cells are termed coinhibitory pathways. Furthermore, it is generally accepted that an additional "signal 3" is also needed for efficient T cell stimulation and polarization. A well-known example is the potent induction of interferon (IFN)-γ-producing T helper type 1 (Th1) cells by interleukin (IL)-12 produced by DCs as response to certain microbial stimuli [6, 9]. Furthermore, both *in vitro* as well as *in vivo* studies have demonstrated that CD40 ligation of CD8+ T cells is necessary for optimal clonal expansion, effector function, and generation of a memory population [10–12]. Raveney and Morgan [13] have suggested that alterations in one of these three signals could shift the balance to tolerance or (auto)immunity. Recently, Kalinski et al. [6, 7] described a potential fourth signal delivered by DCs that results in the upregulation of chemokine receptors on effector T cells and that thus might play part in organ-specific chemotaxis of T cells.

Depending on the cytokines present upon T cell activation, naïve CD4+ T helper (Th) cells can acquire a variety of immune effector phenotypes [14]. In brief, release of IL-12 by DCs promotes a Th type 1 (Th1) response. Th1 cells mediate a cellular as well as delayed-type hypersensibility immune response with proliferation of T cells and production of IFN-γ and IL-2. Furthermore, Th1 cells induce stimulation of CD8+ cytotoxic T cells (CTL). Th2 cells are stimulated through OX40 ligation by DCs, produce mainly IL-4, IL-5, and IL-13, and promote the activation of B cells, which can also be involved in autoimmunity [15]. Tumor-growth factor (TGF)-β, in the absence of proinflammatory cytokines, induces Tregs, while TGF-β, IL-1, and IL-6 are needed for induction of Th17 cells [16]. Tregs are immune suppressive, and hence counteract effector T cells. In contrast, Th17 cells generate an influx of neutrophils and cause allergic or autoimmune reactions.

2.3. Dendritic Cells Inducing T cell Tolerance. DCs are essential for both central and peripheral tolerance [5, 17–20]. Central tolerance occurs in the thymus where thymoid DCs present self-antigens to developing T cells. Subsequently, lymphocytes with autoreactivity above a certain threshold are deleted, a process called clonal deletion. Additionally, naturally occurring Tregs (nTregs) are positively selected by thymoid DCs in the thymus [21]. However, some limitations of central tolerance resulting in escape of potentially autoreactive T cells underlie the need for effective peripheral silencing mechanisms. In this regard, several mechanisms mediated by DCs have been proposed. (i) It has been

suggested that iDCs fail to stimulate T cells sufficiently because of their low expression of MHC molecules and co-stimulatory factors. This results in T cell anergy [1, 22]. (ii) It has also been reported that suboptimal antigen presentation, together with indoleamine 2,3-dioxygenase (IDO) or Fas (CD95) expression by iDCs leads to inhibition of T cell proliferation and T cell deletion [5]. (iii) Furthermore, DCs are able to induce Tregs to preserve immune tolerance to self-antigens [17] as well as to certain foreign antigens [1, 2, 5]. Moreover, IL-10-producing regulatory type 1 T cells (Tr1) are also promoted by DCs, hereby reinforcing peripheral tolerance [17, 23, 24] (for review on Treg subsets, see [21]).

Zehn and Bevan [19] showed that central tolerance accompanied by equal efficient peripheral tolerance is very efficient in withholding high avidity autoreactive T cells. Despite these mechanisms, some low avidity autoreactive T cells may escape and be present in the periphery. Therefore, it has been suggested that their activation can occur by cross-reaction with foreign antigens, subsequently driving T cells to differentiate into effector T cells causing autoimmunity.

3. Role of DCs in the Pathogenesis of Autoimmunity

A healthy immune system recognizes and eliminates invading pathogens, but preserves tolerance for self-antigens. In contrast, autoimmune diseases develop when self-antigens are recognized as foreign by the immune system, resulting in hyperactivity of both cellular and humoral immunity against these antigens. The underlying mechanisms abrogating immune tolerance for self-antigens are still unclear. However, given the central role of DCs in maintaining the balance between (auto)immunity and tolerance, they are believed to play an important role in this process [2, 25].

While neonatal mice who have undergone thymectomy [26] or thymic deletion [27] develop severe systemic autoimmune diseases, similar clinical outcomes in mice were obtained upon the depletion of both cDCs and pDCs. Indeed, Ohnmacht et al. [28] observed that constitutive ablation of DCs in mice leads to the breakdown of tolerance for self-antigens resulting in severe spontaneous autoimmune responses possibly caused by an increased amount of Th1 and Th17 cells. Moreover, a variety of antibodies against both nuclear and tissue-specific autogens was found in these mice. The authors showed that DCs with a short lifespan did not induce an efficient tolerance of CD4+ T cells, which was reflected in the thymus as a decreased negative selection and as a shortage of tolerogenic DCs in the periphery. Albeit that others demonstrated that increasing the lifespan of DCs through inhibition of apoptosis also induced autoimmunity in mice [29], thereby emphasizing the ambiguous role of DCs in immunity as well as tolerance. Of interest, it was recently described that peripheral T cells can reenter the thymus, where they target thymic DCs and medullary thymic epithelial cells. As a consequence, negative selection in the thymus was suppressed with breakthrough of T cells with a high affinity for self-antigens causing autoimmune diseases [30]. Altogether these studies underscore the importance of immune regulation in the thymus and periphery controlling (auto)immunity.

Whereas it is generally accepted that DCs in steady state, although loaded with self-antigens from their environment, do not trigger autoimmunity [5, 18, 31], discrepancies in DC number, phenotype, and function are believed to contribute to disease [2, 32–36]. Indeed, in animal models for type I diabetes, arthritis [17], Wiskott-Aldrich syndrome [20], and systemic lupus erythematosus (SLE) [37], it was shown that increased access of DCs to intracellular autogens—mediated by increased amounts of apoptotic cells or insufficient clearance of these cells—resulted in subsequent autogen presentation and activation of T cells. In an attempt to elucidate possible underlying mechanisms, Sawatani et al. [29] attributed a role in the phagocytic activity and antigen-presenting function of DCs to the dendritic cell-specific transmembrane protein (DC-STAMP). Indeed, in DC-STAMP-deficient mice the authors found increased *in vitro* phagocytosis and antigen presentation by DCs which could give rise to systemic autoimmunity [29]. Because of the high expression of MHC class II and co-stimulatory molecules, mature DCs are utmost equipped to activate T cells. In addition, both mature cDC and pDC produce proinflammatory cytokines, including IL-12p70 and type 1 IFN, respectively, which could contribute to the pathogenesis of autoimmunity [38, 39]. In this perspective, Lech et al. [40] demonstrated that the absence of the Sigirr gene, which is a variant of Toll-like receptor (TLR)/interleukin 1 receptor (Tir) family and suppresses the TLR-mediated pathogen recognition in DCs, resulted in enhanced activation of DCs. This was evidenced by increased expression of proinflammatory mediators and was associated with the development of murine lupus. In inflamed tissues, such as the synovium in rheumatoid arthritis (RA), these proinflammatory signalling molecules are found in high amounts in DCs in the vicinity of T cells. For this, it has been hypothesized that DCs maintain the local autoreactive T cell response [38]. Besides, a correlation exists between the amount of DCs and the concentration of anticitrullinated peptide antibodies in serum of RA patients [38], suggesting a possible regulatory role for DCs in the production of autoantibodies in RA. Furthermore, DCs are described to enhance the formation of ectopic lymphoid tissues in target organs. The underlying mechanism is probably explained by chemotactic cytokines released by DCs leading to lymphoid neogenesis and recruitment of leukocytes in the inflamed tissue, including the synovium [41] and the pancreatic islets [42]. In other studies the formation of ectopic lymphoid structures was ascribed to B cells [16]. DCs can also directly damage surrounding tissues. In this perspective, it was recently shown that monocyte-derived DCs could destroy the cartilage in joints through the production of TNF-α [43].

4. Tolerogenic DCs-Based Treatments

Efforts to bring DC vaccination to the clinic aiming induction of tolerance, were initiated by Dhodapkar et al. who demonstrated that pulsing immature DCs with influenza matrix protein (IMP) and keyhole limpet hemocyanin (KLH)

resulted in a decrease of influenza-specific CD8+ IFN-γ-secreting T cells, while peptide-specific IL-10-secreting T cells appeared [44]. Menges et al. [45] showed in mice that bone marrow-derived DCs treated with TNF-α, so-called semi-mature DCs, were able to suppress the course of experimental autoimmune encephalomyelitis (EAE), the animal model for MS, through the activation of IL-10-secreting Tregs. Unfortunately, the semi-mature phenotype of these DCs is not stable since they produce proinflammatory cytokines upon introduction of a secondary stimulus (e.g., LPS). In contrast, biological molecules and pharmaceutical agents, including vitamin D_3, IL-10, the corticosteroid dexamethasone, and the immunosuppressive drug rapamycine, are known to induce immature DCs with a low immunogenic character, that is, no upregulation of co-stimulatory molecules or secretion of proinflammatory cytokines, so-called tolerogenic DCs (tolDCs). Indeed, treatment of DCs with vitamin D_3 or equivalents resulted in an increased release of IL-10, whereas the expression of co-stimulatory molecules and bioactive IL-12 was downregulated. Moreover, the authors demonstrated that these tolDCs induced tolerance to the allograft in a mouse model [46]. Another example is triptolide, derived from a Chinese herb, which was found to have potent immunosuppressive effects as demonstrated by its prevention of DC migration and release of chemokines as well as subsequent inhibition of T cell activation and proliferation [47, 48]. Treatment of human DCs with the immunoregulatory neuropeptide, vasoactive intestinal peptide (VIP), induces significant production of anti-inflammatory cytokines, such as IL-10, causes a decrease in the expression of the co-stimulatory molecules CD80/86, and inhibits the phagocytic activity by DCs [49, 50]. Importantly, these DCs_{VIP} keep their immature phenotype after exposure to inflammatory signals like TNF-α and LPS. Hence, a stable immature phenotype is generated. In addition, a population of antigen-specific Tr1-like cells, producing both IL-10 and TGF-β and inhibiting the proliferation of Th1 cells, was found. Moreover, CD8+CD28– Tregs were also induced contributing to the antigen-specific tolerance. Vaccination of DCs_{VIP} in mice during development of collagen-induced arthritis (CIA), EAE, and graft-versus-host disease (GVHD) in allogeneic bone marrow transplantation induced organ-specific tolerance and suppressed the course of disease.

Recently, genetic engineering has made its way in the quest for therapeutic possibilities for autoimmune diseases. Indeed, the insertion of new DNA in order to enhance tolDC function has been investigated. For example, by transfection of DNA coding for the Fas-ligand [51] or TNF-related apoptosis-inducing ligand (TRAIL) so-called "killer" DCs could be obtained. These genetically modified DCs efficiently induce T cell apoptosis, suppress autoimmune arthritis, and prevent rejection of donor-specific heart transplants in animal models [52]. In addition, injections of genetically modified IL-4-producing DCs in CIA suppress the development and inflammation level of arthritis. In a study of Kaneko et al. [53], however, these DCs caused an accelerated immune reaction and rejection of the allograft, making these IL-4-producing DCs less attractive for therapeutic use. Alternatively, selective knockout of the expression of DC-characteristic molecules and functions has been intensively investigated. Utilizing RNA interference (RNAi) directed at IL-12p35 in order to generate IL-12-silenced DCs resulted in the prolongation of the intestinal allograft lifespan in rats [54]. Similar results were achieved in animal models after silencing of RelB and NF-κB which resulted in allogeneic donor-specific hyporesponsitivity of the T cells, associated with an inhibition of the cytokine production of Th1 cells, and prolonged survival of the cardiac allograft in mice [55]. Recently, a clinical trial administering monocyte-derived DCs genetically modified with antisense oligonucleotides targeting the transcripts of CD40, CD80, and CD86, thereby selectively reducing their surface expression [56], was performed in type 1 diabetes patients and was proven to be safe, well tolerated, and without any adverse effects [57]. Whether recently identified negative regulators of DC activation, including zDC [58] and FOXO3 [59], hold promise for future DC-based tolerance-inducing strategies remains to be established.

5. Induction of Long-Lasting Immune Tolerance

Ideally, therapies for immunosuppression must also be durable. This means that the ability to regulate the autoimmune response has to be permanent or at least for many years following intervention, for instance, via the generation of self-antigen-specific Tregs.

Indeed, different *in vitro* generated tolDCs, including IL-10-modulated DCs [60] and DCs treated with a combination of dexamethasone and 1α,25-dihydroxyvitamin D_3 [61], were shown to induce Tregs. In addition, Housley et al. [62] demonstrated that activation of PPARγ, a nuclear hormone receptor, in CD103+ DCs from the gut-associated lymphoid tissue (GALT) in mice was important for the regulation of retinoic acid secretion and Treg generation by DCs. This might contribute to the suppression of autoimmunity since other studies [63, 64] reported that CD103+ GALT DCs induce an increased conversion of effector T cells to Tregs in a retinoic acid-dependent manner. Interestingly, some tolDC populations also promote the induction of regulatory B cells (Bregs), underlining suitability for tolerance-inducing strategies [61].

Whereas DCs drive the differentiation of Tregs in order to control immune responses, Tregs also modulate DC phenotype and function [65]. Indeed, Gabryšová et al. [66] showed that the autoimmune response was limited by a negative feedback system started by the antigen-induced differentiation of Th1 cells into IL-10-producing Tregs which on their turn inhibited DC maturation, thereby suppressing Th1 responses and completing the negative feedback loop. Furthermore, following depletion of FoxP3+ T cells, DCs that lack the expression of MHC class II molecules were not able to make cognate interactions with CD4+ T cells resulting in spontaneous and fatal CTL-mediated autoimmunity, indicating the critical suppressive role of the FoxP3+ Treg population in maintaining DCs in a tolerogenic

state [67]. Overall, these findings highlight the importance of the bidirectional crosstalk between DCs and Tregs in maintaining and inducing tolerance.

6. Discussion

The use of tolerogenic DCs as cellular mediators for the induction of tolerance in autoimmune diseases and transplantation is very promising and could in the future complement or even substitute immunosuppressive agents which have important side effects including increased risk of infections. However, some open-standing questions need to be addressed before DC-based vaccines could be implemented in the clinic [68].

A first challenge is the identification of a maturation-resistant subtype of DCs. For instance, while CD8α+ DCs, the mouse equivalents of human myeloid DCs can act tolerogenic by inducing T cell apoptosis via their expression of Fas-ligands [69, 70], others demonstrated that these CD8α+ DCs released high amounts of IL-12 and were able to stimulate CD8+ CTL [71]. Additionally, Waithman et al. [72] described a CD11c+CD207+ skin-derived DC subset presenting self-antigens in the draining lymph nodes and inducing deletion of MHC class I-restricted autoreactive T cells, thereby contributing to tolerance. In contrast, others showed that these skin-derived DCs drive autoimmune tissue destruction. Hence, tolDCs cannot solely be distinguished based on their phenotype but must be carefully investigated regarding their stability and tolerogenic effect, especially after vaccination. Given the risk of *in vivo* reactivation, this is particularly of importance in any pathological state with an underlying inflammatory microenvironment.

Ideally, therapies for immunosuppression must also be (self-) antigen specific and durable. In this respect, Hawiger et al. [73] devised a DC-targeting system. Using a monoclonal antibody targeting DEC-205, a DC-restricted endocytic receptor, the authors delivered a specific antigen to DC. Albeit that initially an extensive T cell proliferation was observed, this was followed by T cell anergy and deletion. With these results, the authors suggested a possible role for inducing antigen-specific peripheral tolerance with this system. Unfortunately, in combination with a DC maturation stimulus, this strategy resulted in immune activation, thereby limiting its clinical use for the treatment of autoimmunity. Hence better insights in the role of distinct DC populations are warranted. In this respect, antigens delivered via antibodies to CLEC9A, a recently discovered C-type lectin receptor which is selectively expressed by CD141+ myeloid DCs, were shown to be a promising strategy to efficiently induce immunity against infections and malignant diseases [74, 75]. Likewise, antigens specifically delivered to migratory DCs, trafficking from peripheral tissues to draining lymph nodes charged with self-antigens, were shown to be superior in generating Tregs *in vivo* and consequently drastically improved the outcome of autoimmune disease [76]. In addition, durable tolerance means that the ability to regulate the autoimmune response has to be permanent or at least for many years following intervention, for instance, via the generation of self-antigen-specific Tregs. For this, increased knowledge with regard to the pharmacokinetic and pharmacodynamic properties of DC-based strategies is imperative. Other related questions that need to be taken into consideration for the success of this approach are the timing of DC therapy (e.g., a prophylactic or a therapeutic treatment regimen) and selection of antigenic peptide(s) for loading DCs. Additionally, parameters such as antigen dose, number of cells, requirements for repetitive DC vaccinations, and the route of administration need to be addressed in clinical application. Finally, ethical issues may also arise, especially with regard to the implementation of experimental therapy for graft acceptance upon transplantation while there is a shortage of organ donations. Note must be taken that patient-specific treatment modalities, including DC-based vaccination, are very expensive and require careful monitoring of treatment-related efficacy and toxicity, individual patient morbidity, and quality of life, as well as societal costs.

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Review Article

Transplant Tolerance: New Insights and Strategies for Long-Term Allograft Acceptance

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One of the greatest advances in medicine during the past century is the introduction of organ transplantation. This therapeutic strategy designed to treat organ failure and organ dysfunction allows to prolong the survival of many patients that are faced with no other treatment option. Today, organ transplantation between genetically dissimilar individuals (allogeneic grafting) is a procedure widely used as a therapeutic alternative in cases of organ failure, hematological disease treatment, and some malignancies. Despite the potential of organ transplantation, the administration of immunosuppressive drugs required for allograft acceptance induces severe immunosuppression in transplanted patients, which leads to serious side effects such as infection with opportunistic pathogens and the occurrence of neoplasias, in addition to the known intrinsic toxicity of these drugs. To solve this setback in allotransplantation, researchers have focused on manipulating the immune response in order to create a state of tolerance rather than unspecific immunosuppression. Here, we describe the different treatments and some of the novel immunotherapeutic strategies undertaken to induce transplantation tolerance.

1. History of Organ Transplantation

Earl C. Padgett first described the phenomenon of allograft rejection in 1932. He used nonrelated skin allografts to cover severely burned patients and reported that none of the skin allografts survived permanently. However, he observed that skin grafts from relatives seemed to survive longer than those from unrelated donors [1]. In 1943, Gibson and Medawar developed the first scientific explanation of the phenomenon of allorejection. They observed that patients who received autografts (tissue from the same individual transplanted to a different part of the body) accepted the tissue with no complications unlike patients that had received a sibling's skin allograft (tissue from a different individual belonging to the same species) who eventually rejected the allograft. In addition, they observed that a second skin transplant with skin from the same donor resulted in more rapid rejection compared with the first skin transplantation. The observation of the accelerated rejection of the second graft from the same donor was convincing evidence that supported the involvement of an immunological process during allograft rejection [2, 3].

In 1948, Medawar and colleagues excluded an important role of antibodies in allograft rejection [4, 5] and designed an experiment to assess whether cellular components of the immune system are responsible for transplant rejection. They injected cells from the allograft-draining lymph node from transplanted mice into mice recently transplanted with skin from the same donor. They observed that mice rejected the allograft as similar to mice transplanted for a second time,

indicating that cellular components of the immune system are responsible for the generation of the immune response against the allograft [3, 6].

Advances achieved in surgical techniques in parallel with improvements in knowledge of the immune mechanisms mediating allograft rejection allowed the first kidney transplant in 1963 [7–10]. Joseph E. Murray and his colleagues at Peter Bent Brigham Hospital in Boston performed the first successful kidney transplant from one twin to another [11]. It was a great advance in medicine, demonstrating that it was possible to perform successful organ transplants in humans, but it was still necessary to solve the problem of rejection between unrelated donors [12].

Since then, different pharmacological treatments have been developed in order to induce an immunosuppressive state that allows the acceptance of an allograft transplant between unrelated donors [1, 13–16]. The first successful cadaveric unrelated kidney transplant was performed in 1962 by Joseph Murray and his group [17]. Murray used azathioprine, an immunosuppressive drug previously tested in dogs [18], which allowed the transplant recipient to survive one year after receiving the kidney transplant [17, 19].

The immunosuppressive effects of cyclosporine A (CsA) were discovered in Switzerland in 1972. Some trials to compare CsA versus azathioprine and steroids were developed and the promising results led to clinical approval for the use of CsA in human transplants in 1980 [20, 21]. The introduction of CsA contributed substantially towards the improvement of allograft and patient survival [22].

The massive development of immunosuppressive drugs opened the door to organ transplantation, extending to other organs such as the liver, lungs, and heart. In parallel with the increased number of organ transplants, several investigators are currently working on developing new immunosuppressive drug protocols that will further improve the outcome and reduce tissue toxicity in transplanted patients [23–26]. However, despite these efforts, currently all immunosuppressive drugs have serious side effects including nephrotoxicity, development of malignancies, and susceptibility to infections by opportunistic pathogens. For this reason, immunologists face a new challenge in developing strategies to reduce or eliminate the use of immunosuppressive drugs in organ transplants. These efforts are being focused on reeducating the immune system or inducing allograft-specific tolerance mechanisms.

2. Immune Tolerance

One of the hallmarks of the adaptive immune system is its ability to recognize a vast number of different antigens. This ability is a consequence of the large lymphocyte repertoire, in which each cell has a different antigen receptor generated by the process of somatic recombination. This process is able to produce an estimate of 10^{15} different lymphocyte clones, each with a different antigen receptor that can hypothetically recognize any naturally occurring structure [27]. Since somatic recombination is a random process, it generates T cell clones that can recognize self-structures or self-peptides (auto-antigens). The mechanism used by the immune system in order to avoid a possible harmful immune response against an individual's own cells and tissues is known as immune tolerance and can be classified into central and peripheral tolerance.

2.1. Central Tolerance. Central tolerance occurs in the thymus and allows the deletion of a major percentage of autoreactive T cells. The thymus is the major site of maturation of T cells and can be anatomically and functionally separated into two zones: the thymic cortex and medulla. The cortex is the region where the process of positive selection occurs and contains densely packed immature thymocytes. The medulla contains loosely packed mature lymphocytes and is the site where the process of negative selection takes place [28].

2.1.1. Positive Selection. After originating in the bone marrow, the early precursors of T cells enter the thymus and migrate into the cortex where most of the subsequent maturation events take place. These T cell precursors do not express the T cell receptor (TCR), CD3, ζ chains, CD4, or CD8 coreceptors and therefore are called $CD4^-CD8^-$ double negative (DN) thymocytes. Within the cortex, DN cells undergo TCR rearrangement and become $CD4^+CD8^+$ double positive (DP) cells, which express the TCR α and β chains as well as both CD4 and CD8 coreceptors.

2.1.2. Negative Selection. Double positive cells are programmed to undergo apoptosis by default unless they receive a "rescue signal" which is provided by cortical thymic epithelial cells (cTEC) that express self-peptide/major histocompatibility complex (MHC). Only thymocytes recognizing self-peptide/MHC complex with low avidity will receive the rescue signals and will continue with the maturation process. The DP clones that are rescued will continue with the process of maturation and will become single positive (SP) cells that express either the CD4 or CD8 coreceptor [29–31].

The acquisition of adequate chemokine receptors allows SP cells to exit the thymic cortex and to enter the medulla. It is in the medulla where they will continue with the negative selection process, which is crucial to central tolerance [29, 32, 33].

One of the questions regarding negative selection is how autoreactive clones that recognize self-peptides that are not normally found in the thymus are controlled. Recent evidence has demonstrated that the AIRE transcription factor is involved in the promiscuous gene expression in mTEC cells that allows an increase in the repertory of auto-antigens presented by antigen presenting cells (APCs) during negative selection [34–38].

As a consequence of positive and negative selection, T cells that leave the thymus and populate peripheral lymphoid tissues are self-MHC restricted and tolerant to many auto-antigens.

2.2. Peripheral Tolerance. Although central tolerance mechanisms are efficient in deleting the auto-reactive T cell clones that recognize self-antigen/MHC complex with high affinity,

some autoreactive T cells are able to bypass this control and exit the thymus [39–41]. In the periphery, these autoreactive clones are able to induce autoimmune responses, generally in response to an inflammatory environment such as one triggered during infection [42, 43]. Therefore, there is a constant threat of potential autoimmune responses due to the escape of auto-reactive T cells clones to the periphery. These potentially harmful auto-reactive cells must be effectively controlled by peripheral tolerance mechanisms.

Peripheral tolerance mechanisms involve the *deletion* of activated effector T cells, *anergy* induction, *clonal exhaustion,* and active *regulation* of effectors T cells [44]. Regulatory T cells (Tregs) mediate active regulation of the immune response preventing autoimmune and inflammatory diseases and restraining responses to infections of viral, bacterial, or parasitic origin. Moreover Tregs can restrain immune responses directed towards tumors or transplanted tissue [42–46].

Two different types of Tregs have been described; natural $CD4^+CD25^+Foxp3^+$ regulatory T cells (nTregs), which are generated in the thymus and regulate immune responses in the periphery, and inducible $CD4^+CD25^+Foxp3^+$ regulatory T cells (iTregs) which develop in the periphery from naïve $CD4^+$ T cells after exposure to antigens in a specific cytokine microenvironment, tolerogenic APCs, or immunosuppressive drugs [44].

Dendritic cells play an important role in establishing peripheral tolerance. These cells are found in mucosal and parenchymal tissues where they function as sentinels in search for pathogens and tissue injury. During infection and tissue damage, immature DCs (iDCs) are activated through different pathogen-associated molecular pattern (PAMP) receptors, which trigger the maturation of DCs. These DCs migrate to the draining lymph nodes where they acquire the capacity to activate naïve T cells [39]. Under steady-state conditions, iDCs constitutively take up and process cellular debris produced as a consequence of normal cell turnover of the tissues. Internalization of self-antigens present in apoptotic cells by peripheral iDCs induces tolerance mechanisms such as the expansion of iTregs that control effector responses and protect cells and tissues from damage during pathogenic autoimmunity [47].

3. Mechanisms of Allograft Rejection

3.1. Clinical Rejection. Despite the advances in transplantation tolerance, the mechanisms that mediate allograft rejection have not yet been fully described. Clinical rejection may occur at any time following transplantation and therefore is classified according to the time in which it occurs after the transplant.

Hyperacute rejection may occur within a few minutes to hours after transplantation. It is due to preformed alloantibodies by the recipient, mainly against MHC antigens, which become deposited in the allograft and induce complement activation and recruitment of inflammatory cells that trigger platelet aggregation, with consequent capillary obstruction and tissue necrosis. This type of rejection is not very common nowadays because it is easily prevented by blood typing and crossmatching prior to transplantation.

Acute rejection occurs days to months after the transplant. It consists of a tissue injury process mediated by alloantibodies and alloreactive T cells, mainly in response to MHC antigens. Acute cellular rejection is due to alloreactive cytotoxic $CD8^+$ T cells that recognize the alloantigens present in the transplanted tissue and carry out its destruction. The lesion occurs mostly in the endothelial cells, which in response to the injury develop a microvascular endothelialitis and arteritis. Antibody-mediated rejection, on the other hand, is characterized by alloantibodies that induce complement activation, neutrophil recruitment, and the consequent inflammation and coagulation activation that results in thrombotic ischemia of the transplanted tissue. This type of rejection was a critical obstacle to overcome in the early steps of organ transplantation; however, today it is well managed by the employment of immunosuppressive drugs.

Chronic rejection is today the main cause of allograft failure. It occurs months or years following transplantation. Organ failure occurs due to chronic inflammation that triggers the proliferation of intimal smooth muscle cells and results in vascular occlusion and ischemic damage. The pathogenesis involves the chronic secretion of cytokines by activated T lymphocytes and the production of alloantibodies that are able to activate the complement system through the classical pathway, thus generating chronic damage [48]. Despite the advances in immunosuppressive therapy, this type of rejection remains unresolved [49, 50] and it is necessary to develop new strategies to improve organ acceptance.

As mentioned above, alloantibodies have an important role in the different types of rejection mechanisms. These antibodies can be directed against HLA (major antigens) or non-HLA molecules (minor antigens). Therefore it is important to detect their presence in order to prevent possible events of organ rejection.

3.2. Immune Mechanisms of Rejection

3.2.1. Ischemic-Reperfusion Injury. When the allograft is recovered from the donor, the organ has to undergo a procedure that necessarily involves the induction of stress. The different sources of stress during the medical procedure, namely, anesthesia, damage by physical factors (temperature and mechanical stress), and ischemia trigger an inflammatory state called "ischemic-reperfusion injury" (IRI). IRI induced by organ manipulation induces the expression of danger-associated molecular patterns (DAMPs), such as heat-shock proteins or HGMB1 that are recognized by pattern recognition receptors (PRRs) localized on epithelial cells and cells of the immune system such as neutrophils, macrophages, and DCs [51, 52].

The recognition of DAMPs by PRRs results in the activation of signaling pathways that activate the inflammasome, that is, synthesis of transcription factors and micro-RNAs, that results in an inflammatory response. The secretion of inflammatory cytokines such as a interleukin (IL)-1 and IL-6, as well as chemokines, and also the complement cascade

activation [53] contribute to the generation of a microenvironment required to activate DCs. Activated DCs carrying the alloantigens from the transplanted organ then migrate to the lymph nodes and induce the activation of alloantigen-specific T cells, thus mounting a specific immune response against the allograft [54].

3.2.2. Allorecognition and T Cell Activation Mechanisms. Today, the cellular events involved in organ rejection are better understood and three key mechanisms have been described that explain the activation of T cells by alloantigens, resulting in allograft rejection. The first mechanism of alloantigen recognition is called *direct presentation*. Donor APCs, mainly DCs present in the allograft, mediate this type of presentation. These donor DCs migrate to the draining lymph nodes where they present alloantigens (in the context of donor MHC molecules) to alloreactive recipient T cells [54]. This type of allopresentation is responsible for the activation of the immune system against the donor allograft in acute rejection. However, this allorecognition mechanism is not permanent since donor DCs are cleared out over time, due to natural cell death.

Indirect presentation is mediated by recipient DCs that process and present different alloantigens from the graft to alloreactive recipient T cells. In contrast with direct presentation, the alloantigens presented by DCs in indirect presentation are processed as exogenous antigens and are therefore presented by APCs in a self-MHC context. This type of alloantigen presentation is responsible for the aforementioned chronic rejection and therefore is the main cause of organ loss, which currently cannot be addressed through prevention or treatment.

The third mechanism involved in allograft recognition is called *semidirect presentation* where donor membrane fragments which carry MHC class I molecules among others are transferred to recipient APCs (Figure 1) [55, 56]. Semidirect presentation is likely to involve cell-to-cell interaction, or release and uptake of small MHC-containing vesicles [57].

4. New Strategies to Induce Long-Term Acceptance to Organ Transplantation

The immune system protects the host from a broad range of pathogens by generating a response mediated by T cells, B cells, and innate immune cells. After the clearance of the pathogen, immune regulation avoids misguided or excessive immune reactions that could damage self-tissues, maintaining or restoring a homeostatic environment. The state of unresponsiveness of the immune system to antigens is known as immune tolerance, and this involves tolerance to self-antigens, which is established and maintained to avoid host damage.

In transplanted patients, prevention of graft rejection is achieved by long-term use of immunosuppressive drugs, which have an effect over the entire immune system, rather than a specific effect over alloreactive T cells. The development of new drugs and protocols of drug combinations is in continuous progress, but drug toxicity, chronic rejection, and immune deficiencies associated with these treatments remain unresolved. Current research is focused on promoting allograft-specific immune tolerance as a means to reduce the dose and number of immunosuppressive drugs administered, thereby allowing the host to react to potential pathogens and malignancies.

The two major approaches to induce transplant tolerance involve, first, the induction of a state of mixed chimerism through the transfer of donor hematopoietic stem cells (HSC) to the recipient, thereby inducing central tolerance to alloantigens and, second, the delivery of alloantigens to the recipient in a "tolerogenic fashion" in order to activate peripheral tolerance mechanisms to the allograft. In the following sections, we will discuss the current research that is being carried out concerning new strategies to induce long-term acceptance of allografts.

4.1. Mixed Chimerism as a Strategy to Induce Allograft Tolerance. Mixed chimerism is defined as the coexistence of donor and recipient hematopoietic cells in an individual after allogeneic bone marrow transplantation (BMT) [58, 59]. To be considered mixed chimerism, donor cells in the blood must represent more than 1% of the total cells as measured by flow cytometry [58, 60]. To induce a state of mixed chimerism, it is necessary to perform a conditioning treatment in order to allow donor HSC bone marrow acceptance. The establishment of mixed chimerism allows the redefinition of immunological "self" previously learned in the thymus. The allogeneic BMT generates a new source of T cells and DCs that induces a relearning of the "new self" state, depleting the possible T cell clones that recognize both allo- and autoantigens [58].

Currently used mixed chimerism protocols induce robust donor-specific tolerance and allow long-term acceptance of fully mismatched skin grafts in murine models [61]. Tolerance maintenance is mediated by intrathymic clonal deletion of alloreactive cells [62–64], mimicking the natural mechanisms to produce self-tolerance. Deletion of host alloreactive T cells depends on the continuous presence of donor DCs in the thymus [62, 63, 65], while donor alloreactive T cells are eliminated intrathymically by clonal deletion. Thus, the new T cell repertoire in chimeras is tolerant to both recipient and donor cells.

Evidence of tolerance induction due to mixed chimerism has been reported in kidney transplant patients. Patients who had received a conventional BMT (usually to treat a hematological malignancy) that later developed organ failure accepted an organ transplant from the same donor with the use of myeloablative conditioning (elimination of recipient HSC). Such patients are able to accept the transplanted organ even across MHC barriers [66–70]. However, myeloablative conditioning is not ethically accepted due to the high risk involved in this type of conditioning.

Nonmyeloablative conditioning has emerged as an alternative to produce tolerance through mixed chimerism. Nonmyeloablative conditioning consists of the administration of sufficient immunosuppression (e.g., antithymocyte globulin, costimulation blockage, and immunosuppressive drugs) to

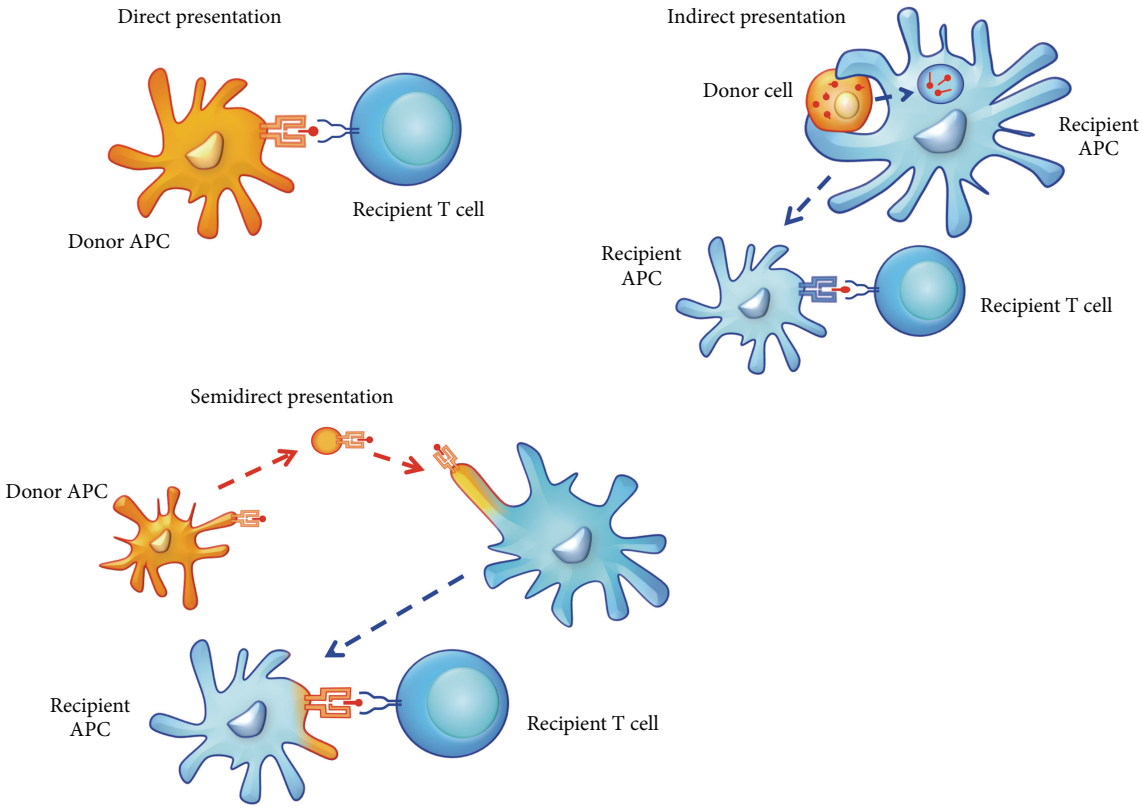

Figure 1: Mechanisms of alloantigen recognition. In direct presentation, donor APCs are able to present alloantigens to alloreactive T cells from the recipient. In indirect presentation, alloantigens are taken up from donor cells by recipient DCs that process and present alloantigens to alloreactive T cells. In semidirect presentation, intact MHC molecules are transferred to recipient DCs that directly present alloantigens through donor-MHC or process and present alloantigens as described for indirect presentation.

allow the engraftment of fully mismatched BMT, but at the same time, minimal enough to avoid toxic secondary effects. Although some physical and pharmacological strategies such as total body irradiation, thymic irradiation, or the use of depleting antibodies are able to induce mixed chimerism; however, it is still necessary to generate conditioning protocols that minimize systemic immunosuppression [58, 71–74].

New approaches have been developed in human and nonhuman primate models in order to induce mixed chimerism in nonmyeloablative conditioning protocols. Using a simultaneous bone marrow and kidney transplantation and a preconditioning protocol consisting in the costimulatory blockade with anti-CD154 antibody, Kawai and coworkers achieved the establishment of mixed chimerism and prolonged renal allograft survival in nonhuman primates [75]. Additionally, using a high-dose BMT and costimulatory blockade, it has been demonstrated the achievement of bone marrow engraftment without cytoreduction in mice [76]. In human, Kawai and coworkers have reported tolerance induction across HLA-mismatched barriers with a peri-conditioning treatment using pharmacological immunosuppression and thymic irradiation. This protocol allowed the removal of long-term immunosuppressive therapy achieving full acceptance of the transplanted organ up to five years after transplant [77]. However, one of the main obstacles in the induction of mixed chimerism using the aforementioned protocol is the presence of the memory T cells that can cross-react with alloantigens [78]. Recently the group of Yamada demonstrated the induction of "delayed tolerance" by performing first, a kidney transplant and second, bone morrow transplantation in addition with $CD8^+$ memory T cell depletion therapy [79].

The use of cellular therapy in nonmyeloablative conditioning protocols could be a valuable strategy to induce mixed chimerism. The principal candidates are immature DCs, regulatory macrophages, apoptotic cells, regulatory T cells, and mesenchymal stromal/stem cells due to their capacity to induce tolerance in antigen-specific fashion, therefore minimizing the possible side effects of non-antigen-specific experimental protocols to achieve mixed chimerism.

4.2. Dendritic Cells and Regulatory Macrophages. Dendritic cells constitute a heterogeneous population of professional, bone-marrow-derived APCs that have the potential to induce both tolerance and immunity [80, 81]. This potential is directly related to DC maturation status, where T cell tolerance is induced by immature DCs that express low surface levels of MHC class II and costimulatory molecules, whereas T cell immunity is generated by mature DCs that express

higher levels of these antigen presenting and costimulatory molecules [82].

Dendritic cells have been well characterized in the context of organ transplantation, where it has been hypothesized that tolerogenic DCs are involved in graft acceptance while immunogenic DCs are key to graft rejection [83]. It has been described that tolerogenic DCs have the capacity to induce or expand Tregs [81, 83–87]. Tolerogenic DCs have been characterized by low levels of expression of CD86, CD40, PD-L2, and high levels of expression of PD-L1 [83, 88–90] and CD80 [91, 92].

A wide variety of strategies and pharmacological agents have been used to generate tolerogenic DCs *in vitro*. Such approaches include the use of cytokines and growth factors (IL-10, TGF-β, GM-CSF) during their differentiation, genetic interference with NF-κB signaling and costimulatory molecules, and exposure to immunosuppressive agents such as CsA, vitamin D3, rapamycin, aspirin, mycophenolate mofetil, sanglifehrin A, deoxyspergualin, and corticosteroids [80, 81, 93–95].

IL-10-treated DCs or DCs genetically modified to overexpress IL-10 induce antigen-specific T cell anergy [96], while very low doses of GM-CSF lead to the development of immature DCs that induce alloantigen-specific T cell unresponsiveness *in vitro* and *in vivo* [97]. It has been described that the culture supernatant obtained from the GM-CSF producing-J558L cell line can be used in order to differentiate and expand immature DCs from bone marrow precursors. Some reports and our unpublished results [98, 99] demonstrated that this supernatant contains similar amounts of GM-CSF and IL-10 and that DCs generated with this supernatant have an immature/tolerogenic phenotype, since they are resistant to lipopolysaccharide (LPS) activation. This demonstrates the importance of immunomodulatory cytokines such as IL-10 in the maturation state of DCs.

On the other hand, the immunosuppressive drugs CsA, tacrolimus, and LF15-0195 inhibit DC maturation by blocking NF-κB signaling [93]. Both vitamin D3 and dexamethasone affect DC differentiation by downregulating their capacity to secrete IL-12p70, which leads to the induction of IL-10-secreting Tregs. In addition, vitamin D3-induced upregulation of PD-L1 in DCs provides inhibitory signals that regulate both central and peripheral tolerance [100] and, importantly, blockade of PD-L1 abolishes the tolerogenic capacity of vitamin D3-generated DCs [93]. Other immunosuppressive drugs such as rapamycin confer DC resistance to maturation in response to a proinflammatory stimulus [101] and promote organ transplant tolerance by inducing the *in vitro* and *in vivo* generation of Tregs [87, 93].

Immature DCs are also used in the generation of mixed chimerism as a strategy to induce transplant tolerance. In mouse models, mixed chimerism and transplant tolerance to a secondary skin allograft in an alloantigen-specific fashion were achieved using sequential doses of irradiated immature DC in bone marrow transplant protocols [102], demonstrating a potential use of DC in future treatments.

In the context of transplants, macrophages have been usually associated with graft rejection and resistance to tolerance induction. It has been demonstrated that these cells are major constituents of inflammatory infiltrates and are a prominent cell type in rejecting allografts [103]. Macrophages are also able to infiltrate heart allografts and contribute to transplant vasculopathy in an animal model of chronic allograft rejection [104]. Moreover, it has been demonstrated that some kidney transplant patients experience episodes of acute rejection even in the presence of T cell depletion therapies and this type of rejection was associated with intense monocytic infiltrations [105]. All these pieces of evidence presented so far support a key role for macrophages in graft damage and rejection [106].

However, in addition to classically activated (M1-polarized) macrophages that promote Th1-type T cell responses and alternatively activated (M2-polarized) macrophages that produce IL-10 and favor Th2-polarized T cell responses, novel macrophage populations with T cell-suppressive properties called "regulatory macrophages" have been described in the literature. The group of Mosser demonstrated that stimulating macrophages in the presence of high-density immune complexes and a TLR ligand resulted in IL-10 producing macrophages [107]. On the other hand, Brem-Exner and coworkers have observed that when macrophages are driven to an activated state by the addition of IFN-γ, these macrophages prevent autoimmune colitis by inducing and expanding Foxp3$^+$ Tregs [106, 108].

Since the discovery of these subsets of "regulatory macrophages," much attention has been paid towards the potential use of these populations in the induction of tolerance in transplants. Evidence directly involving macrophages in the acceptance of transplants was obtained from mice injected with CSF-1 before the transplant. In this study, CSF-1 induced the expansion of the host macrophage pool, reduced donor T cell expansion, and improved GVHD morbidity and mortality after allogeneic hematopoietic cell transplantation [109]. Moreover, *in vitro* generated murine regulatory macrophages have demonstrated to completely suppress polyclonal T cell proliferation through an inducible-nitric-oxide-synthase- (iNOS-) dependent mechanism and the administration of these cells before transplantation significantly prolonged allograft survival in fully immunocompetent recipients in a heterotopic heart transplant model [110]. Recently, human regulatory macrophages were isolated from peripheral blood and characterized by their morphology, cell-surface phenotype, and their capability to inhibit T cell proliferation *in vitro* [54]. These cells have been used in kidney transplantation in human, and their utilization allowed to decrease the level of immunosuppressive drugs to induce operational tolerance to the allograft [111]. All these studies suggest that regulatory macrophages may be used as a potential immune-conditioning therapy for use in solid-organ transplantation in the future.

4.3. Exosomes and Phagosomes as Tools for Alloantigen Delivery. The delivery of alloantigens in a non-immunogenic context constitutes an alternative strategy to reduce the immune response following transplantation since it has been observed that donor-specific allograft tolerance can be induced in rodents by presentation of donor MHC antigens before transplantation [112]. Recent approaches include

the use of exosomes and phagosomes as tools for delivering such alloantigens [86, 113–117].

Exosomes are cell-derived membrane nanovesicles of relatively uniform shape and size (50–100 nm) that can be easily purified from fluids (serum, urine, bronchoalveolar lavage, etc.) by ultracentrifugation [117–120]. Exosomes are formed by reverse budding of the limiting membrane of late endosomes/multivesicular bodies (MVB) fused to the plasma membrane. Exosomes are produced by multiple cell types such as enterocytes, mast cells, DCs, T and B lymphocytes, macrophages, tumor cells, and platelets [121–123].

It has been demonstrated that incubation of DCs with exosomes that carry MHC class II results in an efficient stimulation of T cells even when the DCs are MHC class II-deficient [114, 116]. On the other hand, exosomes from thymocytes have the capacity to induce Tregs that suppress the proliferation of effector T cells *in vitro* and *in vivo* [117].

The use of exosomes in a cardiac allograft transplant model in rats has produced promising results. Treatment with exosomes induced a significant prolongation of allograft survival, and in some recipients long-term graft survival was seen after transplantation [112]. Other reports demonstrate that exosomes derived from mature DCs can trigger effector T cell responses leading to rapid skin graft rejection, while exosomes obtained from immature DCs significantly prolong heart allograft survival [113, 115]. Moreover, a combination of donor exosomes with suboptimal doses of the immunosuppressive drug LF15-0195 induced long-lasting survival of cardiac allografts [113]. These reports demonstrate that exosomes constitute a potentially powerful tool of alloantigen delivery in order to induce immune tolerance in transplantation.

Recently, a protocol of alloantigen administration based on phagosomes has been developed. Phagocytosis of PLGA (polylactic-co-glycolic acid) nanoparticles by immature DCs allows these particles to become sequestered in the phagosome. These PLGA-containing phagosomes display a biochemical composition similar to the plasma membrane of the original phagocytic cell [86, 124]. Therefore, the disruption of PLGA-loaded immature DCs produces PLGA-phagosomes that carry alloantigens and other surface molecules expressed by immature DCs [86]. When these phagosomes are fed to immature DCs from a different strain, almost all DCs were able to capture the phagosomes while remaining immature. DCs expressed low expression levels of MHC class II and CD86 maturation markers, secreted low levels of the activating cytokines IL-2 and IL-12, and showed increased IL-10 secretion [86]. Moreover, *in vivo* studies in mice demonstrated that, when administered intravenously, PLGA-phagosomes were phagocytosed only by spleen DCs and this process did not induce DCs maturation. Additionally, when PLGA-phagosomes were used to treat mice prior to alloimmunization, there was a significant reduction in alloantibody secretion and cellular responses. This effect is specific, since third party allogeneic PLGA-phagosomes did not decrease the alloimmune response (our unpublished results). The decreased humoral and cellular immune responses observed in mice treated with phagosome-based alloantigen delivery prior to alloimmunization constitute important observations that should stimulate the use of allogeneic PLGA-phagosomes as a suitable tool for alloantigen administration in a tolerogenic context.

4.4. Apoptotic Cells. The finding that apoptotic cells exert potent anti-inflammatory and immunoregulatory effects on APCs of the immune system [125] has paved the way for the development of novel apoptotic cell-based therapies that have been used successfully in delaying transplant rejection and treating T cell-mediated autoimmune disorders in murine experimental models.

Cell death is an integral cellular process that occurs by two major events: apoptosis and necrosis. Apoptosis, or programmed cell death, is an energy-dependent process that involves typical cellular morphological changes including cell shrinkage, nuclear condensation, DNA fragmentation, and membrane blebbing. Scattered cells in a tissue undergo apoptosis triggered by stimuli in both physiological and pathological conditions. In contrast, necrosis, associated with pathological tissue injury, is characterized by rapid, disorganized swelling and subsequent release of intracellular components into the local environment [126]. These different pathways leading to cell death may give rise to distinct immunological responses [126, 127]. Generally, apoptotic cells are removed through phagocytosis by resident macrophages and DCs, restraining inflammatory or immune reactions [128], and can actively promote anti-inflammatory and tolerogenic signals [47]. In contrast, debris from cells that die prematurely by necrosis is able to activate proinflammatory and immunostimulatory responses [129].

The molecular mechanisms that guide the recognition of apoptotic cells by phagocytes are complex and have not been entirely elucidated. Apoptotic cells display a series of apoptotic cell-associated molecular patterns (ACAMPs) that serve as "eat me" signals that are recognized by PRRs expressed on the surface of the phagocytes, including DCs [130, 131]. Under steady-state conditions, peripheral DCs take up self-antigens carried by apoptotic cells and induce a state of tolerance that protects cells and tissues from potential damage by pathogenic autoimmune reactions as well as immune responses induced by viral and bacterial infections [47]. A broad variety of factors are likely to determine whether a DC becomes tolerogenic or immunogenic after the uptake of apoptotic cells. For example, it is known that early stage apoptotic cells are more likely to induce tolerance than late stage apoptotic cells [132–134]. Molecules displayed on the surface of apoptotic cells [135], the number of apoptotic cells [136], receptors and secreted cytokines [126, 137], the presence or absence of danger signals [138], and interactions with other cells [47] can all contribute to determine different types of immune responses. Additionally, DC maturation status can play a role in the induction of tolerogenicity or immunogenicity. Immunogenic responses are generally associated with mature DCs, which display high numbers of MHC class II and costimulatory molecules. However, it has been difficult to establish a correlation between the maturity state of a DC and its tolerance-inducing function. Early evidence has indicated that tolerance in the periphery is controlled by immature DCs [139]. However, it is becoming

clear that semimature and mature DCs can also induce antigen-specific tolerance [84, 134, 140].

The initial view that the rapid clearance of apoptotic cells *in vivo* does not elicit inflammatory or immune responses in steady-state conditions was expanded by Voll and collaborators [141] who first described that apoptotic cells exert an active and potent immunosuppressive effect on monocytes, promoting the secretion of IL-10 and reducing the release of the proinflammatory cytokines tumor necrosis factor (TNF)-α, IL-1β, and IL-12. This profound downregulatory effect of apoptotic cells on immunity occurs in professional and nonprofessional phagocytes and in nonphagocytic cells [142].

Several reports have shown that interaction and/or internalization of apoptotic cells by immature DCs does not induce expression of the DC maturation-markers MHC class II, CD40, CD80, CD86, and CD83 *in vitro* or *in vivo*, even after challenge with LPS, CD40 signaling, TNF-α, or monocyte-conditioned medium [143–146]. Additionally, DCs that internalize cells in early apoptosis exhibit a selective decrease in the levels of mRNA and secretion of the proinflammatory cytokines IL-1α, IL-1β, IL-6, IL-12p70, and TNF-α, while secreting normal or increased amounts of immunosuppressive transforming growth factor (TGF)-β1 and IL-10, even in the presence of LPS [147–149]. DCs that acquire antigens from apoptotic cells efficiently present apoptotic cell-derived peptides to CD4 T cells and cross-present the internalized antigen to MHC class I-restricted CD8 cytotoxic T cells [150–153]. However, DCs exposed to apoptotic cells show a decrease in their ability to stimulate T cells, a phenomenon that seems to be related to the inhibitory effect of apoptotic cells on the amount of expression of MHC and costimulatory molecules, rather than to a defect in the antigen processing function of the APC [132, 144, 145, 154].

A report has shown that intestinal DCs with internalized apoptotic cell fragments (from intestinal epithelial cells) travel to mesenteric lymph nodes [155, 156] and DCs with intracellular fragments (probably derived from apoptotic cells) containing a self-antigen produced by parietal cells have been detected near the gastric epithelium and in T cell areas of the stomach-draining lymph nodes [156]. These *in vivo* observations reinforce the concept that internalization of apoptotic cells by DCs in peripheral tissues followed by transportation and presentation of self-peptides to naïve T cells in secondary lymphoid organs plays a critical role in the maintenance of peripheral T cell tolerance [139]. A similar principle could be exploited to restrain the anti-donor T cell response in the transplantation setting. Apoptotic cells carrying the entire repertoire of donor alloantigens can be generated easily *in vitro* by physical (UV-B irradiation) or chemical (incubation with ceramide) treatment of cells expressing MHC class I and class II molecules [121, 125].

Systemic administration of apoptotic cells that carry donor MHC molecules has been used in experimental animal models to inhibit the antidonor response [121, 147, 157], and apparently cells in early apoptosis have advantages for specific targeting of alloantigen to DCs *in vivo* compared to other systems: (i) early apoptotic cells deliver a potent immunosuppressive signal to DCs [143, 145, 147, 154, 158]; (ii) apoptotic leukocytes are a rich source of MHC molecules; (iii) apoptotic cells are easy to prepare [143]; (iv) i.v. administration of apoptotic cells is relatively safe; (v) once injected i.v., blood-borne apoptotic cells are captured efficiently by splenic DCs [147, 153]; (vi) DCs present apoptotic cell-derived allopeptides to T cells [159] and (vii) there is no requirement for prepreparation of DCs loaded with apoptotic cells *in vitro* [160].

In mice, i.v. administration of early apoptotic donor leukocytes before transplantation significantly prolongs the survival of heart allografts [121]. In this model, it has been demonstrated that splenic DCs quickly take up the i.v. injected apoptotic cells, process apoptotic cell-derived peptides onto MHC molecules and mobilize to T-cell areas of the splenic follicle [147]. On the other hand, De Carvalho Bittencourt and collaborators [157] showed in a murine model that i.v. injection of donor apoptotic splenocytes facilitates bone marrow engraftment independently of the origin of the apoptotic bodies. In a recent study, it was shown that administration of donor apoptotic cells decreased the systemic anti-donor T and B cell response and prolonged cardiac allograft survival in mice. Moreover, CD40-CD154 blockade resulted in indefinite graft survival mediated by the generation of Tregs [161].

A better understanding of the mechanisms involved in the interaction of APCs with apoptotic cells could open up new possibilities for the prevention/treatment of the antidonor response or, alternatively, certain autoimmune disorders.

4.5. Regulatory T Cells. As described in Section 2.2, the function of Foxp3$^+$ regulatory T cells is to maintain immune tolerance and to prevent inflammatory diseases. It has been demonstrated that a lack of Tregs causes autoimmunity and deregulated T cell activation profiles in mouse models and human diseases. The impaired function or homeostasis of Tregs has been implicated in type 1 diabetes, rheumatoid arthritis, multiple sclerosis, and systemic lupus erythematosus [162]. Given the critical function of Tregs in the maintenance of immune tolerance and the specific immunomodulatory mechanisms that can effectively inhibit the targeted effector cell population, their use has been proposed as a therapy to induce specific immune tolerance and to reduce the use of immunosuppressive drugs. In murine models, many groups have used unmanipulated host nTregs or *in vitro* expanded nTregs in combination with immunosuppressive drugs or immune ablation as a strategy to generate immune tolerance and allograft acceptance [163]. It has been demonstrated that the injection of purified or *ex vivo* cultured CD4$^+$CD25$^+$Foxp3$^+$ nTregs significantly reduces GVHD [164, 165] and, in combination with bone marrow transplantation, inhibit alloreactive CD4$^+$ and CD8$^+$ T cells and prolong allograft survival [166, 167].

It has been demonstrated that nTregs expressing CD4, CD25 and Foxp3 prevent allograft rejection mediated by CD4$^+$Foxp3$^-$ activated T cells and cytotoxic CD8$^+$ T cells; however, nTregs constitute only 5–10% of peripheral CD4$^+$ T cells. For this reason, protocols to obtain Tregs have been a subject of intense research in transplantation immunology. Several reports indicate that Tregs can be obtained using

different strategies: they can be directly obtained from the host, they can be obtained from the host and expanded *ex vivo*, they can be induced *in vitro* from naïve T cells under appropriate culturing conditions, or they can be induced by polyclonal activation, antigen-specific activation, or allogeneic activation.

It has been described that Tregs can be generated by culturing naïve T cells with a mixture of immature DCs, mature DCs and B lymphocytes in the presence of a combination of TGF-β, retinoic acid, and IL-2 [168, 169]. In addition, alloantigen-specific Tregs can be generated by stimulation of naïve T cells with allogeneic APC and a combination of TGF-β, IL-2, and retinoic acid. These alloantigen specific Tregs present immunosuppressive activity *in vitro*; therefore, they could be used as a specific cellular therapy, and in combination with a regimen of low immunosuppression, they could generate immune tolerance to bone marrow allografts. The utilization of alloantigen-specific Tregs as a conditioning protocol could induce the immune tolerance necessary for subsequent solid organ transplantation [166, 170].

Regulatory T cells have been used in the generation of mixed chimerism with reduced conditioning regimens, where the peripheral T-cell repertoire of the recipient is maintained largely intact and Tregs of donor origin are crucial to the active suppression [170, 171]. Although Tregs have potent effects in murine allograft models, current evidence indicates that Tregs are not capable of inducing prolonged skin allograft tolerance in unmanipulated recipients [166, 167, 172]. However, the therapeutic use of Tregs is an interesting approach in the development of minimum conditioning protocols for transplants.

4.6. Mesenchymal Stromal/Stem Cells. Other immunomodulatory cells with a high potential in future therapies in transplantation are mesenchymal stromal/stem cells (MSCs). It is well known that bone-marrow-derived MSCs have the capacity to migrate to inflammatory sites and regulate the function of most immune cells through direct contact and/or cytokine secretion [54, 173].

Recent reports in animal models and human have addressed the potential role of MSCs in the induction and/or differentiation of different immunosuppressive populations. For instance, it has been shown that murine MSCs can suppress heart graft rejection through the induction of $Foxp3^+$ T cells and the inhibition of alloantibody production [174]. In keeping with this report, the groups of Maccario and Mougiakakos have demonstrated that human MSCs favor the differentiation of CD4 regulatory T-cell subsets from peripheral-blood mononuclear cells in mixed lymphocyte cultures and prevent skin [175] and semiallogeneic heart rejection [176]. Finally, a recent report has shed light into some of the possible mechanisms involved in the immunosuppressive properties of MSCs as they demonstrated that porcine MSCs inhibit alloreactive T cells through the induction of PGE_2 and IDO [177]. Thus, although additional efforts are needed to further understand the mechanisms of the observed immunomodulatory properties of MSCs, this population constitutes a promising weapon for future transplant therapies.

5. Concluding Remarks

Medical and scientific advances achieved since the first steps of organ transplantation have made it an acceptable resource for human medical care. Nonetheless, since the massive development of organ transplantation near the end of the 20th century, there have been few steps made toward the improvement of allograft survival and pharmacological immunosuppression. As an alternative, cell-based therapy offers the opportunity to induce immune tolerance without the adverse effects associated to pharmacological immunosuppression. Here we have described mechanisms related to allograft tolerance and cellular treatments that have been well characterized for their ability to induce immune tolerance. Dendritic cells, regulatory macrophages, apoptotic cells, regulatory T cells, and mesenchymal stromal/stem cells offer a viable alternative for future use in clinical procedures that could greatly benefit patient survival and quality of life in transplanted patients. The next steps of transplantation immunology will most certainly involve the clinical standardization of dosage, administration, and effectiveness, among other parameters, for the potential therapies discussed here. Efforts are now focused on overcoming the challenges that currently limit the use of cell therapy, either alone or in combination with pharmacological tools, with the goal of breaking through the main causes of failure in the current protocols to achieve organ acceptance.

Conflict of Interests

The authors declare no financial or commercial conflict of interests.

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Research Article

Trichostatin A Promotes the Generation and Suppressive Functions of Regulatory T Cells

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Regulatory T cells are a specific subset of lymphocytes that suppress immune responses and play a crucial role in the maintenance of self-tolerance. They can be generated in the thymus as well as in the periphery through differentiation of naïve $CD4^+$ T cells. The forkhead box P3 transcription factor (Foxp3) is a crucial molecule regulating the generation and function of Tregs. Here we show that the *foxp3* gene promoter becomes hyperacetylated in *in vitro* differentiated Tregs compared to naïve $CD4^+$ T cells. We also show that the histone deacetylase inhibitor TSA stimulated the *in vitro* differentiation of naïve $CD4^+$ T cells into Tregs and that this induction was accompanied by a global increase in histone H3 acetylation. Importantly, we also demonstrated that Tregs generated in the presence of TSA have phenotypical and functional differences from the Tregs generated in the absence of TSA. Thus, TSA-generated Tregs showed increased suppressive activities, which could potentially be explained by a mechanism involving the ectonucleotidases CD39 and CD73. Our data show that TSA could potentially be used to enhance the differentiation and suppressive function of $CD4^+Foxp3^+$ Treg cells.

1. Introduction

Regulatory T cells (Treg) are a specific subset of lymphocytes that suppress immune responses and play a crucial role in the maintenance of self-tolerance [1, 2]. Their development and function are programmed by the forkhead box P3 transcription factor Foxp3, which is predominantly expressed in $CD4^+CD25^+$ Treg cells [1, 3]. Tregs actively suppress the activation and expansion of autoreactive immune cells to limit the duration and extent of inflammation. Therefore, a decrease in Treg activity can contribute to autoimmunity and inflammatory diseases [4]. Because of their suppressive capacities, Tregs represent a promising strategy for inducing tolerance to self- and non-self-antigens in such diseases.

In recent years, increasing evidence has demonstrated the role of epigenetic alterations in the etiology of many autoimmune and inflammatory diseases through changes in DNA methylation and histone modifications [5, 6]. Therefore, it is important to determine crucial histone modifications for Treg development and function and to study compounds able to revert or modify epigenetic patterns.

Among histones modifications is acetylation, which occurs at lysine residues mainly on their amino-terminal tails. This posttranslational modification is dynamic and its overall effect on gene expression depends on the balance between histone acetyltransferases (HATs) and histone deacetylases (HDACs) [7, 8]. HDACs typically dampen histone-DNA and histone-non histone protein interactions [9, 10], but they also regulate the function of non-histone proteins [11].

Histone deacetylase inhibitors (HDACi) such as trichostatin A (TSA) are small molecule compounds capable of inhibiting class I, II, and IV HDAC families of enzymes [12].

Previous studies in mice have shown that TSA administration *in vivo* promotes the generation and function of Tregs [13], and has beneficial effects in cardiac allograft transplant, inflammatory bowel disease [13] and lupus models [14]. Unfortunately, none of these studies provided *in vitro* data regarding the effect of TSA on Treg generation from conventional $CD4^+CD25^-$ T cells and their suppressive function, making it difficult to understand whether these are direct or indirect effects. Our study provides evidence that TSA increases the generation of $CD4^+Foxp3^+$ Tregs *in vitro* and gains insight into the regulation of $CD4^+Foxp3^+$ Tregs by the deacetylase inhibitor TSA.

2. Materials and Methods

2.1. Animals. Six- to eight-week-old Foxp3-GFP mice were used for all experiments. All mice were maintained and manipulated according to institutional guidelines at the pathogen-free facility of Fundación Ciencia & Vida after approval by the Ethical Review Committee.

2.2. Antibodies. Anti-CD3ε, anti-CD16/32, anti-CD4-APCH7, anti-CD25-APC, anti-IL-17A-PE, anti-IFNγ-PECy7, and anti-CD39-PE, anti-GARP-PE, anti-CD73-Cy7 were purchased from eBioscience (CA, USA). Anti-CTLA-4-PE was purchased from BD PharMingen (NJ, USA). Anti-IL-10-PE, anti-LAG3-biotin, streptavidin-APC, anti-IL4, and anti-IFNγ were purchased from BioLegend (CA, USA). Anti-H3ac and anti-H3 were purchased from Abcam (MA, USA) and Rabbit IgG from Millipore (MA, USA).

2.3. Flow Cytometry and Cytokine Secretion Analysis. The expression of cell surface markers on T cells was determined by FACS analysis after surface staining with specific anti-mouse antibodies. To determine IL-10 secretion, Treg cells were stimulated for 4 h at 37°C with 50 ng/mL PMA and 1 ug/mL ionomycin (Sigma-Aldrich). Following the reactivation, the supernatants were recovered and immediately analyzed using a mouse BD cytometric assay array (CBA). All data were collected on a FACSCanto II (BD Biosciences) and analyzed with FACS Diva software (BD, New Jersey) or FlowJo software (TreeStar).

2.4. Purification of T Cells and In Vitro T Cell Differentiation. Splenic $CD4^+$ T cells from Foxp3-GFP mice were enriched by MACS purification using the CD4 isolation kit II (Miltenyi Biotec) following the manufacturer's instructions. Naive $CD4^+CD25^-$ T cells were further purified by cell sorting using a FACS ARIA II (Becton Dickinson, NJ, USA). For T helper cell cultures, dendritic cells were enriched by MACS purification using the CD11c microbeads (Miltenyi Biotec). Naive $CD4^+$ T cells were cocultured with dendritic cells at a 5 : 1 ratio, in the presence of 1 μg/mL anti-CD3, 10 μg/mL anti-IL-4, 10 μg/mL anti-IFN-γ and the presence or absence of 10 nM TSA, under polarizing conditions towards iTregs (5 ng/mL TGF-β, 100 U/mL IL-2, and 10 nM retinoic acid) or Th17 cells (5 ng/mL TGF-β, 10 ng/mL IL-6, and 10 ng/mL IL-1β) for 4 days. Before harvesting for chromatin immunoprecipitation (ChIP), naive $CD4^+$ and iTreg cells were purified by cell sorting after surface staining with anti-CD4 and anti-CD25. Cell sorting was gated on the $CD4^+CD25^-Foxp3^-$ (naive) or $CD4^+Foxp3^+$ population (iTregs). For Th17 sorting, cells were stained with anti-CD4 and permeabilized for further anti-IL-17A staining. Sorting was gated on the $CD4^+IL17A^+$ cells.

2.5. Isolation of Splenic DCs. Spleen tissue was fragmented and digested for 45 min at 37°C in the presence of collagenase D (Roche, Germany) and 2 μg/mL of DNAse I (Roche) in PBS plus 10% fetal bovine serum. Undigested fibrous material was removed by filtration through cell strainer. $CD11c^+$ cells were obtained by positive selection using anti-CD11c microbeads (Miltenyi Biotec) according to the manufacturer's instructions.

2.6. Western Blot Analysis. Cells were washed twice with cold phosphate-buffered saline (PBS) and then lysed in RIPA buffer (50 mM Tris, 150 mM NaCl, 0.1% SDS, 0.5% Na deoxycholate, and 1% NP40) supplemented with a protease inhibitor cocktail. Protein concentration from lysates was determined by Bradford assay (Pierce, Rockford, IL, USA). Equal protein amount of cell lysates was loaded on the gels and subjected to SDS-PAGE. The separated proteins were transferred onto PVDF membranes and then analyzed by Western blotting using anti-H3 and anti-H3ac antibodies. Bands were quantified using Quantity One software (Bio-Rad).

2.7. ChIP and DNA Quantification. After cell sorting, cells were cross-linked with 1% formaldehyde for 10 min. The reaction was quenched with 0.125 M glycine for 5 min, and cells were washed twice with PBS, resuspended in cell lysis buffer (10 mM Tris-HCl pH 8.0, 10 mM NaCl, 0.2% NP-40, and a proteinase inhibitor cocktail) for 10 min, and then resuspended in nuclear lysis buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 1% SDS, and a proteinase inhibitor cocktail). Lysate was sonicated with a Branson Sonifier 250, diluted twice in sonication buffer (50 mM HEPES pH 7.9, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% deoxycholic acid, 0.1% SDS, and a proteinase inhibitor cocktail), and precleared with rabbit IgG and protein A agarose (Millipore). 3 ug of the precleared samples was taken as input control and for ChIP reactions, which were performed overnight at 4°C with 1 ug of anti-H3ac or 1 ug of rabbit IgG. Immunocomplexes were isolated with protein A agarose and washed twice with the sonication buffer, once with wash buffer (500 mM LiCl, 100 mM Tris HCl pH 8.0, 1% NP-40, and 0.1% deoxycholic acid), and once with TE (50 mM Tris-HCl pH 8.0 and 2 mM EDTA). Samples were then eluted with 100 uL of the immunoprecipitation elution buffer (50 mM $NaHCO_3$ and 1% SDS), and finally the cross-link was reversed by incubating the samples at 65°C in 200 mM NaCl. Samples were treated with 1 mg/mL proteinase K and the DNA isolated by DNA Clean and Concentrator kit (Zymo Research). For quantification of the immunoprecipitated DNA, KAPA SYBR FAST (KAPA Biosystems) was used according to the manufacturer's

instructions. Immunoprecipitated DNA was quantified by creating a line of best fit from a standard curve using serial dilutions of genomic template DNA to allow normalization of primer sets. Primers for the *foxp3* promoter were as follows: forward, 5′ CCTTGGCAACATGATGGTGGTGAT 3′; reverse, 5′ AAGAAGGGATCAGAAGCCTGCCAT 3′.

2.8. Analysis of Treg Cell Function. For Treg suppression assays, carboxyfluorescein-succinimidyl-ester- (CFSE-, Invitrogen) labeled effector T cells ($CD4^+CD25^-$) were stimulated with 1 mg/mL of antibody against CD3ε (eBioscience) in the presence of $CD11c^+$ dendritic cells at a ratio of 1 : 2 (T effector: dendritic cells) and at a ratio of 1 : 0.5 of T effector: purified $CD4^+Foxp3^+$ Treg cells generated with or without TSA. To rule out that TSA acted upon dendritic cells rather than on the T lymphocytes, suppression assays were performed after activating the T cells in the presence of plate-bound anti-CD3 (0.5 μg/mL and 5 μg/mL) and soluble anti-CD28 (1 μg/mL) and in the absence of dendritic cells. After 72 hours, suppression of proliferation was determined by flow cytometry analysis of CFSE dilution using a FACS Canto II cytometer (BD), and data were analyzed using FACS Diva software (BD).

3. Results

3.1. The foxp3 Promoter Is Hyperacetylated on iTreg Cells. We first sought to analyze the patterns of histone H3 acetylation on differentiated Treg cells. For this purpose, we cultured naive ($CD4^+CD25^-Foxp3^-$) T cells with spleen-derived $CD11c^+$ DC plus α-CD3 mAb under Treg polarizing conditions for 4 days and then cells were sorted to purify the resulting Treg population ($CD4^+CD25^+Foxp3^+$) (Figure 1(a)). We then analyzed by ChIP assay the H3ac mark on the histone H3 of the *foxp3* promoter on both, the sorted naive $CD4^+$ T cells and the *in vitro* iTreg (Figure 1(b)). The results show that when compared to the naive $CD4^+$ T cells, histone H3 is hyperacetylated at the *foxp3* promoter in iTreg cells. To demonstrate that the acetylation of the *foxp3* promoter was related to the differentiation of naive $CD4^+$ T lymphocytes to Treg cells, we also compared the level of H3ac of the *foxp3* promoter on *in vitro* differentiated naive $CD4^+$ T cells to Th17 cells (Figure 1(a)). As expected, the acetylation levels of the *foxp3* promoter did not change on differentiated Th17 cells. Thus, we conclude that the *foxp3* promoter becomes hyperacetylated on histone H3 upon differentiation of naive $CD4^+$ T cells to Treg cells.

3.2. TSA Increases the Generation of Treg In Vitro. Since the acetylation patterns of histone H3 changes during Treg differentiation, we next questioned whether by affecting the acetylation levels of histone H3, Treg differentiation could be pharmacologically modulated. For this purpose, we cultured naive ($CD4^+CD25^-Foxp3^-$) T cells with spleen-derived $CD11c^+$ dendritic cells plus α-CD3 mAb under iTreg polarizing conditions in the presence or absence of the histone deacetylase inhibitor TSA. After 4 days, Foxp3-GFP expression was assessed by FACS, as an indicator of Treg differentiation. Figure 2(a) shows that 69% of the population expressed Foxp3, whereas the percentage increased to 78% when naive T cells were differentiated in the presence of TSA. Statistical analyses on 6 independent experiments indicated that the TSA treatment increased the differentiation of Tregs by 14.8% compared to untreated cultures (Figure 2(b)). To confirm that the mechanism involved in the effect of TSA on Treg differentiation implied changes on the acetylation levels of histone H3, we isolated total protein extracts from iTreg and analyzed the acetylation levels of histone H3. Western blot analyses showed that TSA treatment increased the global levels of acetylated histone H3 on Tregs by nearly two fold compared to untreated cultures (Figures 2(c) and 2(d)). Taken together, these results suggest that TSA stimulates the differentiation of Tregs while at the same time increasing the levels of histone H3 acetylation.

3.3. TSA Upregulates Treg Suppressive Capacity. Next we tested the activity of the Tregs generated in the presence of TSA. For this, $CD4^+Foxp3^+$ Treg cells were generated as indicated above and after 4 days of culture the resultant $Foxp3^+CD4^+$ population was cocultured with CFSE-labeled naive T cells in the presence of $CD11c^+$ DCs and anti-CD3, in a conventional T cell suppression assay. Figure 3 shows that at a ratio of 1 effector to 0.5 Treg, the proliferation of the naive T lymphocytes was clearly suppressed by the Treg. Interestingly, Treg generated in the presence of TSA showed a higher suppressive activity compared to those generated in the absence of the drug. Therefore, these results show that TSA increased the ability of Tregs to suppress the *in vitro* proliferation of CFSE-labeled naive $CD4^+$ T cells. To determine if the effect of TSA on the activity of Tregs was due to a direct effect of the drug on Treg cells, Tregs were generated in the absence of dendritic cells by activating naive T cells with plate-bound anti-CD3 and soluble anti-CD28. The results show that TSA increases the suppressive activity of Tregs even when generated in the absence of dendritic cells (Figures 3(a) and 3(b)). However, Tregs generated in the presence of dendritic cells show higher suppressive activity than those generated using activating antibodies. Moreover, the number of Tregs generated in the presence of dendritic cells needed to achieve 50% suppression is half of those generated in the presence of activating antibodies (ratio 1 : 0.5 in the presence of dendritic cells versus 1 : 1 in the presence of activating antibodies).

3.4. Tregs Generated under TSA Treatment Differentially Express Treg Markers Involved in Their Suppressive Functions. Besides Foxp3 and IL-10 secretion, several additional markers such as CTLA-4 (cytotoxic T-lymphocyte associated molecule-4), GITR (glucocorticoid-induced TNF receptor), and LAG-3 are also expressed on regulatory T cells; however the functional significance of these molecules remains to be defined. Also the ectonucleotidases CD39 and CD73 are expressed in Treg and convert ATP into immunosuppressive adenosine. As TSA increased the differentiation and suppressive functions of Treg cells, we decided to analyze whether TSA was capable of inducing changes in the expression of

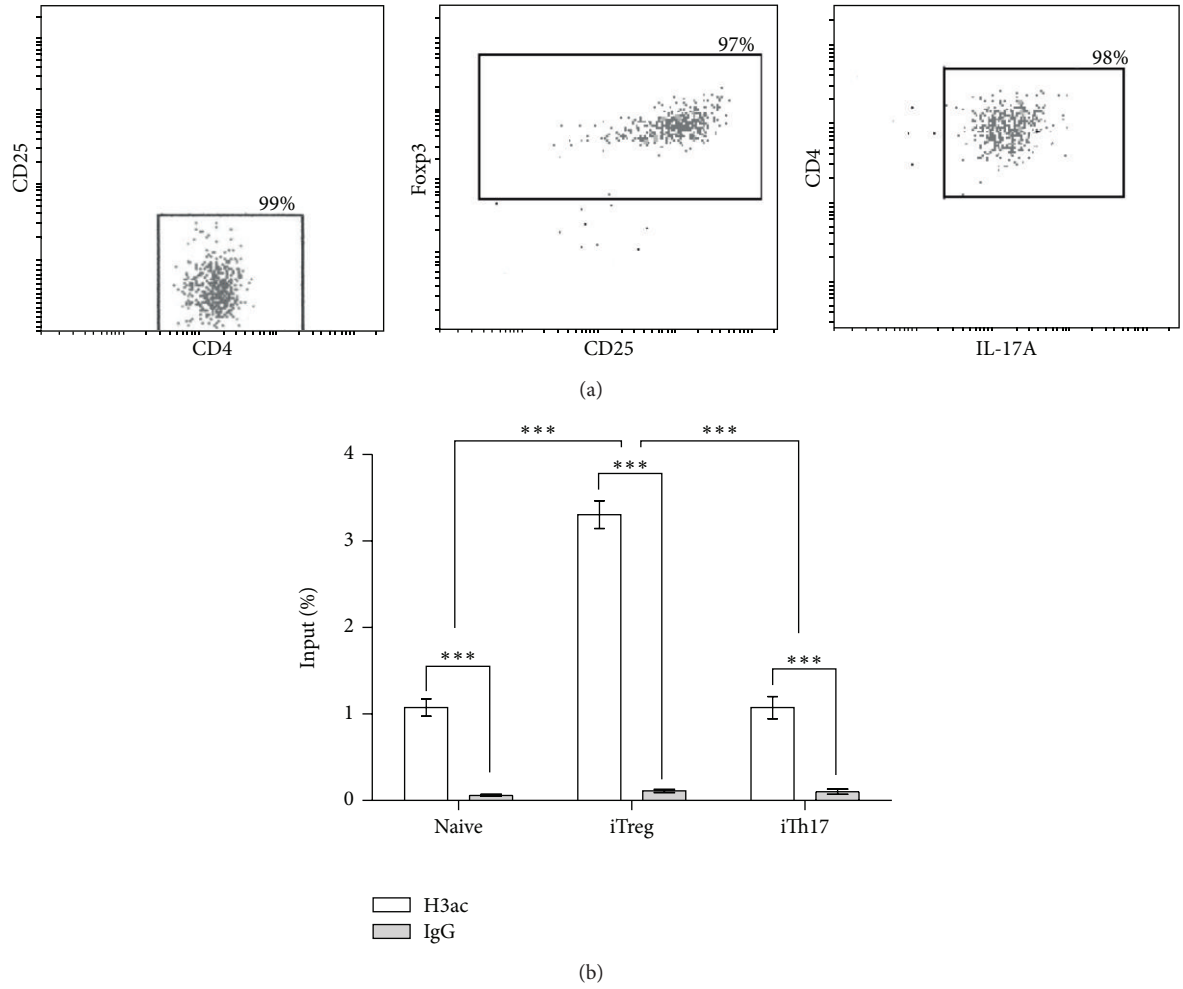

Figure 1: The *foxp3* promoter is hyperacetylated on iTreg cells. (a) Flow cytometry of the sorted naive cells and *in vitro* differentiated Treg and Th17 cells. Numbers indicate the percentage of naive ($CD4^+CD25^-$), Treg ($CD4^+Foxp3^+$), and Th17 ($CD4^+IL\text{-}17^+$) positive cells. (b) chromatin immunoprecipitation assays on *foxp3* promoter. ChIP assays were performed on sorted splenic naive $CD4^+$ T cells and *in vitro* induced Tregs and Th17 cells. DNA fragments bound to acetylated histones were immunoprecipitated using antibodies directed against acetylated histone H3 (H3ac) or a rabbit isotype-matched immunoglobulin G (IgG), as control. Precipitated DNA was quantified by real-time PCR with primers specific for the *foxp3* gene promoter, and the PCR products were set in relation to input DNA. Standard deviation was obtained from three (naive and iTregs) and two (iTh17) independent experiments.

these additional Treg markers. For this purpose, we generated Tregs in the presence or absence of TSA for 4 days as described, and we then stained the cells with specific antibodies. Figure 4 shows that although no significant changes could be detected in the expression of CTLA-4 or GARP, the mean fluorescence of TSA-treated Treg for the ectonucleotidases CD39 and CD73 showed significant increase of 1.7- and 1.4-fold, respectively. We also observed a modest (0.5-fold) decrease in the mean fluorescence of LAG-3. These results indicate that TSA may affect the immune suppressive activity of Treg by specifically increasing the protein levels of the ectonucleotidases CD39 and CD73, the expression of which is driven by Foxp3, the Treg-specific transcription factor. This result may be a clue to the mode of action of TSA on Treg activity. When analyzing IL-10 secretion, we found no differences in Tregs generated in the presence or absence of TSA, when these Tregs were generated in the absence of dendritic cells. However, we found that TSA produced higher IL-10 secretion (2.5 fold) when Tregs were produced in the presence of DCs, probably due to an effect of TSA on DCs rather than on the Tregs.

4. Discussion

It is well known that histone posttranslational modifications play an important role in the regulation of gene expression and that pharmacological control of histone-modifying enzymes could be potentially used to manipulate the differentiation of specific cellular lineages. On the other hand, defects in the development or function of regulatory T cells contribute significantly to the pathogenesis of many inflammatory and autoimmune diseases. Thus, there are important

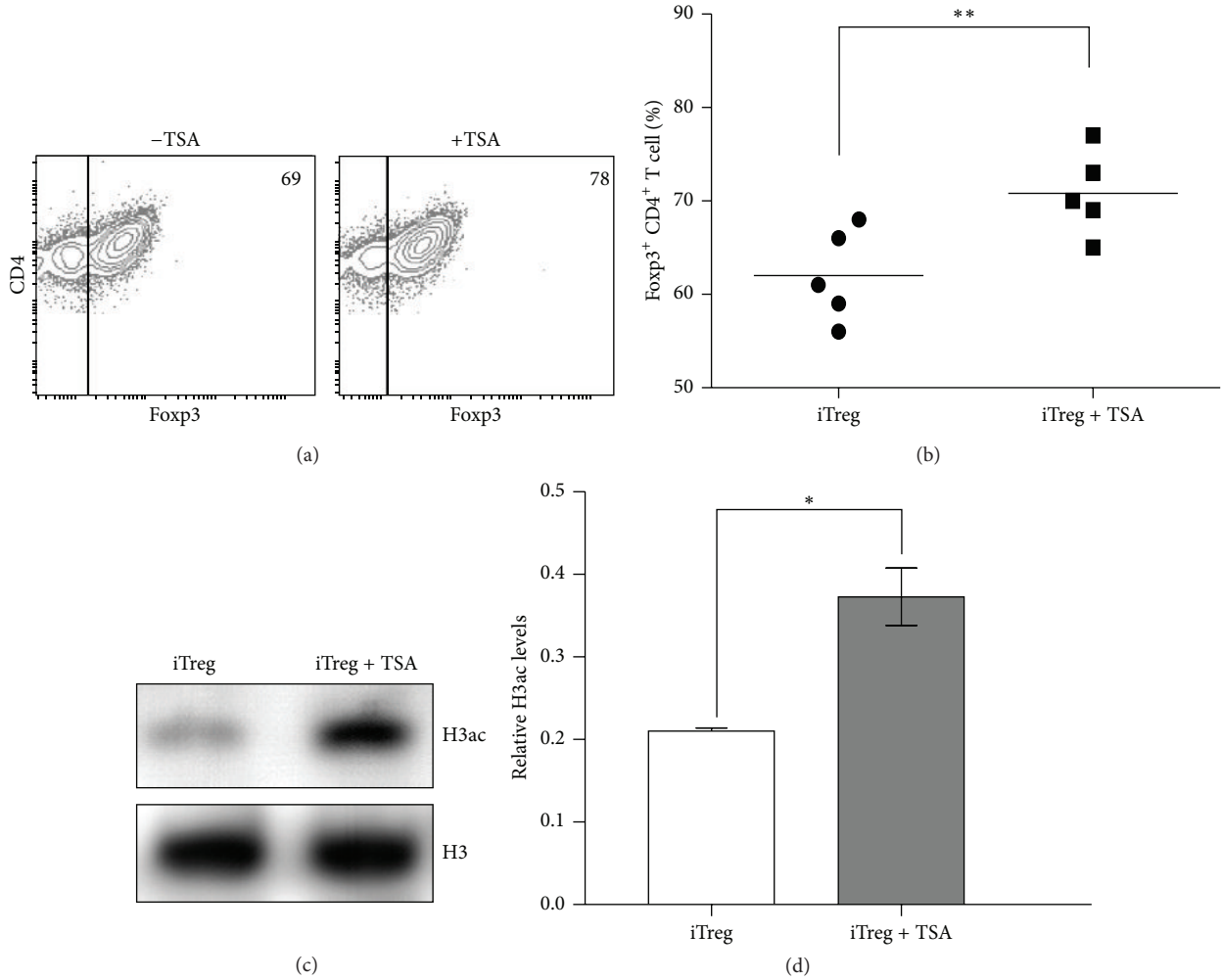

FIGURE 2: TSA increases the generation of Tregs from naive $CD4^+$ T cells. (a) Flow cytometry analysis of $CD4^+Foxp3^+$ cells generated in the absence (left) or presence (right) of 10 nM TSA for 4 days. Numbers indicate the percentage of $CD4^+Foxp3^+$ double positive cells. (b) Scatter plot depicts percentage of $CD4^+Foxp3^+$ double positive cells from five independent experiments in the presence and absence of TSA. $^{**}P < 0.008$ by student's t-tests. (c) Western blot analyses of total protein extracts derived from Tregs differentiated in the absence or presence of 10 nM TSA, as indicated. (d) Quantitation of the acetylation levels relative to the histone H3 with the Quantity One software. Standard deviation was obtained from three independent experiments. $^{*}P < 0.05$ by two-way ANOVA.

attempts to develop new strategies for generating $Foxp3^+$ regulatory T cells as a valuable therapy for these diseases. HDAC inhibitors have previously shown to induce cell-cycle arrest and apoptosis in cancer and $CD4^+$ T cells [15, 16] and to have anti-inflammatory effects on human monocytes [17]. Specifically, the histone deacetylase inhibitor TSA has been shown to modulate inflammatory and immune responses by boosting thymic production of naturally occurring Treg cells and also to increase the suppressive function of Treg *in vivo* [13].

In this study, we analyzed the effect of TSA on the differentiation of naive $CD4^+$ T cells towards a regulatory phenotype and performed a functional and phenotypic characterization of TSA-differentiated Tregs. We observed that TSA stimulates the differentiation of naive T cells towards a Treg phenotype and that this stimulation correlated with hyperacetylation of the global histone H3. This suggests that TSA may act through the hyperacetylation of the histone H3 in the *foxp3* promoter. Interestingly, it has been reported that the Foxp3 protein becomes acetylated as well, preventing its proteasomal degradation [18]. Thus, the increased $CD4^+Foxp3^+$ T cell population produced by TSA could possibly be the result of combined pathways: by the upregulation of the *foxp3* gene expression mediated by the hyperacetylation of the histone H3 on the *foxp3* promoter and, on the other hand, by increasing the Foxp3 protein's half-life. The stimulation of Treg differentiation that we observed by TSA could explain, at least in part, the anti-inflammatory effect of TSA. This could be due to an increase in peripheral Treg cells generation, as it has been suggested by Tao et al. [13].

Since our differentiation experiments were carried out in the presence of $CD11c^+$ dendritic cells we wanted to rule out that TSA affected the DC and thus indirectly Treg activity.

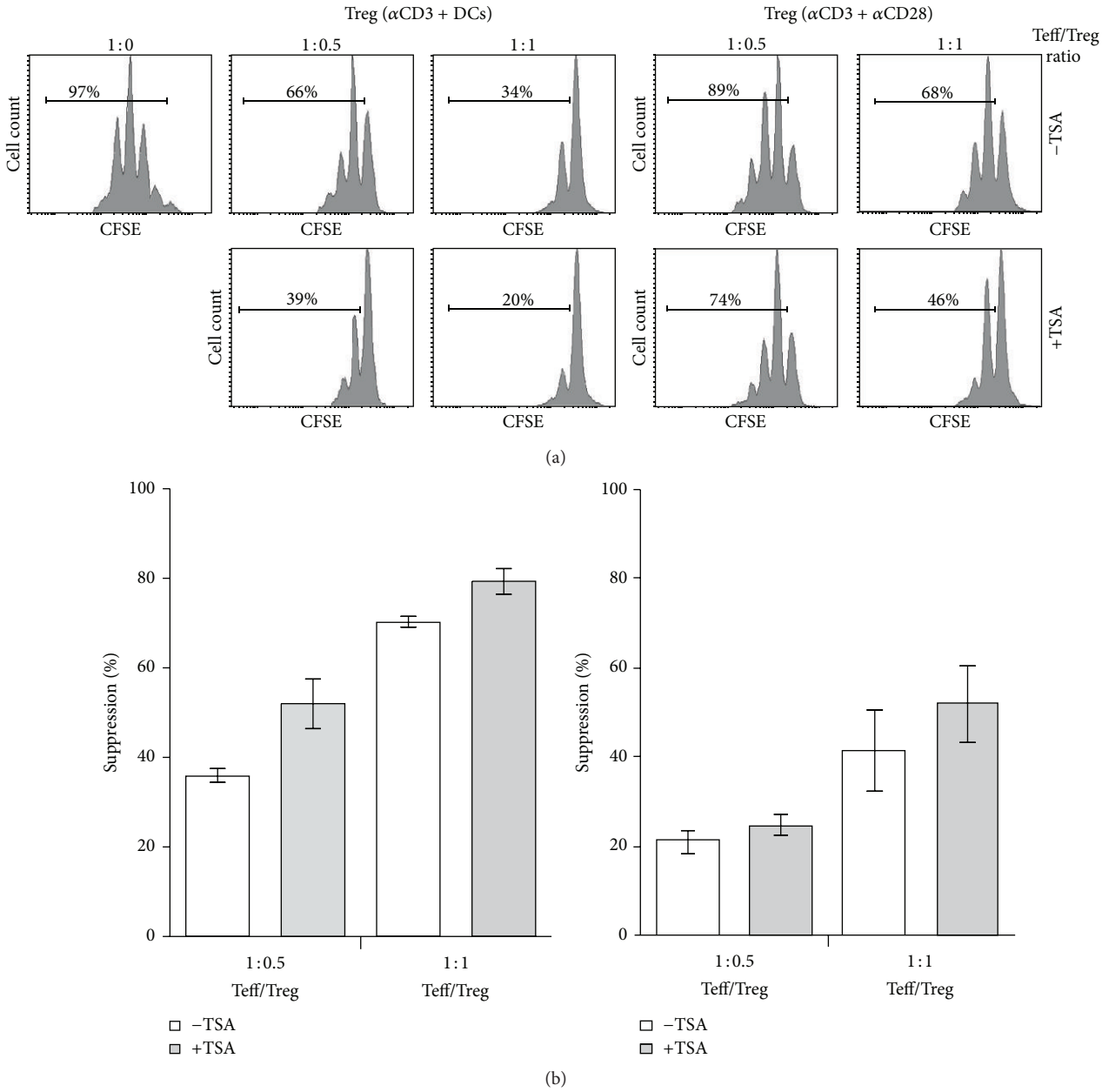

Figure 3: TSA Treg presents higher suppressive capacity. (a) Treg suppression assays of Tregs generated in the presence (left) or absence (right) of dendritic cells, and with (+TSA) or without TSA (−TSA). Tregs from each group were added to CFSE-labeled naive $CD4^+$ T cells cocultivated with dendritic cells plus anti-CD3 antibody. The percentage of proliferating T cells is shown in each plot. Data are representative of three independent experiments. (b) Quantitation of the percentage of suppression of Tregs generated with or without TSA in the presence of anti-CD3 and dendritic cells (left) or plate-bound anti-CD3 and soluble anti-CD28 (right).

Our results show that Tregs generated in the absence of DC but in the presence of activating antibodies also show an increased suppressive activity, indicating that TSA acts on naive T cells. However the results also suggest that dendritic cells may become more tolerogenic in the presence of TSA, since the number of Tregs generated in the presence of TSA and dendritic cells needed to achieve 50% suppression is half of those generated in the presence of activating antibodies (ratio 1 : 0.5 in the presence of dendritic cells versus 1 : 1 in the presence of activating antibodies). Consistently, it has been reported that TSA can change dendritic cells into a tolerogenic phenotype *in vitro* [19], so the evidence points to the fact that both Tregs and dendritic cells are targets for TSA.

Importantly, we also demonstrated that TSA increases Treg functions. Tregs exert their functions in several ways. The widely recognized mechanisms of suppression include the secretion of suppressive soluble factors, cell contact-mediated suppression, and competition for growth factors.

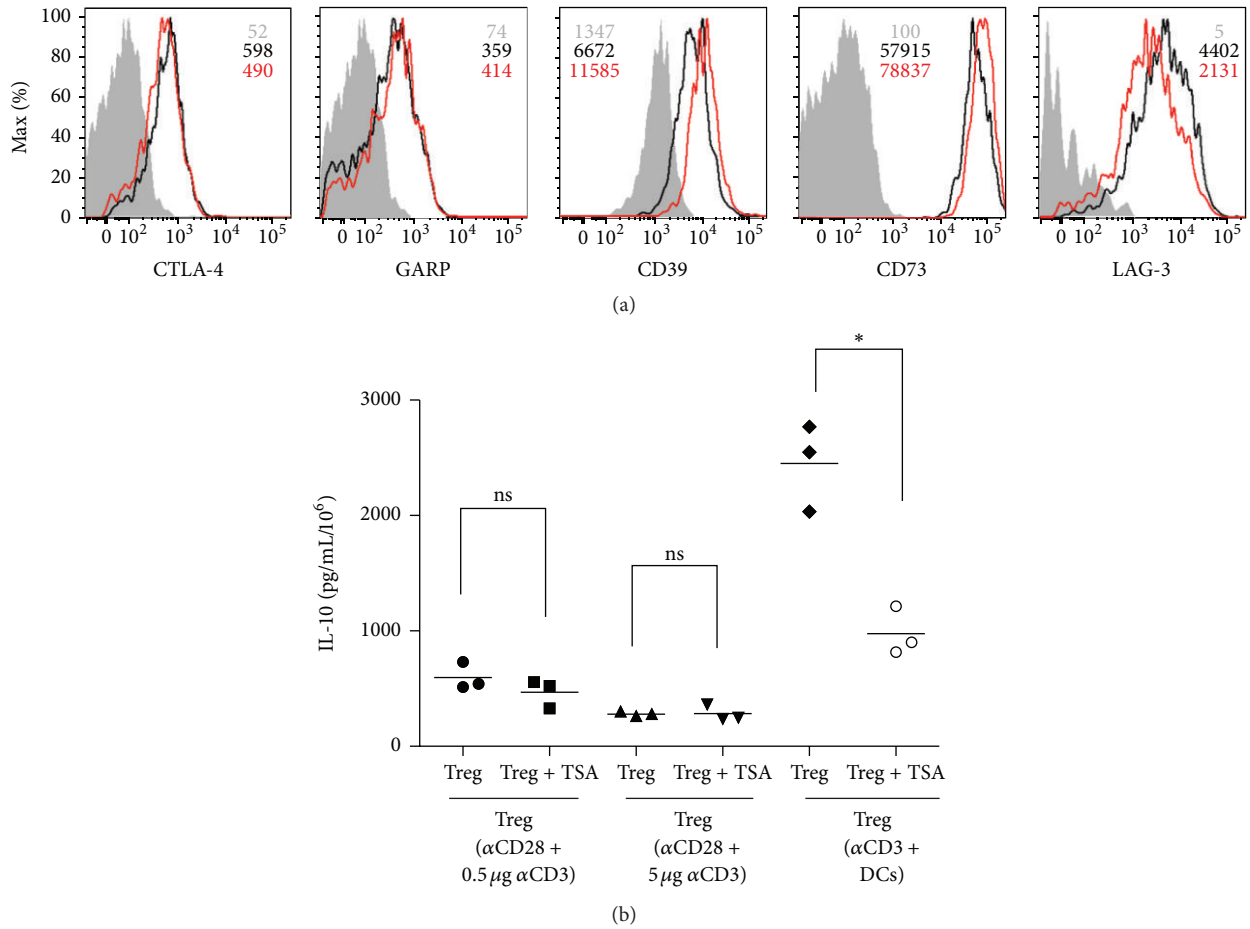

Figure 4: Phenotypical characterization of Tregs generated under TSA treatment. (a) Representative histograms of CTLA-4, GARP, CD39, CD73, and LAG-3 expression on Tregs generated in the absence (black) or presence (red) of TSA. Controls are filled histograms. Average MFI ± SEM was derived from three independent experiments. (b) IL-10 secretion was determined using the CBA method. Each point represents an individual mouse.

The phenotypic characterization of Tregs generated in the presence or absence of TSA gave us some lights on the mechanisms by which TSA could be affecting Treg suppressive functions. We found that TSA increased the expression of CD39 and CD73 ectonucleotidases in Tregs. These ectoenzymes generate pericellular adenosine from extracellular nucleotides that suppress effector T cell functions [20]. The correlation between the TSA effect and the level of these ectoenzymes could explain the higher suppressive activity observed in Treg cells generated under the TSA treatment. In contrast, TSA decreased the expression of LAG-3, a molecule involved in Treg cell suppression of dendritic cell function [21], suggesting that the expression of CD39 and CD73 might compensate this reduction. On the other hand, no differences in the expression of GARP or CTLA-4 were observed between both types of Treg cells. Although we found that TSA could increase the production of IL-10, these effects could only be seen in the condition where Tregs were generated in the presence of dendritic cells, so it could be due to an effect on them. Further work needs to be performed to better understand the Treg cells suppressor mechanisms being affected by TSA. A better understanding of the effect of TSA on Treg cells should provide important tools to therapeutically enhance Treg production and suppressive function. In conclusion, our results showed that the outcome of pharmacological modulation of histone-modifying enzymes on the differentiation of Tregs might be exploited as potential valuable therapy for autoimmune diseases.

Authors' Contribution

Cristian Doñas and Macarena Fritz contributed equally to this work.

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Review Article

Regulatory T Cells in Allogeneic Stem Cell Transplantation

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Growing evidence suggests that cellular adoptive immunotherapy is becoming an attractive though challenging approach in regulating tumor immunity and alloresponses in clinical transplantation. Naturally arising CD4+CD25+Foxp3+ regulatory T cells (Treg) have emerged as a key component in this regard. Over the last decade, a large body of evidence from preclinical models has demonstrated their crucial role in auto- and tumor immunity and has opened the door to their "first-in-man" clinical application. Initial studies in clinical allogeneic stem cell transplantation are very encouraging and may pave the way for other applications. Further improvements in Treg *ex vivo* or *in vivo* expansion technologies will simplify their global clinical application. In this review, we discuss the current knowledge of Treg biology and their potential for cell-based immunotherapy in allogeneic stem cell transplantation.

1. Introduction

In recent years, the great progresses in our understanding of the basic processes that control immune tolerance, as well as the more recent characterization of naturally arising CD4+CD25+Foxp3+ regulatory T cells (Treg), that tip the balance between auto- and tumor immunity, opened the door to their therapeutic application, either by enhancing their activity in autoimmune diseases [1–3], allograft rejection [3], and graft-versus-host disease (GVHD) [4, 5] or by blocking their suppressive activity in tumor immunity [6] and in vaccine development [7]. Treg therapy has the promise of avoiding many of the toxicities observed with current drug regimens. However, many issues on the homeostasis and function of human Treg still need to be addressed. The development of new markers and technologies for Treg identification, antigen-specific isolation, and *in vitro* or *in vivo* expansion by specific stimulation will help to "unlock the power" of Treg and devise novel therapeutic strategies to control untoward immune responses.

In this review, we discuss the current knowledge of Treg biology and their potential for cell-based immunotherapy in allogeneic stem cell transplantation.

2. Biology of Treg

Human natural Treg (nTreg) derive from thymus and are characterized by the coexpression of CD4, high levels of surface CD25 (also known as interleukin-2 receptor α (IL-2Rα)), and intracellular expression of a master switch transcription factor called forkhead box P3 (Foxp3) [8]. Treg can be distinguished from activated CD25+ conventional T cells (Tcon) by their low or absent surface expression of CD127 (also known as IL-7R) [9, 10].

Induced or adaptive Treg (iTreg) can be generated from naïve T cells *in vitro* via specific stimulation conditions or induced in the peripheral lymphoid organs *in vivo*, with transforming growth factor β (TGFβ) playing a pivotal role [11–13].

As demonstrated a decade ago, the transcription factor Foxp3 is indispensable for both nTreg and iTreg development and suppressive function [8, 14, 15]. Absence of functional Foxp3 protein due to mutations in the *Foxp3 gene* results in the development of severe autoimmune disorders as can be observed in the "scurfy" mouse mutant [14] and patients suffering from immune dysregulation, polyendocrinopathy, enteropathy, and X-linked syndrome (IPEX) [15]. Very recent

data revealed another particularly important intracellular protein for proper Treg development, the Helios transcription factor, a member of the Ikaros family, that has been shown to upregulate expression of Foxp3 protein. Furthermore, constant Helios expression throughout Treg expansion can keep Foxp3 highly expressed, which results in a more stable population [16, 17].

Recent studies suggest that nTreg are more stable compared with iTreg. This is related to their different DNA methylation profiles and to other epigenetic regulations of *Foxp3* [18–21]. In particular, a conserved region upstream of exon 1 within the *Foxp3* locus, the so-called *Treg-specific demethylation region (TSDR)*, is completely demethylated in nTreg but fully methylated in Tcon and iTreg [21–23]. The TSDR is a transcription factor binding site, and its enhancer function stabilizes Foxp3 expression in Treg [22, 24].

Thus, due to unstable Foxp3 expression, *in vitro* differentiated human iTreg might not be stable phenotypically and functionally, implying that *in vivo* transfer of iTreg for therapeutic purposes may give unexpected results and should be considered with caution [25].

Significant progress has been made over the last few years in delineating the mechanism of suppression exerted by nTreg [26]. Numerous putative mechanisms have been proposed in the literature that can be subdivided into two categories: dependent on cell-cell contact and/or mediated by cytokines. *In vitro*, nTreg were shown to inhibit the activation of effector CD4+CD25− T cells predominantly by cell-cell contact dependent mechanisms. nTreg express on their surface important molecules for their suppressive function such as cytotoxic-T-lymphocyte-associated-antigen-4 (CTLA-4), membrane-bound TGFβ latency-associated peptide (LAP), glucocorticoid induced tumour necrosis factor receptor (GITR), CD4-related lymphocyte-activation-gene-3 (LAG-3), galectin-1, and CD39. Moreover, after activation, human nTreg were shown to be able to directly kill CD4+ and CD8+ T cells via the secretion of perforin and granzyme B. The role of regulatory cytokines such as IL-10, TGFβ, and, more recently, IL-35 in nTreg-mediated suppression of immune pathologies has mainly been described in *in vivo* experimental models [25].

Also, IL-2 is crucial for nTreg homeostasis, as these cells are highly dependent on exogenous IL-2 for growth *in vitro* and *in vivo* and for their peripheral maintenance and competitive fitness [27, 28]. Moreover, the high expression of CD25 empowers Treg to "consume" local IL-2 and therefore starve actively dividing effector T cells by depleting the IL-2 they need to survive [26].

In addition to directly affecting effector T cell function, nTreg can modulate the maturation and/or function of dendritic cells required for effector T cell activation [26].

To summarize, Treg are a specialized subpopulation of T cells that suppress the activation, expansion, and function of other T cells, thereby maintaining homeostasis through a fine balance between reactivity to foreign and self-antigens. More importantly, strategies to expand this population by *ex vivo* culture *or in vivo* by specific stimulation will help to devise novel therapeutic strategies to control untoward immune responses.

In the allogeneic stem cell transplantation setting, preclinical models demonstrated (as discussed below) that graft-versus-host disease (GVHD) prevention and transplantation tolerance require tipping the balance in favour of Treg against effector T cells.

3. Treg Suppress GVHD in Murine Models

A number of groups have demonstrated that in aggressive murine GVHD models, where bone marrow and GVHD-inducing Tcon were transplanted across complete major histocompatibility complex (MHC) class I and II barriers, lethal acute GVHD was prevented by donor Treg, if cotransplanted at 1:1 ratio with Tcon [4, 5, 29]. This 1:1 ratio was also then evaluated for the impact of Treg on Tcon-associated beneficial graft-versus-tumor (GVT) effect, while maintaining protection from GVHD [30]. Cotransfer of donor Treg induced a profound suppression of the proliferation of Tcon in secondary lymphoid organs by >90% at day 7 and GVHD target tissues at this ratio. *In vitro* analysis of splenic Tcon on day 5 from different experimental groups for their phenotype, activation markers, and cytokine production has shown that Treg do not inhibit activation and functional maturation of Tcon.

In two independent tumor models, including the leukemia line A20, which infiltrates the bone marrow, and BCL1 lymphoma, which primarily invades liver and spleen, Treg did not interfere with the GVT activity of Tcon but permitted the elimination of tumors from all these compartments, resulting in long-term survival of the hosts.

The presence of Treg therefore does not abrogate the activation of Tcon or their cytolytic response. The investigators hypothesized that the significant decrease in Tcon proliferation by Treg controlled acute GVHD, which generally requires a strong alloreactive response. However, this decrease is not complete, and the remaining Tcon are sufficient to effectively mount a GVT effect [30].

Additional studies [31] demonstrated that regulatory suppression of GVHD by murine Treg preserved thymic and lymphoid architecture of the host and can thereby accelerate posttransplant T cell immune reconstitution.

4. Treg and Induction of Transplantation Tolerance

Treg have been shown to mediate transplantation tolerance in murine models of skin, solid organ transplantation [32–34], and, more recently, antigen-specific tolerance to bone marrow allografts. In a semiallogeneic murine model [35], transplanted bone marrow was protected from rejection by host T cells, through injection of host Treg preactivated *in vitro* against donor antigen-presenting cells (APCs). When a third-party bone marrow was cotransplanted, host preactivated Treg preferentially augmented donor chimerism, as assessed 2 weeks after transplantation (most notably at lower Treg to effector T cell ratios).

In another murine MHC-mismatched bone marrow transplantation (BMT) model [36], cotransplantation of donor Treg into sublethally irradiated recipients resulted in decreased rejection of both lineage-committed and multipotential donor hematopoietic progenitors within the first week of the transplantation. Enhanced engraftment with Treg was further shown with increased long-term donor chimerism in animals that received Treg compared with those that received bone marrow transplant only. Importantly, recipients of Treg in this model demonstrated tolerance to host and donor antigens but mounted responses to third-party antigens such as skin allografts and *in vitro* polyclonal T and B cell stimulations.

Unlike generalized immunosuppressive regimens, Treg are long-lived and functional in a dominant and antigen-specific manner. Thus, therapeutic infusion of Treg has the potential to induce long-term donor-specific tolerance without impeding desired immune responses to pathogens and tumors in transplant patients.

5. Treg and Tumor Progression

There is accumulating evidence that Treg may also modulate host T cell activity against tumor-associated antigens, thereby facilitating tumor escape from immunological control. Treg were shown to be expanded in murine tumor models [37]. Moreover, their deletion reinstated an efficient antitumor immune response leading to complete tumor regression [38–40].

Several reports have demonstrated that Treg are expanded in patients with solid tumors [41–45], B cell chronic lymphocytic leukemia (CLL) [46–48], monoclonal gammopathy of undetermined significance (MGUS), and multiple myeloma (MM) [49, 50], Hodgkin lymphoma [51, 52], or non-Hodgkin lymphomas (NHLs) [53], but little is known about the differentiation, origin, antigen specificity, and mechanisms of expansion of Treg in cancer patients.

In CLL, a direct correlation between higher Treg numbers and more aggressive clinical-biological features of the disease, as well as with disease progression, has been described [54]. Moreover, the percentage of circulating Treg appears able to predict the time to first treatment in low-risk patients, thus emerging as a useful biomarker with prognostic power [55, 56]. Interestingly, when patients with CLL were treated with fludarabine frequencies of Treg were normalized, despite an initial transient increase [57]. In another study [58], treatment with lenalidomide resulted, after a transient increase in the percentage of Treg after 3 cycles, in a significant decrease in Treg after 15 cycles of therapy. These data are consistent with the *in vitro* inhibitory effect of lenalidomide on Treg [59] and suggest a mechanism by which this drug may help overcome an important barrier to tumor-specific immunity in cancer patients.

In a recent study in newly diagnosed MM patients [50], increased frequencies of suppressive Treg correlated with lower overall survival of the patients, suggesting a certain role of Treg in facilitation of disease progression and infectious complications. Specifically, patients with higher percentages of Treg (equal or above median 6.16%) lived shorter when compared with those with lower frequencies of Treg (median overall survival 21 months versus not-reached, $P = 0.013$, at median follow-up of 32 months). Interestingly, Treg frequencies were not influenced after chemotherapy with novel antimyeloma agents (CTD regimen: cyclophosphamide, thalidomide, and dexamethasone or MPT regimen: melphalan, prednisone, and thalidomide) nor autologous stem cell transplantation reduced Treg significantly.

Alternatively, suppressive Treg might actually play a beneficial role in Hodgkin lymphoma (HL), which is characterized by a chronic background inflammatory response, important for the proliferation and survival of Hodgkin-Reed-Sternberg (HRS) cells. The frequency of Foxp3+ T cells was determined in lymphoma-afflicted lymph nodes (LNs). Low frequencies of Foxp3+ cells and high frequencies of cytotoxic T/NK lymphocytes (CTLs) in the reactive background of LNs correlated with poor overall survival [52, 60]. However, costaining for Foxp3 and CD4 or CD25 was not performed, limiting the significance of this finding because Foxp3 expression in humans might not be confined to Treg only [61].

As already outlined, a correlation of increased Treg with greater disease burden and poorer overall survival has been reported. Treg are able to recognize tumor-associated self-antigens and to control natural T cell responses against various cancer antigens, which may explain the failure of many cancer vaccines [62]. In addition, a therapeutic cancer vaccine could induce tumor-specific Treg that blunt the expansion and function of antitumor T cells [62]. In line with these results, in an attempt to improve vaccination efficacy against foreign antigens and to break tolerance against self-tumor antigens, various approaches have been developed to deplete or inhibit the activity of Treg [63, 64].

A recent phase I study [65] demonstrated that vaccination with a myeloma-specific vaccine, generated by the fusion of patient-derived MM cells with autologous dendritic cells (DCs), resulted in the expansion of tumor reactive lymphocytes and disease stabilization in a majority of patients with advanced myeloma. Of note, the majority of patients in this trial exhibited a dampening of immunologic response 6 months after vaccination, which suggests the downmodulation of antitumor immunity. Immunosuppressive features prevalent in MM patients, including the increased presence of Treg, likely interfere with vaccine efficacy. In this regard, the same group examined the effect of lenalidomide *in vitro* on the response to the MM tumor vaccine [7]. Stimulation with DC/MM fusions in the presence of lenalidomide resulted in enhanced expansion of T cell expressing IFN-γ, decreased levels of Treg, and, most significantly, an increased capacity of vaccine stimulated T cells to lyse autologous MM cells. Hence, lenalidomide may create an ideal platform for myeloma-specific immunotherapy that acts synergistically with therapeutic vaccination with DC/MM fusions to induce myeloma-specific immunity.

In conclusion, therapeutic vaccination in conjunction with Treg depletion as a mean to augment vaccine response remains an area of further investigation.

6. Treg for Prevention and Treatment of GVHD: First-In-Man Clinical Trials

Given the striking results in murine GVHD and bone marrow graft rejection models, the ready availability of donor Treg together with the known and transient risk period for adverse consequences from alloreactive T cells and the high degree of morbidity and mortality associated with allogeneic stem cell transplantation (SCT), it is not surprising that GVHD prevention has emerged as the first clinical application for human Treg.

In the "first-in-man" clinical trial [66], *in vitro* expanded Treg (average 64% Foxp3+ after expansion) derived from partially HLA-matched third-party umbilical cord blood units were used in 23 patients undergoing double-cord blood transplantation. Treg were administered on day +1 post-transplant (fresh) (doses ranged from 1×10^5 to 3×10^6/kg) and additionally on day +15 (3×10^6/kg) in 13 patients, using cryopreserved Treg expanded from the same cord blood unit. The rates of GVHD and infectious complications were compared with those from 108 historical controls. Importantly, no increase in opportunistic infections and no Treg-related acute toxicities were observed. The authors did report a reduced incidence of grades II–IV acute GVHD (43% versus 61%, $P = 0.05$) in the trial group, but efficacy for the prevention of GVHD could not be demonstrated definitely in this phase I safety and feasibility trial, as standard pharmacologic prophylaxis was coadministered (cyclosporine A/mycophenolate mofetil or sirolimus/mycophenolate mofetil).

In another small phase I safety and feasibility trial [67], Edinger and Hoffmann transfused freshly isolated donor Treg into 9 patients with high risk of leukemia relapse after allogeneic SCT. In this preemptive donor lymphocyte infusion strategy, up to 5×10^6 cells/kg (>50% Foxp3+) were administered after the cessation of pharmacologic GVHD prophylaxis (within a year after SCT). After an observation period of 8 weeks, additional Tcon were administered at the same dose to promote GVL activity. No Treg-related acute toxicity was observed; neither GVHD nor opportunistic infections or early disease relapses occurred after Treg transfusion, despite the absence of pharmacologic immunosuppression. By design, this trial was not suited to prove the efficacy of Treg for the prevention of GVHD because of the low patient number and the lack of control group.

A more recent study [68] demonstrated for the first time that adoptive immunotherapy with freshly purified Treg counteracts the GVHD potential of a high number of donor Tcon in patients receiving an HLA-haploidentical graft. In this trial, 28 patients undergoing haploidentical SCT received freshly isolated donor Treg (average 69% Foxp3+; $n = 24$ with 2×10^6/kg, $n = 4$ with 4×10^6/kg) on day −4, which was followed by transfer of a highly purified CD34+ stem cell graft together with Tcon. Patients did not receive any prophylactic immunosuppression. For safety reasons of this pilot trial, the first group of 4 patients received only 25% Tcon compared to Treg. Because none of these first 4 patients developed acute GVHD, Tcon were then escalated to 50% of the Treg dose. Rapid and stable engraftment was seen, and, surprisingly, only 2 of 26 evaluable patients developed grades II–IV acute GVHD, while the majority of patients remained free of clinically relevant GVHD. At a median follow-up of 11.2 months, no patient developed chronic GVHD. When compared to a dataset of 152 patients receiving haploidentical SCT without Treg transfer, this approach promoted lymphoid reconstitution and improved immunity to opportunistic pathogens. These first clinical data suggest that donor Treg infusion prevents acute GVHD after allogeneic SCT in humans, because lethal GVHD would have otherwise been expected in all patients after the administration of such high donor Tcon numbers in the absence of pharmacologic immunosuppression. Thus, these results are highly encouraging and now demand confirmation in randomized controlled multicenter trials.

Clinical trials exploring the efficacy of Treg for the treatment of GVHD are much more challenging than prevention trials, and therefore it is not surprising that evidence is still sparse.

High Treg numbers and maximum Treg purity would be required to avoid aggravation of GVHD by contaminating Tcon. Since Treg have to be isolated from the stem cell donor and require two to three weeks *in vitro* expansion, cell production may often be too slow for patients with severe and rapidly progressive disease.

In a recent anecdotal report [69] it was suggested that the transfer of *in vitro* expanded donor Treg (90% Foxp3+; single dose of 1×10^5/kg) contributed to the amelioration of chronic GVHD in a single patient and allowed mycophenolate mofetil (MMF) withdrawal and a reduction in steroids. A second patient treated with higher Treg numbers (total dose of 3×10^6/kg over three infusions) for treatment-resistant acute GVHD had no benefit. The last Treg infusion for that patient contained only 40% Foxp3+ cells, and it is questionable whether such a cell product should have been administered in a life-threatening disease caused by donor Tcon.

Edinger and Hoffmann [67] have also transfused *in vitro* expanded Treg (>95% Foxp3+) in a small number of patients with treatment-resistant acute GVHD and found that these cells survive *in vivo* and may ameliorate severe acute gastrointestinal GVHD. Clinical trials are clearly warranted to test the therapeutic potential of donor Treg infusion for ongoing GVHD.

7. Treg and Infectious Complications

Like all therapies, clinical use of *ex vivo* expanded Treg is associated with potential risks. One issue to be investigated in future studies is whether adoptive immunotherapy with Treg compromises general immunity, blunting responses to infectious agents.

In animal models, it has been demonstrated that Treg not only prevent GVHD but also enhance immune reconstitution after bone marrow plus Tcon transplant, by preventing GVHD-induced damage of the thymus and secondary lymphoid organs, thus allowing protection against lethal cytomegalovirus (CMV) infection [31].

Limited safety data have been obtained from initial clinical trials. As described above, the phase I clinical trial by Brunstein et al. [66] reported that Treg infusion in patients who had undergone double umbilical cord blood transplantation did not increase the incidence of fungal, bacterial, or viral infections compared with the control group.

In the clinical trial by di Ianni et al. [68], Treg infusion prior to haploidentical transplantation did not inhibit immune reconstitution. CD4+ and CD8+ cell counts achieved sustained levels quickly, and high frequencies of pathogen-specific CD4+ and CD8+ T cell precursors were detected as early as 2 months after transplantation. Strikingly, the prevention of CMV disease was markedly improved, with no CMV-related deaths, an improvement over 40% of nonleukemic deaths caused by CMV that had previously been reported by this group. Furthermore, seven patients were vaccinated against influenza 3–14 months post-transplant, and five acquired protective antibody titers. It was hypothesized that, as in animal models, in a postconditioning inflammatory environment, Treg are activated by recipient APCs, block alloreactive T cells in an antigen-specific fashion, and allow the expansion of nonalloreactive T cells, which ensures long-term immunity.

8. Drugs That Potentiate Treg

A significant challenge for the use of nTreg in the clinic is the difficulty in isolating sufficient numbers of Treg for clinical application, since circulating numbers of Treg in the peripheral blood are limited (5%–10% of CD4+ T cells) [70, 71]. Current research focuses on the development of large-scale expansion protocols for Treg with higher cell yields [72–74].

The use of granulocyte-colony-stimulating-factor (G-CSF) may also be considered to increase Treg mobilization into the peripheral blood for potential extraction during leukapheresis [75]. Under steady state, human bone marrow contains a large fraction of CD4+CD25+Foxp3+ T cells with regulatory function [76]. Recent data suggests that donor Treg after G-CSF stimulated stem cell mobilization retain their potent suppressive and stable phenotype, and, thus, the adoptive transfer of donor Treg after G-CSF stimulation appears to be feasible and safe [75]. The isolation of donor Treg from the stem cell graft will simplify their global clinical application.

It has been recently shown that expansion of adoptively transferred Treg *in vivo* is critical for their GVHD suppressive activity [77]. This would alleviate the need for cumbersome *ex-vivo* manipulations, thus rendering the therapy more clinically applicable.

Recent data suggests that T cell depletion protocols used to induce tolerance in clinical transplantation, for example, by using total lymphoid irradiation (TLI) and antithymocyte globulin (discussed below) as conditioning regimen [78, 79], alter the balance of residual host T cells subsets to enrich host innate regulatory natural killer T cells (NKT cells) that cooperate with donor Treg and induce IL-4 dependent *in vivo* Treg expansion.

Rapamycin, a small molecule inhibitor of the Akt/mTOR pathway used for the prophylaxis and treatment of GVHD, has been shown *in vitro* to selectively expand or preferentially preserve Treg over Tcon [80], thus attenuating GVHD also by shifting the balance of aggressive to protective type alloimmunity. Data from murine *in vivo* SCT models support the observation of Treg-supportive effects of rapamycin [81], including increased generation of thymic Treg [82] and infiltration into GVHD target organs [83].

As stressed before, *in vivo* homeostasis and expansion of Treg are highly dependent on IL-2. Thus, IL-2 is also investigated as a putative agent for selective expansion of Treg [84]. Recently, a phase I escalation study of IL-2 administration to 29 patients with active steroid-refractory chronic GVHD was performed [85]. Administration of daily subcutaneous low-dose IL-2 rapidly induced preferential and sustained Treg expansion, reversed advanced fibrotic and sclerotic manifestations of chronic GVHD in a substantial proportion of patients, and permitted a substantial reduction in glucocorticoid dose.

Also, the combination of rapamycin plus IL-2 appears to be more effective than rapamycin alone in the prevention or suppression of GVHD by *in vivo* expansion of nTreg, enhanced conversion of iTreg by IL-2, and inhibition of effector T cells [86].

It has also been shown that rabbit-derived anti-T lymphocyte immunoglobulin (ATG) is a potent inducer of iTreg. Lopez et al. [87] and Feng et al. [88] showed that thymocyte-induced ATG (thymoglobulin; Genzyme) converts human effector T cells into iTreg that subsequently suppress the proliferation of autologous responder cells to external stimuli. Ruzek et al. [89] further demonstrated that anti-mouse thymocyte globulin induces Treg in mice, which express several Treg markers (but not Foxp3) and inhibit GVHD. Similarly, we have recently shown that Fresenius anti-T lymphocyte globulin (ATG-F) can generate Treg *in vivo* that suppress mixed lymphocyte culture in patients undergoing allogeneic SCT [90]. These results may pave the way for novel therapeutic potentials; in addition, ATG may be synergized with another Treg inducer to obtain stronger long-term tolerance against the aggressive activity of T cells in allotransplantation and GVHD.

The majority of adoptively transferred Treg maintain their suppressive activity, but a minority of cells lose Foxp3 expression and can differentiate into Tcon [91]. It is conceivable that understanding why cells lose their "Treg-ness" and preventing this dedifferentiation *in vivo* will improve both the safety and efficacy of Treg therapy. Since Foxp3 is the master regulator of Treg function, alterations in Foxp3 expression or activity are likely involved in converting Treg to Tcon. Foxp3 expression is modulated by DNA methylation; therefore, administration of selective demethylating agents may enhance Treg function and fidelity *in vivo* [92].

In preclinical studies, Azacitidine (AzaC) treatment of allotransplanted mice mitigates deleterious GVHD while preserving beneficial GVT effect, by peripheral conversion of alloreactive effector T cells into Foxp3+ Treg and epigenetic modulation of genes downstream of Foxp3 required for the suppressor function of Treg [93]. Thus, the administration

of AzaC after transplantation in humans may provide a simple and relatively nontoxic approach to limit GVHD while preserving the GVT effect and engraftment potential of donor T cells.

It is likely that different immunosuppressive and immunomodulatory agents will be more or less permissive for Treg development and function. For example, cyclosporine A (CsA) was shown to inhibit Treg function. Reduced suppressor function of CsA-exposed Treg was IL-2 dependent and correlated with a decreased number of Foxp3+ T cells both *in vitro* and *in vivo* [81]. On the other hand, these inhibitory effects by CsA on Treg function may have important implications for boosting immune responses to vaccination protocols.

Interestingly, the role of lenalidomide in the modulation of Treg remains unresolved. As discussed above, lenalidomide was shown to inhibit the proliferation and function of Treg *in vitro* [59]. Idler et al. [94] studied the changes in Treg population in patients with CLL treated with lenalidomide over a prolonged period of time and showed that lenalidomide decreased the percentage as well as the absolute number of Treg. However, a recent report by Lee et al. [58], as discussed above, showed that lenalidomide has a biphasic effect on Treg in CLL. Lenalidomide is highly effective in treating newly diagnosed and relapsed/refractory MM. Clinical data indicate that lenalidomide in combination with dexamethasone is highly effective in relapsed/refractory MM following allogeneic SCT, which is associated with an increase in Treg number [95]. However, lenalidomide maintenance after allogeneic SCT as part of first-line treatment in MM was not found feasible by the same group, mainly because of the rapid induction of acute GVHD [96]. Notably, Treg were increased at the ninth cycle, without correlation with clinical parameters. It was suggested that the Treg elevation occurred as a reaction against the immune-stimulating effects of lenalidomide.

Raja et al. [97] have shown that the combination of lenalidomide and dexamethasone increases Treg in patients with previously untreated MM. The data suggest that, in spite of a positive antitumor immune response in patients treated with lenalidomide and dexamethasone, Treg are increased.

The conflicting but compelling data from *in vitro* studies that lenalidomide inhibits Treg suggest that the *in vivo* effects of lenalidomide might be a result of the microenvironment on the immune cells. Thus, the data may be interpreted such that, once lenalidomide induces effector immune responses *in vivo*, there is a negative feedback induced by transformation of the T helper cells into Treg to maintain the immune homeostasis [98].

9. Conclusions

Building on extensive research in Treg biology and preclinical testing of Treg therapeutic efficacy over the past decade, we are now at the point of evaluating the safety and efficacy of Treg therapy in humans. SCT seems a clinical setting suited to prove the efficacy of adoptive Treg therapies and may pave the way for further applications.

As discussed in our review, initial studies in clinical allogeneic SCT are very encouraging and have demonstrated that Treg-based clinical studies are feasible and do not result in toxicity. Human Treg infusion appears to suppress GVHD risk of Tcon while promoting enhanced immune reconstitution and decreasing the incidence of infectious complications.

In patients with malignant diseases, a direct correlation between higher Treg numbers and more aggressive clinical-biological features, as well as with disease progression, has been described in several types of tumors. Elimination or inhibition of Treg might be particularly useful in the context of therapeutic vaccination against tumor-associated antigens, as stressed above.

Finally, further improvements in Treg *ex vivo* or *in vivo* expansion technologies and the use of costimulatory novel compounds to get higher cell yields, as detailed above, will "unlock the power" of Treg and facilitate the broader exploration of Treg therapies, for example, for the treatment of active GVHD or the prevention of graft rejection after solid organ transplantation.

Disclosure

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Research Article

Interferon-γ Triggers Hepatic Stellate Cell-Mediated Immune Regulation through MEK/ERK Signaling Pathway

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Hepatic stellate cells (HSCs) interact with immune cells to actively participate in regulating immune response in the liver which is mediated by the effector molecules, including B7-H1. We demonstrated here that expression of B7-H1 on HSCs was markedly enhanced by interferon-(IFN-) γ stimulation. IFN-γ stimulated HSCs inhibited T-cell proliferation via induction of T-cell apoptosis (22.1% ± 1.6%). This immunosuppressive effect was inhibited by preincubation with an anti-B7-H1 antibody, or inhibitor of the MEK/ERK pathway inhibited IFN-γ mediated expression of B7-H1. Thus, regulation of B7-H1 expression on HSCs by IFN-γ represents an important mechanism that regulates immune responses in the liver favoring tolerogenicity rather than immunogenicity. Involvement of MEK/ERK pathway provides a novel target for therapeutic approaches.

1. Introduction

Hepatic tolerance was initially suggested by spontaneous acceptance of liver transplants a cross major MHC disparity without the requirement for immunosuppressive therapy in many species, as well as by induction of tolerance to antigens delivered by way of portal vein or oral route [1–3]. In addition, the spontaneous acceptance of liver allograft can suppress the rejection of subsequent other organ grafts such as heart or kidney from the same donor [4, 5]. Interestingly, although liver allografts are accepted, hepatocytes transplants are promptly rejected, suggesting a crucial role of liver nonparenchymal cells in protecting parenchymal cells from immune attacks. We have demonstrated that hepatic stellate cells (HSCs), abundant liver tissue stromal cells known for participating in liver fibrogenesis, can protect the cotransplanted islets allografts from rejection [6]. However, quiescent and B7-H1 gene knockout (KO) HSCs lost the protective effect on co-transplanted islet allografts, indicating a critical role of B7-H1 in immune regulatory activity of HSCs, which may represent one of the mechanisms that regulate immune responses in the liver favoring tolerance rather immunogenicity [7]. However, little is known about the regulatory mechanisms of B7-H1 expression in HSCs.

Interferon-(IFN-) γ is a proinflammatory cytokine that is, mainly produced by T cells and natural killer (NK) cells and has been shown to participate in regulation of antiviral and antitumor immunity [8]. Thus, in cancer microenvironment, a cellular process involving the release of inflammatory mediators including IFN-γ [9] and subsequently induction of various cellular proteins such as B7-H1 in cancer cells was reported. Overwhelming data indicate that cancer-associated B7-H1 in murine cancer model facilitated apoptosis of cancer-reactive T cells [10]. B7-H1 expression was enhanced on malignant plasma cells from multiple myeloma patients by IFN-γ and Toll-like receptor stimulation via MEK/ERK-dependent and MyD88/TRAF6-dependent pathways and can inhibit T-cell responses [11]. JAK/STAT pathway was also involved in induction of B7-H1 expression in response to IFN-γ in a human lung cancer cell line [12]. However, it remains unclear whether molecular mechanisms are involved in IFN-γ-induced B7-H1 expression in HSCs.

In this study, we demonstrated that HSCs expressed B7-H1 in response to IFN-γ stimulation in a dose- and time-dependent manner at transcriptional level, and the MEK/ERK pathway is responsible for the IFN-γ-induced expression of B7-H1 in HSCs. And stimulation of HSCs with IFN-γ reduced T-cell proliferation and promoted T-cell apoptosis.

2. Materials and Methods

2.1. Mice and Reagents. C57BL/6 (B6; H-2^b) and BALB/c (H-2^d) mice were purchased from Shanghai Laboratory Animal Center of Chinese Academy of Sciences (Shanghai, China). Stat1 KO (129S6/SvEvTac-Stat^{tm1Rds}) mice were obtained from Taconic (Germantown, NY, USA). IFN-γ R1 KO (B6.129S7-Ifngr1^{tm1Agt}/J) mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). B7-H1 KO mice were kindly provided by Dr. Lieping Chen (Johns Hopkins University Medical School, Baltimore, MD, USA). Animals were fed with standard chow *ad libitum* and were used at 7–9 weeks of age. The animal experimental protocols were in accordance with Chinese Administration Rule of Laboratory Animal. Recombinant IFN-γ, cycloheximide (CHX), actinomycin D (ActD), phorbol myristate acetate (PMA), and U0126 were obtained from Sigma-Aldrich (St. Louis, MO, USA). SP600125 and LY294002 were purchased from Cell Signaling Technology (Danvers, MA, USA).

2.2. Preparation of HSCs. HSCs were isolated from the mice liver nonparenchymal cells as previously described [7]. The liver was perfused through the portal vein with collagenase IV (Life Technologies, Grand Island, NY, USA). The smashed cells were filtered through a nylon mesh. Subsequently, the HSCs were purified by Percoll density gradient centrifugation (Sigma-Aldrich) and cultured in complete medium supplemented with 20% fetal bovine serum for 7 to 14 days unless otherwise indicated. The purity of HSCs ranged from 90% to 95% measured by desmin immunostaining and the typical light microscopic appearance of the lipid droplets.

2.3. Flow Cytometric Analysis. Expression of cell surface molecules was detected on FACScan (BD Biosciences, San Jose, CA, USA) and analyzed using CellQuest software (BD Biosciences). Cells were stained with the following monoclonal antibodies (mAbs): FITC-B7-H1 (eBioscience, San Diego, CA, USA) and FITC-CD3 (BD Biosciences). Isotype-matched irrelevant mAbs were used as negative controls. Apoptosis was assessed by PE-Annexin V staining (BD Biosciences).

2.4. Reverse Transcription Polymerase Chain Reaction (RT-PCR). Total RNA was extracted from HSCs with TRIzol reagent (Life Technologies) according to the manufacturer's instructions. RNA was then reverse-transcribed into cDNA, using random primers and SuperScript II reverse transcriptase (Life Technologies). For semiquantitative RT-PCR, the PCR amplification was performed using Taq DNA polymerase (Life Technologies). PCR products were analyzed on agarose gels stained with ethidium bromide and photographed. B7-H1 cDNA was amplified by RT-PCR using the forward primer 5′-CTGTAGAACGGGAGCTGGAC-3′ and the reverse primer 5′-TGGACTTTCAGCGTGATTCG-3′.

2.5. Western Blot Analyses. HSCs were suspended in lysis buffer (20 mM Tris-HCl, pH 7.8, 50 mM NaCl, 5 mM EGTA, and 1% v/v Triton X-100) containing freshly added protease and phosphatase inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 2 μM aprotinin, 1 mM sodium orthovanadate, and 20 mM glycerophosphate). Lysates were clarified by centrifugation at 4°C and protein concentration determined by Bio-Rad protein assay (Hercules, CA, USA). Equal quantities of proteins were separated by SDS-PAGE, transferred to PVDF membrane, and sequentially incubated with primary antibodies and HRP-conjugated secondary antibodies, followed by enhanced chemiluminescence detection. Anti-phospho-ERK1/2 (Thr202/Tyr204) and anti-ERK2 antibodies were purchased from Cell Signaling Technology.

2.6. Mixed Lymphocyte Reaction (MLR). Nylon wool-eluted spleen T cells (2×10^5) from BALB/c mice were used as responders. γ-irradiated (20 Gy) DCs derived from B6 bone marrow were used as stimulators. Cultures were maintained in complete medium for 3 days at 37°C in 5% humidified CO_2. [^{3}H]-thymidine (0.5 μCi/well) was added for the final 18 hours of culture. Cells were harvested onto glass fiber disks using an automated system, and incorporation of [^{3}H]-thymidine into DNA was assessed by Wallac 1450 liquid scintillation counter (PerkinElmer, Boston, MA, USA). Results were expressed as mean counts per minute (cpm) ± 1 SD. To examine the effect of HSCs on T-cell proliferation, γ-irradiated (50 Gy) HSCs were added into cultures at the beginning of the culture.

2.7. Statistical Analysis. Statistical analysis was performed with Stata 8.0 software (Stata, College Station, TX, USA). The data was given as mean ± 1 SD. Statistical comparisons between groups were performed using a one-way ANOVA followed by a Scheff's test, as appropriate. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. IFN-γ Induces B7-H1 Expression in HSCs. Quiescent HSCs isolated from B6 mice expressed very low B7-H1. However, expression of B7-H1 was markedly upregulated following exposure to IFN-γ. To determine the dose- and time-dependent effects of IFN-γ-induced B7-H1 expression, we treated HSCs with various concentrations of IFN-γ (0.1–200 U/mL) for 24 hours or at the same concentration but various duration. The result showed that increase in expression of B7-H1 was correlated with the IFN-γ concentration (Figure 1(a)). As shown in Figures 1(b) and 1(c), B7-H1 expression initiated to be increased following exposure to IFN-γ for as short as 0.5 hours and reached at the maximum after stimulation for 24–48 hours.

IFN-γ receptor (R) contains IFN-γR1 binding chain and internal IFN-γR2 transducing chain [13]. Expression of B7-H1 on HSCs that were isolated from IFN-γR1 KO mice showed no response to IFN-γ (Figure 1(d)), indicating that B7-H1 is a product of the IFN-γ signaling. This was supported by the

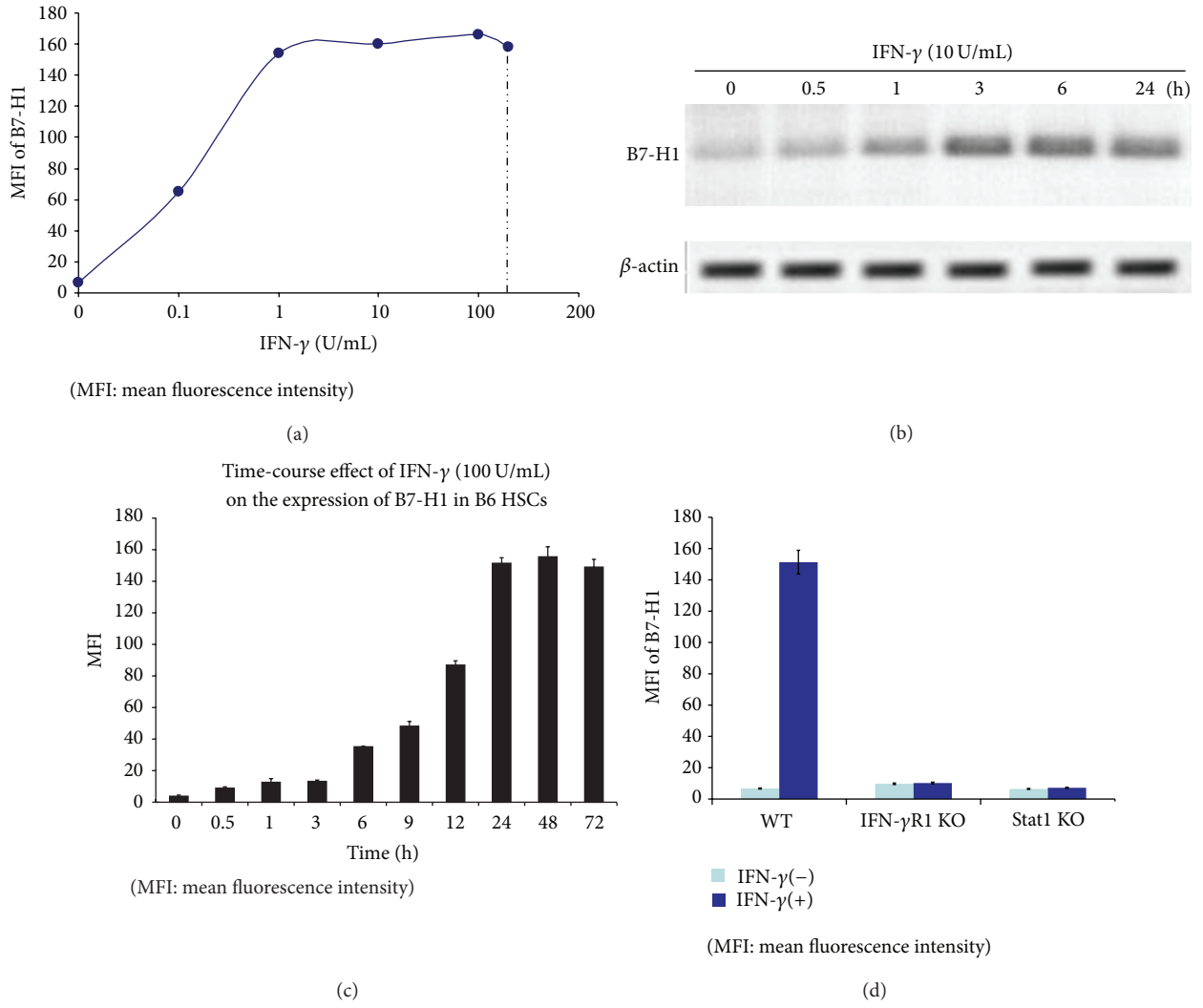

Figure 1: Expression of B7-H1 on HSCs in response to INF-γ stimulation. (a) HSCs isolated from B6 mice were exposed to graded concentrations of IFN-γ (0.1–200 U/mL) for 24 hours in vitro and stained with anti-B7-H1 mAb and analyzed by flow cytometry. (b) HSCs were treated with IFN-γ (10 U/mL) for varying times (0.5–24 hours) and analyzed by RT-PCR. (c) HSCs were incubated with IFN-γ (100 U/mL) for the indicated times, and the expression patterns were analyzed by flow cytometry. (d) HSCs isolated from wild type (WT) or IFN-γR1 KO mice or Stat1 KO mice (all on B6 background) were exposed to IFN-γ (100 U/mL) for 48 hours. Cells were stained using anti-B7-H1 mAb and analyzed by flow cytometry. The data are representative of two separate experiments.

fact that B7-H1 expression in response to IFN-γ stimulation was almost entirely impeded on HSCs isolated from Stat1 KO mice (Figure 1(d)), since Stat1 is a key transcription mediator for IFN-γ signaling [8].

3.2. Involvement of MEK/ERK Pathway in IFN-γ Induced B7-H1 Expression. To understand which signaling pathway is involved in IFN-γ-induced B7-H1 expression in HSCs, we first assessed if RNA synthesis was required in this process by blocking RNA synthesis with ActD. Addition of ActD completely blocked B7-H1 mRNA synthesis in HSCs (Figure 2(a)). However, blocking protein synthesis with CHX had no effect on B7-H1 mRNA synthesis, indicating that de novo protein synthesis is not required for B7-H1 transcription.

We then tried to determine the involved signaling pathways using several transduction pathway inhibitors (Figure 2(b)). Blocking of PI3 K with LY294002 did not reduce B7-H1 expression. However, blocking MEK1/2 with U0126 dramatically downregulated IFN-γ-induced B7-H1 expression. A slight reduction was also observed after blocking JNK with SP600125.

To confirm that IFN-γ induced B7-H1 expression through MEK/ERK pathway in HSCs, we analyzed the phosphorylation of ERK1/2, showing that IFN-γ induced phosphorylation of ERK1/2, which was almost completely blocked by U0126 (Figure 2(c)). Phosphorylation of ERK1/2 was also identified in HSCs without exposure to IFN-γ (Figure 2(c)). These HSCs were activated with culture in vitro for 7 to 14 days. They might produce other factors that participated in

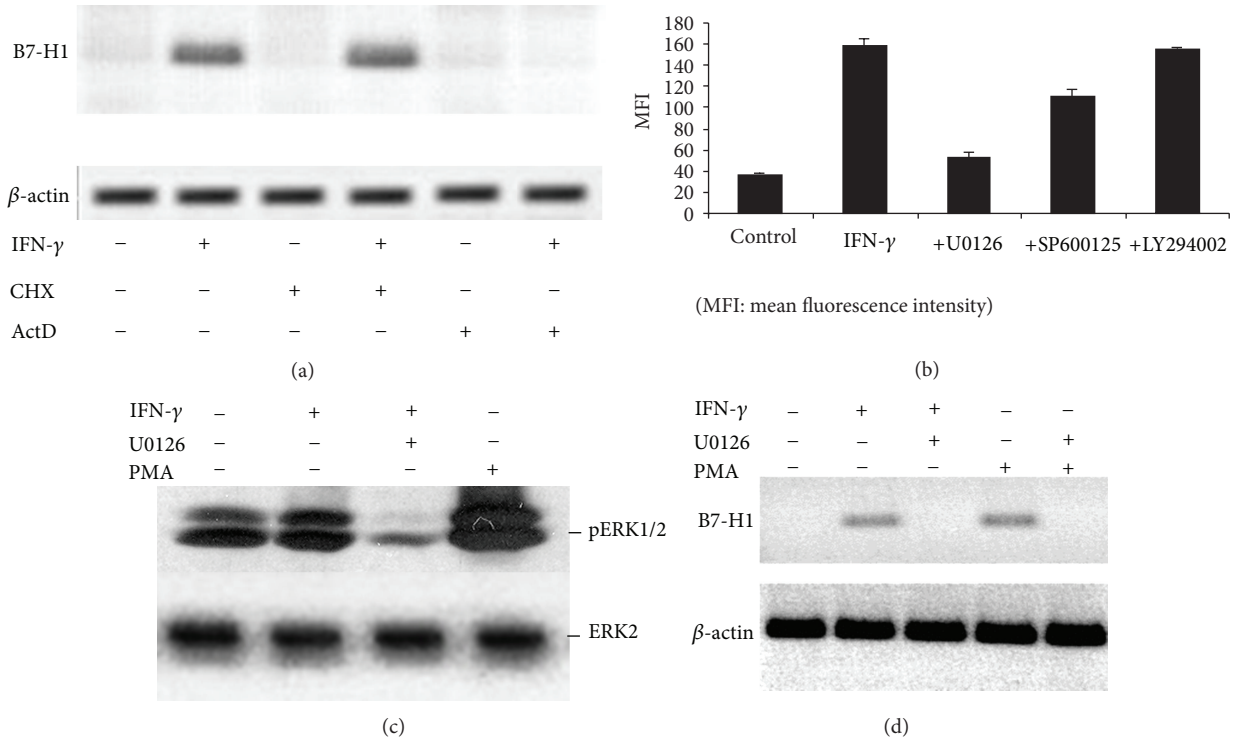

Figure 2: MEK/ERK-dependent B7-H1 expression in HSCs. (a) B7-H1 mRNA levels in HSCs measured by RT-PCR after exposure to IFN-γ (10 U/mL) for 6 hours with or without 10 μg/mL CHX or 10 μg/mL ActD for 90 minutes. (b) Flow cytometric analysis of B7-H1 expression in control HSCs (without IFN-γ stimulation) and after 24 hours incubation with IFN-γ with or without 1 hour pretreatment with signal transduction inhibitors, that is, 100 μM U0126 (MEK1/2), 100 μM SP600125 (JNK), and 100 μM LY294002 (PI3 K). (c) Western blot analysis of ERK1/2 phosphorylation in HSCs after incubation with IFN-γ (10 U/mL) or 1 ng/mL PMA, with or without 1 hour preincubation with 100 μM U0126. (d) RT-PCR analysis of B7-H1 mRNA levels in HSCs exposed for 24 hours to IFN-γ (10 U/mL) or 1 ng/mL PMA with or without 1 hour pre-incubation with 100 μM U0126.

phosphorylation of ERK1/2. Incubation of HSCs in PMA, a known activator of the MEK/ERK pathway, induced B7-H1 expression and ERK1/2 phosphorylation which were also blocked by U0126 (Figures 2(c) and 2(d)).

3.3. T-Cell Inhibition by IFN-γ Stimulated HSCs. To test the ability of HSCs to suppress T-cell responses, HSCs were added into a MLR culture in which BALB/c splenic T cells were stimulated by B6 DCs. HSCs from either B6 or BALB/c mouse livers markedly suppressed thymidine uptake by T cells in a dose dependent fashion (Figure 3(a)), suggesting that the inhibitory effect of HSCs on T-cell response is not MHC specific. The inhibitory effect of HSCs on T-cell response was partially reversed when HSCs were pre-incubated with anti-B7-H1 antibody or with U0126 before being added to the culture (Figure 3(a)). HSCs from IFN-γR1 KO mice and B7-H1 KO mice also lost the capacity to inhibit T-cell response (Figure 3(a)).

We speculated that HSCs induced T-cell hyporesponsiveness may result from apoptotic death of activated T cells. To address this, BALB/c splenic T cells were cultured for 3 days with irradiated allogeneic (B6) DCs in the presence or absence of activated B6 HSCs. Flow cytometric analysis of the cells that were double stained with anti-CD3 mAb and anti-Annexin V mAb confirmed that the activated HSCs enhanced incidence of T-cell apoptosis (Figure 3(b)). Apoptotic T cells markedly decreased when U0126 or anti-B7-H1 antibody was added during pre-incubation (Figure 3(b)). Thus, stimulation of HSCs with IFN-γ reduced T-cell proliferation and promoted T-cell apoptosis, via a MEK/ERK/B7-H1 pathway.

4. Discussion

IFN-γ is an important proinflammatory cytokine mainly produced by T helper 1 cells and NK cells, mediating both innate and adaptive immune responses [14]. Recent accumulating evidence suggests that IFN-γ is also critical for tolerance induction in transplantation [13, 15, 16]. Liver allografts transplanted into wild type (WT) mice achieve long-term survival, whereas no WT allografts survived beyond 14 days in IFN-γ KO recipients or IFN-γR KO allografts in WT recipients [13]. The underlying mechanisms are not completely understood. IFN-γ is an important modulator of cytotoxic T cells, macrophages, and NK cells, as well as the expression of MHC molecules. Many genes, including those for various chemokines, adhesion molecules, and costimulatory molecules, are transcriptionally activated in IFN-γ treated cells. Among these molecules, B7-H1 is broadly expressed on most lymphocyte lineage cells, normal tissue, and a variety of tumor cells by stimulated cytokines [17].

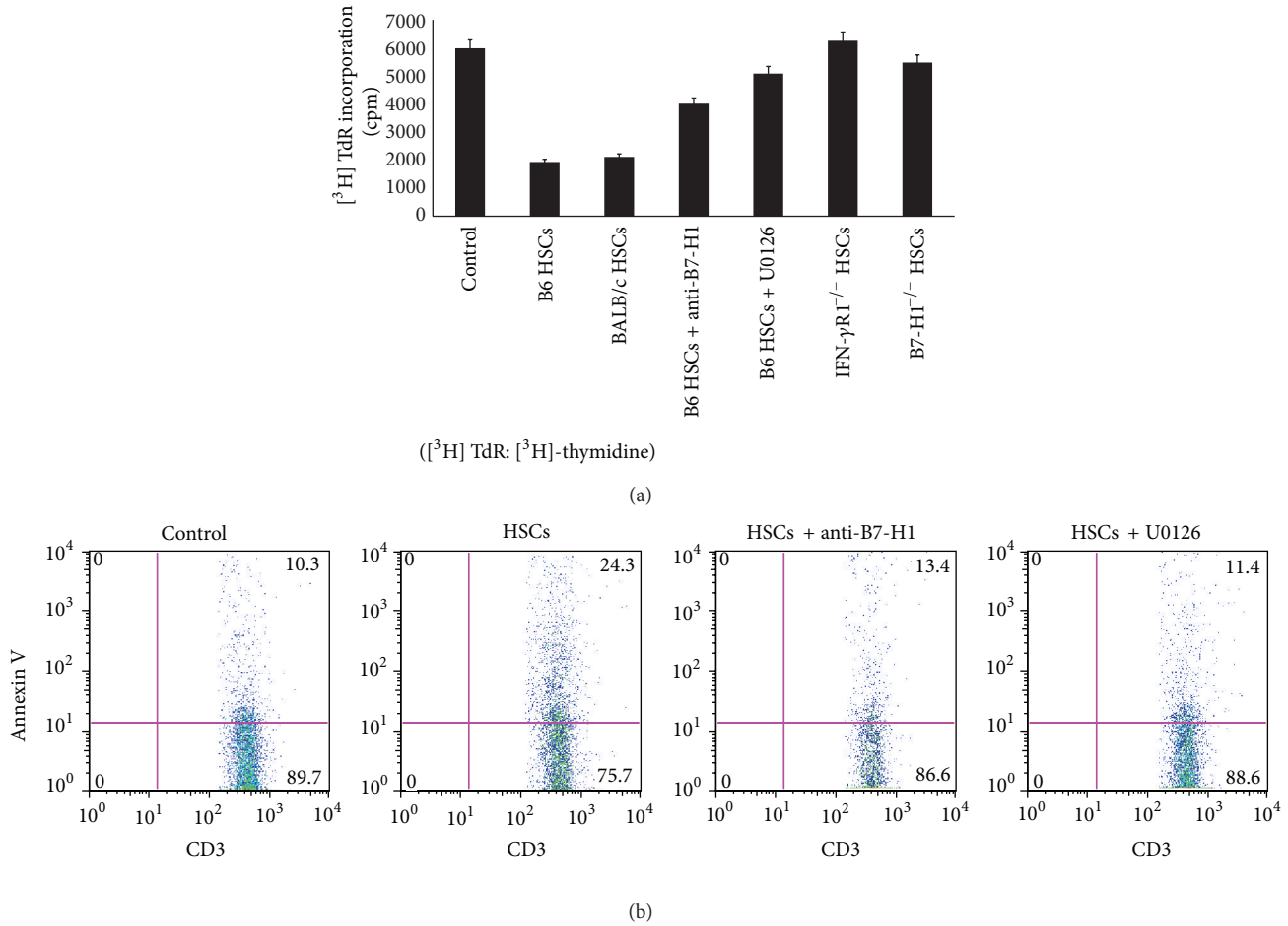

Figure 3: T-cell inhibition by HSCs. (a) B6, BALB/c, IFN-γR1 KO, or B7-H1 KO HSCs cultured in uncoated plastics for 7 days were γ-irradiated (50 Gy) and added at the beginning of an MLR culture in which splenic T cells (2×10^5) from BALB/c mice, and irradiated (20 Gy) B6 DCs were cultured at a final T-cell/DC/HSC ratio of 20 : 2 : 1 for 3 days. In some groups, irradiated HSCs pre-incubated with 100 μM U0126 or anti-B7-H1. Controls were without HSCs. The data are representative of three separate experiments. (b) Cells following cultures for 2 or 3 days were double stained with FITC-anti-CD3 and PE-anti-Annexin V for flow analysis. The data demonstrated Annexin V expression in CD3+ populations. In some groups, irradiated HSCs pre-incubated with 100 μM U0126 or anti-B7-H1. Controls were without HSCs. The data are representative of three separate experiments.

The role of B7-H1 as a coinhibitory ligand is consistent with its ability to interact with its receptor PD-1, which also binds to B7-DC on DCs [18]. There were conflicting data that have been reported on the role of B7-H1. Many studies in mouse islet, corneal, skin, and cardiac transplant models have demonstrated that the PD-1/B7-H1 pathway is required for the induction and maintenance of established graft tolerance [19]. However, in some in vivo settings, B7-H1 can costimulate T-cell responses [20]. We found that HSCs deficient in either B7-H1 or IFN-γR1 largely lost the capacity to inhibit T-cell response, indicating that the immune regulation of HSCs requires IFN-γ stimulation, and that its downstream product, B7-H1, is a crucial effector molecule.

The present study revealed that exposure of HSCs to IFN-γ resulted in a dramatic increase in B7-H1 expression in dose- and time-dependent manners. We tried to inhibit several components of the pathways known to mediate IFN-γ signaling. Inhibition of MEK1/2 almost completely blocked IFN-γ-induced B7-H1 expression by HSCs. A partial inhibition of B7-H1 expression was observed when JNK was blocked with SP600125, but no effect could be observed when the PI3k pathway was blocked. U0126 was also able to block the inhibitory effect of IFN-γ-stimulated HSCs on T cells. Thus, the MEK/ERK pathway seems to be a major contributor responsible for IFN-γ induced expression of B7-H1 in HSCs. Several studies have previously established that, in addition to the classical JAK/Stat pathway, IFN-γ also activates MAPK [8]. p38 MAPK can activate Stat1 through phosphorylation of serine 727 [21]. ERK activates C/EBP dependent gene transcription through IFN-γ [22]. Lee et al. [23] demonstrated that MAPK and PI3 K pathways were involved in induction of B7-H1 expression in response to IFN-γ in dermal fibroblasts. Liu et al. [11] reported that MEK/ERK and MyD88/TRAF6 pathways were important for inducing B7-H1 expression in multiple myeloma plasma cells by IFN-γ and Toll-like receptor stimulation. Our data did not support a role for PI3 K pathway in induction of B7-H1 expression in HSCs, but we found the LPS did not induce B7-H1 expression in

HSCs (data not shown). It is likely that IFN-γ signaling is mediated through different pathways depending on the cell types involved.

In summary, we have shown that B7-H1 is expressed on HSCs, is involved in inhibition of T-cell responses by these HSCs, and is upregulated by IFN-γ through the MEK/ERK pathway. These findings may provide new insights into better understanding of the mechanisms regarding how HSCs participate in hepatic tolerogenicity and help to develop novel strategies for induction of transplantation tolerance.

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Research Article

Depletion of Regulatory T Cells in a Mouse Experimental Glioma Model through Anti-CD25 Treatment Results in the Infiltration of Non-Immunosuppressive Myeloid Cells in the Brain

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The recruitment and activation of regulatory T cells (Tregs) in the micro-environment of malignant brain tumors has detrimental effects on antitumoral immune responses. Hence, local elimination of Tregs within the tumor micro-environment represents a highly valuable tool from both a fundamental and clinical perspective. In the syngeneic experimental GL261 murine glioma model, Tregs were prophylactically eliminated through treatment with PC61, an anti-CD25 mAb. This resulted in specific elimination of CD4+CD25hiFoxp3+ Treg within brain-infiltrating lymphocytes and complete protection against subsequent orthotopic GL261 tumor challenge. Interestingly, PC61-treated mice also showed a pronounced infiltration of CD11b+ myeloid cells in the brain. Phenotypically, these cells could not be considered as Gr-1+ myeloid-derived suppressor cells (MDSC) but were identified as F4/80+ macrophages and granulocytes.

1. Introduction

Escape from immunosurveillance has now been widely accepted as a hallmark of cancer. In early stages of malignancy, antitumor responses are mounted and are in many cases successful to eradicate the malignant cells. However, as malignancy progresses, few tumor cells escape the immunosurveillance, finally leading to clinically detectable tumors that are often very hard to cure [1]. This concept is also applicable in patients diagnosed with high-grade glioma. Malignant glioma cells have acquired a broad arsenal of strategies by which antitumor immunity can be countered and even reversed. Without any doubt, recruitment, expansion, and activation of Treg towards the tumor site is one of the dominant immunosuppressive mechanisms handled by glioma cells. Under physiological conditions, Tregs represent a final but crucial line of defence against the onset of autoimmunity caused by autoreactive T cells that have escaped the mechanisms of central tolerance in the thymus. The presence and detrimental contribution of Treg to antitumor immunity in the context of malignant glioma has been extensively documented both in clinical settings and in experimental models [2–7]. Furthermore, research in murine glioma models (such as the syngeneic GL261 model in C57BL/6 mice) recently focused on the development of strategies that allow (specific) elimination or silencing of tumor-induced Treg. In this perspective, low-dose cyclophosphamide treatment, CTLA-4 blockade, and STAT3 inhibition are promising [8–11]. We and others previously reported that a widely used rat monoclonal antibody (mAb), clone PC61, directed against the alpha chain of the mouse IL-2 receptor (CD25), which is highly expressed on natural Foxp3+ Treg,

efficiently dampens their activity and restores the endogenous clearance of GL261 tumor cells by the immune system of the host mice [12, 13]. In the study presented here, local immunomonitoring revealed that prophylactic anti-CD25 treatment resulted in a pronounced infiltration of CD11b+ myeloid cells in the brain of glioma-bearing mice. Flow cytometric phenotyping revealed that these myeloid cells could not be classified as Gr-1+ MDSC but rather as F4/80+ macrophages and granulocytes. To our view, this is the first report describing that the depletion of Treg in an experimental (glioma) tumor model through PC61 treatment results in local infiltration of nonimmunosuppressive myeloid cells.

2. Materials and Methods

2.1. GL261 Brain Tumor Model. C57BL/6 mice were orthotopically challenged in the striatum with syngeneic GL261 cells or firefly luciferase- (Fluc-) transduced GL261 cells through stereotaxic surgery as previously described. Bioluminescence imaging was performed with an IVIS 100 system (Xenogen, Alameda, CA, USA) in the Small Animal Imaging Center of the KULeuven as described in [14]. All animal experiments were performed with permission of the Ethical Committee of the KULeuven on laboratory animal welfare.

2.2. Treatment with Anti-CD25 Monoclonal Antibody . Three weeks prior to tumor challenge, 250 microgram of the anti-CD25 mAb clone PC61 (Bioceros B.V., Utrecht, The Netherlands) was administered intraperitoneally in a volume of 200 microliter sterile saline. Polyclonal rat IgG (Rockland, Gilbertsville, USA) was used as control.

2.3. Immunomonitoring. Brain-infiltrating immune cells were isolated as previously described [12]. Sorting of CD11b+ and CD11b− cells was performed using paramagnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). Multicolor flow cytometry was performed using anti-mouse CD8a-FITC (53-6.7), CD4-PerCP-Cy5.5 (GK1.5), CD25-PE (7D4), Foxp3-APC (cFJK-16s), CD45− PerCP-Cy5.5 (30F11), CD11b-PE (M1/70), Ly6G/Gr-1-FITC (RB6-8C5), and F4/80-APC (BM8) mAbs (all from eBioscience, San Diego, CA, USA). For intracellular Foxp3 staining, the manufacturer's protocol was followed. For acquisition, a FacsCanto II flowcytometer (BD Biosciences, San Jose, CA, USA) was used, and data analysis was performed with FacsDiva software (BD).

Fluorescence minus one gating strategy was applied. Absolute cell numbers of each specific cell population were calculated by multiplying the relative cell numbers (as percent of total) with the total number of cells in a livegate. Cytospins were prepared on 2×10^5 brain-infiltrating cells per sample by resuspending pelleted cells in 2 mL of sterile saline. The microscope slides were loaded with 500 microliter cell suspension, spun, and May-Grünwald Giemase stained. Analysis was performed with StereoInvestigator software (Microbrightfield, Magdeburg, Germany). For each sample, cell counts were performed in triplicates on a field of view (FOV) under 40x magnification.

2.4. Statistical Analysis. All data are represented as mean ± standard deviation. Nonparametric Mann-Whitney *U* testing was used. Statistics were calculated with Graphpad Prism software (Graphpad Software Inc., San Diego, CA, USA). In figures, $*$ and $**$, respectively, indicate P values of <0.05 and <0.01.

3. Results

3.1. Single-Dose Prophylactic Administration of Anti-CD25 Monoclonal Antibody PC61 Lowers CD4+CD25hiFoxP3+ Treg Infiltration in the Brain but Not CD4+CD25+Foxp3− and CD8+CD25−/+ Lymphocytes. Treatment of C57BL/6 mice with a single dose of the anti-CD25 mAb clone PC61 prior to orthotopic glioma challenge significantly lowers the number of brain-infiltrating CD4+CD25hiFoxp3+ Treg as compared to IgG control animals when assessed 14 days after tumor challenge ($11,2 \pm 1,92 \times 10^3$ *versus* $19,6 \pm 5,12 \times 10^3$, $P < 0.05$). In contrast, the number of both CD4+CD25+Foxp3− cells ($6,09 \pm 0,76 \times 10^4$ *versus* $2,24 \pm 0,50 \times 10^4$, $P < 0.01$) and CD4+CD25− cells ($53,4 \pm 9,37 \times 10^4$ *versus* $16,1 \pm 3,01 \times 10^4$, $P < 0,01$) was significantly increased in PC61 treated mice. Likewise and irrespective of CD25 expression, CD8+ T cells were significantly increased ($P < 0,01$) in PC61-treated mice (Figure 1(a)). PC61-treated mice were completely protected against subsequent tumor challenge (survival up to 50 days after tumor challenge) compared to IgG-treated controls that displayed a median survival of 22 days (Figure 1(b)). To exclude the efficiency of the tumor challenges as variable, *in vivo* optical imaging was performed on mice that were challenged with Fluc-transduced GL261 cells, revealing that the initial tumor mass was comparable in all mice (Figure 1(c)). Furthermore, direct specific effects of the PC61 mAb on the GL261 tumor cells were excluded as expression of CD25 was absent on the GL261 glioma cells (data not shown).

3.2. Anti-CD25 Treatment Results in Local Infiltration of Non-immunosuppressive Myeloid Cells in Glioma-Bearing Mice. PC61 treatment resulted in a pronounced increase (up to 3-fold more) of brain-infiltrating CD45+CD11b+ myeloid cells compared with IgG-treated control animals. Further characterization of this cell population was performed by analyzing the surface expression of Ly6G (Gr-1) and F4/80. In control mice, more Gr-1+ MDSCs were present within the CD45+CD11b+ cell population than in anti-CD25-treated mice ($12.3 \pm 2.8\%$ *versus* $4.7 \pm 1.6\%$, $P < 0.05$). The proportion of F4/80+ macrophages/granulocytes among CD45+CD11b+ myeloid cells was increased in PC61-treated mice ($82.9 \pm 3.53\%$) compared with IgG-treated mice ($57.2 \pm 8.23\%$, $P < 0,05$). A representative flow-cytometric analysis for both PC61 and control IgG-treated mice is depicted in Figure 2(a). In naïve mice, both Gr-1+ MDSC and F4/80+ macrophages/granulocytes were virtually absent (data not shown). These results were confirmed by cell counting on cytospins revealing higher macrophage

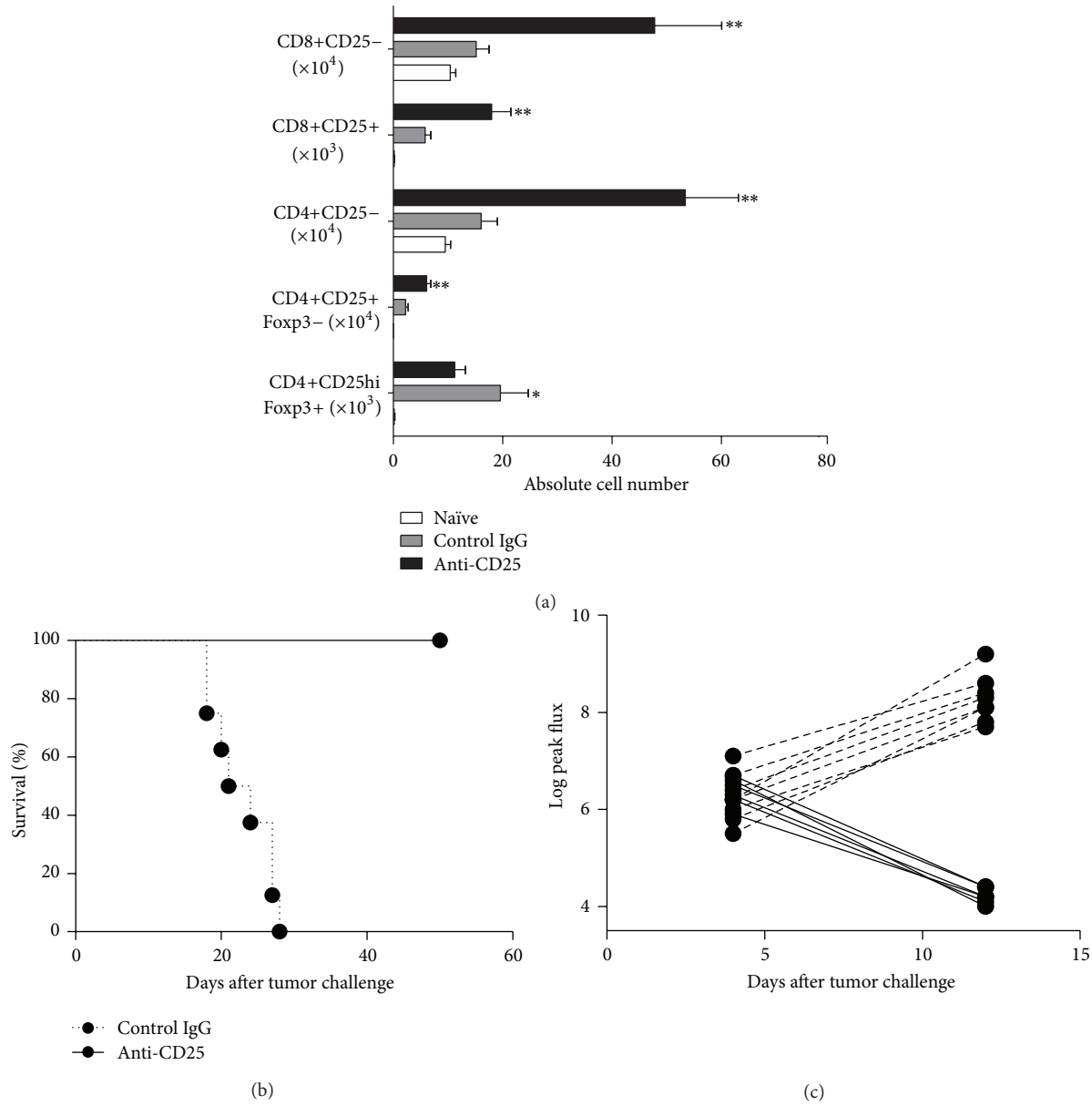

Figure 1: PC61 treatment specifically eliminates brain-infiltrating CD4+CD25hiFoxp3+ Treg. (a) Two weeks after tumor challenge, animals that were either prophylactically treated with PC61 anti-CD25 mAb (black bars, $n = 11$) or with control rat IgG (grey bars, $n = 5$) were sacrificed, and the brain-infiltrating CD4+ and CD8+ lymphocytes were analyzed by flow cytometry for expression of CD25 and Foxp3. Results are presented as absolute numbers. (b) Kaplan-Meier survival graph with PC61-treated mice (solid line, $n = 9$) and rat IgG control mice (dotted line, $n = 8$). Mice were tumor challenged on day 0. In one experiment, mice were challenged with Fluc-transduced GL261 cells. (c) Tumor burden was assessed through bioluminescence imaging on days 4 and 12 after tumor challenge of both PC61-treated mice (solid lines, $n = 6$) and control IgG-treated mice (dashed lines, $n = 8$). Data are presented as LOG (peak flux).

counts in PC61-treated mice (9.47 ± 0.95 cells/FOV, $P < 0.01$) compared with control IgG-treated mice (2.92 ± 0.38 cells/FOV). Similarly, when granulocytes were considered, PC61 treatment induced a significant higher influx (8.93 ± 1.35 cells/FOV, $P < 0.01$) compared with IgG-treated mice (4.42±0.61 cells/FOV) (Figure 2(b)). Representative cytospin pictures are shown in Figure 2(b). In absolute numbers (Figure 3), PC61-treated mice harbored significantly more CD45+CD11b+ cells compared to control IgG-treated mice ($9.60 \pm 2.07 \times 10^6$ *versus* $1.39 \pm 0.43 \times 10^6$, $P < 0.05$). In control mice but not in PC61-treated mice, a higher influx of Gr-1+CD45+CD11b+ MDSC was noted compared to naïve animals ($3.44 \pm 0.63 \times 10^5$ *versus* $0.38 \pm 0.09 \times 10^5$, $P < 0.05$). PC61 treatment resulted in an increased infiltration of F4/80+CD45+CD11b+ macrophages/granulocytes compared to control animals ($6.21 \pm 0.69 \times 10^6$ *versus* $1.09 \pm 0.30 \times 10^6$, $P < 0.01$). In the brain of naïve mice, virtually no myeloid cells were detected.

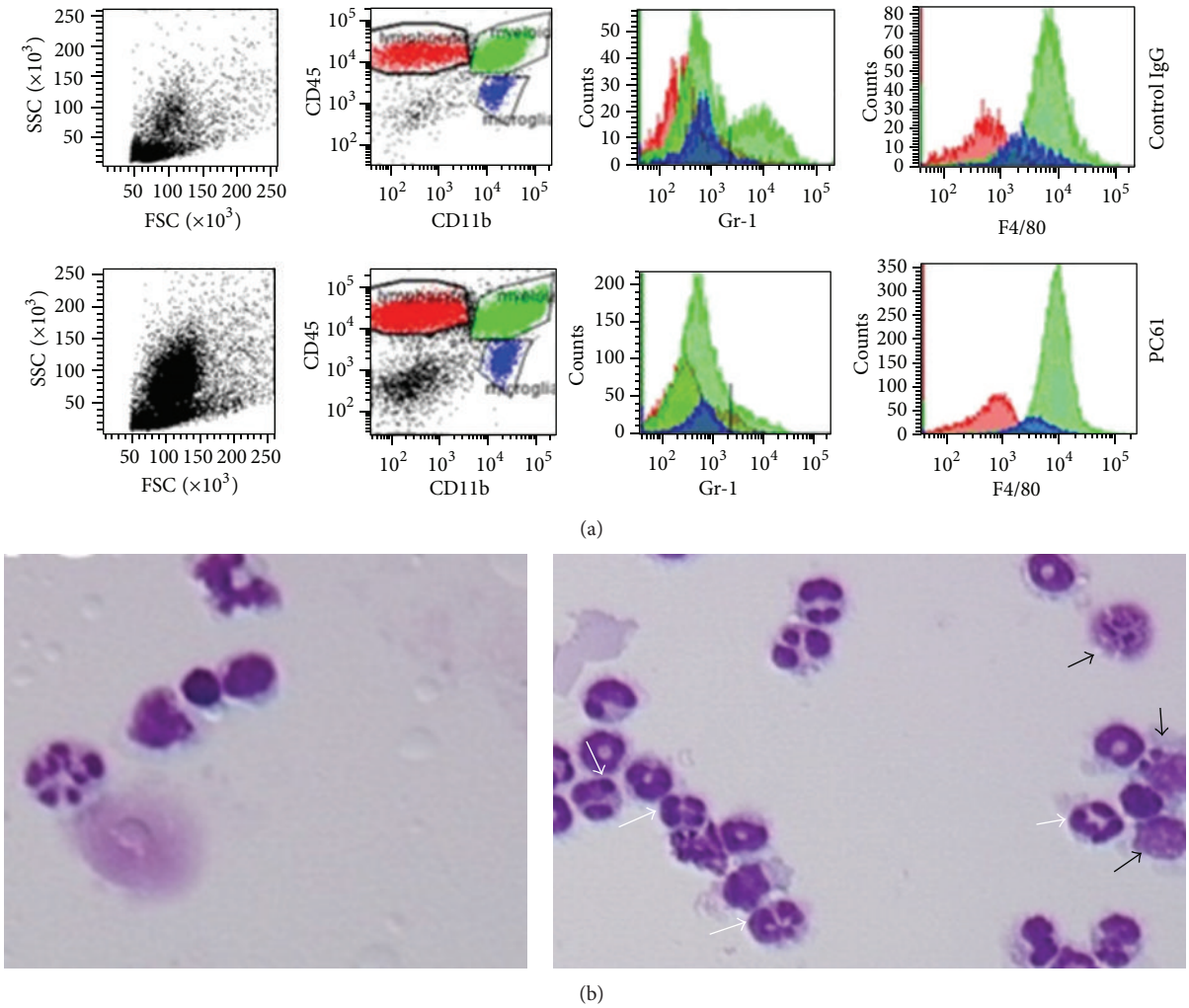

Figure 2: PC61 treatment of glioma-bearing mice leads to local infiltration with macrophages and granulocytes. (a) Flowcytometric phenotyping of brain-infiltrating myeloid cells in control (upper row) and PC61-treated mice (lower row). Based on the expression of CD45 and CD11b, three populations of brain-infiltrating cells were identified: CD45+CD11b− lymphocytes (in red), CD45+CD11b+ myeloid cells (in green), and CD45-CD11b+ microglial cells (in blue). Within the CD45+CD11b+ myeloid cells, expression of Ly6G (Gr-1) and F4/80 was measured. (b) Morphologic analysis of brain-infiltrating myeloid cells on cytospins. Detail from a representative FOV (40x) from control IgG-treated mice (left) and mice that were PC61 treated (right). Macrophages (black arrows) and granulocytes (white arrows) are highlighted.

4. Discussion

Together with others, we previously showed that treatment of mice with PC61, a widely used rat anti-mouse CD25 mAb, prolonged overall survival of tumor-bearing mice [15]. Moreover, the elimination of CD25hiFoxp3+ Tregs resulting from PC61 treatment was shown to be essential for effective vaccination with tumor lysate-pulsed dendritic cells and in combination with prophylactic DC vaccination, even resulted in the establishment of long-term immunity [12, 13].

In this study, we demonstrated that PC61 anti-CD25 mAb treatment of glioma bearing mice resulted in a decrease of brain-infiltrating Tregs only, whereas the CD4+ and CD8+ effector cells were increased and could exert their cytotoxic activity on the tumor cells. The complete protection of PC61-treated mice against tumor challenge underscores again the dominant immunosuppressive role of Treg in this model. Recently, Wainwright et al. reported that most of the brain-infiltrating Tregs in this model were FoxP3+ thymus-derived natural Treg [16].

To our view, this is the first report describing the marked infiltration of myeloid cells into the brain of GL261 tumor-bearing mice in which CD25 expressing Tregs were targeted with PC61. As was expected taking into account the beneficial effect on survival of PC61 treatment, we excluded that these cells were Gr-1+ MDSC [17]. On the contrary, PC61-treated mice even displayed less MDSC than control-treated mice. This could be explained by the fact that Tregs can induce or attract MDSC towards the tumor site (or vice versa), together building an immunosuppressive network [18]. In our hands, the brain-infiltrating myeloid cell pool consisted of both F4/80+ macrophages and granulocytes. Preliminary functional *in vitro* data reveal that these cells clearly exhibit phagocytic activity towards fluorescently labeled GL261

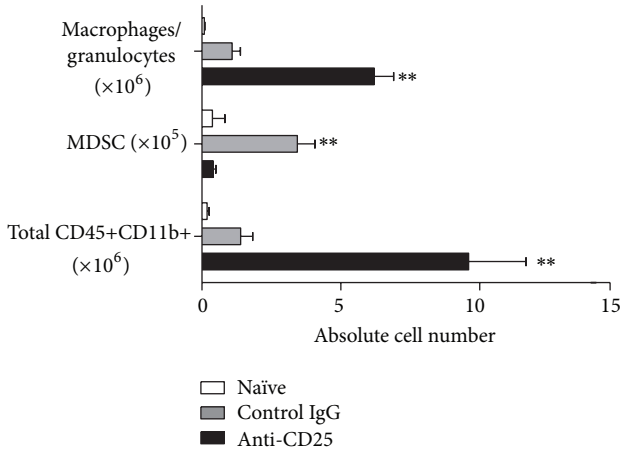

Figure 3: Quantitative analysis of myeloid cell infiltration in the brain of PC61-treated mice. Absolute numbers of brain-infiltrating total CD45+CD11b+ myeloid cells, Gr-1+ MDSC, and F4/80+ granulocytes/macrophages were calculated in PC61 (black bars, $n = 9$) and control IgG- (grey bars, $n = 7$) treated mice 14 days after tumor challenge. Naive mice were included as controls (white bars, $n = 3$).

tumor cell lysate and dextran. A more in-depth analysis of *in vitro* antitumor activity of sorted CD45+CD11b+ cells would complete this picture. Moreover, Setiady et al. elegantly demonstrated that *in vivo* depletion of CD4+Foxp3+ Tregs through PC61 treatment is mediated through antibody-dependent-cellular phagocytosis by CD16+ (FcgammaRIII+) phagocytes (including both macrophages and granulocytes). In this perspective, it would be informative to run our model with PC61 treatment in FcgammaRIII$^{-/-}$ mice [19]. Taken together, the data presented here further elucidate the local immunological events in the brain of GL261 tumor-bearing mice in which Tregs were targeted through treatment with anti-CD25 mAb PC61.

From a clinical perspective, Sampson et al. demonstrated in a pilot study that treatment with daclizumab, a humanized anti-CD25 therapeutic mAb, depletes Treg and correlates with enhanced immunity in glioblastoma patients [20]. Thus, Treg depletion in the context of glioma immunotherapy definitely warrants further investigation.

Acknowledgments

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Review Article

Potential of Immunoglobulin A to Prevent Allergic Asthma

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Allergic asthma is characterized by bronchial hyperresponsiveness, a defective barrier function, and eosinophilic lower airway inflammation in response to allergens. The inflammation is dominated by Th2 cells and IgE molecules and supplemented with Th17 cells in severe asthma. In contrast, in healthy individuals, allergen-specific IgA and IgG4 molecules are found but no IgE, and their T cells fail to proliferate in response to allergens, probably because of the development of regulatory processes that actively suppress responses to allergens. The presence of allergen-specific secretory IgA has drawn little attention so far, although a few epidemiological studies point at a reverse association between IgA levels and the incidence of allergic airway disease. This review highlights the latest literature on the role of mucosal IgA in protection against allergic airway disease, the mechanisms described to induce secretory IgA, and the role of (mucosal) dendritic cells in this process. Finally, we discuss how this information can be used to translate into the development of new therapies for allergic diseases based on, or supplemented with, IgA boosting strategies.

1. Introduction

Allergic asthma is a major health problem worldwide, causing episodes of wheezing, coughing, and breathlessness in susceptible individuals after repeated inhalation of harmless environmental allergens, such as house-dust mites (HDMs), molds, plant pollen, and animal dander [1, 2]. Currently approximately 300 million people worldwide suffer from asthma, with estimates suggesting that asthma prevalence increases globally by 50% every decade. Prevalence has reached a peak in developed countries, but rates are rising in developing regions (Africa, Latin America, and parts of Asia) as they become more westernized. This has reduced the global differences in prevalence; however, the global burden of asthma and allergies continues to rise, and new therapies are warranted [3, 4].

Interestingly, negative associations are found between the prevalence of allergic asthma and growing up on traditional European farms or rural tropical areas, usually exposed to higher ambient concentrations of microbial pollutants or higher rates of parasitic infections [5, 6]. Therefore, it was suggested that a reduced microbial exposure during childhood, due to changes in lifestyle, vaccination patterns, and/or improved hygiene, has contributed to the global increases in hyperinflammatory diseases. Insufficient microbial exposure may result in deficient maturation of the regulatory arm of the immune system, causing a disbalance of the immune system, allowing for uncontrolled expression of inflammatory responses against innocuous antigens later in life ("hygiene hypothesis") [7].

Currently used medication against asthma is aimed at symptom relieve and does not restore this immune disbalance. As a consequence, treatment is chronic and sometimes resulting in severe side effects. New therapies should focus on reducing inflammatory responses against allergens at an early age preventing the onset of structural damage and changes to the lungs, by targeting natural tolerizing mechanisms as found in healthy individuals. In this review, we will focus on one of these mechanisms and describe the potential of inducing IgA responses by modulation of

dendritic cell function and controlling unwanted allergic responses.

2. Immune Responses against Allergens

2.1. Inflammatory Reactions against Inhaled Allergens in Allergic Asthma: Th2 Cells and IgE. Allergic asthma is a chronic inflammation of the airways controlled by effector Th2 cells and characterized by eosinophilic airway inflammation and high levels of allergen-specific IgE antibodies, hallmarks of a persistent Th2 response [2] (Figure 3(a)). Upon encounter with the allergen, effector responses can be divided into immediate and late phase reactions. The immediate allergic inflammatory reaction is initiated by crosslinking of IgE molecules which are bound to IgE receptors on basophils and mast cells. As a result, these cells will degranulate and release preformed mediators from vesicles or secrete cytokines (IL-6, TNFα, MIP1α) [9], causing immediate vascular permeability, blood vessel dilation, bronchoconstriction, and smooth muscle contraction [10]. This immediate reaction may be followed by the late phase response, initiated by inflammatory cytokines and type 2 cytokines, such as IL-4, IL-5, Il-9, and IL-13, which recruit and activate eosinophils and basophils and induce gobleT-Cell metaplasia and overproduction of mucus [11, 12]. In severe forms of asthma, also Th17 cells are found, which enhance the effects of the Th2 cytokines and recruit neutrophils and other inflammatory leukocytes [13–15]. In addition to distortion of immunological pathways during allergen sensitization and challenge, also aberrant structural airway remodeling is involved in the development of asthma. Some groups have even suggested that the airway structural changes occur before the deranged immune response is present. Indeed, basement membrane thickening is detectable in children younger than three years old with persistent wheezing before the diagnosis of asthma [16, 17]. Airway remodeling includes marked changes in the airway wall, like epithelial injury, extracellular matrix deposition under the epithelial basal membrane, gobleT-Cell hyperplasia, and increased smooth muscle mass. These changes lead to a defective physical and functional barrier of the airway epithelium in severe asthma. Various studies point at dysfunctional injury/repair mechanisms in response to damaging stimuli and/or respiratory viruses in asthmatics, which may only be in part explained by allergic airways inflammation [18, 19].

Dendritic cells (DCs) play a crucial role in the described processes leading to asthma pathogenesis (Figure 3(a)). Immature DCs reside in peripheral and mucosal tissues, such as the lungs, where they continuously sample the environment for foreign soluble antigens and small particles, including inhaled allergens [20, 21]. DCs express different types of pattern recognition receptors (PRRs), such as toll-like receptors (TLRs), NOD-like receptors, and C-type lectin receptors, that allow the recognition of different classes of molecules broadly shared by pathogens (pathogen-associated molecular patterns (PAMPs)) [22, 23]. Upon encounter of danger signals, DCs become activated and migrate to the draining lymph nodes [24], where they activate antigen-specific naïve Th cells and drive their development in effector T helper cells, such as Th1, Th2, Th17, or regulatory T (Treg) cells [25, 26]. Various studies have demonstrated that DCs are necessary for inducing allergic sensitization [27], for driving the development of Th2 immunity and eosinophilia [28–30], and are crucial for maintaining the inflammatory processes in the airways as well as bronchial hyperreactivity and chronic airway remodeling [31, 32]. After allergen uptake, the function of DCs is strongly influenced by signals encountered during their stay in the peripheral tissues, which can include microbial signals induced by the ligation of pattern recognition receptors (PRRs) on the DCs or alarming signals from structural cells like local epithelial cells of the airways [33–35]. Crosstalk between airway epithelial cells and DCs may form a critical link for the induction and continuation of allergic inflammation in the lungs as several EC-derived molecules can influence DC migration, differentiation, and function [20, 33, 36].

2.2. "Tolerizing" Immune Responses to Allergens in Healthy Individuals: Treg Cells and IgA. In healthy individuals, T-cell responses to allergens are commonly observed, yet are usually dominated by anergy or by regulatory T (Treg) cells that can suppress various effector Th cell subsets [37, 38]. Allergen-specific Treg cells can suppress Th2 cells by cell-cell contact or release of the anti-inflammatory and immunoregulatory cytokines IL-10 and transforming growth factor (TGF)-β. In almost all patients with asthma, one can find the counterregulatory Treg cells, but these fail to or insufficiently suppress allergic inflammation [39]. It has therefore been suggested that asthma may result from aberrant of defective Treg mechanisms.

Humoral responses of healthy individuals consist of mainly low IgG1, IgG4, and secretory IgA (sIgA) antibodies to allergens in the presence or absence of low amounts of IgE [37, 40, 41]. Although the presence of allergen-specific IgA has drawn relatively little attention so far, it is still unclear what its relative role is in the protection (or exacerbation) of allergic disease [42]. Although most individuals with immunoglobulin A (IgA) deficiency are asymptomatic, allergic disorders appear to be more common among patients with IgA deficiency [43]. Indeed, Balzar et al. found lower IgA levels in bronchoalveolar lavage of severe asthmatics than in healthy subjects, which correlated with lung function and asthma symptoms [44]. In contrast, high salivary secretory IgA levels were associated with less development of allergic symptoms in sensitized Swedish children [45]. Furthermore, high levels of specific IgA antibodies in salivary of sensitized infants were associated with significantly less late-onset wheezing [46]. In addition, allergic patients who naturally develop tolerance responses towards cow's milk concomitantly undergo a shift towards IgA dominance in serum [47]. Moreover, in an experimental setting, Schwarze was able to protect mice against the development of eosinophilic airway inflammation and hyperresponsiveness by treating with antigen-specific IgA during challenge [48].

Taken together, these data show an inverse relationship between IgA and allergy development, suggesting a protective role for IgA in allergic diseases such as asthma.

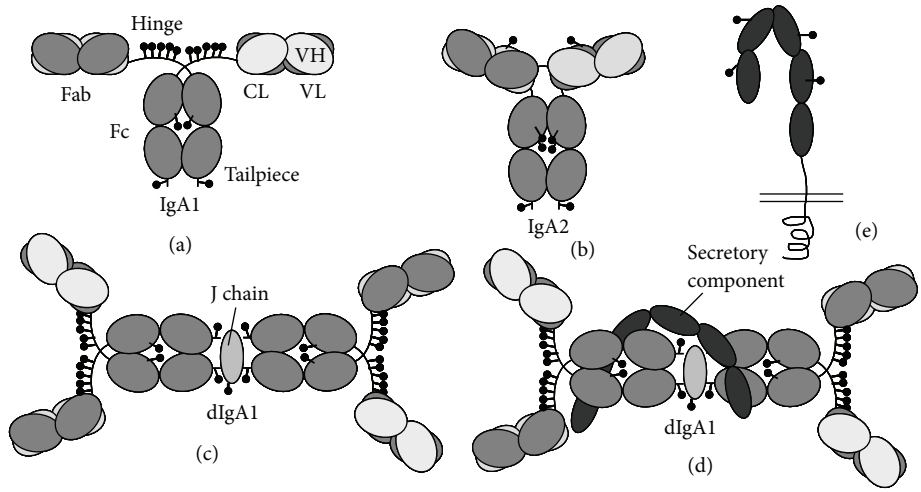


Figure 1: Human IgA structure. Schematic diagrams of (a) IgA subclass 1 (IgA1), (b) IgA2, (c) dimeric IgA1 (dIgA1), (d) secretory IgA1, and (e) polymeric immunoglobulin receptor (pIgR) (adapted from [8]).

3. Immunoglobulin A Antibodies and Its Functions

3.1. Isoforms and Receptors. The antibody IgA can occur as a monomer (Figures 1(a) and 1(b)), but also in dimeric or even polymeric forms through interactions with the joining chain (J-chain) (Figure 1(c)). All these different forms are mainly found in the circulation, while secretory IgA (sIgA) is only found at mucosal surfaces and is generated by the binding of dimeric IgA via the J-chain to the polymeric immunoglobulin receptor (pIgR) at the basolateral side of the epithelium which is subsequently transported to the luminal side (Figures 1(d) and 1(e)). Here, IgA is released at the mucosal surface (lumen) by cleavage from the pIgR. In this process part of the pIgR, called the secretory component (SC), remains attached to the IgA molecule, and together they form the molecule secretory IgA (SIgA). Mouse and human IgA biology differ in several aspects. In human serum, IgA occurs mainly in a monomeric form, while in mice polymeric IgA is the main isotype in serum. Furthermore, human IgA, but not mouse IgA, is divided into closely related subclasses, IgA1 and IgA2, of which the later one is less susceptible for proteolytic degradation (Figures 1(a) and 1(b)). In serum, the subclass IgA1 is dominant, while in secretions the main isoform found is IgA2, although both IgA1 and IgA2 can be detected as SIgA [49].

IgA has been described to interact with various host receptors, that is, pIgR, FcαRI (CD89), transferrin receptor (CD71), asialoglycoprotein receptor (ASGPR), and Fcα/μR. The consequences after ligation are not very clear for most of these receptors. However, the FcαRI allows both inhibitory and activating signals and therefore is considered to be important for the role of IgA in preserving homeostasis and tolerance at mucosal sites [8, 50]. Moreover, FcαRI is the only IgA Fc receptor expressed on (blood) myeloid cells, including DCs, monocytes/macrophages, neutrophils, and eosinophils. In mucosal areas in steady state conditions, only few cells are positive for FcαRI. Intriguingly, this receptor has not been identified in mice. Although this receptor is associated with an immunoreceptor tyrosine-based activation motifs (ITAM), its signaling can be activating as well as inhibitory. This depends on the ligand and subsequent configuration (involving Syk or SHP-1 phosphatase) of the ITAM, resulting in an activating or an inhibitory ITAM motif. The inhibitory ITAM (ITAMi) pathway takes place in the absence of receptor coaggregation and of an immunoreceptor tyrosine-based inhibitory motif (ITIM), which is known for inhibiting immune responses. All forms of IgA can ligate to FcαRI, but they differ in their binding capacities. Monomeric IgA only binds with low affinity to the FcαRI and activates the ITAMi, which does not lead to cell activation or degranulation/oxidative burst (in the case of granulocytes) [51]. In contrast, IgA complexes show a stronger binding and subsequent activating signal, resulting in cell activation [52, 53].

3.2. Effector Functions of IgA. IgA is classically known for neutralizing toxins and bacteria (viruses) at mucosal surfaces [54, 55], by interfering with their motility, by competing for epithelial adhesion sites, and by improving the viscoelastic properties of the airway secretions [56]. The SC protects SIgA from proteolytic degradation and is involved in establishing local interactions with bronchial mucus, thereby contributing to the "trapping" and removal of the antigen ("immune exclusion") [57]. Interestingly, it has been suggested that IgA can also directly reduce inflammatory responses by inhibiting effector functions of inflammatory cells. For example, anti-FcαRI Fab treatment, by initiating ITAMi signaling, suppressed manifestations of allergic asthma in FcαRI transgenic mice immunized with anti-IgE immune complexes [58]. Triggering ITAMi signaling also prevented marked inflammation and leukocyte infiltration in kidney inflammation models such as glomerulonephritis [59]. Furthermore, *in vitro* crosslinking of FcαRI on human monocyte-derived DCs leads to internalization of IgA complexes and antigen presentation, resulting in DC maturation and IL-10 production

[60, 61]. (Serum) IgA ligation on monocytes also induces IL-10 expression [62] and inhibits inflammatory cytokine (IL-6 and TNFα) release [63, 64]. Importantly, IgA has only limited capacity to activate the complement system, in contrast to IgG and IgM. Furthermore, it can competitively block the IgG-mediated activation of complement [8, 65]. Of note, a few specific diseases are associated with an increase in serum IgA levels, often paralleled by IgA tissue deposition [66]. In IgA nephropathy, the formation of aggregated IgA immune complexes in the kidney causes severe inflammatory responses [67, 68]. However, there are indications that in these patients, glycosylation (e.g., sialylation) of the circulating IgA antibodies is abnormal, which may explain the pathogenic potential [53].

Collectively, these data suggest that under homeostatic conditions, secretory IgA contributes to the maintenance of mucosal tolerance by dampening immune responses. Therefore, IgA can have a role in preventing the development of hyperinflammatory responses towards environmental allergens that otherwise could cause allergic inflammation as observed in allergic rhinitis or asthma.

4. Regulation of Immunoglobulin A Responses

4.1. T-Cell-Dependent and T-Cell-Independent IgA Class Switching. Humoral responses in the mouse are mediated by at least three different subpopulations of mature B cells. These B cells can acquire the expression of various antibody isotypes, including IgA, by undergoing class switch recombination (CSR). In contrast to other isotypes, IgA class switching can occur both via a conventional T-Cell-dependent (TD) pathway and an alternative T-cell-independent (TI) pathway (Figure 2). Follicular (or B-2) B cells, located in the spleen, lymph nodes, and the Peyer's Patches respond to T-cell-dependent antigens and can acquire the expression of various isotypes by undergoing class switch recombination (CSR). The resulting IgA^+-B cells will migrate to the draining effector sites. TD class switch is induced by CD40-CD40L ligation and specific cytokines secreted by T cells as a result of activation by DCs or other APCs. The major cytokine signal for α-CSR is TGF-β with contributions from IL-2, IL-4, IL-5, IL-6, IL-10, and IL-21 [69–72] (Figure 2 TD pathway).

Nonfollicular B cells, such as the splenic marginal zone B cells and the B-1 cells, which are mostly enriched in the peritoneal and pleural cavity and the lamina propria of the small and large intestines, primarily respond to T-cell-independent antigens and secrete natural or polyspecific antibodies [73]. The alternative TI pathway occurs locally at effector sites and is a much faster mechanism to generate IgA. TI class switching is induced independently of CD40-CD40L engagement and needs alternative costimulatory signals, such as B-cell activating factor of the TNF family (BAFF, also known as BLyS), a proliferation-inducing ligand (APRIL), retinoic acid (RA), TGF-β, nitric oxide (NO), and/or IL-6. These IgA costimulatory factors can be produced by both resident epithelial cells of mucosal organs and by local DCs. In fact, mucosal DCs, from Peyer's Patches (PPs), gut lamina propria [74], or lungs [75], are the primary APCs able to drive TI IgA class switching. Once CSR has taken place, most of these factors, including BAFF and APRIL, further enhance both TD and TI IgA responses by providing survival signals, and/or inducing plasma cell differentiation and IgA secretion, pointing at an additional role of structural cells and DCs at a later stage of IgA development [69–71] (Figure 2 TI pathway).

It is increasingly clear that gut IgA-producing B cells can be both generated from follicular B cells or the B-1 cells by complementary pathways, requiring different signals to undergo IgA switching which links back to their capacity to respond to TD or TI antigens. For example, peritoneal B-1 cells more readily switch to IgA *in vitro* in response to BAFF, TLR ligation, and TGF-β, while follicular B cells or peritoneal B-2 cells require cytokines like IL-4, IL-5, or anti-IgD dextran [76]. Interestingly, B-1 cells can switch to all immunoglobulins *in vitro*, while *in vivo* studies with SCID mice or irradiated mice reconstituted with bone marrow or peritoneal cavity cells have suggested that B-1 cells preferentially switch to IgA [77, 78], where they accounted for most of the gut IgA plasma cells. However, studies in gnotobiotic allotype Ig chimeric mice (allowing the distinction between Abs derived from B1 and B2 cells based on different allotypes) suggested that in normal immunocompetent mice intestinal B-2 cells contributed for most of the IgA found in the gut in response to gut bacteria [79]. Importantly, also in the respiratory tract and their draining lymph nodes local B-1 cells have been demonstrated [80]; however, the respective role of B-1 or follicular B cells in the production of IgA has not been studied yet. The relative role of B-1 versus B-2 cells in IgA-mediated immunity is reviewed elsewhere [72].

Theoretically, it might be possible to induce *in situ* CSR of existing allergen-specific IgE^+ B cells into $IgA_2{}^+$ cells. Because $C_H\alpha_2$ is the last exon located downstream from $C_H\varepsilon$ in the human heavy chain locus, this may be the only alternative for CSR in IgE^+ B cells. Shifting the allergen-specific antibody response from IgE to IgA2 would result in neutralization of allergen in the mucosal lumen, before it could interact with IgE, and could therefore constitute a therapeutic target. Although this has not been tested nor reported yet, based on the role of IL-21 and TGF-β in IgA class switching [81], they may contribute to induce IgA2 production in already class-switched B cells.

4.2. Role of Mucosal Dendritic Cells and TLRs. Mucosal conditioning of DCs occurs via resident tissue-derived factors, such as thymic stromal lymphopoietin (TSLP), IFN-β, RA, and TGF-β, but also by ligation of toll-like receptor (TLR) ligands expressed by (commensal) bacteria [82–84] (Figure 2). Epithelial cells release these DC conditioning factors in addition to other IgA stimulatory factors in response to TLR ligands [85]. In the gut lamina propria, several specialized DC subsets are described with an enhanced intrinsic capacity to drive IgA CSR. For example, Tip DCs express inducible nitric oxide synthase (iNOS) in response to TLR signaling and initiate TI IgA production by releasing BAFF and APRIL [86]. $CD11c^{hi}CD11b^{hi}$ DCs induce TI IgA production upon sensing bacteria through TLR5, a process that elicits release of RA and IL-6 [87]. $CD103^+$ DCs are known for driving the differentiation of $FoxP3^+$ Treg cells. As the main RA

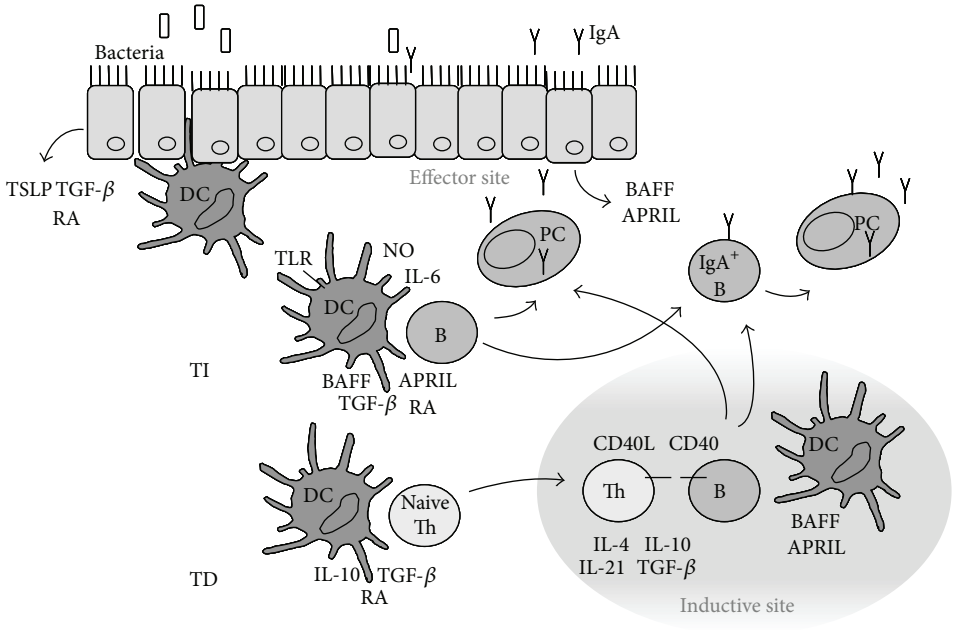
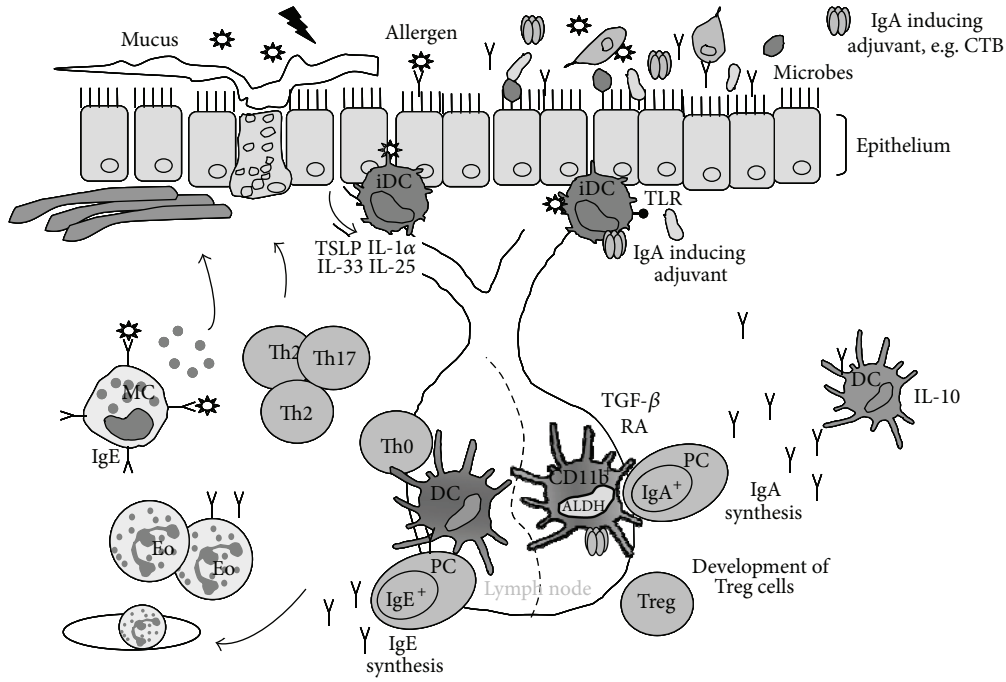


Figure 2: After conditioning by tissue-derived factors and TLR ligands, DCs activate T cells which provide for costimulatory signals (CD40-CD40L) and specific cytokines (TD), or DCs provide for alternative costimulatory signals, like BAFF and APRIL (TI) to initiate class switch recombination and expression of IgA by the mature B cells (IgA^+ B cell). From the inductive site, the IgA^+-B cells will migrate to the draining effector sites, where, in response to additional signals, they become plasma cells (PCs) and start to secrete IgA.



(a) Allergic airway inflammation (b) Tolerogenic mucosal responses

Figure 3: Crosstalk between epithelial cells and dendritic cells (DCs) in the lungs determines the balance between immunity and tolerance. (a) Allergen exposure in susceptible individuals may result in epithelial damage, initiating a cascade of immunological events leading to inflammation and allergic symptoms. (b) In contrast, IgA-inducing agents, such as CTB, alone or in combination with microbial exposure, prevent the development of allergic inflammation, by protecting the epithelial barrier and by effecting the function of various immune cells via DC. Immature DC (iDC), T helper 2 cell (Th2), T helper 17 cell (Th17), eosinophil (Eo), masT-Cell (MC), and plasma cell (PC).

producing subset, they are also responsible for imprinting gut-homing molecules on B cells and support IgA synthesis [74]. How these DCs acquire their tolerogenic properties is not yet fully understood, but a role for microbial activation was suggested [88, 89]. Recently, it was published that *in vitro* mouse CD11b^{hi} lung DCs induce IgA more efficiently than CD103^{+} lung DCs [90]. The use of CD11b as a marker for lung DCs is however confusing, as CD11b is not only found on a subset of conventional (c)DCs, but also on the population of monocyte-derived DCs (moDCs) that are recruited to the lungs at times of inflammation [91].

In addition to the conventional mouse DC subsets which drive IgA synthesis and are portrayed in the previous paragraph, also a plasmacytoid (p)DC subset has been described [92]. These pDCs differ from cDCs in expressing lower amounts of CD11c, yet they produce very high amounts of IFNα. Although both mouse gut lymph node-derived pDCs and cDCs were able to support B-cell IgA production, pDCs were more superior *in vitro* due to type 1 IFN dependent enhanced APRIL and BAFF expression [93]. Also in humans, IFNα producing pDCs seem to be more advanced in supporting B-cell proliferation and differentiation into antibody producing cells, including IgA, compared to myeloid DCs (mDC, grossly equivalent to mouse cDC subset) [94, 95].

Altogether DCs form a crucial cell type in the differentiation of IgA responses. Although by different mechanisms, both cDCs and pDCs can promote Ig responses, and their IgA inducing capacity can be enhanced by local factors produced by mucosal tissues as well as by (local) microbial products such as TLR ligands.

4.3. Early Priming and the Microbiota. The establishment of commensal flora in the intestine, and most likely also the respiratory tract [96], starts at birth and is considered to be crucial for stimulating and directing the development of the host immune system. Animals raised under germ-free conditions have an undeveloped immune system with fewer germinal centers and decreased number of IgA-producing plasma cells [97]. Interestingly, gut microbiota is necessary for a protective immune system, including mucosal IgA responses, in the airways. In response to OVA, germ-free (GF) mice developed more severe features of airway inflammation compared to control specific pathogen free (SPF) mice, which could be reversed by recolonization of GF mice with complex commensal flora. Furthermore, the absence of commensal bacteria was associated with less pDCs and attenuated production of IgA in the airways [98]. Human studies have also suggested the link between commensals and allergy. Indeed, children who developed allergy had significantly less diverse gut microbiota and lower levels of salivary SIgA [99], while, intestinal colonization by *Staphylococcus aureus* was associated with high circulating IgA levels and with a lower frequency of eczema [100]. The exact number and diversity of an individual's community of commensals seem to be determined by factors occurring in early childhood [101]. Several mouse and human studies have shown that early life (prenatal, preconception) exposure to environments characterized by a diverse and concentrated microbial milieu such as traditional farming sites may protect against the development of allergic diseases [102–105]. Breast milk contains many Igs and may have a collective tolerogenic effect acting via sIgA, cytokines, and/or immune complexes [106].

A rich microbial environment contributes to mucosal tolerance and protective IgA responses, which are associated with protection against allergic asthma. The ideal candidate for an adjuvant stimulating protective IgA responses and thereby preventing development of allergic asthma could therefore be a microbial-derived molecule.

5. Strategy for Allergy Intervention via Induction of IgA

5.1. Cholera Toxin B, an IgA Inducing Adjuvant. Cholera toxin is the most widely experimentally used mucosal adjuvant, potentiating serum and local immune responses to coadministered antigens [107]. The enterotoxin Cholera Toxin is produced by the bacterium *Vibrio cholerae* and consists of an A and B subunit, each with distinct effects on cells of the immune system. The A subunit is known for its toxic (side) effects: after entering the cell cytosol, the A subunit triggers electrolyte efflux via activation of adenylate cyclase and increased cyclic AMP (cAMP) production, resulting in severe watery diarrhea. The B subunit of CT (CTB) is more considered as a nontoxic subunit, as it is not linked to the activation of cAMP and its adjuvant activity seems to be mainly associated with immunoregulatory events [107, 108]. For example, feeding of CTB conjugated to myelin basic protein before or after disease induction protected rats from experimental autoimmune encephalomyelitis [109], and nasal administration of CTB insulin significantly delayed incidence of spontaneous diabetes in NOD mice [110]. In these models, protection against autoimmunity by CTB/Ag conjugates was associated with the formation of Treg cells expressing IL-10 and/or TGF-β [109]. The tolerizing effect of CTB has also been shown to extend to other immune-mediated diseases. In a delayed type hypersensitivity model, (prolonged) oral treatment with low doses of OVA conjugated to CTB prevented sensitization and suppressed IgE antibody responses in sensitized mice [111]. Furthermore, intranasal pretreatment of CTB linked to the BetV1, a major allergen of birch pollen, prevented sensitization to the antigen by shifting the Th2 response towards Th1 and the induction of allergen-specific IgA responses [112]. Likewise, we found that CTB administration in the lungs stimulates local secretory IgA responses which protected against the development of allergic airway inflammation (AAI), while mice deficient for polymeric Ig receptor (pIgR) and lacking SIgA were not [113]. Interestingly, the primary action of CTB as an adjuvant may be primarily mediated through a direct effect on APCs such as DCs. Upon *in vivo* administration, CTB mainly effected cDCs and not so much pDCs, while adoptive transfer of *in vitro* generated CTB treated DCs was sufficient to enhance IgA responses in mouse lungs [113]. Furthermore, *in vitro* cocultures of CTB exposed bone marrow-derived DCs and B cells also resulted in the induction of IgA production. These

in vitro experiments suggest that asynergism between CTB and MyD88-dependent TLR signals selectively imprints an IgA inducing phenotype in DCs, characterized by RALDH1 and TGF-β expression [114]. Upon exposure to CTB *in vivo*, ALDH activity was mainly enhanced in the CD11b$^+$ DCs, which may include mo-DCs (Figure 3(b)). Also the experiments with the bone marrow-derived DCs differentiated in GM-CSF, which are a good model for these inflammatory DCs, may suggest that (CD11b$^+$) moDCs could be responsible for CTB induced IgA responses in the mouse airways. This is certainly a possibility as CTB seems to work best at inducing IgA responses when accompanied by some degree of LPS. LPS is a known trigger of moDC recruitment [115]. Until we have more specific depleting antibodies or transgenic mouse strains to selectively deplete moDCs, we can however only speculate at this stage whether this is true.

If we are to exploit the full potential of IgA as an immunomodulatory immunoglobulin in allergic asthma and other immune mediated diseases, the role of different DC subsets in the regulation of humoral IgA responses and modulation by adjuvants should be studied in more detail.

5.2. Allergen-Specific Immunotherapy. Allergen-specific immunotherapy (SIT) represents the only curative treatment of allergic diseases currently available and involves the incremental delivery of the allergen to which the individual is sensitive [116]. Successful IT components of the regulatory network such as Treg cells and the cytokine IL-10 are elevated, while allergen-specific IgE levels are reduced. It is hypothesized that the enhanced immunoregulatory network is instrumental in suppressing allergen-specific effector T cells which are responsible for many of the characteristics of allergic diseases. IL-10 does not only contribute to T-Cell tolerance but also potently suppresses total and allergen-specific IgE, and it simultaneously increases IgG4 and IgA production in cultures [117, 118]. Interestingly, successful immunotherapy is also associated with increases in IgA responses *in vivo*. In a 2-year double blind trial, grass-pollen immunotherapy induced a shift in allergen-specific antibody response towards IgA2, which correlated with increased local TGF-β expression and induced monocyte IL-10 expression [119]. Another study using sublingual grass-pollen immunotherapy (SLIT) reported increases in allergen-specific IgG4 and IgA [120].

In its current form, SIT has major drawbacks and cannot compete with treatment on the basis of symptom relief (antihistamines and corticosteroids) for many asthma patients. High concentrations of allergen extract need to be administered on a long term (~5 years) and regular basis. This introduces a risk of potentially life-threatening allergic reactions [121]. Therefore, it might be interesting to apply the use of tolerogenic adjuvants, specifically inducing Treg cells and/or secretory IgA to improve efficacy and safety of SIT. For example, when the allergen is coupled to the adjuvant CTB, it will be efficiently targeted to the DC [113], allowing the use of lower allergen doses and decreasing the risk of anaphylactic shocks. Future experiments in mouse models for true allergens, like birch pollen or house-dust mite, experimental SIT models, and (cells from) allergic patients will need to point out the usefulness of application of the class of "mucosal" adjuvants in current SIT protocols.

5.3. Boosting IgA as a Preventive Strategy. The establishment of commensal flora in the intestine and respiratory tract starts at birth and is considered to be crucial for stimulating and directing the development of the host immune system, including the mucosal IgA response [97, 122, 123]. In our *in vitro* coculture system, we confirmed the role for microbial-derived TLR ligands in the conditioning of DCs for stimulating IgA responses. Interestingly, the mucosal adjuvant CTB does not only enhance IgA induction by TLR-ligand primed DCs, but also initiates IgA production in the case of low dose exposure to MyD88-activating signals which are insufficient to induce IgA on their own [114]. This is interesting considering the hypothesis that decreased or altered microbial exposure associated with an affluent life style is contributing to the increase in asthma prevalence during the last decades. Only recently we have started to appreciate the importance of the microbiota on human health, and restoring or manipulating disrupted host-microbiota relationship has become a potent strategy for treating inflammatory diseases, including asthma [124]. CTB could contribute to broad antibody repertoire and sufficient mucosal IgA levels in people with impaired or delayed IgA synthesis, by reducing the threshold for microbial signals or providing the necessary cosignals, to maintain mucosal immunity and local homeostasis (Figure 3).

It was shown that children who developed allergy had less diverse (gut and airway) microbiota [99] and decreased serum or mucosal IgA responses [45, 46, 100] compared to healthy controls. Studies that measured IgA levels at different time points showed an increase over time which may be due to microbial exposure and microbiota development [45, 100]. Especially during the first months and year, events such as mode of birth delivery, type of "first" milk (breast versus formula milk), and microbial exposure will determine the composition of microbiota [122]. This suggests that particularly during this early period in life impaired IgA responses may allow for sensitization and/or development of allergic symptoms. By the time that allergic (asthma) patients have reached adulthood, impaired IgA responses may be restored to normal levels, but the "damage" has already occurred and allergen-specific inflammatory responses have developed. Indeed, in a cohort of adult allergic asthmatic patients, we did not find reduced (secretory) IgA levels in nasal washes compared to nonallergic controls (Gloudemans et al., unpublished observations). Alternatively, in a fraction of the allergic infants with a slowly developing mucosal IgA repertoire, allergy symptoms may relieve together with the establishment of a fully developed IgA response. Thus, one should keep in mind that the association between IgA and asthma may be misinterpreted using an adult cohort.

6. Words of Caution

The effector function of IgA is very much depending on local (inflammatory) factors present and needs to be carefully examined before applying IgA enhancing therapy. For

example, IgA immune complexes bind with stronger affinity, subsequently resulting in cell activation and elimination of the pathogen [52, 53]. In certain cases, IgA complexes can even cause severe inflammation and pathology, like in immune complex glomerulonephritis [68, 125]. Importantly, eosinophils and neutrophils express receptors for IgA that can activate the cells upon binding of IgA immune complexes [51, 126], resulting in activation and/or degranulation of the cell [50, 127]. Therefore, also in severe asthma, where in addition to eosinophils also neutrophils are important mediators, IgA may aggravate the inflammation instead of promoting tolerance. Interestingly, both in patients with IgA nephropathy and in patients with asthma, abnormal glycosylation of the IgA antibody or the FcαRI receptor was found, which may allow exacerbated immune responses and disease development instead [128, 129].

In addition to the isoform of IgA and local tissue factors, reactivity or specificity of the antibody will determine the receptor binding affinity and thus the immunological effect of Ig-receptor ligation. Primitive or polyreactive (natural) IgA antibodies are sufficient to protect the host from excess mucosal immune stimulation by harmless commensal bacteria and may protect against some noninvasive parasites [130, 131]. However, affinity maturation of IgA is necessary to provide protection from more invasive commensal bacteria and from true pathogens. Thus, there seems to be a correlation between the "sophistication" of the IgA response and the aggressiveness of the subsequent immune response, at least in the gut [132]. However, it still remains unclear how this applies for environmental particles in the lung, such as inhaled allergens. Therefore, to evaluate the efficacy of IgA-based treatment against allergic diseases, not only the level of mucosal IgA responses need to be carefully studied in health and disease, but also aspects such as the affinity and reactivity of the antibodies should be taken into account.

Although local IgA induction during specific immunotherapy may have potential to improve the treatment of allergic airway inflammation, based on the dynamics of the development of IgA responses in life and the functional duality of the IgA-receptor interaction, it seems essential to stimulate IgA responses under noninflammatory conditions. Therefore, we hypothesize that the development of protective mucosal IgA responses will occur best in the context of a homeostatic environment, through the activation of dedicated mDCs and/or mo-DCs, aiming at the induction of a fully developed mucosal IgA repertoire in time and preventing the development of inflammatory responses to allergens (Figure 3).

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Research Article

Synergy between Vitamin D_3 and Toll-Like Receptor Agonists Regulates Human Dendritic Cell Response during Maturation

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Human dendritic cells (DC) can be differentiated from blood monocytes in the presence of GM-CSF and IL-4 and matured by lipopolysaccharide (LPS). Vitamin D_3 inhibits the maturation of human DC measured by changes in surface expression of HLA-DR, CD14, CD40, CD80, CD83, and CD86. We here examine the function of vitamin D_3 during DC maturation. One of the earliest changes to LPS-induced maturation was an increase in CD83 expression. Vitamin D_3 inhibited the increase in expression of HLA-DR, CD40, CD80, CD83, and CD86 and the decrease in expression of CD14, which was paralleled morphologically by vitamin D_3-induced inhibition of dendritic cell differentiation. Vitamin D_3 acted in synergy with the TLR agonists LPS and peptidoglycan (PGN) in inducing IL-6, IL-8, and IL-10, whereas vitamin D_3 completely inhibited LPS-induced secretion of IL-12. The synergy occurred at concentrations where neither vitamin D_3 nor the TLR agonists alone induced measurable cytokine secretion. Both LPS and PGN enhanced the level of the vitamin D_3 receptor (VDR). Taken together, these data demonstrated that vitamin D_3 and TLR agonists acted in synergy to alter secretion of cytokines from human DC in a direction that may provide an anti-inflammatory environment.

1. Introduction

Dendritic cells (DC) are highly specialized, professional antigen-presenting cells (APC) that orchestrate the immune response via integration of a variety of signals [1]. Immunosuppressive and anti-inflammatory compounds like IL-10 [2], 1α,25-dihydroxyvitamin D_3 [3–5], and TGF-β [6] induce DC with tolerogenic properties *in vitro*. These DC are generally characterized by an immature or semimature phenotype, with low expression of costimulatory molecules. In addition, tolerogenic DC produce low amounts of proinflammatory cytokines and high amounts of anti-inflammatory cytokines.

Interactions between DC and regulatory T cells (Treg) facilitate the immunosuppression, immature DC induce Treg, and vice versa Treg prepare DC to become immunosuppressive [7, 8].

The biological active form of vitamin D is mediated by the active hormonal form 1α,25-dihydroxyvitamin D_3 (hereafter referred to as vitamin D_3). Its intracellular transcriptional effects are mediated through binding to the vitamin D receptor (VDR), which subsequently functions as a transcription factor. VDR is constitutively expressed in APC, such as macrophages and DC, and is inducible in activated T lymphocytes [9–11].

Several studies have demonstrated immunosuppressive effects of vitamin D_3 on the functions of DC. *In vitro* maturation of both human and mouse DC in presence of vitamin D_3 lead to reduced expression of MHC-II and the costimulatory molecules CD40, CD80, and CD86 resulting in an enhanced production of IL-10 and a reduced secretion of IL-12 [12, 13].

Vitamin D_3 deficiency has been associated with a higher rate of several diseases, including multiple sclerosis (MS) [14, 15]. Moreover, administration of vitamin D_3 in the animal model experimental autoimmune encephalitis (EAE) suppressed the development and progression of disease [16], and vitamin D_3 has also been shown to ameliorate several other models of autoimmune diseases [17, 18].

Infection with Gram-negative bacteria provides lipopolysaccharide (LPS), which contains pathogen-associated molecular patterns (PAMP) that function as a Toll-like receptor (TLR) ligand. LPS is detected by TLR4 and is one of the major components used for inducing maturation of monocyte-derived DC *in vitro* [19]. TLR are expressed by

many APC and activate an intracellular signaling pathway that leads to transcriptional activation of proinflammatory genes and innate effector molecules [20].

It is feasible that PAMP and TLR ligands interfere with vitamin D_3 functions in the immune system. This would be consistent with the fact that individuals with severe vitamin D_3 deficiencies have an increase susceptibility to intracellular infections [21]. A recent study shows that the influence of vitamin D_3 on TLR4 ligand-induced activation of APC is dependent on the order of VDR and TLR4 engagement [9]. To further study the interplay between TLR agonists and vitamin D_3, we examined the maturation and cytokine profile of DC differentiated *in vitro* from human peripheral blood monocytes.

2. Materials and Methods

2.1. Ethical Approval. The study was conducted in accordance with the Ethical Declaration of Helsinki. The project was approved by the local Ethics Committee on Biomedical Research Ethics (j. no20090210).

2.2. Isolation of Peripheral Blood Mononuclear Cells. Human peripheral blood mononuclear cells (PBMC) were isolated from buffy coats (Blood bank, Aarhus University Hospital, Skejby, Denmark) of healthy donors using Ficoll-Paque PLUS (GE Healthcare BioScience AB, Uppsala, Sweden) with density gradient centrifugation according to the manufacturer's procedure. PBMC were cryopreserved in 90% heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich, Saint Louis, USA) supplemented with 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Saint Louis, MO, USA) at a concentration of 10^7 cells/mL and stored at −80°C until use.

2.3. Isolation of $CD14^+$ Monocytes by EasySep Negative Selection. For the isolation of monocytes, PBMC were rapidly thawed and resuspended in phosphate-buffered saline (PBS) supplemented with 2% FBS and 1 mM EDTA. From these cells, monocytes were purified by negative immunomagnetic depletion using the EasySep Human Monocyte Enrichment Kit according to manufacturer's instructions (cat. no 19059, Stemcell Technologies, Grenoble, France). In brief, cells were resuspended at a concentration of 5×10^7 in PBS (without Mg^{2+} and Ca^{2+}) + 2% FBS + 1 mM EDTA. First, cells were labeled with EasySep Human Monocyte Enrichment Cocktail for 10 min at 4°C, and then the EasySep D Magnetic Particles for Monocytes were incubated with the cell suspension for 5 min at 4°C. Finally, the suspension was placed into the EasySep Magnet, and the desired negatively selected untouched monocytes were collected. To increase purity, the magnetic isolation procedure was repeated once, and the harvested cells were kept cold. The purity of the untouched monocytes was evaluated by flow cytometry for CD14 expression. Cells were collected and labeled with FITC-conjugated mouse anti-CD14 monoclonal antibody (TÜK4, DAKO Denmark A/S, Glostrup, Denmark). Cell populations were analyzed on a Cytomics FC500 flow cytometer (Beckman-Coulter) and with FlowJo software (Tree Star, Ashland, USA). The purity ranged from 85 to 95% (data not shown).

2.4. Generation of Monocyte-Derived Dendritic Cells. The purified monocytes were washed twice and cultured in 6-well plates (Techno Plastic Products) at a density of 0.5–0.7×10^6 cells/mL in 1.2 mL of RPMI 1640 medium (Gibco, LifeTechnologies, UK) supplemented with 2% normal human serum type AB (NHS AB), 2 mM glutamine, and 20 μg/mL gentamicin (Gibco). The cultures were established in the presence of 100 ng/mL recombinant human GM-CSF (Bayer Healthcare AG, Leverkusen, Germany or Peprotech, London, UK) and 20 ng/mL recombinant human IL-4 (Immunotools GmbH, Friesoythe, Germany). The plates were incubated at 37°C with 5% CO_2 for six days. On day 3, the cultures were supplemented with 360 μL fresh medium supplemented with 100 ng/mL GM-CSF and 20 ng/mL IL-4. On day 5, 1.56 mL fresh medium supplemented with 100 ng/mL GM-CSF and 20 ng/mL IL-4 was added to the cultures together with 0, 20, or 100 nM of 1α,25-dihydroxyvitamin D_3 (Sigma-Aldrich) as indicated. On day 6, either mock (medium), bacterial LPS (*E. coli* O111:B4, LPS ultrapure cat. no. tlrl-3pelps, InvivoGen, San Diego, USA) in concentrations of 0, 0.01, 0.1, 1, 10, or 100 ng/mL, or PGN (from *S. aureus*, cat. no. tlrl-pgnsa, InvivoGen) in concentrations of 0, 0.1, 0.5, 1, 5, or 10 μg/mL was added during the last 24 hrs of culture. On day 7, all supernatants were collected and frozen at −70°C and all cells were harvested for subsequent phenotypic and functional analyses.

2.5. Morphological Examination. In order to examine the morphological maturation of the cells, viability and morphology were evaluated by light microscopy (Leica Microsystems) of the cells in the 6-well plates at a magnification of ×10 or ×40 prior to harvest.

2.6. Flow Cytometric Analyses. Harvested cells were washed twice in PBS and resuspended in 100 μL PBS supplemented with 0.5% bovine albumin serum (BSA) and 0.1% sodium azide for staining. Cells were incubated for 10 min at room temperature with an Fc receptor blocking solution (BioLegend, San Diego, USA). Next, cells were incubated at 4°C for 30 min with the following monoclonal mouse-anti-human antibodies (mAb): anti-HLA-DR FITC (L243, BD Biosciences), anti-CD14 FITC (TÜK4, DAKO), anti-CD40 PE (5C3, BD Biosciences), anti-CD80 PE (MAB104, Beckman Coulter), anti-CD83 PE-Cy5 (HB15e, BD Biosciences), and anti-CD86 PE-Cy5 (2331 FUN-1, BD Biosciences). After staining, cells were washed twice in PBS supplemented with 0.5% BSA and 0.1% sodium azide and resuspended in PBS with 0.99% paraformaldehyde. Flow cytometry was performed on a Cytomics FC-500 flow cytometer (Beckman Coulter), and all subsequent analyses were made in FlowJo software (Tree Star).

2.7. Quantification of Cytokines. For cytokine assessment, supernatants from DC cultures were thawed and centrifuged shortly and the content of IL-6, IL-8, IL-10, and IL-12p70

was measured with enzyme-linked immunosorbent assay (ELISA). Concentrations of IL-6, IL-8, IL-10, and IL-12p70 were measured using DuoSet ELISA kits (R&D Systems) according to the manufacturer's instructions. The spectrophotometer Versamax ELISA microplate reader (Molecular Devices, LLC, Sunnyvale, USA) and Softmax Pro software (Molecular Devices) were used to measure and analyze the samples.

2.8. Western Blotting Analysis/Cell Lysis and Immunoblotting. Whole-cell extracts were prepared using 1x lysis buffer (Cell Signaling Technology, Beverly, MA, USA) supplemented with 1 mM PMSF, 5 mM NaF, and Complete Mini Protease Inhibitor (Roche Diagnostics, Basel, Switzerland) at a concentration recommended by the manufacturer. Lysates were centrifuged at 2.600 ×g for 5 min, followed by 20.000 ×g for 10 min, and whole-cell extracts were immediately frozen at −70°C. Proteins were separated in XT Criterion 12% gels (BioRad Laboratories Inc, Hercules, CA, USA) using XT MOPS running buffer (Bio-Rad) for 1 h and 30 min at 175 V and subsequently transferred to nitrocellulose membranes for 1 h and 45 min at 300 mA. Detection of VDR was performed using anti-VDR mAb (sc-13133) (Santa Cruz Biotechnology Inc, Dallas, TX, USA) diluted 1 : 1000, and GAPDH was detected using anti-GAPDH antibody (Santa Cruz Biotechnology) diluted 1 : 2000. The secondary antibodies were horseradish peroxidase-conjugated rabbit anti-mouse or swine anti-rabbit antibody (Dako) diluted 1 : 2000. All antibodies were diluted in 5% skimmed milk in TBS with 0.1% Tween-20. Ponceau-S staining was performed as a loading control. Immunoblots were developed using Super Signal West Femto Maximum Sensitivity Substrate (Thermo Scientific, Rockford, IL, USA).

3. Results

3.1. LPS Induces Rapid Maturation of DC, Which Is Inhibited by Vitamin D_3. As expected, vitamin D_3 added 24 hrs prior to LPS treatment inhibited LPS-induced maturation measured by the expression of surface molecules 24 hrs after the addition of LPS (Figure 1(a)). Conversely, vitamin D_3 prevented the downregulation of CD14 expression, indicating a block in maturation of immature DC (Figure 1(a)). To further explore the kinetics, cells were examined 4 hrs after addition of LPS. In the absence of vitamin D_3, DC had already upregulated CD83 and downregulated CD14 after 4 hrs of LPS treatment, whereas only minor or no changes were observed for HLA-DR, CD80, CD86, and CD40. Thus, upregulation of CD83 appears to be an early event during DC maturation. The presence of vitamin D_3 partially inhibited upregulation of CD83 almost to the same degree as observed after 24 hrs.

The early induction of DC maturation was morphologically visible as dendritic formations as early as 4 hrs after the addition of LPS, but more pronounced after 24 hrs. The presence of vitamin D_3 inhibited the outgrowth of these processes (Figure 1(b)).

3.2. Vitamin D_3 Is Necessary for LPS-Induced Secretion of IL-6, IL-8, and IL-10. The presence of vitamin D_3 inhibited the maturation of differentiated DC. To examine whether vitamin D_3 influenced the cytokine secretion induced by LPS, differentiated DC were treated with various concentrations of LPS and monitored for the secretion of IL-6, IL-8, and IL-10 in the absence or presence of either 20 or 100 nM vitamin D_3. These concentrations of vitamin D_3 did not induce cytokine secretion from DC. Although LPS induced a dose-dependent secretion of IL-6, IL-8, and IL-10, the presence of vitamin D_3 was able to further enhance this secretion (Figures 2(a)–2(c)). Importantly, at low doses of LPS, cytokines were only induced in the presence of vitamin D_3, despite the fact that neither LPS alone nor vitamin D_3 alone induced measurable cytokines. These data demonstrated that vitamin D_3 acted in synergy with LPS to induce the secretion of IL-6, IL-8, and IL-10.

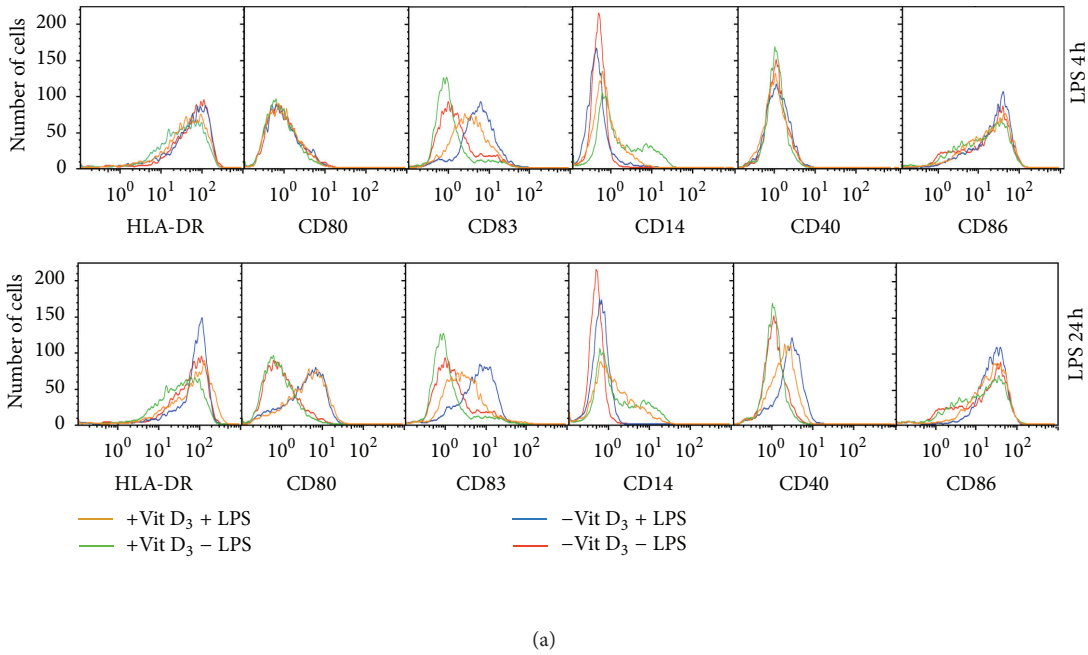
3.3. Vitamin D_3 Inhibits LPS-Induced IL-12 Secretion. Since vitamin D_3 is thought to inhibit the proinflammatory response, we next examined whether IL-12, a cytokine that promotes a Th1-like response, was affected by vitamin D_3. The presence of LPS at concentrations above 1 ng/mL induced high levels of IL-12p70 (Figure 2(d)). In the presence of either 20 or 100 nM vitamin D_3, the detection of LPS-induced IL-12p70 was completely abolished. This demonstrated that vitamin D_3 is a potent inhibitor of IL-12.

3.4. Vitamin D_3 Is Necessary for PGN-Induced Secretion of IL-6, IL-8, and IL-10. To test whether LPS was the only TLR ligand able to induce cytokines and act in synergy with vitamin D_3, differentiated DC were treated with the TLR2 agonist PGN. PGN was able to induce high levels of IL-8 and low levels of IL-6 and IL-10 at high concentrations of PGN (Figures 3(a)–3(c)). However, in the presence of vitamin D_3, PGN-induced cytokine secretion from differentiated DC was synergistically increased for IL-6, IL-8, and IL-10 (Figures 3(a)–3(c)). In contrast to LPS, PGN did not induce IL-12 (Figure 3(d)). This indicated that PGN, similar to LPS, induced cytokine secretion from DC in synergy with vitamin D_3.

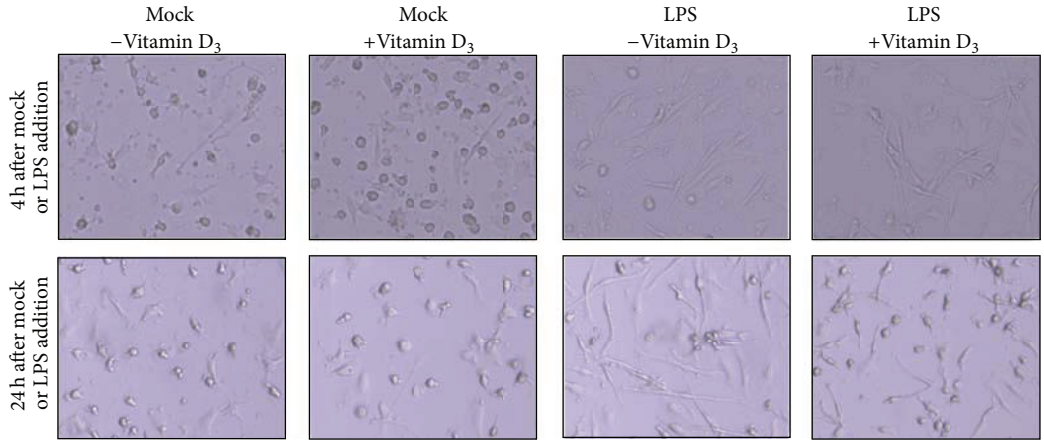
3.5. LPS and PGN Enhance Expression of the Vitamin D Receptor. Vitamin D_3 exerts its function after binding to an intracellular receptor, VDR. To examine whether LPS could increase the sensitivity of DC for vitamin D_3, the protein level of VDR was measured by western blotting. In the absence of vitamin D_3 or TLR agonists, a low level of VDR was detectable (Figure 4). However, the presence of LPS or PGN increased the level of VDR, which was not further affected by the presence of vitamin D_3 (Figure 4). This indicated that LPS or PGN was able to enhance the sensitivity of DC for vitamin D_3 by increasing the expression of its receptor.

4. Discussion

The generation of Treg is in part controlled by the maturation of DC. In mice, treatment with vitamin D_3 induces a regulatory T-cell profile with increased expression of IL-10, TGF-β, FoxP3, and CTLA-4 and a significant reduction of IL-12p70, IL-23p19, IL-6, and IL-17 [22]. We also observed



(a)



(b)

Figure 1: Vitamin D_3 inhibits LPS-induced DC maturation. (a) DC were differentiated in the presence or absence of vitamin D_3 and matured with LPS for 4 hrs (upper panel) or 24 hrs (lower panel) prior to flow cytometric analysis. DC were treated without vitamin D_3 (vit D) and LPS (red curve), without vitamin D_3 and with LPS (blue curve), with vitamin D_3 and without LPS (green curve), and with both vitamin D_3 and LPS (yellow curve) and were stained with fluorescence-conjugated antibodies HLA-DR(FITC), CD80(PE), CD83(PC5), CD14(FITC), CD40(PE), and CD86(PC5). Histograms represent collection of 5,000 events. (b) Morphology of the DC was examined by light microscopy at ×10 magnification at 4 and 24 hrs after mock or LPS treatment. Experiments are representatives of at least two independent experiments.

that vitamin D_3 promoted a regulatory profile, promoting IL-10 and inhibiting IL-12p70. Surprisingly, we found that in synergy with LPS or PGN, vitamin D_3 increased IL-6 in cultures of differentiated DC. Whether the detection of IL-6 indicates a pro- or an anti-inflammatory profile is, however, not clear. IL-6 may be associated with a soluble form of the IL-6 receptor and this complex may mediate proinflammatory reactions through a process known as transsignaling [23]. Blockade of IL-6 transsignaling completely protected $gp130^{F/F}$ knock-in mutant mice from LPS hypersensitivity, suggesting cross-talk between JAK/STAT and TLR pathways [24]. On the other hand, the classical binding of IL-6 to gp130/IL-6 receptor complexes on the cell surface may promote anti-inflammatory/regenerative reactions [23]. Thus, further investigations may elucidate which one of these scenarios is supported by vitamin D_3.

Importantly, we found that, at very low levels of LPS, vitamin D_3 promoted the secretion of IL-8 in synergy with LPS. That is, neither the concentration of 0.01 ng/mL of LPS nor the presence of vitamin D_3 alone was able to induce this cytokine. However, when added together, vitamin D_3 and LPS acted in synergy and induced in the order of 2–4 ng of IL-8. IL-8 is a major chemoattractant for the recruitment of polymorphonuclear leukocytes that serve as part of the first

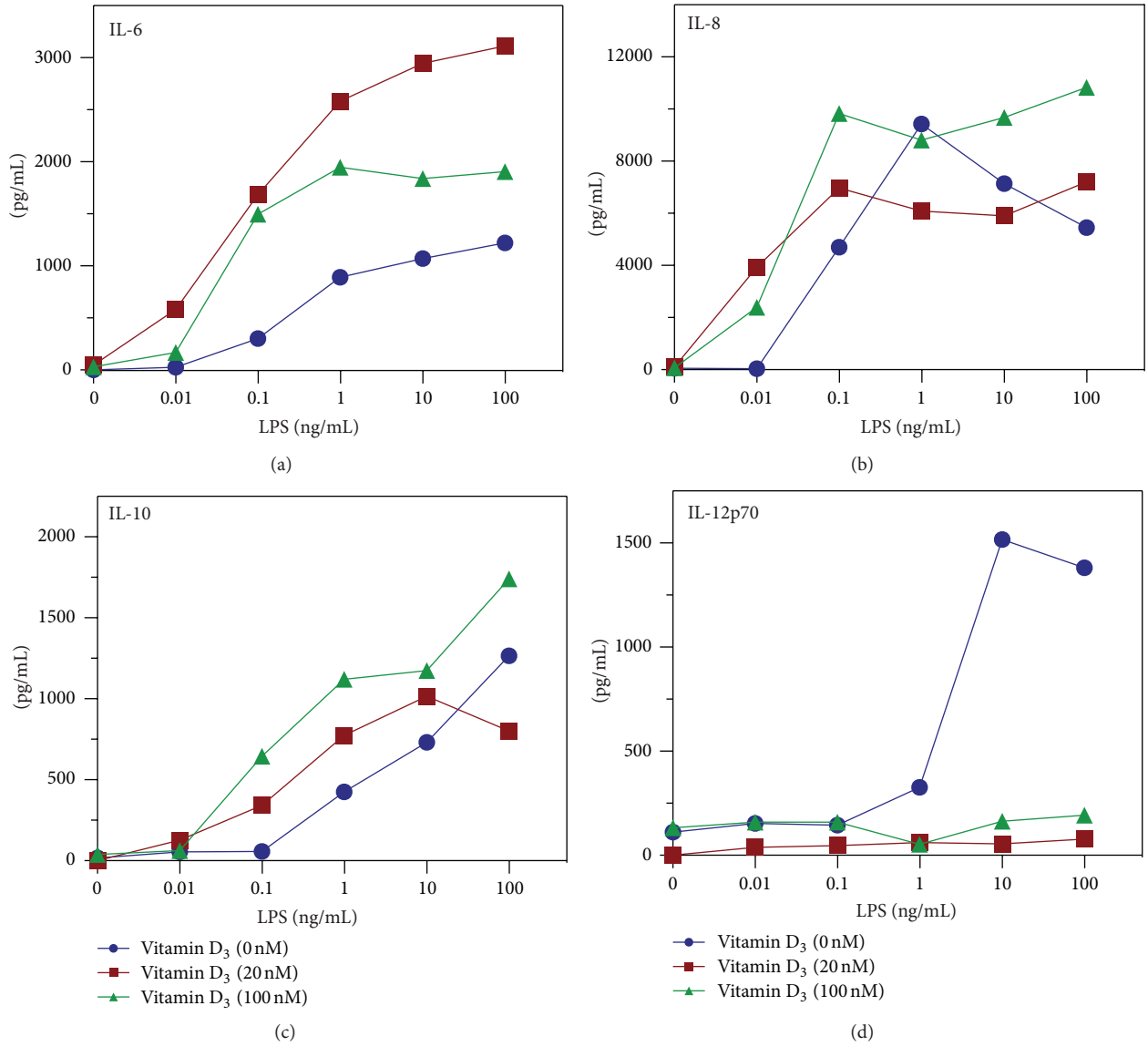


FIGURE 2: Vitamin D_3 is necessary for LPS-induced secretion of IL-6, IL-8, and IL-10. DC were stimulated with 0, 20, or 100 nM of vitamin D_3 (vitamin D) and 10-fold dilutions of LPS (from 100 to 0.01 ng/mL). Cell-culture supernatants were assessed for IL-6 (a), IL-8 (b), IL-10 (c), and IL-12 (d) by ELISA. Experiment is representative of at least two independent experiments.

line of defense against intruding bacteria. This emphasizes that besides its effect on the adaptive immune system, vitamin D_3 also has important functions for the innate immune response.

IL-8 has previously been shown to be induced by the transformed cell line THP-1 in the presence of vitamin D_3 and agonists of TLR2, TLR3, TLR4, NOD1, and NOD2 [25]. However, other studies have reported that vitamin D_3 inhibited IL-8 production in response to IL-1α in human peripheral blood mononuclear cells, keratinocytes, and fibroblasts [26, 27], although TLR and IL-1R are both signals through MyD88. Inhibition of IL-8 by vitamin D_3 has also been observed in primary cultures of human periodontal ligament cells stimulated with *P. gingivalis*, a Gram-negative bacteria containing LPS [28]. Thus, the cell type, the presence of other cytokines, and potentially virulence factors from microorganisms may all be important for modulating the control exerted by vitamin D_3 on IL-8 secretion.

Although LPS is frequently used to mature monocyte-derived human DC, other TLR agonist may serve the same functions. LPS is a component of the outer membrane of the Gram-negative bacterial cell wall, whereas Gram-positive bacteria are characterized by a cell wall containing PGN, a ligand for TLR2. In agreement with the observed functions of LPS, PGN also acted in synergy with vitamin D_3 for the secretion of IL-6, IL-8, and IL-10 but was unable to induce IL-12p70 as did LPS. This indicates that PGN from Gram-positive bacteria may also have the ability to prime the response towards a regulatory T-cell profile and that PGN also induces chemoattractants for recruiting polymorphonuclear leukocytes.

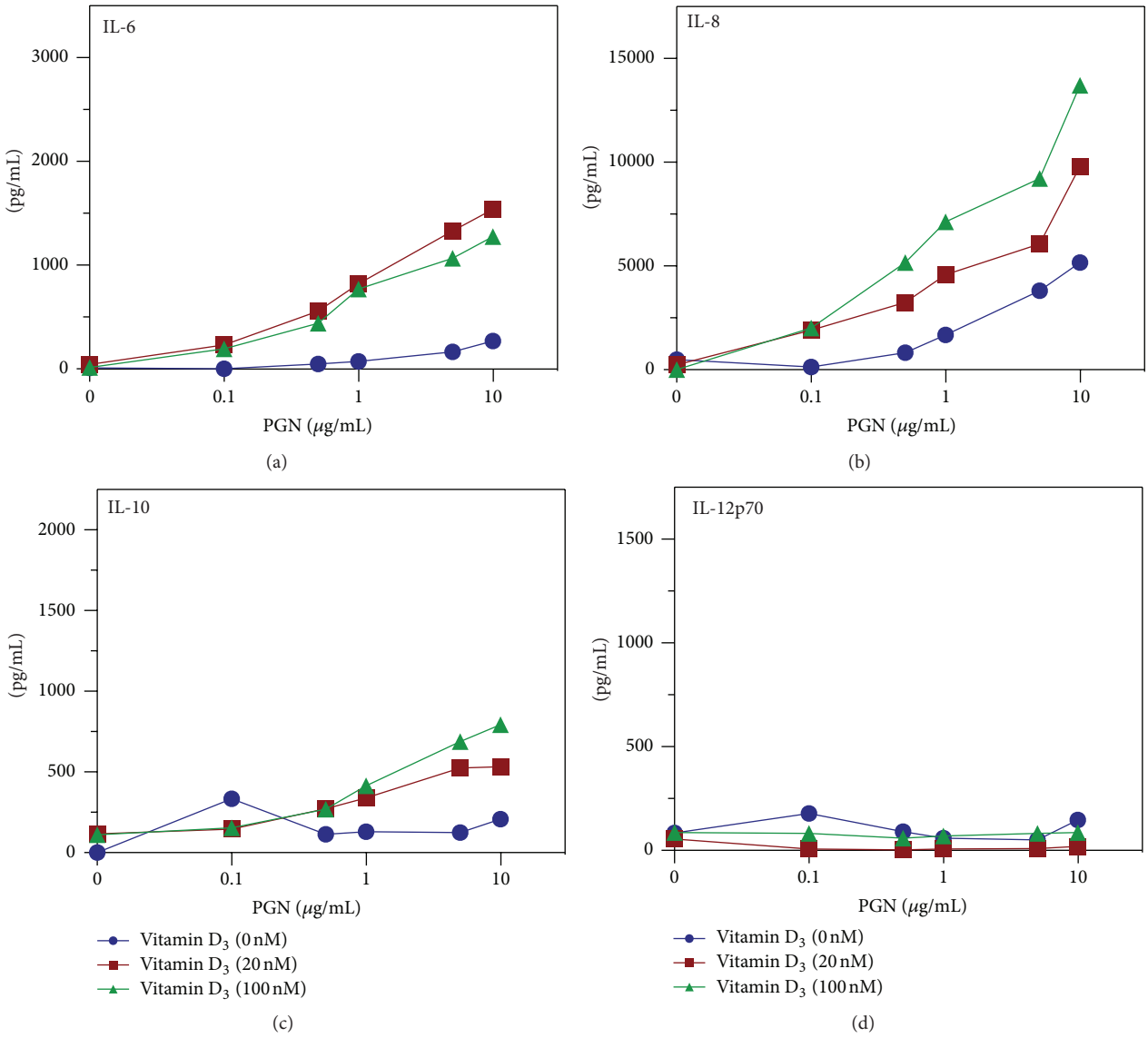


FIGURE 3: Vitamin D_3 is necessary for PGN-induced secretion of IL-6, IL-8, and IL-10. DC were stimulated with 0, 20 or 100 nM of vitamin D_3 and dilutions of PGN (from 0.1 to 10 μg/ml). Cell-culture supernatants were assessed for IL-6 (a), IL-8 (b), IL-10 (c), and IL-12 (d) by ELISA. Experiment is representative of at least two independent experiments.

The level of VDR in part determines the sensitivity of DC for vitamin D_3. Liu et al. found that the *M. tuberculosis*-derived lipopeptide, which is a TLR2/1 ligand, induced the expression of VDR. In addition, they observed that vitamin D_3 upregulated the antimicrobial peptide cathelicidin, which is important for the killing of intracellular bacteria [19]. However, since most bacteria are extracellular, we examined the general Gram-negative and Gram-positive TLR ligands: LPS (TLR4) and PGN (TLR2). We found that these TLR ligands were also able to increase the level of VDR. The shifting by vitamin D_3 of the immune response towards a regulatory profile as opposed to a proinflammatory response may be of benefit for the invading microorganisms. In this view, the upregulation of VDR by both LPS and PGN may further enhance the effect of vitamin D_3 and thus further prevent the antimicrobial defense. The inhibition of IL-8 induction by *P. gingivalis* [28], as mentioned previously, further indicates that the microorganism may have evolved mechanisms to avoid part of these host innate responses. Whether this is a general feature remains to be investigated. Conversely, we speculate that these effects of vitamin D_3 may also prevent unnecessary inflammatory reactions towards commensal microorganisms present in the gut. The shifting towards a regulatory profile by vitamin D_3 may also be of benefit during graft-versus-host disease (GVHD) [29]. However, whether the anti-inflammatory actions of vitamin D_3 can be enhanced in an *in vivo* setting by the addition of low levels of TLR agonists remains to be examined.

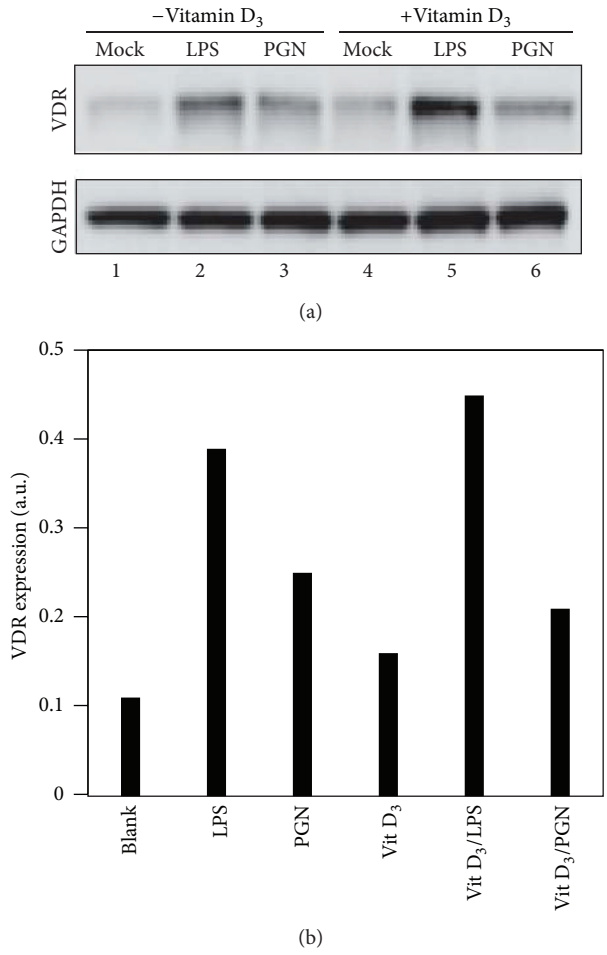


FIGURE 4: LPS and PGN act in synergy with vitamin D_3 to enhance expression of the vitamin D receptor. (a) The levels of VDR protein in DC were measured by the western blotting of whole-cell lysates from DC cultures stimulated with either mock, 10 ng/mL LPS, or 10 μg/mL PGN in the absence or presence of 100 nM vitamin D_3. GAPDH was used as a loading control. (b) The band intensity in (a) was quantified and normalized to GAPDH as a VDR/GAPDH ratio (VDR expression).

5. Conclusions

In conclusion, human DC maturation is inhibited by vitamin D_3. LPS and PGN (TLR4 and TLR2 agonists) increase the level of VDR and act in synergy with vitamin D_3 for induction of IL-6, IL-8, and IL-10, whereas LPS induction of IL-12 is inhibited by vitamin D_3. PGN does not induce measurable IL-12. Thus, vitamin D_3 synergizes with TLR agonists in modulating human DC cytokine secretion during maturation. This may generate an anti-inflammatory environment that favors the induction of regulatory cells.

Acknowledgment

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Review Article

Understanding Dendritic Cells and Their Role in Cutaneous Carcinoma and Cancer Immunotherapy

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Dendritic cells (DC) represent a diverse group of professional antigen-presenting cells that serve to link the innate and adaptive immune systems. Their capacity to initiate a robust and antigen-specific immune response has made them the ideal candidates for cancer immunotherapies. To date, the clinical impact of DC immunotherapy has been limited, which may, in part, be explained by the complex nature of DC biology. Multiple distinct subsets of DCs have been identified in the skin, where they can be broadly subcategorized into epidermal Langerhans cells (LC), myeloid-derived dermal dendritic cells (mDC) and plasmacytoid dendritic cells (pDC). Each subset is functionally unique and may activate alternate branches of the immune system. This may be relevant for the treatment of squamous cell carcinoma, where we have shown that the tumor microenvironment may preferentially suppress the activity of mDCs, while LCs remain potent stimulators of immunity. Here, we provide an in depth analysis of DC biology, with a particular focus on skin DCs and their role in cutaneous carcinoma. We further explore the current approaches to DC immunotherapy and provide evidence for the targeting of LCs as a promising new strategy in the treatment of skin cancer.

1. Introduction

Dendritic cells (DC) represent a small subset of immune cells that are derived from the bone marrow and are found in nearly every tissue in the human body [1]. Originally described by Steinman and Cohn in 1973 [2], these cells were found to play a critical role in linking the innate and the adaptive immune systems. This is achieved via the unique ability of DCs to sample the surrounding environment and transmit the collected information to T and B cells of the adaptive immune system [3]. DCs are considered to be professional antigen-presenting cells based on their ability to present antigen in the context of MHC class II and costimulatory molecules. They are, therefore, extremely efficient stimulators of immunity and are thought to be key players in initiating the body's immune response.

DC immunity often begins in the peripheral tissues such as the skin, where sentinel cells containing non-clonal recognition receptors will respond to specific pathogen-associated molecular patterns (PAMPs) with the secretion of protective cytokines [4]. Alternatively, peripheral DCs may ingest and process foreign antigens, followed by migration through the afferent lymphatics to the nearby lymph nodes. Antigen-derived peptides will then be loaded onto a major histocompatibility complex (MHC) for presentation to naive T cells in the lymphoid tissue [1]. Binding of T cells to the MHC-antigen complex and costimulatory molecules on the DC surface results in the activation and subsequent differentiation of T cells into effector cells capable of launching an antigen-specific response. This process is thought to be highly efficient, with only small numbers of DCs required to launch a large and successful immune attack [5]. Furthermore, nonactivated, immature DCs will also contribute to immune function through the constitutive presentation of self-antigen. Interaction with these DCs will trigger T cell deletion and the differentiation of regulatory or

suppressor T cells, which effectively limits immune reactivity and generates self-tolerance. This ensures a well-controlled and targeted immune response which is limited to foreign invaders [6].

The potential for DCs to amplify immune function in an antigen-specific manner makes them ideal candidates for cancer immunotherapy, which attempts to eradicate tumors through the manipulation of the body's own innate immune mechanisms [7]. Mouse models have demonstrated DC tumor presentation to be an essential step in the generation of antitumor immunity; however, tumor cells themselves have been found to be poor antigen presenters [8]. Accordingly, many different DC vaccination strategies have been developed thus far, with the aim of inducing tumor-specific effector T cell responses. This may not only reduce tumor cell mass, but could also generate immunological memory, thereby preventing tumor cell relapse [9]. Such therapies may prove to be of particular importance in skin cancers, given the role of skin as a barrier to foreign invasion and the high prevalence of DCs found within the dermal and epidermal tissue [10]. Unfortunately, current approaches to DC vaccination in the treatment of human neoplasms have been largely unsuccessful. In order to better elucidate the possible mechanisms for vaccine failure, and to move forward with more effective immunotherapies, a comprehensive understanding of DC biology and its relationship to immune reactivity is required. The purpose of this paper is hence twofold: to provide an in depth analysis of DC biology, with a particular focus on skin DCs and their role in nonmelanoma skin cancers, and to highlight the various therapeutic strategies and future directions of DC immunotherapy.

2. DC Biology and Plasticity

The ability of DCs to interact with foreign antigens and initiate an immune response highlights their role as gatekeepers of the immune system. Moreover, the particular origin of a given DC, and the precise nature of a T cell interaction, can elicit a distinct pattern of differentiation [7]. For instance, DCs that reside in the lymph node typically present antigen to naive CD4+ T cells, which in turn will stimulate the production of interleukin-2 (IL-2) resulting in clonal expansion. In contrast, peripherally located DCs will often present antigen to already activated CD4+ cells, leading to the generation of effector cells [7].

The majority of DCs originate from CD34+ hematopoietic stem cells in the bone marrow, before entering into the circulation and depositing as immature cells in target tissues. These generally include the sites of antigen entry, such as the skin and the lining of the GI tract [11]. Alternatively, DCs may arise from monocyte precursors during times of physiologic stress. As mentioned previously, immature DCs can bind T cells and may induce immune tolerance through T cell deletion or the expansion of regulatory or suppressor T cells. They are also highly efficient at antigen recognition and processing through specialized pathogen recognition receptors (PRR) located on their cell surfaces, which respond to a variety of PAMPs [12]. Additionally, immature DCs are characterized by high levels of MHC class II molecules, which are accumulated in endosomal compartments, to facilitate antigen loading and transport to the cell surface. They also express low levels of chemokine receptors, such as CCR7, which mediate migration to the nearby lymph nodes [13].

Immature DCs will respond to distinct environmental signals and undergo a highly regulated maturation process resulting in activated cells capable of launching an immune response. This process is associated with the downregulation of the DC's antigen-capture capabilities, as well as increased surface expression of MHC II and other costimulatory molecules [13]. Particularly, the cross-linking of costimulatory molecule CD 40 is thought to be an essential stimulus for further DC maturation [14]. Mature DCs will also have an increased ability to secrete chemokines in order to attract naive T and B cells, and release specific cytokines to activate those cells bound to the antigen-MHC complex on their surface. Additionally, mature DCs are typically found in the lymph nodes following the acquisition of CCR7 [15].

While the aforementioned changes occur as necessary elements in the DC maturation process, this does not imply that maturation itself gives rise to a homogenous DC phenotype. In fact, different environmental signals produced by various microbes and nearby immune cells may contribute to the induction of unique DC phenotypes, which will ultimately shape the nature of the immune response [16]. For example, while most microbes activate DCs through PRRs, certain microbes and their associated PAMPs will actually block DC maturation. Similarly, cytokines secreted by mast cells and natural killer (NK) cells found in the tissue microenvironment may stimulate the maturation of distinct inflammatory DCs which give rise to unique T cell populations [17]. Thus depending on the cellular origins and location of an immature DC, the surrounding microenvironment, and the precise nature of a given maturation signal, multiple different DC subsets can develop which will have their own particular effect on the immune cell population and corresponding immune response.

3. DC Subsets in Skin

Current research has identified four main subsets of DCs found in the human skin, which can be distinguished based on the differential expression of surface molecules in the steady state. These include the following: (1) CD1a+, CD207(Langerin)+ epidermal Langerhans cells; (2) CD11c+, CD1c(BDCA-1)+, CD14− dermal DCs; (3) CD11c+, CD1c+, CD14+ dermal DCs; and (4) CD11c−, CD303(BDCA-2)+ plasmacytoid DCs [18, 19] (Figure 1). In addition, a fifth DC subset has recently been identified in human skin, known as CD141+(BDCA-3)+, XCR1+ DCs [20, 21]. For the purpose of this paper, we will briefly discuss each subset, with a focus on the unique role it may play in shaping the immune response.

3.1. Langerhans Cells (LCs). LCs are generally found in the basal and supra-basal layers of the epidermis, where they form a dense network of cells which account for approximately 2–4% of the total epidermal cell population [22, 23]. They are characterized by a unique cytoplasmic organelle known as a Birbeck granule. While the precise function of

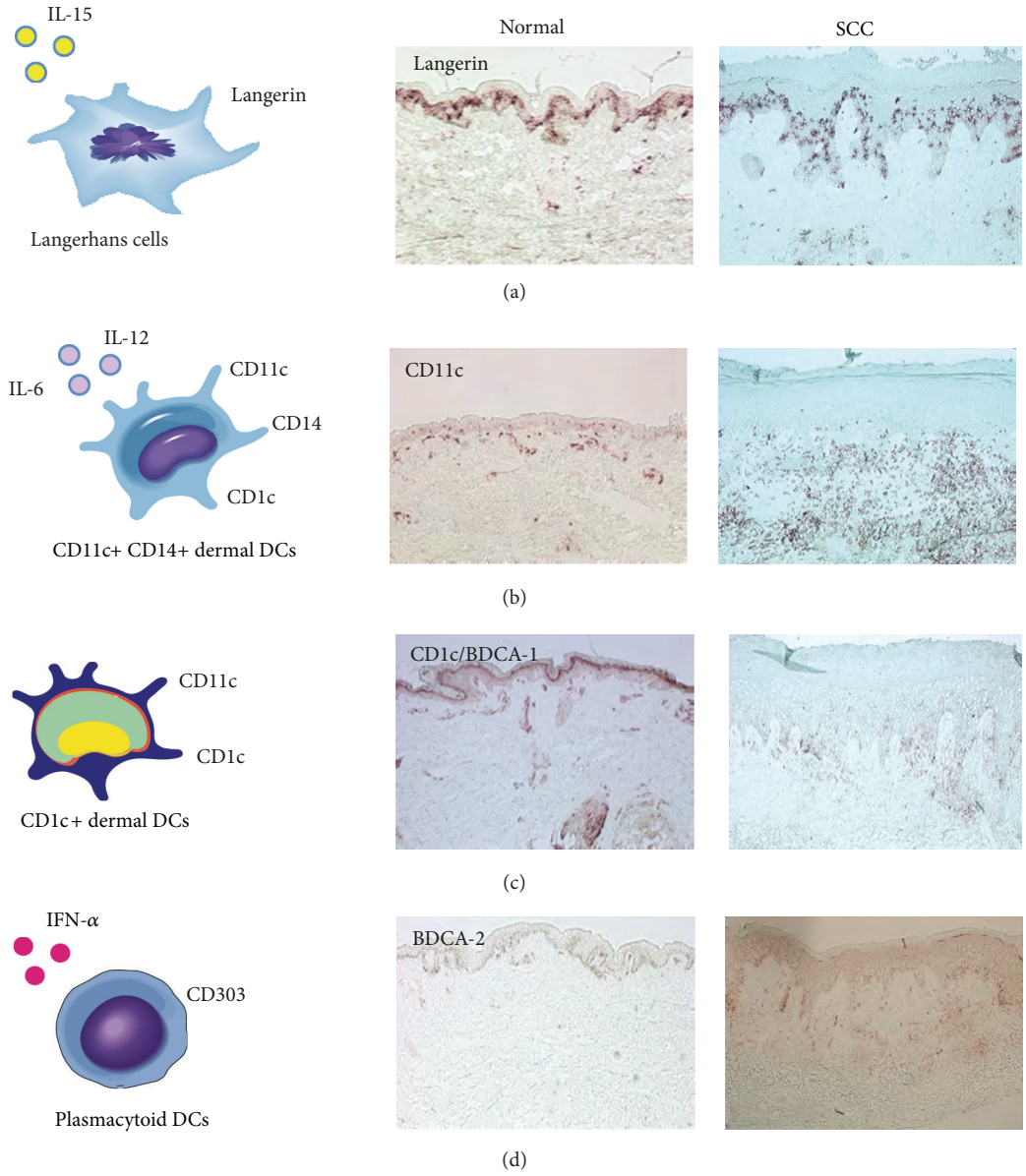

Figure 1: The distribution of cutaneous dendritic cell subsets in normal skin versus human squamous cell carcinoma (SCC). The human skin contains four main subsets of DCs which can be distinguished based on the differential expression of surface molecules in the steady state (left). Representative immunohistochemistry (right) demonstrating the relative distribution of DC subset markers: (a) CD207/Langerin, (b) CD11c, (c) CD1c/BDCA-1, and (d) CD303/BDCA-2.

Birbeck granules remains unclear, they are thought to play a role in receptor-mediated endocytosis and the transport of cellular materials into the extracellular space [24]. Another hallmark of LCs is the expression of a specific lectin molecule, namely Langerin/CD207, which is capable of binding sugar moieties commonly found on a variety of microorganisms [10]. Although the vast majority of LCs are thought to be derived from bone marrow precursors, recent studies have identified a novel pathway by which CD14+ cells resident in the dermis will acquire LC features following treatment with TGF-β [25, 26]. LC differentiation may thus be somewhat dependent on the cytokine microenvironment of the skin at a given point in time.

LCs are thought to be key players in the initiation of cellular immunity through the stimulation of a predominantly CD8+- or NK-cell-mediated response (Figure 2(a)). LCs express a distinct set of toll-like receptors (TLRs; TLR 1, 2, 3, 6, and 10) which, when activated, result in the secretion of IL-15, a cytokine known to preferentially drive the proliferation of CD8+ T cells [27–29]. Additionally, LCs are capable of cross-presenting foreign antigens to CD8+ T cells with a greater degree of efficiency when compared to the other DC subsets, resulting in a more robust proliferation of naive CD8+ T cells [30]. This is thought to be an essential step in the initiation of a highly specific cytotoxic T cell (CTL) response, which may be critical for targeting cancer cells.

In addition to their effect on CD8+ T cells, LCs may play a secondary role in the polarization of naive CD4+ T cells towards a Th2 predominant immune response through the secretion of type 2 cytokines such as IL-4, IL-5, and IL-13 [27].

Additionally, we have recently shown that LCs from normal human skin are capable of inducing distinct IL-22 producing CD4+ T cells (Th22) from naive CD4+ T cells *in vitro* [31]. IL-22 is thought to act mainly on epithelial cells as a key mediator of keratinocyte proliferation and epidermal hyperplasia. Additionally, we demonstrated LCs from human SCC can effectively stimulate Th1 and CD8+ T cell line expansion, which may be beneficial to antitumor immunity [32]. We are currently actively engaged in studies concerning LC-mediated T cell polarization and discovering novel means of harnessing these effects to bolster anticancer immunity.

3.2. Myeloid Dendritic Cells (mDCs). MDCs are usually found within the extracellular matrix, in the upper portion of the reticular dermis. In the past, mDCs were typically identified using an intracellular marker known as coagulation factor XIIIa. Recent studies, however, have shown this molecule to be more commonly associated with dermal macrophages [33]. Currently, mDCs are thought to be best characterized by the presence of the transmembrane integrin molecule CD11c, found at high levels on almost all human mDCs [33]. Myeloid DCs are often grossly subdivided into 2 populations based upon the differential expression of surface markers CD1c (BCDA-1) and CD14 [18, 34]. Each population is thought to represent a distinct entity with its own particular function within the greater immune environment.

The most common mDC subtype found in normal human dermis can be identified through the use of the monoclonal antibody CD1c, also known as blood dendritic cell antigen (BDCA)-1. CD1c+ CD14− DCs are thought to be relatively immature cells that are capable of inducing only a mild T cell response [19]. Following the appropriate maturation signals, however, the immunostimulatory capacity of CD1c+ CD14− cells is greatly increased. Additionally, CD1c+ CD14− cells are thought to have a heightened sensitivity for the expression of surface receptor molecule CCR7 in response to foreign antigen detection, as well as an increased capacity for migration to nearby lymph nodes in response to the lymph node chemokine CCL19 [35]. Recently it has been suggested that in the resting state, CD1c+ CD14− DCs may in fact be tolerogenic [19].

For their part, CD1c+ CD14+ DCs are thought to be critical in the regulation of humoral immunity (Figure 2(c)). They express a novel combination of TLRs (TLR 2, 4, 5, 6, 8, and 10) which respond mainly to bacterial PAMPs, triggering the release of IL-6 and IL-12. This, in turn, serves to stimulate CD40-activated naive B cells resulting in the secretion of large amounts of IgM [30]. Additionally, only CD4+ T cells primed by CD14+ DCs are able to induce isotype switching in naive B cells. The production of mature plasma cells with antigen-specific IgG and IgA is therefore largely dependent on CD14+ DC interaction [36]. Similarly, CD4+ T cells primed by CD14+ DC will secrete high levels of CXCL13, a chemokine which promotes the homing of B cells to the follicular center [30].

More recently, CD14+ DCs have also been shown to induce the differentiation of a novel CD4+ T cell subtype, the IL-21 producing T follicular helper cell (Tfh). This is thought to be mediated by the release of IL-12 from CD14+ DCs, which polarizes naive T cells towards Tfh. These cells are typically found within the B cell follicle and are thought to play a role in the antigen-specific activation of naive or memory B cells. This results in the formation of unique antigen-associated germinal centers, which facilitate the transformation of B cells into high-affinity antibody secreting cells [37].

Additionally, we have studied the function of mDCs taken from human SCC and normal human skin and have found that SCC-associated mDCs were poor stimulators of T cell proliferation. This was true despite the fact that these cells demonstrated a mature phenotype, evidenced by the expression of cell surface molecules MHC II, CD80, CD83, and CD86 [38]. This effect may in part be explained by increased levels of the immunosuppressive cytokines TGF-β, IL-10 and VEGF-A, found in the tumor microenvironment. Alternatively, we have also shown that the SCC microenvironment is associated with an increased percentage of Foxp3 regulatory T cells (Tregs), which may directly inhibit the function of CD4+ and CD8+ effector T cells. Furthermore, DCs cocultured with Tregs have been shown to downregulate the expression of costimulatory molecules, which may impair their ability to stimulate T cell proliferation, thereby contributing to immune system dysfunction [39–41].

3.3. Plasmacytoid DCs. PDCs represent an additional population of resident dermal DCs initially identified based on their morphologic similarity to plasma cells [42]. They are often considered to be the primary foot soldiers of the innate immune system, due to their tremendous potential to produce interferon-α (IFNα) in response to viral invasion [43] (Figure 2(b)). They are characterized by the elevated expression of cell surface marker CD303 (BDCA-2), as well as a distinct set of TLRs, TLR7, and TLR9, which are specialized in the detection of viral components [44]. Furthermore, pDCs contain large stores of MHC class I molecules which enable rapid activation of CD8+ T cells to target viral antigens. PDCs are also thought to contribute secondarily to the induction of plasma cells from activated B cells, as well as the generation of immune tolerance [45, 46]. While the precise role of skin-resident pDCs has yet to be fully elucidated, recent studies have found these cells to be upregulated in the presence of cutaneous carcinomas, particularly in the juxtatumoral dermis, within 100 microns of SCC nests [38, 47]. This may reflect the importance of pDCs in the generation of a functional antitumor response, making them ideal candidates for future immunotherapy efforts.

3.4. CD141+(BDCA-3)+, XCR1+ DCs. Currently, a novel subtype of human mDC has been identified in the skin, known as CD141+, XCR1+ DCs [20, 21, 48, 49]. These cells are marked by the unique coexpression of both CD141 (BDCA-3) and XCR1; however their precise phenotypic characterization remains somewhat unclear. For instance, Chu et al. have

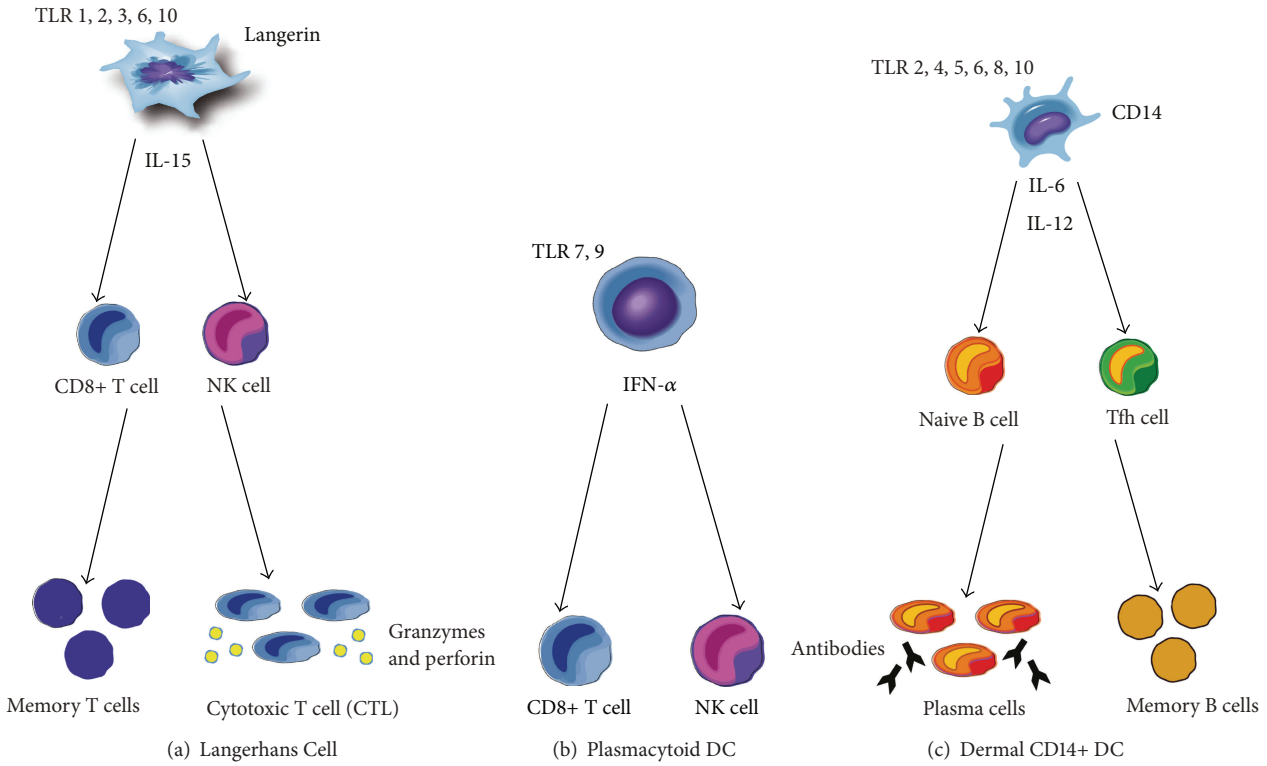

Figure 2: Different DC subsets have unique roles in shaping the immune response. (a) LCs are key mediators of cellular immunity and will preferentially activate CD8+ T cells and NK cells through the secretion of IL-15. LCs are also capable of cross-presenting foreign antigen, which results in a robust proliferation of naive CD8+ T cells and the generation of a highly specific cytotoxic T cell (CTL) response. This may contribute to the formation of immunologic memory. (b) PDCs respond to foreign antigen with the release of large amounts in IFN-α. This serves to activate a predominantly CD8+ and NK cell response. (c) Dermal CD14+ DCs are critical in the regulation of humoral immunity. When activated, they secrete IL-6 and IL-12, which promotes IgM secretion from naive B cells and the generation of mature antibody-secreting plasma cells. IL-12 will also trigger the differentiation of T follicular helper cells (Tfh) from CD4+ T cells, which may contribute to the formation of memory B cells.

shown CD141+ DCs to be both CD11c and CD14 positive, and CD1a negative, with intermediate levels of CD1c expression [20]. In contrast, Haniffa et al. demonstrated these cells to be CD14 and CD207 negative, with low levels of CD1c, and low to intermediate expression of both CD11c and CD1a [48]. Although the exact nature of this apparent discrepancy has yet to be fully elucidated, one possible explanation includes the dramatically different mechanism of DC collection used in either study. Alternatively, these studies may indeed be describing two distinct subsets of CD141+ DCs, which may be present concurrently in the skin.

Despite these phenotypic differences, CD141+ DCs, on a whole, are thought to possess a critical and robust ability to cross-present both self- and foreign antigen [20, 48, 49]. They have been shown to play a key role in the activation of CD25+ Tregs through both the presentation of self-antigen and the secretion of high levels of IL-10, a known immunosuppressive cytokine [20]. They may therefore be essential players in the maintenance of tissue homeostasis and the induction of immune tolerance [20]. Conversely, CD141+ DCs have also been shown to effectively cross-present soluble antigen in pathological states. This results in the generation of a powerful pro-inflammatory response [48, 49]. CD141+ DCs may therefore serve a dual role in the promotion of immune tolerance in the resting state, as well as the stimulation of immune activity in response to foreign invasion. To date, the presence and function of CD141+ DCs in the cancer microenvironment has yet to be fully explored. Further research is thus needed in order to better understand the role of CD141+ DCs in immune function, and their potential impact on cutaneous carcinomas.

4. DCs and the Tumor Microenvironment

DCs can be found in almost all human tumors, and their ability to uptake antigen and initiate an aggressive immune response makes them attractive targets for cancer immunotherapies. Moreover, while the immune system has the innate ability to recognize and attack cancer cells, tumors often evade detection by downregulating antigen presentation and impairing DC function [7]. The effective restoration of DC activity may therefore prove critical in successful tumor detection and the generation of a potent antitumor response.

Tumors are thought to impair antigen presentation and the establishment of a tumor-specific immune response

through a variety of mechanisms. For instance, tumor cells often secrete IL-6 and macrophage colony-stimulating factor (M-CSF), which may shift the differentiation of monocytes towards macrophages rather than DCs. This effectively inhibits the priming of tumor-specific T cells [50]. Furthermore, tumor cells may interfere with DC maturation through the secretion of IL-10, which results in the induction of antigen-specific anergy [51]. Tumor-derived factors have also been shown to alter the maturation pathway of DCs to produce cells which indirectly promote tumor growth. This can be accomplished through the expression of OX40 ligand on DCs, which shifts the immune response towards Th2 production. The subsequent secretion of type 2 cytokines such as IL-4 and IL-13 may actually serve to accelerate tumor growth and prevent tumor cell apoptosis [52].

In addition, the expression of specific tumor-generated surface receptors may prevent recognition and phagocytosis by DCs. For example, while the tumor glycoproteins carcinoembryonic antigen and mucin 1 are capable of being endocytosed by DCs, they become confined to early endosomes within cells, thus preventing processing and presentation to T cells [53]. Similarly, tumor-derived lactoferrin and CD47, which have been shown to interact with signal regulatory protein-α on phagocytes, will bind DCs and release inhibitory signals that will prevent phagocytosis. In fact, mouse tumor models that added a CD47-blocking antibody to the therapeutic regimen saw a marked improvement in tumor eradication, further supporting the importance of proper DC function in the generation of tumor immunity [54].

5. DCs and Cutaneous Carcinomas

Given the rich network of DCs in the skin, these cells are often thought to be the first immune cells to encounter tumor antigens from cutaneous cancers such as squamous cell carcinoma (SCC) and basal cell carcinoma (BCC). Initiating tumor immunity may therefore be critically dependent on the proper functioning of DCs as antigen presenters, with the ability to stimulate T cell proliferation and polarization. Indeed, we and others have previously shown SCC lesions often display significantly reduced quantities of both LCs and CD11c+ dermal DCs, indicating a disruption in DC-generated immunity [38, 55].

We have previously studied both the phenotype and function of mDCs extracted from SCC lesions and have evaluated these cells in the context of mDCs taken from peritumoral or healthy skin. We found that tumor-associated mDCs were poor stimulators of T cell proliferation when compared to their peritumoral or healthy skin counterparts. Furthermore, we found comparable levels of the maturation markers CD83 and CD86 amongst all 3 cell types, suggesting the impairment in tumor-associated T cell activation was not the result of defective DC maturation [38]. Consistent with our findings, tumor-associated mDCs extracted from BCC lesions have also been shown to be deficient activators of the T cell response when compared to normal cutaneous mDCs [56]. While the exact mechanisms underlying this apparent discrepancy are not yet fully understood, they may in part be due to the increased expression of immunosuppressive cytokines IL-10 and TGF-β, which we observed in the SCC microenvironment. Additionally, we have shown the prevalence of Foxp3+ Tregs to be upregulated in cutaneous SCC lesions, which may directly inhibit the antitumor response through the suppression of CD8+ effector T cells. Furthermore, Tregs may impair DC function by inhibiting the expression of costimulatory molecules, which will interfere with DC antigen-presenting ability [39–41]. This may serve as a further factor contributing to the observed dysfunction of mature mDCs in cutaneous carcinomas.

Other unique features of the SCC microenvironment, which may play a role in the suppression of tumor-associated mDCs, include the presence of a novel CD11c+ mDC subtype known as TIP-DCs [38]. These specialized cells are characterized by the secretion of TNF and iNOS, which serve to catalyze the production of nitric oxide (NO) from L-arginine. This may result in a direct immunosuppressive effect, as elevated levels of NO have been associated with the inhibition of activated T cell proliferation [57]. Alternatively, we have previously shown mDCs in the tumor microenvironment demonstrate elevated levels of CD200 receptor expression. They may therefore be increasingly vulnerable to the effects of immunosuppressive molecules such as CD200 [58]. Lastly, we have demonstrated a prominent influx of tumor-associated macrophages in SCC lesions, which we have shown may be directly contributing to tumor growth and carcinogenesis [59]. This is mediated by the secretion of VEGF-C and MMP 9 and 11, which serve to promote tumor lymphangiogenesis and the infiltration of surrounding tissues [60–62]. Further research is currently needed in order to better understand the intricacies of the SCC microenvironment, and how they may result in the suppression of associated mDC function.

In contrast to the diminished T cell response seen with mDCs, LCs harvested from SCC lesions have actually been shown to have an increased ability to stimulate CD4+ and CD8+ T cell proliferation *in vitro* when compared to LCs from matched, nontumor bearing skin [32]. They can also efficiently polarize T cell activity towards a predominantly Th1 response, as shown by the increased expression of IFN-γ. Furthermore, subsequent study revealed that nontumor LCs cultured in the presence of tumor supernatant (TSN) demonstrate an enhanced proliferation of both CD8+ and CD4+ T cells, with a shift towards a Th1 and CD8+ T cell response [32]. This suggests that the SCC microenvironment may actually serve to promote, rather than inhibit, LC activation and the initiation of the antitumor response. This stimulatory effect is appreciated despite the fact that the tumor environment is composed largely of the immunosuppressive cytokines IL-10 and TGF-β [38].

Additionally, we studied the effects of TSN on *in vitro* generated LCs and mDCs derived from CD34+ hematopoietic progenitors. We found that, similar to LCs extracted from peritumoral or healthy skin, the inclusion of TSN in the culture media effectively augmented LC-dependent T cell proliferation and Th1 polarization. However, this was not the case for mDCs, which demonstrated a markedly suppressed T cell response following treatment with TSN [32]. These results support the notion that epidermal LCs are a unique subset

of DCs which, unlike other members of the DC family, may be resistant to the immunosuppressive effects of cutaneous carcinomas. They may therefore serve as critical players in the generation of SCC targeted immunotherapy.

Given the coexistence of LCs and SCC in the human epidermis, and the enhanced ability of tumor-derived LCs to initiate type 1 immune responses *in vitro*, the question remains as to why LCs fail to prevent SCC tumor growth *in vivo*. One possible explanation for this finding is the dramatically reduced number of LCs found in both lesional and peritumoral skin [38, 55, 63]. Moreover, these cells may have impaired patterns of migration and defective mechanisms of T cell priming in the draining lymph nodes [64, 65]. The application of TSN directly to SCC lesions in mice resulted in a markedly diminished *in vivo* migration of LCs to draining lymph nodes [55, 66]. Additionally, it is worth noting that much of our knowledge concerning LC function in SCC is derived from the *ex vivo* study of migrating cells taken from preexisting tumors. Thus, the role of LCs in the tumor initiation stage is largely unknown and may be significantly different from our current observations. Accordingly, recent studies demonstrating the role of LCs in the initiation and promotion of chemically induced mouse cutaneous SCC may be of interest [67, 68].

As mentioned previously, another distinctive component of the SCC tumor microenvironment is the presence of relatively large quantities of pDCs [38]. These cells are thought to be beneficial for tumor eradication due to their inherent ability to produce large amounts of IFN-α in response to foreign antigen. Additionally, it has recently been shown that pDCs are capable of recognizing, processing, and cross-presenting foreign antigen to CD8+ T cells [69, 70]. Although pDCs were found to uptake reduced quantities of antigen when compared to their mDC counterparts, these findings support the notion that pDCs may still be effective mediators of the antitumor immune response [71]. Accordingly, it has been shown that the elevated amounts of pDCs are indeed associated with increased clearance of BCC lesions following treatment with imiquimod [47]. Further research is needed in order to more accurately define the role of pDCs in human cutaneous carcinomas, as well as their potential therapeutic value.

6. Therapeutic Implications

The ability of DCs to link the innate and adaptive immune systems, and to generate and amplify the immune response, has made them attractive targets for tumor immunotherapy. This is particularly the case for cutaneous carcinomas, given the high prevalence of DCs in the skin, and the existence of specialized subsets with highly efficient antigen-presenting mechanisms [10]. In order for immunotherapies to be maximally effective, however, a thorough understanding of DC biology and function is required. To date, many different therapeutic approaches have been studied, with some promising initial results but limited clinical applicability. Several key DC-based therapies currently undergoing investigation for the treatment of cutaneous carcinomas include *in vivo* or epicutaneous immunization, *ex vivo* DC vaccination, and immunomodulatory therapies such as imiquimod and diphenylcyclopropenone (DPCP) administration (Figure 3).

6.1. In Vivo and Epicutaneous Immunization. Direct *in vivo* DC vaccination involves the targeted delivery of tumor antigens to DCs through the use of chimeric proteins, which will fuse tumor antigens to antibodies specific for a given DC receptor, such as CD205 or Langerin/CD207 [72]. DC maturation signals are also commonly coadministered in order to ensure the induction of antigen-specific immunity rather than tolerance. This strategy has been shown to effectively elicit a potent CD4+ and CD8+ T cell response in mouse tumor models [73]. It also enables direct targeting of specific DC subsets by tailoring antibodies to distinct cell surface molecules. With respect to cutaneous carcinomas, targeting LC-specific molecules such as Langerin may be the key to generating the desired tumor-specific CD8+ T cell response with the induction of high-avidity CTL clones [27, 30, 32]. Additionally, direct *in vivo* delivery has the added benefit of allowing tumor-associated LCs to be activated by the local factors present in the SCC microenvironment, a step which we have shown may help bolster subsequent T cell activation [32].

Alternatively, *in vivo* antigen delivery can be accomplished through the direct application of protein antigen to barrier-disrupted skin, a method known as epicutaneous immunization [74]. This simple and noninvasive method will preferentially target LCs due to their predominance in the epidermis, allowing relatively easy access to topical antigen. Furthermore, disruption of the skin barrier has a pro-inflammatory effect, which serves as an adjuvant to recruit and activate LCs, and induces migration to nearby lymph nodes [75]. In mouse melanoma models, it has been shown that epicutaneous immunization is a powerful and efficient strategy for the activation of antigen-specific CD4+ and CD8+ T cell proliferation. It has also been shown to promote the induction of IFN-γ secreting CD8+ effector cells, which ultimately led to the successful inhibition of tumor growth [76]. Moreover, this effective antitumor response is dependent on the proper functioning of LCs, as evidenced by the markedly impaired tumor immunity seen in LC knockout mice [76]. Recent clinical trials using peptide antigen on barrier-disrupted skin in melanoma patients demonstrate the efficacious development of targeted CTL responses against tumor antigen, with the subsequent regression in tumor size and burden for a majority of patients [77]. Targeting LCs via skin immunization may therefore prove to be an important new therapy for the treatment of cutaneous carcinoma.

6.2. Ex Vivo Generated DC Vaccines. In order to generate DC vaccines *ex vivo,* DCs must first be cultured from hematopoietic progenitor cells or peripheral blood monocytes. In the past, DCs were most commonly derived from CD34+ progenitors treated *in vitro* with granulocyte-macrophage colony-stimulating factor (GM-CSF) and tumor necrosis factor α (TNF-α) [78]. More recently, however, the preferred method of obtaining immature DCs is from peripheral blood CD14+ monocytes under tumor-free conditions, which are subsequently treated with GM-CSF and IL-4 [79]. As yet,

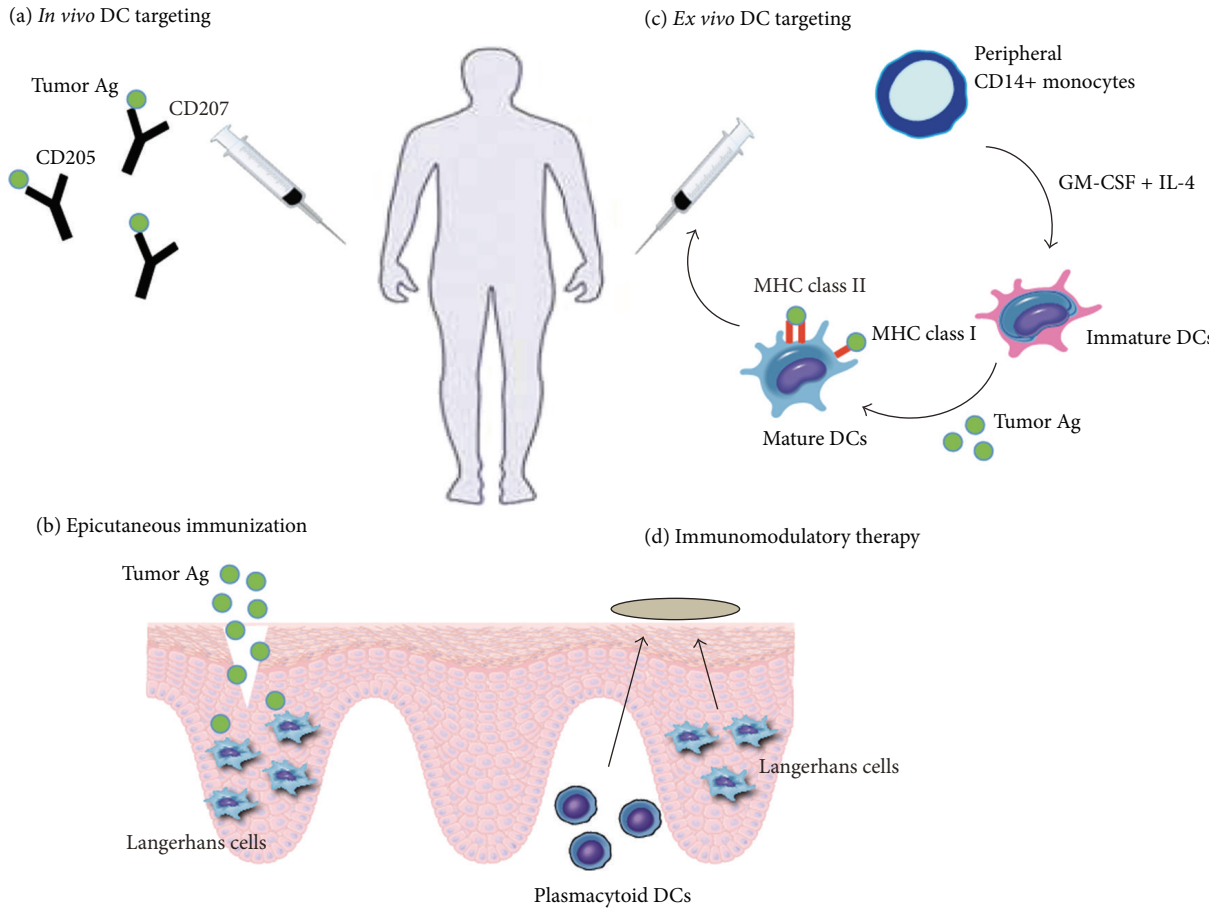

FIGURE 3: Current strategies for DC immunotherapy in the treatment of cutaneous carcinoma. (a) Direct *in vivo* DC vaccination involves the targeted delivery of tumor antigen to DCs by linking them to antibodies specific for a given DC surface molecule. Examples include CD205 for mDCs or Langerin/CD207 for LCs. (b) Epicutaneous immunization allows for the direct application of protein antigen to a disruption in the skin barrier, which will preferentially target LCs due to their predominance in the epidermis. (c) *Ex vivo* DC vaccination relies on the *in vitro* generation of immature DCs from hematopoietic progenitors or peripheral blood monocytes. DCs are then loaded with tumor antigen and reinfused into the patient. (d) Immunomodulatory therapy involves the application of topical agents that may directly regulate the immune response. Often they act as adjuvants to induce the activation and migration of LCs to nearby lymph nodes. Other effects include the TLR7/8-dependent recruitment of pDCs to the tumor region following imiquimod treatment.

there is no standardized method of DC preparation; thus monocytes may also be treated with alternative cytokines such as IFN-α, TNF-α, and IL-15 in combination with GM-CSF [80, 81]. Depending on the combination of cytokines used, this method allows for the preferential differentiation of distinct DC subsets, including LCs and mDCs [82]. Following the isolation of immature DCs, specific tumor antigens must be selected and loaded onto cells and then treated with the appropriate adjuvants to induce DC maturation.

Previous melanoma trials have shown that vaccination with *ex vivo* monocyte-derived DCs may result in the successful induction of tumor-specific CTL responses *in vivo* [83–85]. Furthermore, vaccination with mature mDCs has been associated with the generation of a detectable *in vitro* antigen-specific Th1 immune response [86]. Despite these results, the clinical impact of melanoma mDC vaccination remains relatively limited, with only a small minority of trials showing significant tumor regression [1, 87, 88]. In fact, the most common outcome seen with DC vaccination is the induction of an expanded antigen-specific immunity in the absence of any discernible clinical response [88].

There are several possible explanations as to why recent trials have failed to translate the immune response into an effective therapeutic outcome. First, injected DCs often fail to migrate to local lymph nodes, which may impair their ability to mount a functional immune response [89]. Additionally, proper immune function may be disrupted by the tumor microenvironment through the direct impairment of mDCs by immunosuppressive molecules such as IL-10, TGF-β, VEGF-A, and CD200 [38, 58]. This may be compounded by the inhibition of effector T cell function via Tregs and immune suppressive molecules such as CTLA-4 and PD-1 [38–40, 58, 90]. Lastly, the quality of DCs may be insufficient to generate a potent antitumor response *in vivo*, resulting instead in low avidity T cells which may be incapable of overcoming the immunosuppressive effects of the tumor

environment [91]. In this respect, the cytokine combination used to differentiate monocytes may prove critical. Treatment with GM-CSF and IL-15 will preferentially elicit LC-type DCs, which have been shown to be more efficient in priming melanoma-antigen-specific CD8+ T cells *in vitro* than their mDC counterparts [81, 92]. This may result in the generation of powerful CD8+ effector cells, with an increased ability to target tumor antigen [92].

Similarly, our findings reflect the enhanced ability of LC-type DCs to stimulate CD8+ T cell proliferation *ex vivo,* in the presence of SCC-derived factors. This leads to the generation of a robust effector CD8+ T cell response, evidenced by the increased expression of IFN-γ [32]. LCs are therefore potent immune activators which may be resistant to inhibition from the surrounding tumor microenvironment. This occurs in direct contrast to monocyte-derived DCs, whose activity was suppressed by the tumor microenvironment. This is further reinforced by previous studies which have demonstrated that, when compared to monocyte-derived DCs, LCs are far more efficient at cross-presenting tumor antigen to CD8+ cells. This step is thought to be critical in the activation of a subsequent antigen-specific CTL response, which is necessary for successful antitumor immunity [82, 93]. Additionally, cross-presentation permits antigen loading with tumor-derived proteins rather than peptides, which allows for the generation of multiple antigenic epitopes and promotes a more powerful immune response. Furthermore, CD8+ T cells primed by LCs will show higher avidity binding and express higher levels of cytotoxic molecules such as granzymes and perforin, as compared to those primed by mDCs [88]. Accordingly, they will have a markedly improved capacity to kill target cells. This lends support to the rationale of using DC vaccines with the generation of a predominantly LC rather than mDC-like population in the treatment of cutaneous carcinoma [32].

In addition, given the powerful capacity of CD141+ DCs to cross-present foreign antigen, these cells may prove to be an important new source for the generation of *ex vivo* derived DC vaccines. Recent studies have indeed shown the successful generation of CD141+, XCR1+ DCs from induced pluripotent stem cells [94]. Furthermore, these cells were capable of effectively cross-priming cytotoxic T lymphocytes against Melan A tumor-associated antigen [94]. Although much more research is currently needed, CD141+ DCs represent attractive new targets in *ex vivo* DC vaccination therapy.

6.3. Immunomodulatory Therapies. Imiquimod belongs to a class of drug known as imidazoquinolines and is an immune modulator currently approved for the treatment of a variety of cutaneous diseases such as external genital warts, actinic keratosis, and superficial BCC [95]. Although its precise mechanism of action remains unclear, imiquimod is thought to possess both antiangiogenic and proapoptotic properties and may regulate the immune response through the activation of TLR7 and TLR8 [96]. This results in the secretion of pro-inflammatory cytokines such as IFN-α, IL-6, and TNF-α.

Imiquimod treatment is associated with the recruitment of large amounts of pDCs into the dermis and tumor microenvironment. This has been shown to result in an effective, NK-cell-dependent tumor regression in mouse melanoma models [97]. Furthermore, tumor clearance was found to be directly dependent on the corresponding pDC infiltration, with a positive correlation present between pDC numbers and the degree of tumor regression. Accordingly, imiquimod-treated pDC knockout mice were found to be incapable of successful tumor eradication [98]. The exact nature of the relationship between imiquimod treatment and pDC-dependent tumor clearance has yet to be defined; however several studies have shown that imiquimod is able to induce malignant cell apoptosis through TLR7/IFN-α-dependent pathways [99, 100]. Importantly, human pDCs have been found to respond to imiquimod-associated TLR7/8 stimulation with an increased expression of apoptotic factors TRAIL and FasL, as well as an increased release of cytotoxic granzyme molecules [101]. Imiquimod therapy has also been associated with elevated amounts of pDCs in human BCC lesions, resulting in more efficient tumor clearance [47].

An alternative mechanism that may play a role in imiquimod-mediated tumor clearance is the use of imiquimod as an adjuvant agent [102]. Multiple skin immunization studies have shown that DC adjuvants are required in order to achieve a more robust CD8+ effector T cell response [76, 102, 103]. This is thought to be due to the induction of skin inflammation, which promotes the activation and migration of LCs to nearby lymph nodes. Indeed, the application of imiquimod in conjunction with epicutaneous immunization has been associated with improved antitumor effects and more efficient tumor clearance [76].

Other similar immunomodulatory therapies include the use of the topical medication DPCP, which is currently approved for the treatment of alopecia areata and recalcitrant warts [104, 105]. DPCP is thought to act as a hapten or local irritant, which can induce a potent contact sensitization response, triggering the activation of epidermal LCs. Additionally, DPCP has been shown to have local immunomodulatory effects and may stimulate strong proliferative T cell responses in mice following exposure to contact allergens [106]. Recent reports have demonstrated DPCP administration alone may effectively clear skin metastases in malignant melanoma, presumably through DC activation and lymphocyte-mediated tumor destruction [107, 108]. Additionally, the adjuvant properties of DPCP have been shown to increase the efficacy of epicutaneous immunotherapy, making this drug an attractive candidate for future study [109].

7. Conclusions and Future Directions

For the past decade, DCs have been vigorously studied in clinical trials in order to evaluate the possibility of generating a therapeutic immune response directed against a variety of cancers. Unfortunately, while tumor-specific responses have indeed been measured in some cases, most trials have shown minimal clinical success with respect to tumor regression and overall survival rates [110]. This may, in part, be due to a limited or incomplete understanding of the role of DCs in the regulation of immunity. Moreover, predicted immune

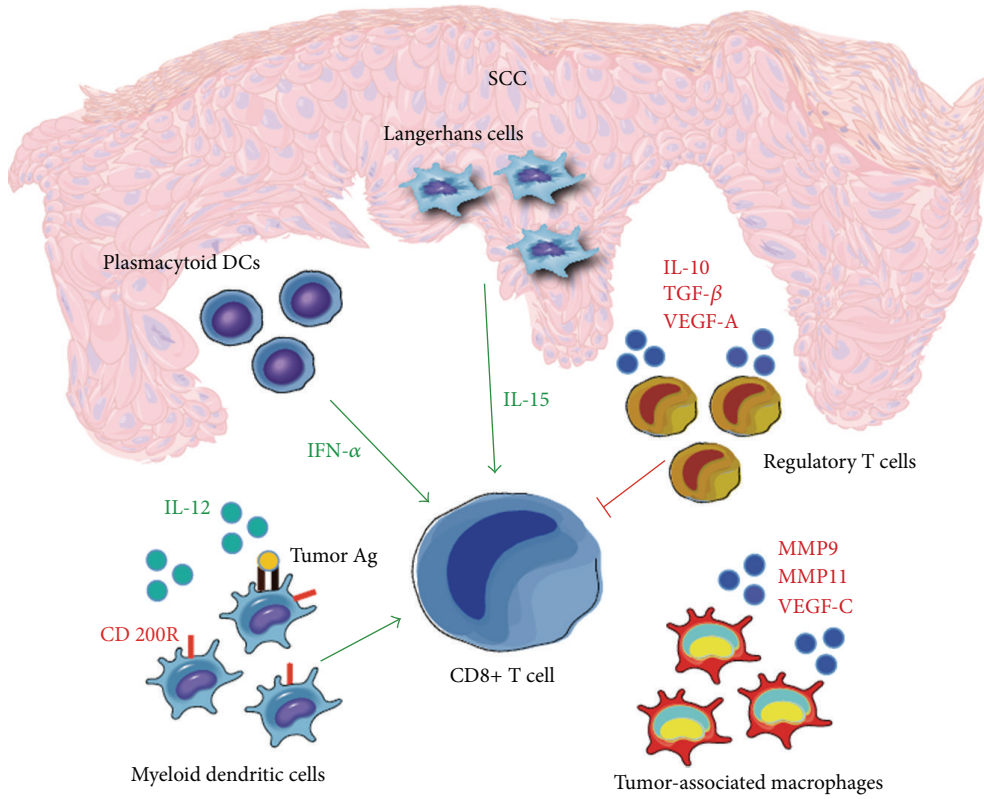

Figure 4: The SCC microenvironment may impact the immune response. The SCC microenvironment is dynamic and involves a complex interplay of both pro- and anti-inflammatory signals. It is associated with an elevated number of IFN-α secreting pDCs, and an increased capacity for LCs to stimulate CD8+ T cells. These may serve to enhance the immune response and promote tumor immunity. Conversely, we have also shown the tumor microenvironment to contain an increased number of regulatory T cells, tumor-associated macrophages, and immune suppressive molecules such as IL-10, TGF-β, and VEGF-A. These may be contributing to tumor growth and immune dysfunction through the suppression of mDC and CD8+ T cell activity.

responses may be dramatically altered in the presence of intricate and complex tumor microenvironments [32, 38] (Figure 4).

Recently, the therapeutic use of cancer vaccination has experienced a revival, owing in large part to the encouraging results seen with a number of clinical trials. Phase III trials for the treatment of metastatic prostate cancer with sipuleucel-T, a cellular product containing enriched antigen-presenting cells cultured with prostatic acid phosphatase and GM-CSF, resulted in a 4-month prolongation of the median survival time [111]. Similarly, a phase III metastatic melanoma trial, which compared peptide vaccination in combination with IL-2 therapy to IL-2 therapy alone, showed a significant improvement in overall response rate and progression-free survival in those patients who had received the vaccine [112].

These studies provide definitive evidence that DC immunotherapy can be exploited to yield clinically significant results. Additionally, using the skin as a therapeutic target allows for a relatively simple and minimally invasive means of investigating cancer biology. The comparative ease with which we can obtain tumor samples enables both the evaluation of clinical efficacy, and the extensive analysis of underlying tumor mechanisms and the corresponding immune reaction. Accordingly, recent years have shown considerable progress in the field of DC biology, with a greater understanding of how unique DC subsets may be interacting with tumor microenvironments to shape the immune response. This raises the possibility of developing novel therapeutic strategies, which may result in vastly improved clinical outcomes. More specifically, we believe that targeting LCs in the treatment of cutaneous carcinomas may be critical for the induction of a potent and antigen-specific CTL response, which may be resistant to the immunosuppressive effects of the local tumor environment [32]. Likewise, the addition of an appropriate adjuvant may prove beneficial in bolstering antitumor activity [76]. Although further research is needed in order to successfully translate DC biology into medicine, we believe the use of LCs in DC-based immunotherapy represents a promising new immunological approach for the treatment of cutaneous carcinomas.

Conflict of Interests

The authors have no conflict of interests to declare.

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