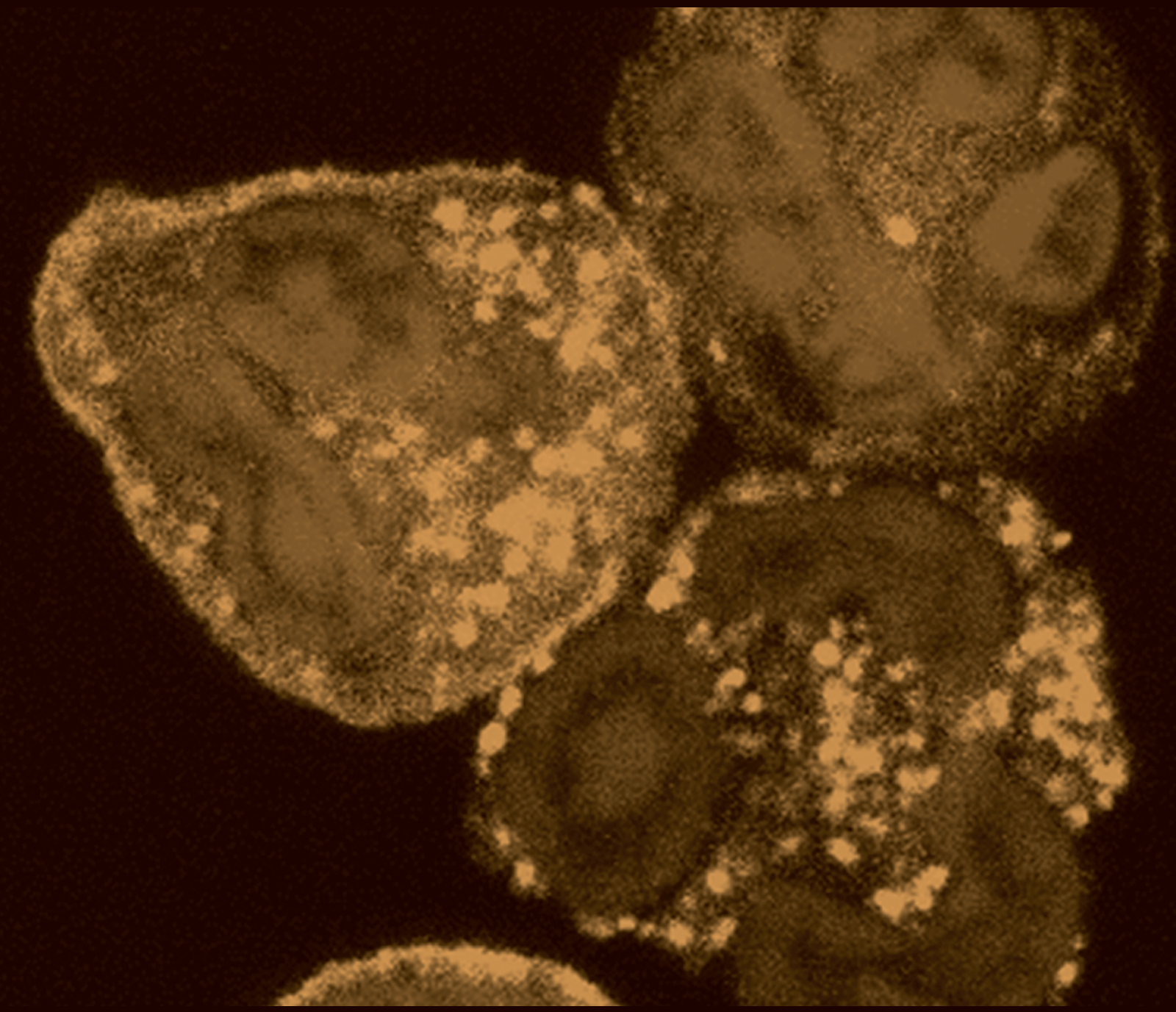
Mediators of Inflammation

Macrophage-Mediated Inflammatory Disorders

Guest Editors: I-Ming Jou, Chiou-Feng Lin, Kuen-Jer Tsai, and Sung-Jen Wei



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Editorial Board

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Editorial

Macrophage-Mediated Inflammatory Disorders

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Mediators of inflammation induced by macrophages are critical for a variety of human inflammatory disorders, such as sepsis-related multiple organ dysfunction/multiple organ failure, microbial infection, acute brain/lung/hepatic/renal injuries, neurodegenerative disorders, tumorigenesis, osteoporosis/osteonecrosis, cardiovascular and metabolic diseases, and autoimmune diseases. Most of these cases are proinflammatory and pathogenic for disease progression, once activated macrophages actively secrete and cause an imbalance of cytokines, chemokines, and mediators of inflammation. The primary focus of this special issue is on the current and updated knowledge of macrophage-mediated inflammatory disorders, particularly on the pathogenesis of the macrophage activation syndrome, mediators of inflammation and anti-inflammation, and strategies against such effects. Lots of papers were submitted and reviewed by Editors and Reviewers, and twenty-four papers are accepted for publication. The brief introductions of these papers are as follows.

Due to the profile of released mediators (such as cytokines, chemokines, and growth factors), neoplastic cells modulate the activity of immune system, directly affecting its components both locally and peripherally. A. Eljaszewicz et al. reviewed, in the paper entitled *"Collaborating with the enemy: function of macrophages in the development of neoplastic disease,"* the specific functions of macrophages in the development of neoplastic disease.

The study called *"Regulatory role of GSK-3β on NF-κB, nitric oxide, and TNF-α in group A streptococcal infection,"* investigates the interaction between GSK-3β, NF-κB, and possible related inflammatory mediators in vitro and in a mouse model. The results revealed that GAS could activate NF-κB, followed by an increased expression of inducible nitric oxide synthase (iNOS) and NO production in a murine macrophage cell line. Y.-T. Chang et al. demonstrated that the inhibition of GSK-3β to moderate the inflammatory effect might be an alternative therapeutic strategy against GAS infection.

During the early and short inflammatory phase, macrophages exert proinflammatory functions like antigen-presenting phagocytosis and the production of inflammatory cytokines and growth factors that facilitate the resolution of inflammation. However, persistence of proinflammatory activity and altered function of macrophages result in the development of chronic inflammatory diseases such as AD. In *"Role of macrophages in the pathogenesis of atopic dermatitis,"* S. Kasraie and T. Werfel highlight the new findings on dysregulated function of macrophages, the importance of these cells in the pathogenesis of AD in general, and the contribution of these cells in enhanced susceptibility against microbial infections in particular.

C. E. Boorsma et al. in *"Macrophage heterogeneity in respiratory diseases"* provided an overview of what macrophage phenotypes have been described, what their known functions are, what is known about their presence in the different obstructive and restrictive respiratory diseases (asthma, COPD, and pulmonary fibrosis), and how they are thought to contribute to the etiology and resolution of these diseases.

In asthma, an important role for innate immunity is increasingly being recognized. Key innate immune cells in the lungs are macrophages. In *"Characterization of macrophage phenotypes in three murine models of house-dust-mite-induced*

asthma" C. Draijer et al. demonstrated the balance between macrophage phenotypes changes as the severity of allergic airway inflammation increases. Influencing this imbalanced relationship could be a novel approach to treat asthma.

Neuropathic syndromes which are evoked by lesions to the peripheral or central nervous system are extremely difficult to treat, and available drugs rarely joint an antihyperalgesic effect with a neurorestorative effect. N-Palmitoylethanolamine (PEA) exerts antinociceptive effects in several animal models and inhibits peripheral inflammation in rodents. In *"Palmitoylethanolamide is a disease-modifying agent in peripheral neuropathy: pain relief and neuroprotection share a PPAR-alpha-mediated mechanism*" these results from L. D. C. Mannelli et al. strongly suggest that PEA, via a PPAR-α-mediated mechanism, can directly intervene in the nervous tissue alterations responsible for pain, starting to prevent macrophage infiltration.

Several studies have provided evidence with regard to the neuroprotection benefit, so hyperbaric oxygen (HBO) therapy incases stroke, and HBO also promotes bone marrow stem cells (BMSCs) proliferation and mobilization. In the paper entitled *"Long course hyperbaric oxygen stimulates neurogenesis and attenuates inflammation after ischemic stroke"* Y.-S. Lee et al. find that mobilization of BMSCs to an ischemic area is more improved in long course HBO treatments, suggesting the duration of therapy is crucial for promoting the homing of BMSCs to ischemic brain by HBO therapies. HBO also can stimulate expression of trophic factors and improve neurogenesis and gliosis.

Indoleamine 2,3-dioxygenase 1 (IDO1), the L-tryptophan-degrading enzyme, plays a key role in the immunomodulatory effects on several types of immune cells. Originally known for its regulatory function during pregnancy and chronic inflammation in tumorigenesis, the activity of IDO1 seems to modify the inflammatory state of infectious diseases. Y. Murakami et al., in *"Remarkable role of indoleamine 2,3-dioxygenase and tryptophan metabolites in infectious diseases: potential role in macrophage-mediated inflammatory diseases,"* offer insights into new therapeutic strategies in the modulation of viral infection and several immune-related disorders.

Effective repair of damaged tissues and organs requires the coordinated action of several cell types, including infiltrating inflammatory cells and resident cells. In *"Macrophage plasticity and the role of inflammation in skeletal muscle repair,"* Y. Kharraz et al. summarize how ordered changes in macrophage polarization, from M1 to M2 subtypes, can differently affect muscle stem cell (satellite cell) functions. In addition, they highlight some of the new mechanisms underlying macrophage plasticity and briefly discuss the emerging implications of lymphocytes and other inflammatory cell types in normal versus pathological muscle repair.

As a facultative intracellular pathogen, *Staphylococcus aureus* invades macrophages and then promotes the cytoprotection of infected cells thus stabilizing safe niche for silent persistence. This process occurs through the upregulation of crucial antiapoptotic genes, in particular, myeloid cell leukemia-1 (Mcl-1). In *"The role of Mcl-1 in S. aureus-induced cytoprotection of infected macrophages,"* J. Koziel et al. report that *S. aureus* is hijacking the Mcl-1-dependent inhibition of apoptosis to prevent the elimination of infected host cells, thus allowing the intracellular persistence of the pathogen, its dissemination by infected macrophages, and the progression of staphylococci diseases.

The P2X7 purinergic receptor is a ligand-gated cation channel expressed on leukocytes including microglia. This study aimed to determine if P2X7 activation induces the uptake of organic cations, reactive oxygen species (ROS) formation, and death in the murine microglial EOC13 cell line. In *"P2X7 receptor activation induces reactive oxygen species formation and cell death in murine EOC13 microglia,"* R. Bartlett et al. demonstrate P2X7 activation induces the uptake of organic cations, ROS formation, and death in EOC13 microglia.

In *"Pivotal roles of monocytes/macrophages in stroke"* the reports from T. Chiba and K. Umegaki suggest that inflammation might directly affect the onset of stroke. Microglial cells and blood-derived monocytes/macrophages play important roles in inflammation in both onset and aggravation of stroke lesions.

Macrophages play crucial roles in atherosclerotic immune responses. Recent investigation into macrophage autophagy in atherosclerosis has demonstrated a novel pathway through which these cells contribute to vascular inflammation. In *"Macrophage autophagy in atherosclerosis,"* M. C. Maiuri et al. discuss the role of macrophages and autophagy in atherosclerosis and the emerging evidence demonstrating the contribution of macrophage autophagy to vascular pathology. Finally, they show how autophagy could be targeted for therapeutic utility.

Dexamethasone (Dex) has been used to reduce inflammation in preterm infants with assistive ventilation and to prevent chronic lung diseases. In *"The role of glucocorticoid receptors in dexamethasone-induced apoptosis of neuroprogenitor cells in the hippocampus of rat Pups,"* C.-I. Sze et al. indicate early administration of Dex results in apoptosis of neural progenitor cells in the hippocampus, and this is mediated through glucocorticoid receptors.

Macrophages are innate immune cells derived from monocytes, which, in turn, arise from myeloid precursor cells in the bone marrow. Macrophages have many important roles in the innate and adaptive immune response, as well as in tissue homeostasis. In *"Alternatively activated macrophages in types 1 and 2 diabetes,"* A. Espinoza-Jiménez et al. review the advantages and disadvantages of two macrophage populations with regard to their roles in types 1 and 2 diabetes.

Macrophage migration inhibitory factor (MIF) is a proinflammatory cytokine, and the predictive role and pathogenic mechanism of MIF deregulation during kidney infections involving acute kidney injury (AKI) are not currently known. In *"Urinary macrophage migration inhibitory factor serves as a potential biomarker for acute kidney injury in patients with acute pyelonephritis,"* M.-Y. Hong et al. showed that elevated urinary MIF levels accompanied the development of AKI during kidney infection in patients with acute pyelonephritis (APN). An elevated urinary MIF level, along with elevated IL-1β and KIM-1 levels, is speculated to be a potential biomarker for the presence of AKI in APN patients.

Peroxisome proliferator-activated receptors (PPARs) are shown to modulate the pathological status of sepsis by regulating the release of high mobility group box 1 (HMGB1), a well-known late proinflammatory mediator of sepsis. In *"Activation of peroxisome proliferator-activated receptor γ by rosiglitazone inhibits lipopolysaccharide-induced release of high mobility group box 1,"* J. S. Hwang et al. showed PPARs play an important role in the cellular response to inflammation by inhibiting HMGB1 release.

In the paper entitled *"Macrophages, inflammation, and tumor suppressors: ARF, a new player in the game,"* P. G. Través et al. provide an overview of the immunobiology of tumor-associated macrophages as well as what is known about tumor suppressors in the context of immune responses. Recent advances regarding the role of the tumor suppressor ARF as a regulator of inflammation and macrophage polarization are also reviewed.

Monocytes express many cell surface markers indicative of their inflammatory and activation status. Whether these markers are affected by diabetes and its complications is not known and was investigated in this study. In *"Alterations in monocyte CD16 in association with diabetes complications,"* D. Min et al. provide the evidence suggesting that the circulating monocyte phenotype is altered by diabetic complications status. These changes may be causally related to and could potentially be used to predict susceptibility to diabetic complications.

Inflammation is implicated in the development and rupture of atheromatous plaques, and there is considerable evidence supporting the involvement of adipocytokines in this inflammatory process. In *"Increased expression of visfatin in monocytes and macrophages in male acute myocardial infarction patients,"* C.-A. Chiu et al. provide another explanation about leukocytes mediated visfatin that may play a pathogenesis role in coronary vulnerable plaques rupture.

The lung is exposed to a vast array of inhaled antigens, particulate matter, and pollution. Cells present in the airways must therefore be maintained in a generally suppressive phenotype so that excessive responses to nonserious irritants do not occur; these result in bystander damage to lung architecture, influx of immune cells to the airways, and consequent impairment of gas exchange. In *"Macrophage-mediated inflammation and disease: a focus on the lung,"* E. G. Findlay and T. Hussell discuss the mechanisms behind this macrophage-mediated pathology, in the context of a number of inflammatory pulmonary disorders.

Most tissues harbor resident mononuclear phagocytes, that is, dendritic cells and macrophages. In *"Tissues use resident dendritic cells and macrophages to maintain homeostasis and to regain homeostasis upon tissue injury: the immunoregulatory role of changing tissue environments,"* M. Lech et al. report that organ- and disease phase-specific microenvironments determine macrophage and dendritic cell heterogeneity in a temporal and spatial manner, which assures their support to maintain and regain homeostasis in whatever condition. Mononuclear phagocytes contributions to tissue pathologies relate to their central roles in orchestrating all stages of host defense and wound healing, which often become maladaptive processes, especially in sterile and/or diffuse tissue injuries.

Different monocytic subsets are important in inflammation and tissue remodeling; although heart failure (HF) is associated with local and systemic inflammation, their roles in HF are yet unknown. In *"Changes in the monocytic subsets $CD14^{dim}CD16^{+}$ and $CD14^{++}CD16^{-}$ in chronic systolic heart failure patients,"* O. Amir et al. indicate the inverse association between EDD values and the expansion of $CD14^{dim}$ $CD16^{+}$ monocytes that can produce IL-13 which could be explained as a measure to counterbalance adverse remodeling, which is a central process in HF.

Traditional risk factors for metabolic disorders, including the waist circumstance, body mass index (BMI), triglyceride (TG), and ratio of TG to high density lipoprotein (HDL) cholesterol, were closely correlated with homoeostasis model assessment (HOMA) index in patients with nondiabetic RA. In *"Increased toll-like receptor 2 expression in peptidoglycan-treated blood monocytes is associated with insulin resistance in patients with nondiabetic rheumatoid arthritis"* S.-W. Wang et al. show the expressions of TLR2 in peripheral blood monocytes, following stimulation with peptidoglycan which is known as a TLR2 agonist, were closely correlated with the HOMA index, TNF-α and IL-6 concentrations. Accordingly, TLR-2 receptor and its related inflammatory cytokines could be potential therapeutic targets in managing insulin resistance in RA patients.

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Review Article

Collaborating with the Enemy: Function of Macrophages in the Development of Neoplastic Disease

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Due to the profile of released mediators (such as cytokines, chemokines, growth factors, etc.), neoplastic cells modulate the activity of immune system, directly affecting its components both locally and peripherally. This is reflected by the limited antineoplastic activity of the immune system (immunosuppressive effect), induction of tolerance to neoplastic antigens, and the promotion of processes associated with the proliferation of neoplastic tissue. Most of these responses are macrophages dependent, since these cells show proangiogenic properties, attenuate the adaptive response (anergization of naïve T lymphocytes, induction of Treg cell formation, polarization of immune response towards Th2, etc.), and support invasion and metastases formation. Tumor-associated macrophages (TAMs), a predominant component of leukocytic infiltrate, "cooperate" with the neoplastic tissue, leading to the intensified proliferation and the immune escape of the latter. This paper characterizes the function of macrophages in the development of neoplastic disease.

1. Introduction

Human body is exposed to a continuous influence of carcinogenic factors (physical, chemical, and biological), representing one of the reasons for the development of genetic mutations. Cells possess an array of mechanisms able to prevent mutations, as well as to repair DNA defects and eliminate genetically altered cells, for example, by the means of apoptosis [1, 2]. Disorders of this complicated protective system lead to the development of neoplastic cells, which, in turn, may be eliminated by an array of immunological mechanisms, including those affected by the innate immune system (monocytes, macrophages, NK cells, cytokines, etc.) and the adaptive immunity (induction of T and B lymphocytes). In order to eliminate neoplastic cells, the cells of the immune system must recognize them as "foreign." The principles of recognition and the mechanisms of the immunological response are similar to those induced by foreign (bacterial, viral) or own antigens (autoantigens). Foreign antigens are highly immunogenic; that is, they induce immune response aimed at the rapid elimination of the infectious factors. These processes lead to the selection of a pool of immunocompetent cells, specialized in the destruction of a given factor. As previously mentioned, neoplastic cells originate from genetically altered cells of own tissues and therefore contain components that induce various degrees of immune tolerance, protecting

them against the elimination by immunological mechanisms. On the other hand, neoplastic cells are characterized by a genetic instability [3], which manifests by changes in antigenic profile, which are not observed in the normal tissue. In some cases, this is accompanied by the overexpression of genes that remain inactive or exhibit low-level activity under normal conditions; many of the factors produced in this manner act as immunomodulators. It is widely known that neoplastic tissue can directly modulate its growth environment as a result of the activity of secreted cytokines and chemokines. This may be due to the following:

(a) chemotactic effect on leukocytes, including monocytes and macrophages;

(b) suppression of the activity of the immune system;

(c) regulation of neovascularization processes [4].

2. Chemotactic Factors for Monocytes/Macrophages Released by Neoplastic Cells

Some factors synthesized and released by neoplastic cells can induce leukocyte chemotaxis, including peripheral monocytes and macrophages located in the surrounding tissues. These cells represent the predominant component of leukocytic infiltrate of many tumors and, due to their pleotropic biological activity control, the majority of immunological processes proceeding in the region of neoplastic growth. Chemotaxis of monocytes and macrophages is a receptor-dependent process [5], directly associated with the polarization of these cells towards pro- or anti-inflammatory cells.

Monocyte chemotactic protein-1 (MCP-1/CCL2), showing affinity to CCR2 receptor, constitutes one of the principal monocyte/macrophage chemokines [6, 7]. Moreover, the concentration of CCL2 correlates positively with the stage of the tumor in cancer of urinary bladder and breast. High level of this cytokine is associated with poor prognosis; it is observed in patients with higher clinical stages of the tumor [8, 9]. In contrast, a proportional decrease in the concentration of CCL2 is observed with increasing stages of gastric malignancies, both in the serum and in neoplastic tissue. Perhaps, this phenomenon results from enhanced "consumption" of MCP-1 and concurrent unchanged level of synthesis [10]. Interestingly, MCP-1 can be released also by macrophages located in the region of neoplastic growth [11]. This suggests direct involvement of these cells in the recruitment of peripheral monocytes. It should be noted, however, that the impaired expression of CCR2 receptor on the surface of tumor-associated macrophages leads to the reversal of the effect exerted on these cells by the discussed chemokine. Plausibly, this is one of the mechanisms enabling the maintenance of rich macrophage infiltrate in the region of neoplastic growth [12]. Expression of MCP-1 correlates positively with the level of vascular endothelial growth factor (VEGF), TNF-α, and IL-8, which suggests its involvement in the processes of neovascularization [11]. The migration of peripheral monocytes to the site of neoplastic growth can be also induced by monocyte chemotactic protein-2 (MCP-2/CCL-8) and monocyte chemotactic protein-3 (MCP-3/CCL-7). These cytokines show both a structural similarity to MCP-1 and an affinity to the CCR2 receptor [13]. The chemotaxis of monocytes is also induced by CCL5 (RANTES), whose level of expression correlates with the degree of macrophage infiltration and lymph node metastases of neoplastic cells [14]. RANTES and CCL2 stimulate monocytes to secrete metalloproteinase-9 (MMP-9) and metalloproteinase-19 (MMP-19), which suggests indirect involvement of the discussed chemokines in the degradation of basal membrane, and hence in the process of neovascularization [15, 16]. Furthermore, high concentration of CCL5 increases the probability of metastases in patients with gastric malignancies [17]. Also, factors released by the neoplastic cells, such as VEGF, IL-8, and angiopoietin-2 (Ang-2), exert chemotactic effect on monocytes/macrophages; additionally, they are involved in the processes of angiogenesis.

3. Regulation of the Process of Neovascularization

Angiogenesis, although physiologically necessary, underlies a number of diseases. Formation of new blood vessels is critical for neoplastic growth and results from the predominance of proangiogenic factors over those inhibiting angiogenesis [18]. Enhanced angiogenesis can be observed in very early stages of malignant growth [19, 20]. Molecules such as (VEGF), interleukin-8 (IL-8/CXCL8), basic fibroblast growth factor (bFGF), angiopoietin-1 (Ang-1), and angiopoietin-2 (Ang-2) are the main mediators of neovascularization released by the neoplastic cells, including the cells of gastric malignancies.

VEGF is synthesized and secreted by many types of neoplasms [21–23] and although high levels of this molecule can be observed both in the plasma and in the serum of patients, the serum concentration of VEGF is higher. This results from the secretory activity of thrombocytes, which release high amounts of this factor during the coagulation of blood [17]. The production of VEGF in human macrophages is regulated by NFκB [24]. VEGF acts in the receptor-dependent manner [25], inducing the chemotaxis of peripheral monocytes as a result of interacting with VEGF-R1 [26]. In response to VEGF, activated monocytes/macrophages synthesize molecules, for example, metalloproteinase-9 [27, 28], which, as previously mentioned, is involved in the processes of angiogenesis. Although tumor-associated macrophages (TAM's) constitute the principal source of MMP9 in the zone of neoplastic growth, it should be noted that this molecule may be also synthesized by neoplastic cells, stromal neutrophils, fibroblasts, and mastocytes [29]. Moreover, the activity of VEGF leads to an increased permeability of blood vessels within the neoplastic tissue [30], proliferation of vascular endothelial cells [31], and the inhibition of dendritic cell maturation [32]. The majority of these processes are associated with the activation of VEGF-R2 receptor [33]. Therefore, the VEGF/VEGF-R2 system is connected with the initiation of neovascularization processes.

Similarly to VEGF, interleukin-8 is a chemokine with proangiogenic activity. High expression levels of this molecule

have been observed in various types of neoplasms, directly correlated with the vascularization of proliferating tissue and poor prognosis, being the highest in the advanced stages of tumor development. Gastric cancer cells also show the expression of A (CXCR1) and B (CXCR2) receptors for IL-8. During *in vitro* IL-8 stimulation, they show increased expression of epidermal growth factor receptor (EGFR), MMP-9, VEGF, and IL-8 [34]. Similarly to VEGF, interleukin-8 induces the migration of monocytes/macrophages to the site of neoplastic growth.

Basic fibroblast growth factor (bFGF) is one of the strongest stimulators of angiogenesis [35], acting via FGF-R1 and FGF-R2 receptors. Its expression is observed in many types of neoplasms [36]. High level of bFGF correlates positively with poor prognosis, and its expression in neoplastic cells is associated with the vascularization of the tumor. Moreover, this factor stimulates the chemotaxis of macrophages [37], which acquire the potential to synthesize and secrete this molecule in response to mediators released by neoplastic cells [38]. This suggests indirect TAMs-dependent influence of bFGF on the processes of tumor neovascularization.

Angiopoetin-1 (Ang-1) and angiopoetin-2 (Ang-2) are the main representatives of angiopoietin family. The activity of Ang-2 during the development of neoplastic disease is associated with the progression of the disease and the neovascularization of the tumor [39]. Angiopoietin-1 activates Tie-2 receptor (angiopoietin receptor), in this way stimulating *in vitro* migration of endothelial cells; moreover, it recruits pericytes into the newly formed vessels in order to stabilize their structure. In contrast, angiopoietin-2 is a natural antagonist of Tie-2 receptor. Ang-2 inhibits the maturation of vessels resulting from Ang-1 activity in a VEGF-independent manner and causes their regression. Therefore, it plays a regulatory function [34]. The activity of Ang-2 leads to the destabilization of vessels, which is necessary for the initiation of neovascularization process [40]. Angiopoietin-2 exerts positive effects on the processes of angiogenesis through VEGF involvement [34]. Interestingly, VEGF causes an increase in Ang-1 expression, but it does not modulate the synthesis of Ang-2 [41]. The level of Ang-2 expression correlates significantly with the clinical stage of disease (lymph node and organ metastases) while the expression of Ang-1 is significantly higher in poorly differentiated tumors [40]. The angiopoietin/Tie-2 system is involved in the remodeling and maturation of blood vessels and is, therefore, complementary to the activity of VEGF [34]. Moreover, the evaluation of Ang-1, Ang-2, and Tie-2 serum concentrations seems to be useful in preoperative differentiation of malignant thyroid tumors [42]. Additionally, angiopoietins are able to stimulate the chemotaxis of Tie-2-positive peripheral monocytes, which, constituting cells with pro-angiogenic potential, support the proliferation of neoplastic tissue [43].

4. Immunosuppressive Effect

Cancer cells and tumor-infiltrating leukocytes (primarily macrophages) modulate the activity of the immune system, also by means of immunosuppression, *via* the profile of released factors (cytokines and chemokines). IL-10 and TGF-β are released by these cells and show an array of immunosuppressive effects, for example:

(a) blockade of the activity of cytotoxic NK cells [44], macrophages, and cytotoxic T lymphocytes ($CD8^+$) [45];

(b) reduced expression of class II MHC molecule on the surface of antigen presenting cells [46];

(c) polarization of immune response towards Th2 [47];

(d) inhibition of dendritic cell maturation [48];

(e) inhibition of certain functions of T lymphocytes [49];

(f) stimulation of tumor cell B7-H3 expression [50].

Together with a strong anti-inflammatory signal, neoplastic cell-released chemotactic factors for monocytes and macrophages induce their differentiation into MII macrophages, and hence into cells showing an array of functions promoting the proliferation of neoplastic tissue. Consequently, it should be noted that aside from direct immunosuppressive activity, growing neoplasm induces anti-inflammatory activity of infiltrating cells, thus escaping from the control of the immune system.

Macrophages are terminally differentiated cells of bone marrow origin that reside in tissues and are derived from peripheral monocytes (Figure 1). Depending on the activating factor, monocytes and macrophages can be involved in an array of biological processes, such as:

(a) presentation of antigen;

(b) cytotoxicity;

(c) phagocytosis;

(d) secretion of biologically active molecules;

(e) control of inflammatory processes;

(f) rearrangement and reconstruction of destroyed tissues [51].

As previously mentioned, the proliferation of neoplastic tissue modulates the activity of immune cells, including the function of monocytes and macrophages. It is widely known that macrophages residing at the site of neoplastic growth, referred to as TAMs, constitute the predominant component of infiltrate in many neoplasms, including gastric malignancies [52–54]. Due to their pleotropic biological properties, TAMs can have both progressive and regressive effects on the development of neoplastic tissue. Moreover, they control primary and secondary immune responses. The pro- or anti-neoplastic activity of macrophages is directly associated with their pro- or anti-inflammatory activity, respectively, and tightly depends upon monocyte-activating factors, which define the relevant polarization of these cells (Figure 1).

5. Activity of Monocytes

As peripheral cells, monocytes do not have direct contact with a neoplastic tissue. Indirectly, however, they are subject to its immunomodulatory effect, responding to chemotactic

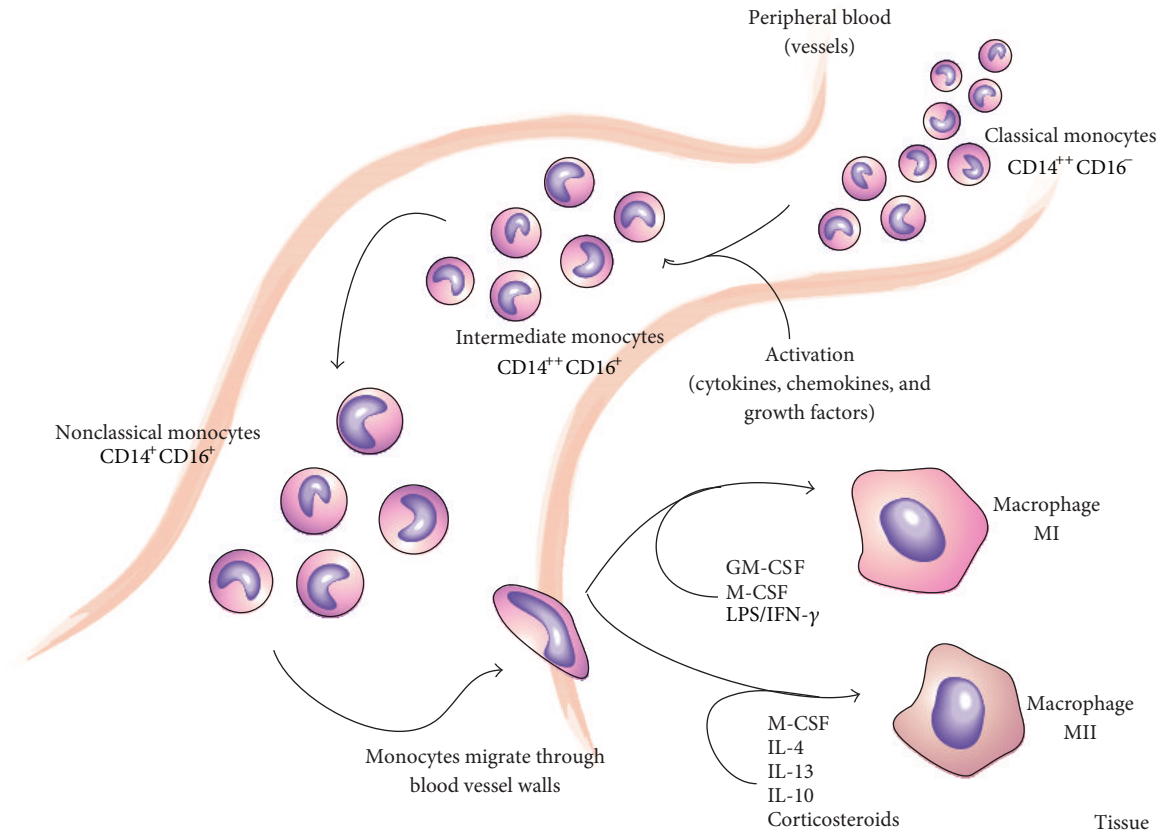

Figure 1: Differentiation of monocytes towards macrophages.

factors and neoplastic antigens present in peripheral blood, as well as to circulating neoplastic cells. All these factors directly induce the differentiation of monocytes to macrophages and their polarization towards pro- or anti-inflammatory cells. In the case of some malignancies, such as colorectal cancer, elevated monocyte count is considered as an independent prognostic factor [55].

Monocytes are heterogeneous population of cells in terms of morphology, phenotype, and effector properties. On the basis of the level of expression of lipopolysaccharide (LPS) receptor (CD14) and Fcγ receptor III (CD16), they can be classified into three well-characterized subpopulations

(a) those showing strong expression of CD14 receptor and lacking the expression of CD16 receptor ($CD14^{++}CD16^{-}$);

(b) those showing strong expression of CD14 and the presence of CD16 receptor ($CD14^{++}CD16^{+}$);

(c) those showing weaker expression of CD16 receptor than in the above-mentioned groups expression of CD14 receptor, with simultaneous ($CD14^{+}CD16^{+}$) [56].

Monocytes with $CD14^{++}CD16^{-}$ phenotype are referred to as classical monocytes and correspond to 85–95% of all peripheral monocytes under physiological conditions. The remaining two populations showing strong expression of CD16 receptor differ from each other in terms of CD14 expression level and, under physiological conditions, represent up to 15% of peripheral monocytes [57, 58]. Despite phenotypic similarities associated with the expression of CD16 receptor, stronger, as compared to classical monocytes, expression of HLA-DR, CD86, and CD54, and lower level of CD64 expression, both of the aforementioned subpopulations of monocytes show different biological activities [59]. An increase in the fraction of $CD14^{+}CD16^{+}$subpopulation of peripheral monocytes was observed during infections and inflammatory processes [60, 61], in septic states [62], and in some types of neoplasms [63, 64]. $CD14^{+}CD16^{+}$ cells are referred to as proinflammatory monocytes, because in contrast to classical monocytes upon stimulation they synthesize and release high amounts of tumor necrosis factor-alpha (TNF-α) without concomitant secretion of IL-10 or with low secretion of this cytokine [65]. The cells from this population show an array of similarities to tissue macrophages, and they are, therefore, postulated to be more mature and macrophage-like cells than the classical monocytes [66, 67]. Higher fraction of monocytes from $CD14^{++}CD16^{+}$ subpopulation has been observed in a number of conditions, including septic neonatal states [68] and gastric malignancies [69]. Additionally, compared to the proinflammatory subpopulation and classical monocytes, these cells show a higher expression of CD11b and TLR4 [59]. Moreover, they differ from the $CD14^{+}CD16^{+}$ subpopulation

in terms of characteristics such as higher phagocytic activity [70] and the presence of anti-inflammatory properties, constituting the principal source of IL-10 amongst peripheral monocytes. The presence of this subpopulation in peripheral blood is thought to constitute an intermediate stage in the differentiation of monocytes to macrophages [59].

In spite of the lack of direct contact with neoplastic tissue, peripheral monocytes represent an interesting object during the assessment of the developmental stage of the disease. As previously mentioned, they undergo a continuous stimulation by chemokines and cytokines released by neoplastic cells and tumor-infiltrating cells, as well as by neoplastic antigens and circulating neoplastic cells.

$CD14^+CD16^+$ cells are the main subpopulation of monocytes showing *in vitro* antineoplastic activity, which is directly associated with the enhanced synthesis and secretion of cytokines such as TNF-α, IL-12p40, and IL-12p70, lack of synthesis and release of IL-10, enhanced synthesis of reactive nitrogen species, and higher cytotoxic and cytostatic activities [71]. Obviously, IL-12p40 and IL-12p70 do not exert direct anti-neoplastic effect. The influence of IL-12p70 is associated with the activation of IFN-γ synthesis in lymphocytes, which in turn contributes to the polarization of immune response towards Th1, that is, a proinflammatory response against neoplastic cells. Additionally, IL-12p70 activates cytotoxic T lymphocytes ($CD3^+CD8^+$) and NK cells, both showing an array of antineoplastic properties [72]. IL-12p40 is a chemotactic factor for monocytes, which differentiate into macrophages and migrate to the tissue. Therefore, IL-12p40 enhances the infiltration of macrophages into the site of neoplastic proliferation [73], where, as pro-inflammatory cells, they can exert many antineoplastic effects. The results of *in vitro* studies suggest that increased fraction of $CD16^+$ monocytes in patients with malignancies can represent a natural consequence of immune response against neoplastic tissue as well as against circulating neoplastic cells. Spontaneous increase in this population of peripheral monocytes was also observed *in vivo*, including gastrointestinal malignancies [64].

Additionally, a decrease in the fraction of the subpopulation 1 of T-helper lymphocytes (Th1) was observed in peripheral blood of cancer patients in relation to the subpopulation 2 (Th2); this was associated with lower plasma concentration of molecules such as IL-2 and IFN-γ, and higher level of cytokines, such as IL-4, IL-10, and IL-13, as compared to healthy individuals [74]. In view of the elevated concentration of anti-inflammatory factors, circulating monocytes should gain anti-inflammatory properties and differentiate into MII macrophages, which are involved in the processes of neovascularization, among others. Proangiogenic activity is also characteristic for monocytes that show the surface expression of angiopoietin receptor (Tie-2), although this receptor is mostly expressed on epithelial cells and is considered as a specific feature of vascular epithelial cells [75]. Tie-2^+ monocytes represent a separate population of cells referred to as the Tie-2-expressing monocytes (TEMs). Even though their physiological fraction in peripheral blood is low and corresponds to only 1-2% of peripheral leukocytes, approximately 20% of circulating monocytes are Tie-2^+ [43, 76]. An increase in the fraction of Tie-2-expressing monocytes, even up to 10% of all peripheral blood mononuclear cells (PBMCs), has been observed in cancer patients. Murine model studies revealed strong pro-angiogenic properties of TEMs during the processes of neoplastic tissue neovascularization. Moreover, these cells are considered the precursors of pro-angiogenic tissue macrophages [76], which correspond to up to 30% of all TAMs in certain parts of the neoplastic tissue [77].

6. Macrophage Activity

Macrophages are the predominant cells of the leukocytic infiltrate of many neoplasms and are able to polarize their immune response in both a pro- or anti-inflammatory direction. Monocytes, activated by microorganisms or their parts, certain pro-inflammatory cytokines (e.g., IFN-γ), GM-CSF, and M-CSF, migrate into tissues and differentiate into pro-inflammatory cells, referred to as MI macrophages, which are involved in the destruction of microorganisms and neoplastic cells, among others (Table 1). Their function includes the activation of immune system and the support of adaptive response by means of:

(a) enhanced synthesis and secretion of pro-inflammatory cytokines such as TNF-α, IL-1, IL-6, IL-12, and IL-23 [78–80];

(b) enhanced synthesis and secretion of chemokines such as CCL5, CCL8, CXCL2, and CXCL4 [81–83];

(c) polarization of immune response towards Th1 and/or Th17 [84];

(d) high capacity for presentation of antigen to antigen-naive T lymphocytes;

(e) cytotoxic potential [85].

Monocytes activated by factors such as IL-4, IL-13, IL-10, and M-CSF show typical activities of anti-inflammatory cells; when present in tissues, they are referred to as MII macrophages (Table 1). These cells are characterized by:

(a) enhanced synthesis and secretion of anti-inflammatory cytokines such as IL-10, TGF-β, and IL-1RA;

(b) enhanced synthesis and secretion of chemokines such as CCL16, CCL18, and CCL22;

(c) polarization of immune response towards Th2;

(d) induction of T-regulatory (Treg) lymphocyte formation;

(e) low capacity for the presentation of antigen to antigen-naive T lymphocytes;

(f) strong expression of arginase-1 (its activity alters the metabolism of L-arginine into ornithine and polyamines, which results in the blockade of inducible nitric oxide synthase (iNOS));

(g) lack of cytotoxic activity;

(h) higher expression of certain membrane receptors, including type 2 Fc receptor for IgG (Fc-R2, CD23),

Table 1: Macrophage subsets.

Populations	Inducing agents	Functions
MI	GM-CSF; IFN-γ + LPS; TNF-α	(i) High capacity for antigen presentation (ii) Th1 polarization (iii) Defense against bacteria (iv) Tumor suppression (v) Immunostimulation (vi) Ability to induce a cytotoxic effect
MII		
MIIa	IL-4; IL-13	(i) Th2 polarization
MIIb	Immune complex;	(ii) Down-regulation of adaptive immunity
	IL-1R agonists; TLR ligands	(iii) Tumor growth promotion
MIIc	IL-10; TGF-β;	(iv) Proangiogenic
	glucocorticoids	(v) Tissue remodeling and repair

mannose receptor (MR), and receptor for LPS (CD14) [85].

The principal tasks of MII include the suppression of adaptive response, inhibition of cytotoxic cell activity, rearrangement and reconstruction of destroyed tissues, and their neovascularization. Therefore, MII macrophages play a regulatory function in pro-inflammatory response *via* the control of MI cell-dependent activities [86]. Consequently, the maintenance of body homeostasis requires maintaining a proper ratio of both discussed subpopulations of macrophages, namely, MI and MII. This balance is disturbed during such pathological conditions as the proliferation of neoplastic cells, leading to the impaired activity of immune system and uncontrolled progression of the disease. This is unambiguously associated with the immunomodulatory effect of proliferating neoplasm. However, the secretory activity of macrophages residing within the tumor should not be forgotten. The activity of macrophages in the course of neoplastic disease has been studied extensively as they can exert both progressive (MII macrophages) and regressive (MI macrophages) effects on the development of neoplastic tissue. The balance between MI and MII macrophages seems to be controlled by NFκB signaling, because targeting of this transcription factor switched macrophages from an MII to an MI phenotype. The consequence of which is the regression of tumor tissue *in vitro* [87].

As pro-inflammatory cells, macrophages are involved in stromal remodeling releasing a slate of pro-inflammatory factors that constitute a signal of danger for immune cells. This macrophage activity may result in the following:

(a) activation of cytotoxic T lymphocytes and NK cells;

(b) influx of dendritic cells;

(c) migration and differentiation of monocytes in a pro-inflammatory direction.

Activated MI macrophages synthesize and release IL-12, which, as was mentioned previously, shows indirect antineoplastic activity. Additionally, it exerts stimulatory effect on NK cells, inducing the synthesis and secretion of IFN-γ [88] and enhancing their cytotoxic potential. Stimulated with this cytokine, macrophages release factors such as ROI, IL-1, IL-6, arginase, and TNF-α, that is, pro-inflammatory factors exerting cytostatic and cytotoxic effects upon neoplastic cells. Moreover, these cells show a strong cytotoxic activity in both the antibody-dependent (ADCC) and antibody-independent (MTC) mechanisms [89].

Unfortunately, the majority of macrophages infiltrating neoplastic tissue have phenotype characteristic for MII [85], and thus they present an array of activities promoting the growth of neoplastic tissue. Consequently, tumor-associated macrophages are considered to constitute MII-like cells. The level of their infiltration is used as an independent prognostic factor in many tumor types. However, it should be noted that in the case of some malignancies, for example, colorectal and gastric cancers, higher fraction of these cells does not necessarily correlate negatively with patients' survival. The activity of TAMs in gastric malignancies can vary depending on tumor region. For example, higher infiltration of TAMs to the region of tumor cell nests is associated with improved survival. Despite the small fraction of nest TAMs, as compared to their overall count in the other regions of the tumor, enhanced apoptosis of neoplastic cells was observed along with a higher activity of cytotoxic T lymphocytes. Consequently, it should be emphasized that antineoplastic activities controlled by macrophages with MI phenotype can be induced in certain regions of neoplastic tissue [52]. However, they represent a minority of pro-neoplastic activities of MII macrophages, associated with the following:

(a) suppression of adaptive response;

(b) promotion of tumor growth;

(c) promotion of the metastases of neoplastic cells;

(d) involvement in the recruitment of peripheral monocytes and macrophages from the surrounding tissues.

The MII macrophages release an array of anti-inflammatory factors, such as IL-10 and prostanoids, causing the

attenuation of type Th1 immune response, as well as an impaired activity of cytotoxic T lymphocytes and NK cells. Moreover, they secrete a variety of specific cytokines (e.g., CCL17 and CCL22), inducing the inflow of regulatory T cells and Th2 subpopulation of helper lymphocytes. These effects are reflected by the suppression of pro-inflammatory activities of the immune system, that is, by the inhibition of activities oriented against neoplastic cells [86]. Moreover, tumor-associated macrophages are capable of modifying ζ subunit of TCR receptor (TCR-ζ) of T-helper lymphocytes [90–92], which plays a crucial role in the activation of the latter cells [93]. The disorders of TCR-ζ expression or inactivation of this subunit are reflected by the anergy of T lymphocytes, leading to their apoptosis.

Intensified proliferation of neoplastic tissue is associated with an increased requirement for nutrients and growth factors and leads to the hypoxia of the tumor. High efficiency of pro-neoplastic MII macrophage activity is associated with their ability to accumulate within the oxygen-deficient regions of the tissue. TAMs synthesize and release an array of growth and pro-angiogenic factors that are concurrently chemotactic factors for monocytes and macrophages, including VEGF, bFGF, CXCL8, PDGF, EGF, and TGF-β [89, 94]. PDGF promotes the proliferation of neoplastic tissue; additionally, it acts as a pro-angiogenic factor and recruits pericytes stabilizing the newly formed vessels [95]. EGF stimulates neoplastic cells to synthesize and release M-CSF. Aside from the chemotaxis of macrophages from the surrounding tissues, M-CSF induces the differentiation and migration of peripheral monocytes. This constitutes one of the mechanisms behind the enhanced infiltration of macrophages into the tumor, which is in turn reflected by an enhanced synthesis of EGF. EGF/M-CSF feedback cycle leads to macrophage-dependent, growth factor-induced intensive proliferation of neoplastic tissue [96].

Macrophages present at the invasive front of the tumor (margin TAMs) participate in the creation of promoting environment for neoplastic cells, enabling them to reach vascular and lymphatic system. As previously mentioned, TAMs constitute a source of metalloproteinases (such as MMP-2 and MMP-9) and an urokinase-type plasminogen activator (uPA) [97], which facilitate tumor invasion due to their involvement in the degradation of basal membrane and extracellular matrix. This process seems to a large extent to be EGF and M-CSF dependent. The blockade of EGF and M-CSF activities is reflected by inhibited migration of neoplastic cells and macrophages, respectively [98, 99]. Therefore, the M-CSF-stimulated TAMs induce the migration of neoplastic cells on EGF-dependent pathway [100]. Most likely, this process is also modulated by IL-4, which polarizes macrophages towards promoting the invasiveness and spread of the tumor; these functions are blocked in the lack of IL-4 [101, 102]. The invasiveness of neoplastic cells can be also modulated in TNF-α-dependent manner [103], with macrophages being the principal source of TNF-α [104, 105]. The migration of neoplastic cells through the stroma is markedly more efficient if supported by collagen fibers (type I collagen) [106], formed with the involvement of TAMs. These fibers expand towards blood vessels [107], significantly facilitating the migration of neoplastic cells and thus promoting the formation of distant metastases. In summary, due to their pro-angiogenic activity, synthesis of collagen fibers, and the induction of neoplastic cell migration, TAMs actively promote the invasion and spread of the tumor.

7. Conclusion

The process of neoplastic tissue proliferation is directly related to the modulatory effect on the immune system. Monocytes/macrophages are particularly susceptible to this effect. This results in a switch from a protective function into one that promotes neoplastic proliferation. In the case of many malignancies (e.g., breast, prostate, or endometrial cancer), high percentage of TAMs is associated with poor prognosis. In practice, high percentage of tumor-associated macrophages may be an independent prognostic factor, providing a thorough examination of all the regions of the tumor and a precise assessment of the phenotype of these cells. TAMs also seem to be a promising target for antineoplastic therapy, the aim of which should be, for example, the reversal of the unfavorable balance between MI and MII macrophages [87]. On the other hand, monocytes/macrophages can be used as a delivery system of anticancer agents to tumors [108].

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Research Article

Regulatory Role of GSK-3β on NF-κB, Nitric Oxide, and TNF-α in Group A Streptococcal Infection

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Group A streptococcus (GAS) imposes a great burden on humans. Efforts to minimize the associated morbidity and mortality represent a critical issue. Glycogen synthase kinase-3β (GSK-3β) is known to regulate inflammatory response in infectious diseases. However, the regulation of GSK-3β in GAS infection is still unknown. The present study investigates the interaction between GSK-3β, NF-κB, and possible related inflammatory mediators in vitro and in a mouse model. The results revealed that GAS could activate NF-κB, followed by an increased expression of inducible nitric oxide synthase (iNOS) and NO production in a murine macrophage cell line. Activation of GSK-3β occurred after GAS infection, and inhibition of GSK-3β reduced iNOS expression and NO production. Furthermore, GSK-3β inhibitors reduced NF-κB activation and subsequent TNF-α production, which indicates that GSK-3β acts upstream of NF-κB in GAS-infected macrophages. Similar to the in vitro findings, administration of GSK-3β inhibitor in an air pouch GAS infection mouse model significantly reduced the level of serum TNF-α and improved the survival rate. The inhibition of GSK-3β to moderate the inflammatory effect might be an alternative therapeutic strategy against GAS infection.

1. Introduction

Group A streptococcus (GAS; *Streptococcus pyogenes*) is an important clinical pathogen in humans and induces a wide spectrum of clinical presentations including pharyngitis, erysipelas, necrotizing fasciitis, toxic shock syndrome, sepsis, and even mortality [1]. Numerous virulence factors of GAS, including surface molecules such as M protein, hyaluronic acid capsule, and C5a peptidase, or exotoxins, such as pyrogenic exotoxins, streptokinase, hyaluronidase, and streptolysins O and S, enable bacteria to resist phagocytosis in macrophages, escape from complement-mediated destruction, retard the influx of inflammatory cells, or trigger overreaction of immune system [2–6]. Despite advanced supportive care and effective antibiotic treatment, invasive GAS diseases are still responsible for at least 163,000 deaths each year in the world [2, 7, 8]. These facts highlight the urgent need for therapeutic strategies beyond effective antibiotic treatment. In patients with acute invasive GAS infection, the magnitude of elevation of IL-6 and TNF-α is closely related to the severity of systemic manifestations of the disease. Severe invasive cases suffering from toxic shock and/or necrotizing fasciitis have significantly higher frequencies of IL-2-, IL-6-, and TNF-α-producing cells in their circulation as compared to nonsevere invasive cases [9, 10]. Evidence suggests that the induction of cytokine production is, at least in part, due to superantigens of GAS. After the activation by superantigens, production of cytokines (e.g., IL-1, TNF-α, IL-6, and IFN-γ), which mediate shock and tissue injury,

is initiated. Streptococcal pyrogenic exotoxin (SPE) B is able to cleave pre-IL-1β to release active form IL-1β [11]. Besides, peptidoglycan, lipoteichoic acid, and killed organisms are capable of inducing TNF production by mononuclear cells *in vitro* [12, 13]. Thus, clinical management to control the exacerbated inflammatory response caused by GAS infection may diminish collateral tissue damage and further reduce morbidity and mortality.

Glycogen synthase kinase-3 (GSK-3), a serine/threonine protein kinase, is involved in the regulation of many intracellular functions, including cell division, apoptosis, cell fate during embryonic development, signal pathways stimulated by insulin and many growth factors, and even the dysregulation of disease processes of cancer, diabetes, and neurodegenerative diseases [14–17]. In addition, GSK-3 is critical in either promoting [18] or repressing [19] the activity of NF-κB which indicates its possible regulatory role in inflammation. Either GSK-3 inhibitors or siRNA could reduce the production of TNF-α and IL-6 and enhance IL-10 production in monocytes after stimulation by lipopolysaccharide (LPS) [20]. GSK-3β was also shown to regulate the STAT3-mediated IL-6 production in LPS-stimulated glial cells [21]. Furthermore, GSK-3 negatively regulated mycobacterium-induced IL-10 production and the subsequent IFN-γ secretion in monocytes [22]. In animal model of sepsis, treatment with GSK-3 inhibitors could suppress NF-κB-dependent proinflammatory cytokine expression and provide protection from organ injury and endotoxin shock [20, 23, 24]. However, most of the previous studies investigating the role of GSK-3 in infection involved a LPS-induced sepsis model. Few studies have investigated the role of GSK in live bacterial infection, which is more representative of the clinical condition [25–28]. Also, less is known about the role of GSK in Gram-positive bacterial infection.

The macrophage is one of the frontline components in human innate immunity. It plays an important role in clearance of invasive pathogens through phagocytosis. Interference with macrophage function results in higher mortality rate in the GAS-infected animal model [29]. This implies an essential role of macrophages against GAS infection. In search of the possible role of GSK-3β in GAS-induced inflammatory response, we evaluated the activity of GSK-3β and subsequent inflammatory mediators in a mouse macrophage cell line and in the mouse model. Our results demonstrate that GAS infection induces GSK-3β activity, NF-κB nuclear translocation, iNOS expression, and NO and TNF-α production. Inhibition of GSK-3β can negatively regulate the activity of NF-κB and the production of NO and TNF-α. The effects of GSK-3β inhibitor were also observed in GAS-infected mice.

2. Material and Methods

2.1. Mice. BALB/c mice were purchased from the Jackson Laboratory, Bar Harbor, Maine, and maintained on standard laboratory food and water *ad libitum* in our animal center. Their progeny, ranging from 8 to 10 weeks of age, were used for experiments. The animal use protocol had been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC).

2.2. Bacterial Strain. *S. pyogenes* NZ131 (type M49, T14) was obtained from Dr. D. R. Martin, New Zealand Communicable Disease Center, Porirua. This strain does not contain phage-specific *spe*A and *spe*C genes.

2.3. Air Pouch Model of Infection. In our previous studies, we have established a mouse model of GAS infection using an air pouch [30, 31]. Mice were anesthetized by ether inhalation and then injected subcutaneously with 2 mL of air to form an air pouch on their back. Bacterial suspension was inoculated into the air pouch. Mice infected with GAS developed bacteremia and disseminated into the kidney, liver, and spleen. Mice died within few days (usually 3–7 days) after GAS infection. This model therefore serves as an animal model of sepsis.

2.4. GAS Infection of Macrophages. Mouse macrophage cell line RAW 264.7 was cultured in DMEM with 10% FBS in 5% CO_2 at 37°C. RAW 264.7 cells were seeded at 2×10^5/well in 24-well plate containing medium without antibiotics. The next day, NZ131 cultures grown in TSBY were harvested at midlogarithmic phase and added to RAW 264.7 monolayers at different multiplicity of infection (MOI) of 10, 50, and 100. After 60 min of incubation at 37°C, nonadherent extracellular bacteria were eliminated by removing the culture medium and washing by PBS. Adherent extracellular bacteria were subsequently killed by incubation with fresh medium containing 10 μg/mL penicillin G. At specific time points after infection, supernatants were collected for ELISA, and whole cell extracts were prepared for Western blot analysis.

2.5. ELISA. The levels of TNF-α in serum or cell culture supernatant were measured by ELISA kits (R&D system), according to the manufacturer's instructions. All measurements were carried out in triplicates.

2.6. Western Blot Analysis. Whole cell extracts were separated using SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membrane. After blocking, blots were developed with rabbit antibodies against total and phosphorylated (Ser9) GSK-3β, total GS and phosphorylated (Ser641) GS, and mouse antibodies against iNOS. Mouse antibodies specific for GAPDH or α-tubulin were used for internal control. Finally, blots were hybridized with horseradish-peroxidase-(HRP-) conjugated goat anti-rabbit immunoglobulin G (IgG) or goat anti-mouse IgG, incubation with enhanced chemiluminescence (ECL) solution, and exposure to X-ray film.

2.7. Immunocytochemical Staining. Cells collected at various time points were fixed in 4% formaldehyde for 10 min. Adequately diluted anti-NF-κB p65 antibodies were applied. After reaction with primary antibody at room temperature for 1 h, FITC-labeled secondary antibodies were applied for additional 1 h. DAPI was used for nuclear staining. The

positive cells in three fields (under magnification ×200) of each culture were measured.

2.8. NO Analysis. After GAS infection for 24 h with or without GSK-3β inhibitors, supernatant of cell culture was collected. Then, 50 μL Griess reagent was mixed with 50 μL sample for measurement of nitrite level, according to the manufacturer's instructions.

2.9. Luciferase Reporter Assay. For the NF-κB reporter assay, cells were transiently cotransfected with NF-κB promoter-driven luciferase reporter (0.2 μg) and 0.004 μg of *Renilla* luciferase-expressing plasmid (pRL-TK; Promega) using the Gene Jammer transfection reagent (Stratagene). At 24 h after the transfection, cells were infected with NZ131 for 1 h and then replaced with medium containing antibiotics. Cells were then harvested for the luciferase assay (Dual-Glo; Promega). The firefly luciferase activity was normalized to the *Renilla* luciferase activity to evaluate transfection efficiencies.

2.10. Antibody and Reagent. Mouse monoclonal antibody specific for NF-κB p65 was obtained from Chemicon International, Inc. Monoclonal anti-mouse iNOS was from BD Biosciences. Peroxidase-conjugated goat anti-mouse IgG and goat anti-rabbit IgG were from Invitrogen Corp. Antibodies against phospho GSK-3β (Ser9), GSK-3β, phospho-glycogen synthase (GS) (Ser641), and GS were from Cell Signaling Technology, Inc. Antibody against GAPDH was from Millipore Corporation. Antibody against α-tubulin was from Santa Cruz Biotechnology Inc. Pyrrolidine dithio- carbamate (PDTC), 3-(2,4-dichlorophenyl)-4-(1-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione (SB216763) and 3-[(3-chloro-4-hydroxyphenyl)amino]-4-(2-nitrophenyl)-1H-pyrrol-2,5-dione (SB415286) were from Tocris Bioscience. 6-Bromoindirubin-3'-oxime (BIO), lithium chloride (LiCl) and ammonium chloride (NH4Cl) were from Sigma-Aldrich Co. Blocking antibodies specific for TLR2 and isotype-matched antibody control were from eBioscience, Inc.

2.11. Cell Viability. At 24 h after GAS infection and treatment with or without GSK-3β inhibitors, RAW 264.7 cells were flushed with culture medium in 6-well plates. Then, the whole culture medium was aspirated. The live and dead cells in culture medium were calculated directly under microscope after staining with trypan blue.

2.12. Mouse Survival Rate after GAS Infection. After inoculation with GAS into air pouch, various dosages of GSK-3β inhibitors were injected into the peritoneal cavity at different time points. The survival of mice after infection was observed every 24 h for 10 days.

2.13. Statistics. All statistics were performed using the two-tailed Student's t-test by the software of GraphPad Prism 5.01. The P values < 0.05 were considered significant. The mouse survival rate was analyzed by the Kaplan-Meier method.

3. Results

3.1. GAS Infection Induces the Activation of NF-κB and the Increased Expression of iNOS and NO Production in RAW264.7 Cells. NF-κB is a ubiquitous transcription factor and plays an important role in the host response to pathogenic organisms. The activation of NF-κB can induce the expression of a number of molecules involved in inflammatory response, such as cytokines and iNOS [32, 33]. Infection of human respiratory epithelial cells with GAS induced the activation of NF-κB [34]. To address if infection of macrophages with GAS could also induce the activation of NF-κB, murine macrophage RAW 264.7 cells were infected with GAS NZ131 strain at MOI of 10 for various times. By using immunocytochemical staining and calculating the fold of NF-κB nuclear translocation, the nuclear translocation of NF-κB increased gradually after GAS infection and marked activation of NF-κB was observed 4 h after infection (Figures 1(a) and 1(b)). Similar trend of NF-κB activation was also observed when the activity was measured by luciferase reporter assay (Figure 1(c)).

To further evaluate the expression of iNOS and the subsequent production of NO, we determined the time kinetics and dose response of GAS by Western blotting and Griess reagent. The results revealed that GAS induced the expression of iNOS in a time-dependent manner (Figure 1(d)). The NO production was increased at 12 h with MOI of 50 or 100, and at 24 h with MOI of 10 (Figure 1(e)). To further clarify whether the iNOS expression and NO production were mainly through a NF-κB-dependent pathway, RAW 264.7 cells were treated with GAS in the presence or absence of NF-κB inhibitor, PDTC. Inhibition of NF-κB activity caused suppression of iNOS and NO production (Figures 1(f) and 1(g)). These results reveal an essential role of NF-κB in regulation of iNOS and NO expression in GAS infection.

3.2. GAS Infection Activates GSK-3β and Inhibiting GSK-3β Reduces the Expression of iNOS and the NO Production in RAW264.7 Cells. Since GSK-3β was revealed to act upstream of NF-κB [16, 18, 35, 36], we investigated whether GSK-3β may regulate NF-κB during GAS infection and might be a potential therapeutic target. The dephosphorylation of GSK-3β at serine 9 was observed within 2 h after GAS treatment, which indicates the induced activation of GSK-3β in RAW 264.7 cells after GAS infection (Figure 2(a)). The phosphorylated GS, a GSK-specific substrate, also increased at 2 h after infection (Supplemental Figure 1 in supplementary material available online at http://dx.doi.org/10.1155/2013/720689). These results suggest that GAS infection could induce GSK-3β activation. When RAW 264.7 cells were stimulated with heat-inactivated GAS, there was only slight activation of GSK-3β (Supplemental Figure 2).

To further explore the role of GSK-3β, we found that the expression of iNOS was reduced by GSK-3β inhibitors (Figure 2(b)). Although the inhibition of iNOS expression was not apparent with lithium treatment as compared with SB compounds and BIO treatment, the NO production was significantly suppressed by lithium (Figure 2(c)). BIO could efficiently suppress the NO production. When measuring the

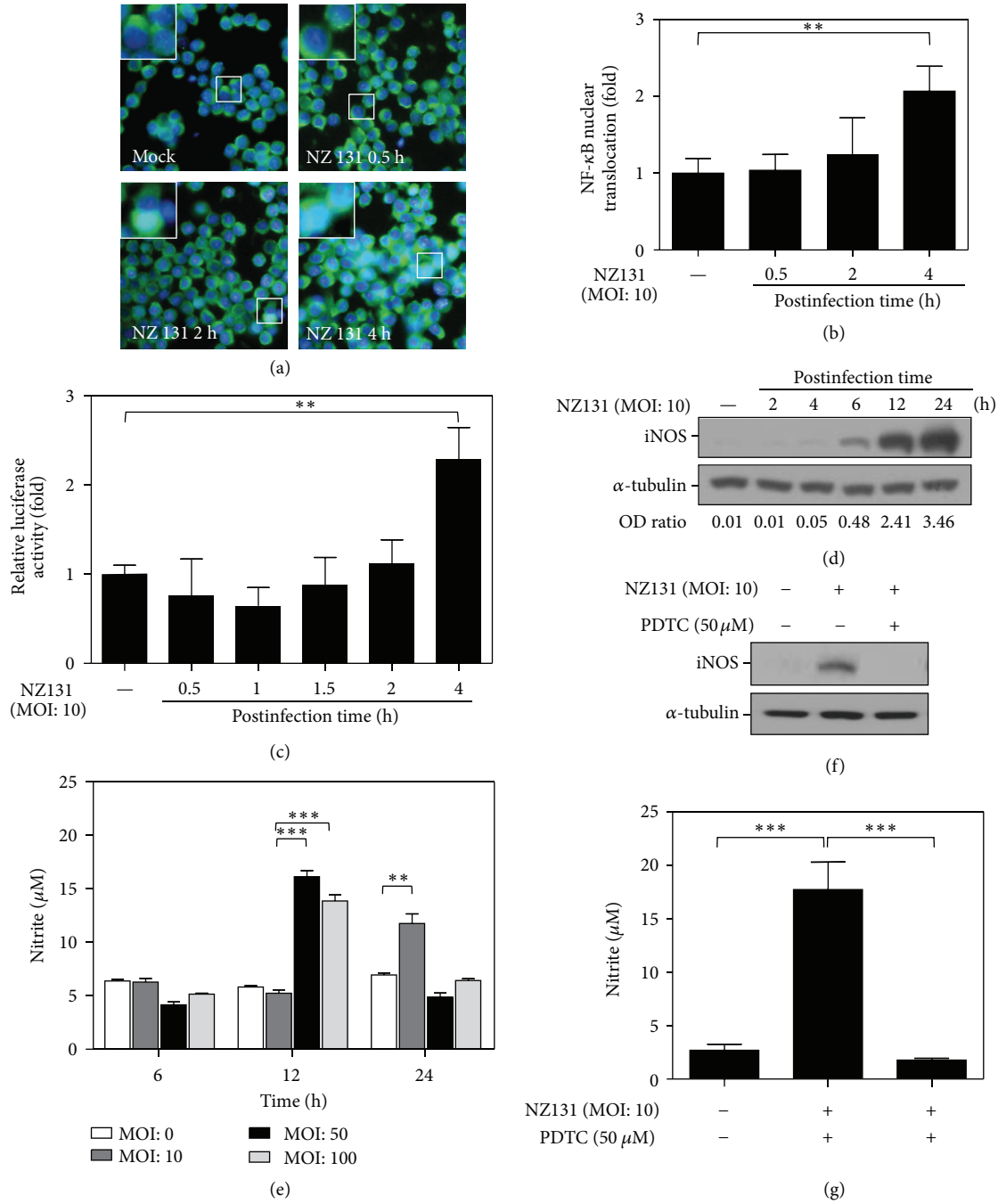

Figure 1: NZ131 infection induces the activation of NF-κB and the increased expression of iNOS and NO in macrophages. (a) Using immunocytochemical staining for detection of NF-κB p65 and DAPI for nuclear staining, the nuclear translocation of NF-κB in RAW 264.7 cells (2×10^5 cells/well in 24-well culture plate) after NZ131 infection (MOI: 10) at different time points was detected. Representative fields of NF-κB translocation in RAW 264.7 cells are shown. (b) The bar chart graph is the summary of (a) for the ratio of the change of NF-κB nuclear translocation after NZ131 infection at different time points. (c) RAW 264.7 cells were cotransfected with NF-κB promoter-driven luciferase reporter and *Renilla* luciferase-expressing plasmid for 24 h. Then, RAW 264.7 cells were infected with NZ131 (MOI: 10) for 1 h. Luciferase activity was used to determine the dynamic change of NF-κB activity 0.5–4 h after infection and the relative luciferase activity is the activity when compared with cells without NZ131 infection. (d) Western blot analysis was used to determine the expression of iNOS in RAW 264.7 cells stimulated by NZ131 (MOI: 10) for the indicated time points. (e) Griess reagent was used to determine the NO production in RAW 264.7 cells after treatment with different MOI of NZ131 (MOI: 0, 10, 50, or 100) at various time periods (6, 12, and 24 h after infection). (f) The expression of iNOS was determined by Western blot analysis with or without NF-κB inhibitor, PDTC (50 μM), for 6 h after NZ131 infection (MOI: 10). (g) The NO production was measured by Griess reagent 24 h after NZ131 infection (MOI: 10) with or without the concomitant treatment of PDTC. The data are means ± SD of the results obtained from three individual experiments. $^{**}P < 0.01$; $^{***}P < 0.001$, comparisons between the indicated groups.

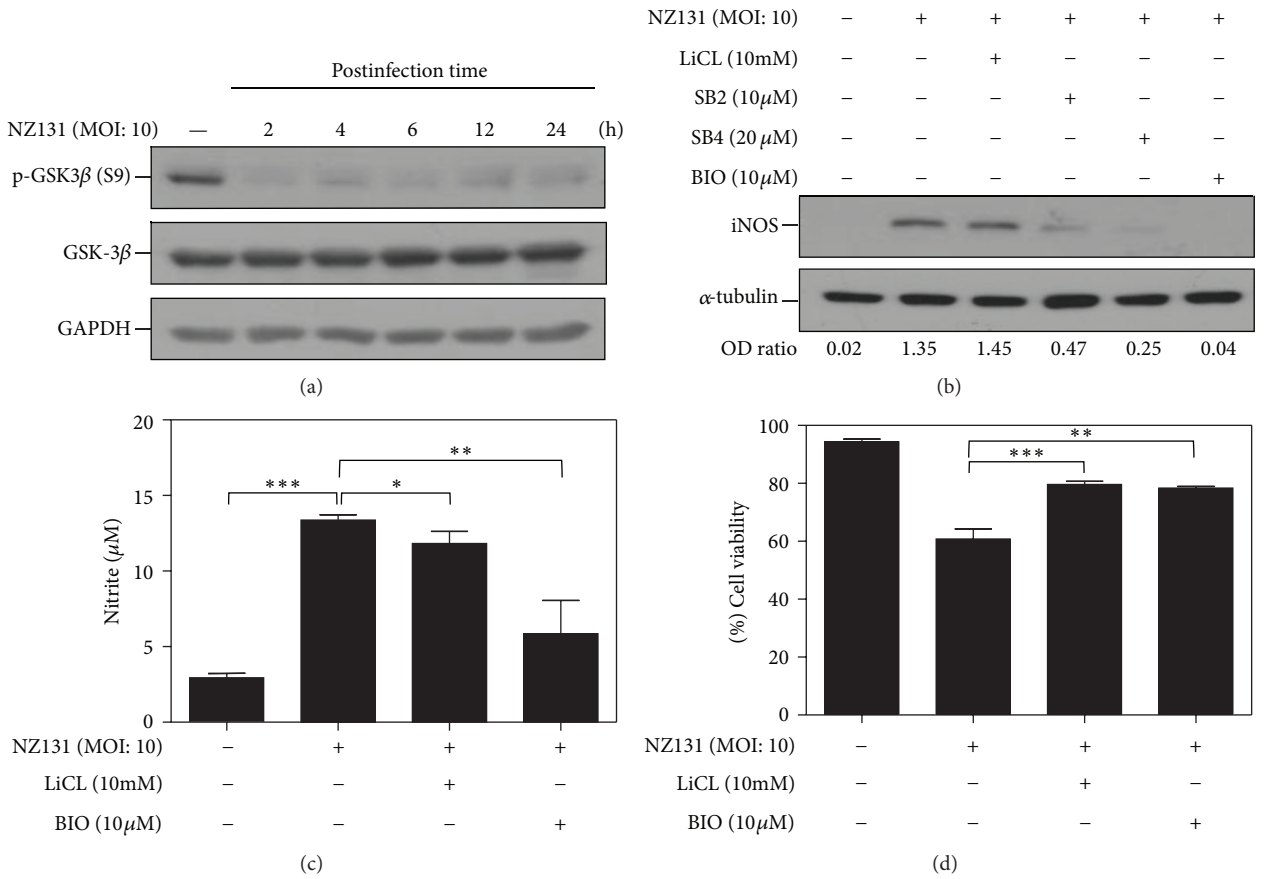

Figure 2: GSK-3β is activated in macrophages after stimulation by NZ131 and inhibiting GSK-3β reduces iNOS expression and NO production. (a) Western blot analysis was used to detect the expression of phospho-GSK-3β at Ser9 in RAW 264.7 cells (MOI: 10) at the indicated time points. (b) After pretreatment with various GSK-3β inhibitors (LiCl, SB216763, SB415286, and BIO), Western blot analysis was used to determine the expression of iNOS in RAW 264.7 cells at 6 h after NZ131 infection. (c) After NZ131 stimulation (24 h, MOI: 10), Griess reagent was used to determine the NO production in RAW 264.7 cells pretreated with GSK-3β inhibitors (LiCl and BIO). (d) Percentage of cell viability was determined 24 h after NZ131 infection with or without concomitant treatment with GSK-3β inhibitors (LiCl and BIO). The data are means ± SD of the results obtained from three individual experiments. $^{*}P < 0.05$, $^{**}P < 0.01$; $^{***}P < 0.001$, comparisons between the indicated groups.

cell viability under GAS infection with or without GSK-3β inhibition, GAS infection resulted in 29.23% cell death and treatment with GSK-3β inhibitors, lithium and BIO, could improve cell viability (Figure 2(a)). Thus, the reduction of NO production was not due to the reduced cell numbers. These results confirm that GSK-3β activation is involved in GAS infection and inhibition of GSK-3β reduces NO production, which implies the possible therapeutic application in GAS infection.

3.3. Inhibition of GSK-3β Activity Downregulates the NF-κB Activation and TNF-α Production in GAS-Infected RAW 264.7 Cells. To further determine the essential role of GSK-3β in GAS infection, we examined the effect of GSK-3β inhibition on NF-κB activation and TNF-α production. Using immunocytochemistry to detect NF-κB p65 after GAS infection, nuclear translocation of NF-κB was reduced after inhibiting GSK-3β by various inhibitors (Figure 3(a)). The ratio of NF-κB nuclear translocation after GSK-3β inhibitor treatment was even lower than the basal level (Figure 3(b)). To investigate whether TNF-α production was also regulated by NF-κB, we treated RAW 264.7 cells with PDTC under GAS infection. The TNF-α production was significantly suppressed by NF-κB inhibition (Figure 3(c)). This implies that GAS induces TNF-α expression via NF-κB activation. Furthermore, when treated with BIO and lithium, the production of TNF-α was also reduced in GAS infection (Figure 3(d) and Supplemental Figure 3). These results indicate that GSK-3β is involved in GAS-induced NF-κB activation and TNF-α production, and inhibition of GSK-3β can lessen the GAS-induced inflammatory response.

3.4. GAS Internalization and TLR-2 Signaling Regulate TNF-α Production and iNOS Expression. GAS is known to survive and replicate in the macrophage and intracellular GAS can mediate NF-κB activity [37]. Besides, cell wall components of Gram-positive bacteria can induce inflammatory response through TLR-2 [38]. We next determined whether GAS invasion or adherence to the plasma membrane can regulate TNF-α and iNOS expression. We found that either inhibition

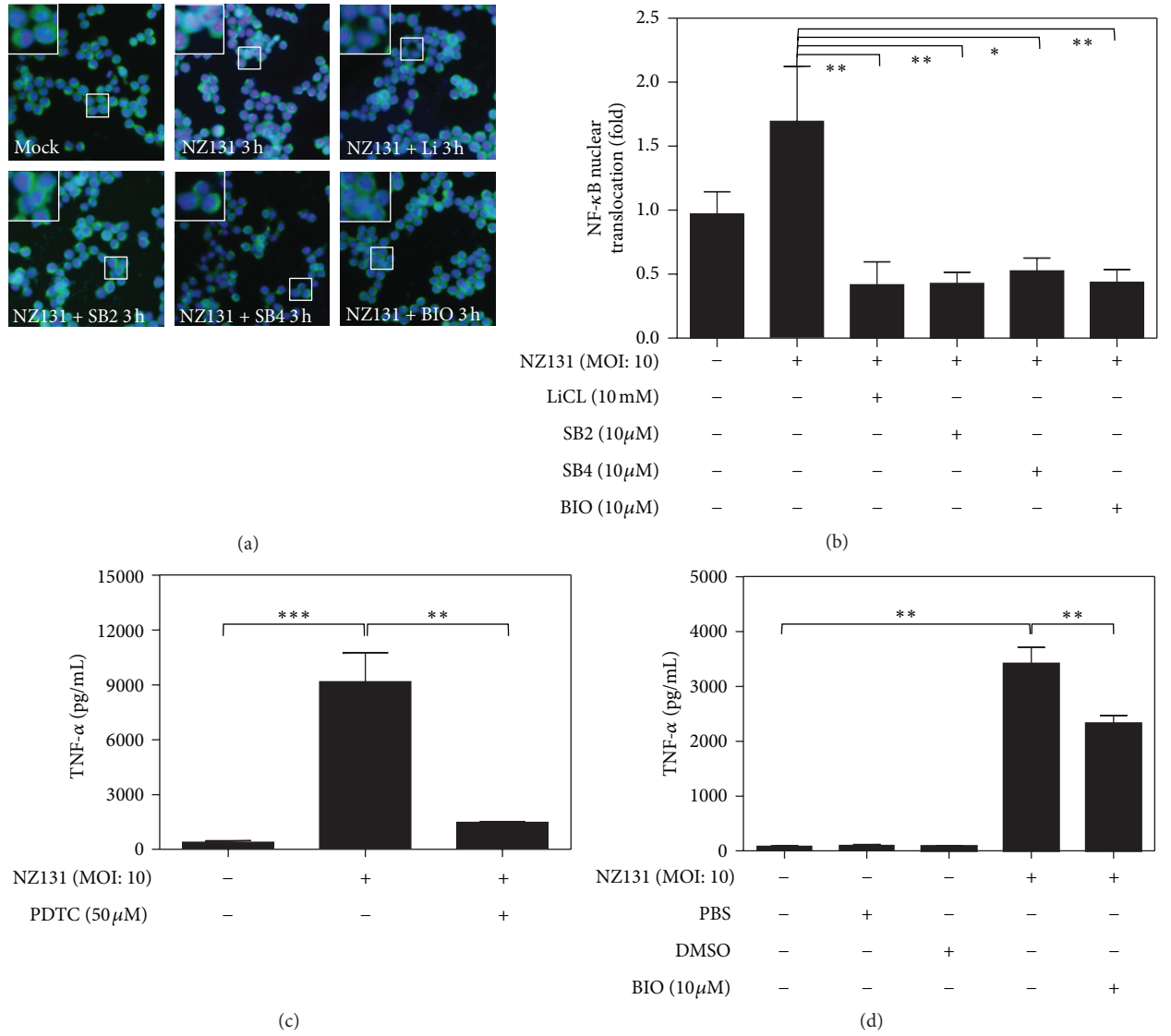

Figure 3: Inhibition of GSK-3β downregulates NF-κB nuclear translocation and proinflammatory cytokine TNF-α production in macrophages after stimulation by NZ131. (a) Immunocytochemical staining for NF-κB p65 and DAPI for nuclear staining were used to determine the nuclear translocation of NF-κB in RAW 264.7 cells (2×10^5 cells/well in 24-well culture plate) 3 h after NZ131 infection (MOI: 10). Representative fields of NF-κB translocation in RAW 264.7 cells are shown. (b) The bar chart graph is the summary of (a) for the ratio of the change of NF-κB nuclear translocation after NZ131 infection and pretreatment with various GSK-3β inhibitors (LiCl, SB2, SB4, and BIO). The data for each treated or untreated group is the average result calculated by six randomly selected fields in one experiment. ((c) and (d)) The concentrations of TNF-α in RAW 264.7 cell culture supernatant 24 h after NZ131 stimulation and pretreatment with NF-κB inhibitor (PDTC) or GSK-3β inhibitor (BIO) were determined.

of endocytosis or blocking TLR-2 signaling can partially suppress TNF-α and iNOS expression (Supplemental Figures 3 and 4). These results suggest that GAS internalization and TLR-2 signaling may, at least in part, participate in the regulation of GAS-induced inflammatory response.

3.5. Inhibition of GSK-3β Activity Provides Protection of Sepsis Induced by GAS and Suppresses Serum TNF-α Level in Mouse Model. Since the inhibition of GSK-3β led to reduced NF-κB activation and TNF-α production *in vitro*, we next determined whether this protective effect via GSK-3β inhibition could be observed *in vivo*. Administration of lithium, a drug which has been used for bipolar disorder, improved the survival rate in the treated group ($P = 0.05$) (Figure 4(a)). Lithium treatment after GAS infection could significantly suppress TNF-α production (Figure 4(b)). Thus, inhibition of GSK-3β could reduce the level of TNF-α and increase survival in the GAS-induced sepsis model.

4. Discussion

Group A streptococcus is an important human pathogen and carries a high clinical burden worldwide [7]. Reducing the related complications and mortality and morbidity caused by

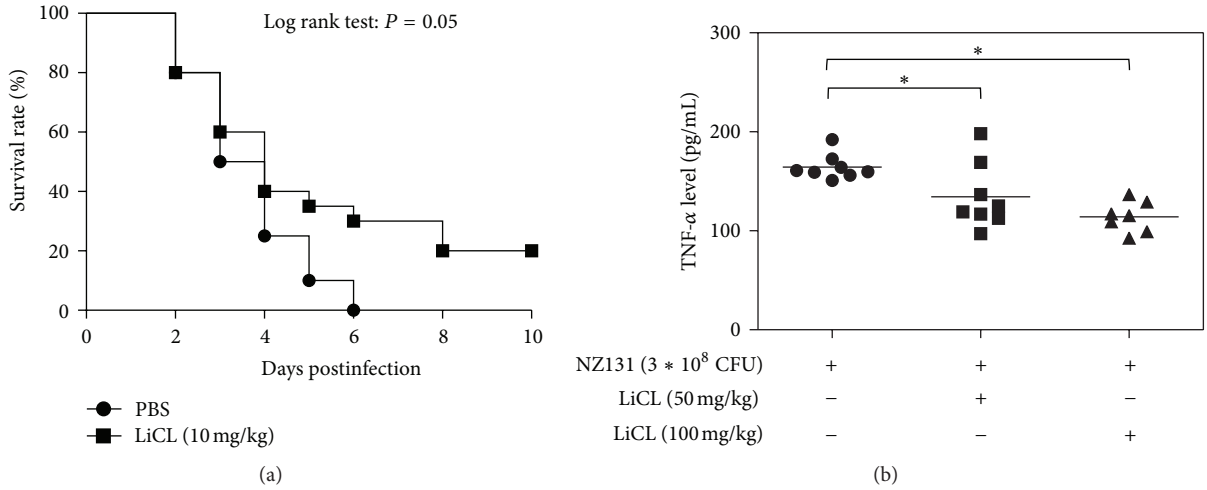

Figure 4: GSK-3 inhibitor, LiCl, can reduce the mortality induced by NZ131 infection in mice and suppress the TNF-α level in sera after NZ131 infection. (a) LiCl (10 mg/kg) was intraperitoneally administered immediately and at 12, 24, and 36 h after the inoculation of NZ131 (3×10^8 CFU) into the air pouch created on the back of BALB/c mice ($n = 20$ per group). The Kaplan-Meier method was used to determine the difference of survival rate after NZ131 infection. P value was analyzed by log rank test ($P = 0.05$). (b) LiCl was given intraperitoneally once immediately after inoculation of NZ131 into air pouch and mice were sacrificed 24 h after infection when serum was collected for measurement of TNF-α ($n = 8$ in NZ131 alone group, $n = 8$ in LiCl 50 mg/kg group, and $n = 7$ in 100 mg/kg group).

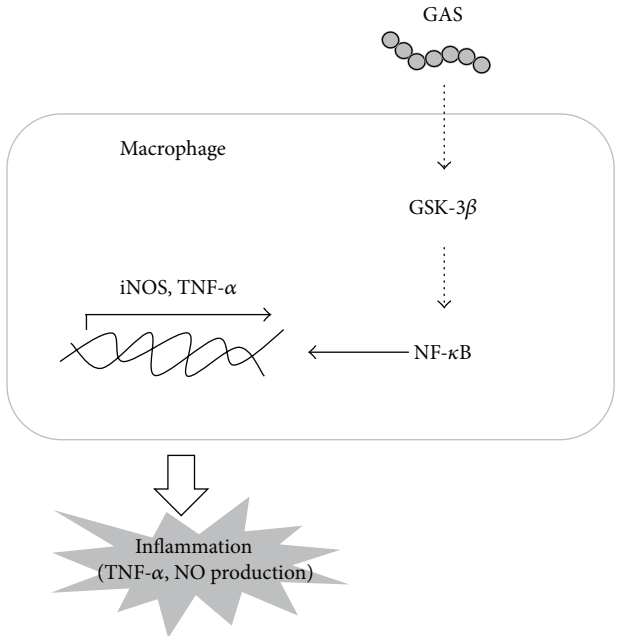

Figure 5: The signaling pathway of group A streptococcus-induced GSK-3β activation, which leads to increased NF-κB nuclear translocation and upregulation of TNF-α and NO production in macrophages.

GAS infection remains a critical issue. GSK-3, a key enzyme in glycogen metabolism, is involved in many intracellular functions [15]. In recent studies of shock and inflammation [16, 20, 23, 36], GSK-3β inhibitors can not only suppress the production of proinflammatory cytokines but also increase anti-inflammatory cytokines. GSK-3β inhibitors also provide a survival advantage and can attenuate organ injury in animal models of sepsis. However, the protective effect of GSK-3β inhibition was mostly demonstrated in LPS-induced sepsis models and only a few studies explored the possible effect of GSK-3β inhibition against live bacteria, which is more similar to clinical conditions [26]. In the present work, we proved that GAS infection induced the activation of GSK-3β and increased nuclear translocation of NF-κB, as well as iNOS expression and NO and TNF-α production. Inhibiting the activity of GSK-3β was able to downregulate the activation of NF-κB and suppress the subsequent inflammatory mediators, including iNOS, NO, and TNF-α induced by GAS infection. This result is also consistent with previous findings that GSK-3β acts upstream of NF-κB [16, 18, 35, 36] and the present study indicates that GAS activates macrophages through a GSK-3β-NF-κB-dependent pathway (Figure 5). Previous report showed that heat-inactivated *Staphylococcus aureus* could also mediate GSK-3β activity [39]. In the present study, the GSK-3β activity was only slightly increased after stimulation with heat-inactivated GAS (Supplemental Figure 2). Whether GAS-induced GSK-3β activity could be related to some secreted proteins, in addition to cell wall components, remains to be explored. Furthermore, the identities of the upstream molecules or even the recognition receptor are not clear. Previous studies [40, 41] indicated that the recognition of GAS and subsequent signal transduction was MyD88 dependent. Thus, further studies are needed to clarify this crosstalk between GSK-3β and MyD88 and NF-κB.

GSK-3β is known to regulate the main eukaryotic transcription factor NF-κB, which is involved in many intracellular processes. After activation of NF-κB, its subunit of p50/p65 translocates from cytoplasm to the nucleus and initiates target gene transcription, including proinflammatory cytokines, chemokines, adhesion molecules, matrix metalloproteases (MMPs), and iNOS [42–44]. Various reports [16, 20, 45, 46] have shown that GSK-3β is able to affect NF-κB activity by several different mechanisms: (1) through phosphorylation of IκB, (2) facilitating translocation of p50/p65

to the nucleus and the binding to DNA, (3) phosphorylation of p65, or (4) through CREB. In our present work, we proved that NF-κB plays an important role in GAS-induced inflammatory responses. After treatment with various GSK-3β inhibitors in GAS-infected RAW 264.7 cells, the ratio of nuclear translocation of NF-κB decreased and the levels were even lower than the noninfected group. NF-κB is known to mediate many proinflammatory responses during bacterial infection [46], including GAS [34]. Our results indicate that NF-κB play a central role in regulation of TNF-α and iNOS expression. We also found that GSK-3β, GAS internalization, and TLR-2 all mediate TNF-α and iNOS expression. The detailed mechanism of the interrelationship between GSK-3β, GAS internalization, and TLR-2 signaling leading to NF-κB activation remains to be clarified.

In the LPS-induced sepsis model, GSK-3β phosphorylation at Ser9 was found to be initially reduced but recovered to baseline level after a short period of time [47–49]. In contrast, our data revealed prolonged dephosphorylation of GSK-3β at Ser9 even 24 h after stimulation by GAS. We found that production of TNF-α and NO persisted 24 h after infection. TNF-α plays a critical role in modulating the cytokine cascade and the fate of macrophages [50]. Deletion of TNF receptor p60 or p80 facilitates LPS-induced apoptosis [51] but desensitizes Fas ligand-induced apoptosis [52]. The dual role of NO in regulation of inflammation is also recognized. Different levels of NO production or the duration to NO exposure are detrimental factors in mediating the degree of inflammation and tissue injury [53]. The pathophysiological role of persistent activation of GSK-3β in GAS-stimulated macrophages and the regulation of subsequent inflammatory reactions and cell fate still need further investigation.

Sepsis is a complex interaction between pathogen and host. Sepsis is characterized as the burst production of cytokines, chemokines, and NO. The transcription of these proinflammatory markers occurs mainly through the activation of NF-κB and leads to subsequent tissue hypoperfusion, organ injury, and dysregulation of the coagulation system [54]. In patients with GAS infection, higher circulation level of cytokines, such as TNF-α and IL-6, also correlated with the disease severity [10]. Thus, blockade of the activity of NF-κB and the production of proinflammatory mediators might prevent further morbidity and mortality of sepsis. In clinical trials targeting proinflammatory cytokines, such as TNF-α or IL-1β, in patients with sepsis have not yielded the desirable outcomes [55–57]. In previous studies, the administration of GSK-3β inhibitors revealed promising benefits for survival in animal models [16, 20, 23, 36]. In the present study, we also show that GSK-3β inhibition provides survival benefit in GAS infection. Live bacterial infection involves the multiplication of bacteria, the production of multiple exotoxins and the various evoked inflammatory pathway after infection. The GAS strain we used is a SPE B-producing strain which could cause the dysregulation of host immune system and facilitate invasion of bacteria [58]. If treatment of GSK-3β inhibitors can only suppress the production of proinflammatory cytokines without also blocking SPE B-induced pathogenic effects, persistent replication and dissemination of bacteria would be expected and this will lessen the therapeutic effect of GSK-3β inhibitor. However, due to the pleiotropic effect of GSK-3β inhibition including anti-inflammation, antiapoptosis, and tissue regeneration [45], GSK-3β inhibition may be a superior therapeutic strategy to those utilizing monoclonal antibody antagonists in the management of sepsis in combination with antibiotic treatment.

5. Conclusions

In conclusion, GAS infection in macrophages can mediate the activation of the GSK-3β-NF-κB signaling pathway and the subsequent production of TNF-α and NO. Inhibition of GSK-3β can downregulate NF-κB and its associated inflammatory response. In animal model studies, GSK-3β inhibition can reduce TNF-α production and lower the mortality when compared to a control group. Since GSK-3β inhibition can provide anti-inflammatory effects *in vitro* and *in vivo*, it could offset cytokine storm-related tissue injury during sepsis and might therefore act as an alternative therapeutic strategy beyond antibiotic treatment.

Conflict of Interests

No conflict of interests should be declared.

Acknowledgments

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Review Article

Role of Macrophages in the Pathogenesis of Atopic Dermatitis

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Atopic dermatitis (AD) is one of the most common and most intensively studied chronic inflammatory skin diseases. Several cofactors, such as an impaired skin barrier function, modifications of the immune system, and a complex genetic background, direct the course of AD. Within this complex network, macrophages play a pivotal role in enhanced susceptibility to cutaneous infections and act as central connecting components in the pathogenesis of AD on the cellular level. In AD, macrophages are known to accumulate in acutely and chronically inflamed skin. During the early and short inflammatory phase, macrophages exert proinflammatory functions like antigen-presenting phagocytosis and the production of inflammatory cytokines and growth factors that facilitate the resolution of inflammation. However, persistence of pro-inflammatory activity and altered function of macrophages result in the development of chronic inflammatory diseases such as AD. The exact mechanism of macrophages activation in these processes is not yet completely understood. Further studies should be performed to clarify the dysregulated mechanism of macrophages activation in AD, and this would allow us to target these cells with versatile functions for therapeutic purpose and improve and control the disease. In this paper, we highlight the new findings on dysregulated function of macrophages and the importance of these cells in the pathogenesis of AD in general and the contribution of these cells in enhanced susceptibility against microbial infections in particular.

1. Introduction

Besides providing a structural barrier, the skin contains several immune cells that can be activated by invading pathogens or skin damage. One of the most important immune cells involved in inflammation and wound healing is the macrophage, which exhibits different immunological functions in the skin, including phagocytosis and antigen presentation. Furthermore, macrophages produce many cytokines and chemokines that stimulate new capillary growth, collagen synthesis, and fibrosis [1]. This immune cell is thought to orchestrate the resolution of inflammation and the wound healing process throughout the different phases such as haemostasis, inflammation, proliferation, angiogenesis, and reepithelialisation as well as remodeling [1–3].

Researchers have long known that macrophages residing in or migrating to different tissues or sites of infection and damage have distinct appearances and cell surface phenotypes; for example, Kupffer cells (liver resident macrophages) appear microscopically different than splenic red pulp macrophages. Until recently, phenotyping macrophages and other related mononuclear phagocytes, including the many dendritic cells (DCs) subtypes, with cell surface markers such as CD11b, CD68, macrophage antigen-2, and F4/80, has been the mainstay of macrophage characterization. However, the last decade has provided new ways of phenotyping macrophages based on their gene-expression profile in response to specific stimuli. By far, the most often-used terms in gene-expression-based macrophage phenotyping are classically activated macrophages (CAMs) (also called M1) and alternatively activated macrophages (AAMs) (M2), which are thought to have characteristic gene-expression profiles defined by markers linked to the stimulation conditions used to generate the subtype—toll-like receptor (TLR) stimulation, bacterial infection, and interferon-(IFN-)γ stimulation for

CAMs and IL-4/IL-13 for AAMs. It is not surprising that given tendencies of immunologists for cell categorization, CAMs and AAMs have been atomized into smaller tranches such as M1a and M2a and M2b. A major question, therefore, concerns the function of the different macrophage types in different homeostatic, infection, and tissue-repair scenarios. Surprisingly, little is known about the functions of individual AAM-associated genes in comparison with CAM-associated macrophage-inflammatory and tissue-remodeling products. However, the gap in knowledge concerning AAM effector functions is closing rapidly with recent publications investigating the effects of deletion of two AAM-associated effector genes, *Arg1* and Retnla. Furthermore, correlations between mouse and human tissue macrophages and their representative subtypes are lacking and are a major barrier to understanding human immunity [4].

Macrophages play key roles in inflammation [5]. During the onset of the inflammatory process, these phagocytic cells become activated and have destructive effects. Macrophage activation, which involves the induction of more than 400 genes, results in an increased capacity to eliminate bacteria and to regulate many other cells through the release of cytokines and chemokines. However, excessive activation has damaging effects, such as septic shock, which can lead to multiple organ dysfunction syndrome and death. In other situations, persistence of proinflammatory activity results in the development of chronic inflammation including chronic inflammatory skin diseases such as psoriasis and atopic dermatitis (AD) [5].

AD is one of the most frequent chronic inflammatory skin diseases with an increasing prevalence affecting 10%–20% of children and 1%–3% of adults in industrial countries [6, 7]. It has a significant impact on the quality of life of patients and their families, and the economic impact is estimated to be billions of dollars [8].

Patients with AD have frequent bacterial and viral skin infections. The most predominant bacteria on AD skin are *Staphylococcus aureus*, constituting 90% of the bacterial microflora on lesional skin and importantly colonizing normal-appearing skin [9]. Many studies have shown that the extent of *S. aureus* colonization positively correlates with the disease activity of AD [10].

Several cofactors, such as an impaired skin barrier function, modifications of the immune system, and a complex genetic background, direct the course of AD [11–13].

Activation of T lymphocytes, DCs, macrophages, keratinocytes, mast cells, and eosinophils is characteristic of AD skin inflammatory responses.

Clinically unaffected skin in AD is not normal. It is frequently dry and has a greater irritant skin response than normal healthy skin. Microscopic studies reveal a sparse perivascular T-cell infiltrate in unaffected AD skin that is not seen in normal healthy skin. Acute AD skin lesions present to the physician as intensely pruritic, erythematous papules associated with excoriation and serous exudation. There is a marked infiltration of $CD4^+$ activated memory T cells in acute AD. Antigen-presenting cells (e.g., Langerhans cells (LCs), inflammatory dendritic epidermal cells (IDECs), and macrophages) in lesional and, to a lesser extent, in nonlesional skin bear IgE molecules. Mast cell degranulation can be observed.

Chronic AD skin lesions have undergone tissue remodeling caused by chronic inflammation. These skin lesions are associated with thickened plaques with increased skin markings (lichenification), increased collagen deposition in the dermis, and dry fibrotic papules.

Macrophages dominate the dermal mononuclear cell infiltrate. Eosinophils also contribute to the inflammatory response, and T cells remain present, although in smaller numbers than seen in acute AD [14].

Within this complex network, antigen-presenting cells such as dendritic cells (DCs) and macrophages play a pivotal role as central connecting components on the cellular level.

Monocytes are important previous cells of macrophages that are involved in skin inflammation of AD [15]. Monocytes invade the dermis and differentiate into macrophages, which can also act as antigen-presenting cells (APCs) [16].

In AD, macrophages are known to accumulate in acutely and chronically inflamed skin [17]. In this paper, we highlight the new findings on dysregulated function of macrophages and the importance of these cells in the pathogenesis of AD in general and the contribution of these cells in enhanced susceptibility against microbial infections in particular.

2. Tissue-Specific Macrophage during Cutaneous Inflammation in AD

Mononuclear phagocytes include tissue-resident cells, such as macrophages and DCs as well as blood monocytes and myeloid progenitors. These progenitors travel through the blood and lymphatic circulation to seed both lymphoid and nonlymphoid tissues, where they develop further, acquiring specific effector functions. DCs are uniquely specialised to detect perturbations originating from both outside and inside the organism. DCs possess the capacity to respond to infectious or noninfectious stress signals, and, following stimulation, they first initiate and then regulate adaptive immunity. DCs function is highly plastic; they can adapt their functional characteristics appropriately, when homing to tissue microenvironments as varied as the skin, the lung, or the gut mucosa. Similarly, when macrophages seed different tissues, they must also adapt and respond to the specific microenvironment. Macrophages and DCs are derived from myeloid bone marrow progenitors and reach the tissues via the blood, yet occupy distinct functional niches; so, it is highly pertinent to determine their precise lineage and progenitors. Once identified, it should be possible to answer long-standing questions concerning when and where in the body specific DC or macrophages commitment occurs and so better understand their differing immune properties *in vivo* and perhaps how they might be better manipulated therapeutically.

Analysis of the origins of mononuclear phagocytes and their pathways of differentiation have been hampered for decades by a lack of molecular markers with defined specificity for particular precursors or subpopulations. To date, much of our understanding of human DC is based on *in vitro*

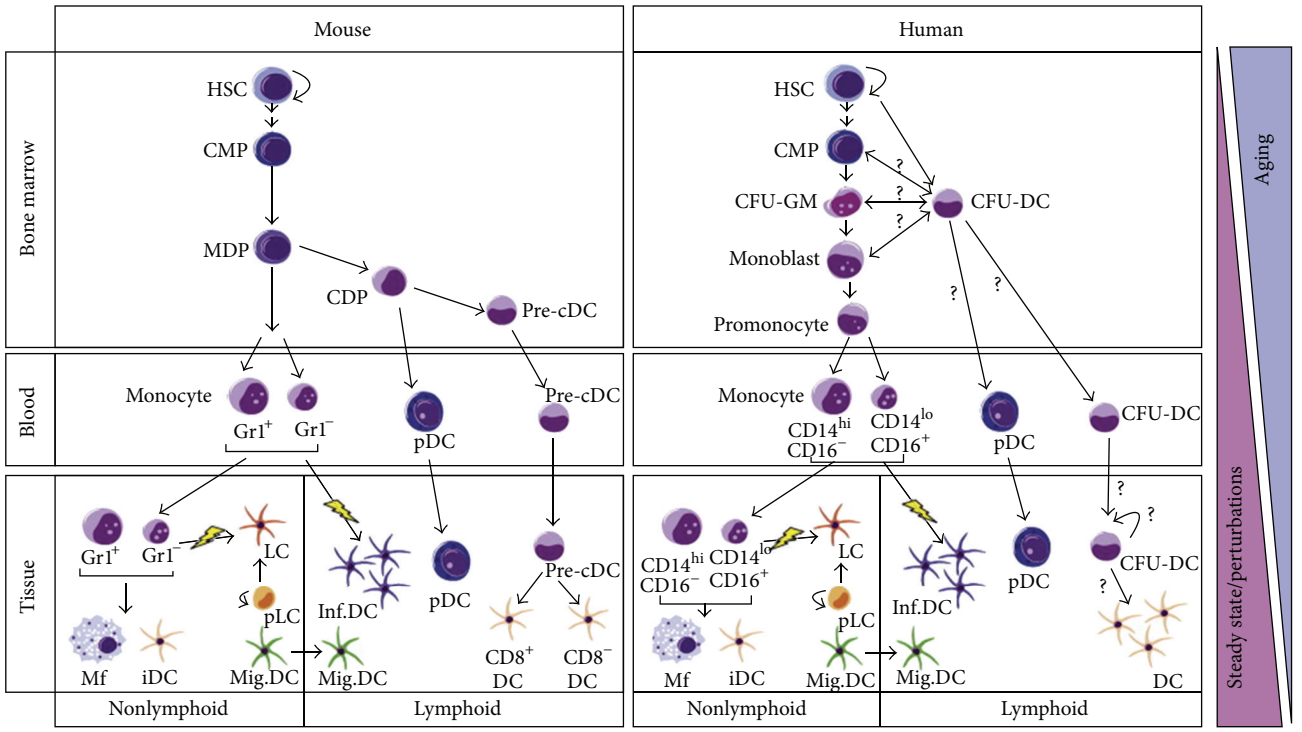

Figure 1: Mononuclear phagocyte differentiation in mouse and human. Hematopoiesis in mouse and human begins from the hematopoietic stem cells (HSCs). The HSCs are self-renewing with clonogenic and multipotent features, giving rise to all blood and tissue-resident immune cells. A very small number of HSCs generate immune cells *de novo* through a multistep differentiation process passing through multilineage progenitors first and to committed progenitors later. All of this process is strictly regulated according to physiological requirements, be it in the steady state or in response to external perturbations, such as infection. Aging also profoundly affects the function of the immune system. The phenomenon is attributable mainly to changes in the HSC compartment that probably gradually reduces its capacity for self-renewal, leading to a progressive reduction in the numbers of immune cells. Myeloid differentiation starts from a common myeloid progenitor (CMP) in the bone marrow. In the mouse, the destiny of CMP is better characterized compared to that of the human counterpart. The CMP generates macrophage-(Mf-) dendritic cell (DC) precursors (MDPs), which are considered the direct progenitor of $Gr1^+$ and $Gr1^-$ monocytes in the blood. Besides monocytes, the MDP differentiates into the common DC precursor (CDP), which in turn generates plasmacytoid DC (pDC) and progenitors for conventional DC (pre-cDC). pDC and myeloid DC diverge at the CDP stage. Pre-cDCs migrate out of the BM through the blood circulation into secondary lymphoid tissues (spleen and lymph nodes), where they replenish both $CD8^+$ and $CD8^-$ DC in the tissues. In humans, CMPs differentiate to granulocyte-macrophage (CFU-GM) precursor, which give rise to monocytes ($CD14^{hi}CD16^-$ and $CD14^{lo}CD16^+$) through subsequent differentiation steps, monoblast first, followed by promonocyte. A DC precursor (CFU-DC) derived from $CD34^+$ HSC with unique differentiation potential towards DC has been identified in bone marrow of the mouse, suggesting that a DC progenitor might exist in humans. Blood monocytes ($Gr1^+$ and $Gr1^-$ in mouse, $CD14^{hi}CD16^-$ and $CD14^{lo}CD16^+$ in humans) migrate to nonlymphoid tissues and generate macrophages (Mfs) and interstitial DC (iDC). In the presence of environmental perturbation *in vivo* or of cytokines *in vitro*, $Gr1^+$ or $Gr1^-$ monocytes in mice and $CD16^+$ or $CD16^-$ monocytes in humans differentiate into Langerhans cells (LCs) in the epidermis, as well as inflammatory DC (Inf.DC). LCs are generated by a dermal long-term precursor (pLC) in the steady state. Finally, migratory DC (Mig.DC) moves between nonlymphoid and lymphoid compartments.

generated cells; however, it is still unknown to what extent they faithfully reproduce the phenotype and function of tissue DC. There is an urgent need to identify and characterise DC progenitors from human blood and to use these "untouched" cells to better understand specific DC functional capabilities. It is possible that certain blood monocyte subpopulations, such as $CD14^+CD16^+$ monocytes, might retain some functional characteristics of DC; for example, they can exhibit enormous plasticity and heterogeneity and may have a role in a range of human diseases (Figure 1) [18].

Macrophages and DCs may play a role in chronicity of AD [11]. However, so far, only limited data are documented on the distribution of macrophages in the skin during cutaneous inflammation.

Kiekens et al. [17] characterized monocytes-derived cells in affected lesional AD skin, compared with nonaffected AD skin and healthy skin. They showed that there was an increase in macrophage numbers in acutely and chronically inflamed AD skin, whereas absolute DC numbers were unchanged, compared with nonlesional AD skin.

The macrophage markers RFD7 (mature tissue phagocyte marker) and CD68 show similar expression patterns during acute and chronic cutaneous inflammation. The total number of $RFD7^+$ macrophages was lower than the number of $CD68^+$ macrophages [17].

Healthy human skin macrophages are known to express CD36, and functionally CD36 is linked to phagocytosis of apoptotic cells [19, 20].

In acutely and chronically inflamed AD skin, Kiekens et al. [17] found increased expression of CD36 by macrophages. In inflamed tissue, many immune cells go into apoptosis after fulfilling their effector function and need to be removed efficiently. Increased expression of CD36 by macrophages may be linked to the removal of apoptotic cells [17].

Human monocyte-derived DCs express mannose receptors (MRs), as was shown by *in vitro* studies, and these cells use the MR for efficient antigen uptake [21]. In peripheral tissues such as the skin, antigen uptake is an important feature of resident macrophages and immature DCs. Both macrophages and DCs express MR in cutaneous inflammation; in nonlesional skin, their number is significantly increased compared with healthy skin. MRs are expressed mainly by macrophages in inflamed AD skin [17].

This can be explained by the fact that macrophages and not DC numbers increase in inflamed AD skin.

Furthermore, phenotypically heterogeneous and overlapping macrophage and DC populations are present in inflamed AD skin. The classic macrophage marker CD68 and prototypic DC marker CD1a could bind to the same cell subpopulation in the dermis of inflamed AD skin [17]. Kiekens et al. [17] demonstrated that, within tissue-specific macrophage populations, further subpopulations are present and that monocyte-derived cells may express markers for both DCs and macrophages. Their results point to the existence of a heterogeneous pool of macrophage/dendritic cell-like cells, from which subpopulations of dermal macrophages and DCs arise [17].

A recent study by Sugaya et al. [22] indicates that the numbers of $CD163^+$ cells (alternatively activated macrophages marker) in lesional skin of AD were significantly larger than in normal skin. Interestingly, the number and distribution of $CD163^+$ cells were quite similar to those of $CD68^+$ cells which were consistent with a previous report [22].

Since in AD research most emphasis has been put on the regulatory role of T cells, little attention has been paid to the monocyte-derived macrophages and their potential role; no conclusive data are available on the distribution and clear phenotype of these cells in the skin of AD patients.

Therefore, further studies should be conducted in order to address the exact function of macrophages during different phases of the skin inflammation.

3. Phagocytosis

Individuals with AD frequently present recurrent infections from pyogenic bacteria or from intracellular microorganisms. The mononuclear and polymorphonuclear neutrophilic phagocytes participate in the innate defense, acting quickly against different agents. These cells initially present chemotactic activity, migrating towards the chemotactic factors and then to the area where the immune response takes place. Following this, phagocytosis occurs, which consists in the ingestion and digestion of the pathogenic organisms, with subsequent elimination of their inactivated products [23].

The high frequency of infections in individuals with AD suggests immune disorders, possibly involving the alterations of neutrophilic and mononuclear phagocytes. However, these alterations have not been fully understood in monocyte-derived macrophages [23, 24].

Forte et al. [25] observed a deficiency in the activity of mononuclear phagocytes in five patients with AD [25]. In another study, they evaluated phagocytes in 19 patients with AD and demonstrated that there was a reduction in the phagocytic activity by mononuclear phagocytes in patients with AD in all age groups studied. In the case of neutrophils, the same deficiency was observed only in patients with AD over 12 years of age [23].

Their data demonstrated a reduction in chemotactic response and phagocytic activity by neutrophilic and/or mononuclear phagocytes in the majority of patients with moderate to severe AD. Their results were coherent with the clinical data concerning the higher incidence of infections by pyogenic bacteria and fungi in patients with AD, which are microorganisms that require defense by the phagocytes [23].

The recurrent infections by pyogenic bacteria or by intracellular organisms that occur in AD suggest that phagocytic activity disorders occur with greater frequency.

4. Pattern Recognition Receptors (PRRs)

The major players in the detection of invading pathogens are the recently identified TLRs. The success of TLRs to function as major sensors of invading pathogens is their ability to identify a range of conserved microbial motifs termed "pathogen-associated molecular patterns" (PAMPs). Innate recognition of PAMPs by TLRs can initiate a cascade of signaling pathways that eventually culminate in the induction of a wide range of immune and inflammatory genes. The most important products of these genes include chemokines and adhesion molecules which result in the recruitment of circulating monocytes from the bloodstream and the production of inflammatory cytokines such as tumour necrosis factor (TNF), interleukin-1 (IL-1), and IFN which mount an inflammatory immune response. As well as their initiation of the innate immune response, there is increasing evidence to suggest that TLRs can also play a role in other macrophage functions such as phagocytosis, antigen processing, and presentation and initiation of the adaptive immune response [26].

Evidence has shown that most of the ten TLRs are expressed on macrophages. In an early study where the mRNA expression of TLRs 1–5 was analysed in a fresh human leukocyte population containing monocytes, T lymphocytes, natural killer (NK) cells, DC, and polymorphonuclear (PMN) cells, TLR-1 was found to be ubiquitously expressed, whereas TLR-2, TLR-4, and TLR-5 were found on monocytes, DCs, and PMNs, and the expression of TLR3 appeared to be exclusively expressed on DCs [27, 28]. Although macrophages were not analysed in this study, it is important to note that the expression of TLRs on monocytes can induce their activation so that they differentiate into either macrophages or DCs [29]. Further analysis has revealed that TLR-6, TLR-7, and TLR-8 are also expressed on freshly isolated human monocytes, whereas TLR-9 and TLR-10 have been shown to be expressed on certain subsets of human DCs [29, 30].

To add to the complexity, TLR expression appears to differ between mouse and human. For example, human TLR-3 appears to be exclusively expressed on DCs, whereas it is expressed and strongly induced in macrophages from mice. TLR-4 is expressed strongly on monocytes and macrophages in both species; however, TLR-4 mRNA expression increases upon LPS stimulation in human macrophages, whereas TLR-4 mRNA is downregulated in response to LPS in murine macrophages [31]. In addition, TLR-9 appears to be almost exclusively expressed on plasmacytoid DCs in both humans and mice; however, in response to LPS, TLR-9 expression can be upregulated in murine macrophages [29, 32]. Mice fail to express TLR-10; however, they express additional TLRs such as TLR-11, TLR-12, and TLR-13 which are absent in humans [33].

The mechanisms that promote the enhanced susceptibility to cutaneous infections in AD are complex interactions among several factors. These contributing factors include skin barrier dysfunction, reduced skin lipid content, and abnormalities of the innate immune response. Some of the innate immune defects observed in AD are primary defects such as epithelial barrier defects and defects in signaling or expression of innate receptors. Others may be secondary to the effects of the adaptive immune response. For example, deficiencies in antimicrobial peptides (AMPs) may be due to the overexpression of Th2 cytokines such as interleukin-(IL-) 4 and IL-13 [7, 34].

The innate immune system protects the host from pathogens and initiates the repair process following injury or trauma. It senses microbes through a group of germline encoded proteins, named pattern-recognition receptors (PRRs).

Host recognition of bacterial pathogens including *S. aureus* is mediated in part by PRRs, including membrane-bound toll-like receptors (TLRs) and intracellular nucleotide-binding oligomerization domain receptors (NLRs).

TLRs act as PRRs comprising a family of at least 10 receptors in humans with distinct recognition profiles [35]. In this context, TLR-2 has emerged as a principle receptor in combating Gram-positive bacteria, especially *S. aureus* [36, 37]. Of the key cells which express TLR-2 are monocytes and macrophages, and they contribute to eliminate pathogens.

TLR-2 forms heterodimers with TLR-1 and TLR-6 to interact with a rather broad spectrum of ligands. Studies using knockout mice identified TLR-1 as the coreceptor required for the recognition of triacylated lipoproteins and lipopeptides such as Pam3Cys [36]. Diacylated components such as lipoteichoic acid (LTA), which is a component of the cell wall of *S. aureus*, interact with TLR-2/TLR-6 heterodimers [36, 37]. Peptidoglycan is a major constituent of the cell wall of Gram-positive bacteria, which induces signal transduction via TLR-2, nucleotide-binding oligomerization domain (NOD) 1 (card4), and NOD2 (card15) receptors, respectively.

NOD molecules, including NOD1 and NOD2, are a family of intracellular pattern recognition proteins involved in bacterial detection [38, 39]. In this context, children with impetiginized AD were found to have increased levels of the TLR-2 ligand LTA in lesional skin that correlated with lesional Eczema Area and Severity Index scores and *S. aureus* colony-forming units. Importantly, the amounts of LTA detected in lesional skin were sufficient to exert biological effects on various cell types *in vitro* [40].

There is emerging evidence that supports a general impairment of TLR-2 expression and TLR-2-mediated proinflammatory cytokines in monocytes and macrophages from AD patients [41, 42].

We could show that macrophages from patients with AD expressed significantly less TLR-2 compared with healthy controls, whereas the expression patterns of TLR-1 and TLR-6 were not altered. Macrophages had a reduced capacity to produce proinflammatory cytokines such as IL-6, IL-8, and IL-1β after stimulation with TLR-2 ligands, which might contribute to the enhanced susceptibility to skin infections with *S. aureus* in AD [42]. Interestingly, weak TLR-2 and TLR-4 signals in the context of allergen exposure in the skin and lung, respectively, had previously been shown to promote a Th2-biased immune response [43]. Therefore, weak TLR-2 responses may not only render AD patients incapable of eradicating *S. aureus* colonizing their skin, but may also promote a Th2 response.

Genetic TLR-2 polymorphisms have been shown to affect the severity of AD. A high frequency (12%) of adult AD patients was found to carry the TLR-2 R753Q single-nucleotide polymorphism (SNP). These patients suffered from a more severe phenotype compared with AD patients without this mutation [44]. These data suggest that the TLR-2 polymorphism R753Q increases the susceptibility to infections and chronic colonization with various pathogens, including *S. aureus*.

In addition, we could show functional differences in TLR-2 responsiveness of monocytes from AD patients with the TLR-2 R753Q mutation compared with wild-type AD patients [45].

More recently, Nod2, an NLR protein which senses muramyl dipeptide produced during the synthesis and/or degradation of peptidoglycan, has been implicated in the host response to *S. aureus* [46]. While TLR2 and Nod2 induce immune responses via the activation of the transcription factor NF-κB and MAP kinases [47], another group of NLRs that include Nlrp3 and Nlrc4 are critical for the activation of caspase-1 and IL-1β secretion in response to bacterial and endogenous stimuli in macrophages [48].

Previous studies have shown that IL-1β signaling and the adaptor protein Asc play a critical role in the clearance of *S. aureus* infection in the skin through monocytes and macrophages [49].

Muñoz-Planillo et al. [49] showed that *S. aureus* hemolysins including α-toxin circumvent the requirement of ATP and the P2X7 receptor to induce caspase-1 activation in macrophages via Nlrp3 inflammasome.

We recently showed that staphylococcal α-toxin contributes to the Th1 polarization by induction of CXCL10 in macrophages. However, macrophages from patients AD show a reduced CXCL10 expression in response to staphylococcal α-toxin [50]. Our data support the hypothesis that the contribution of macrophages in the pathogenesis of AD is linked to the presence of distinct alterations in their capacity to

respond to the staphylococcal exotoxin α-toxin and that these abnormalities can modulate the amplification and persistence of chronic skin inflammation [50].

The mechanism of macrophages activation by staphylococcal α-toxin through inflammasome in monocytes and macrophages from patients with AD is not investigated yet.

Inflammasome-dependent mechanisms which may be altered in patients with resistant AD may contribute to the chronification of the disease and the susceptibility of patients with AD to cutaneous microbial colonization and infections.

Taken together, these data partially explain how macrophages contribute to skin colonization and infection with *S. aureus* and play a crucial role in chronic skin inflammation in AD.

5. The Cytokine and Chemokine Network

Monocytes and macrophages are source of many cytokines and chemokines which play a fundamental role in pathogenesis of many chronic inflammatory diseases such as AD (see Table 1) [51–53].

Diversity and plasticity are hallmarks of cells of the monocyte-macrophage lineage. In response to IFNs, TLR engagement, or IL-4/IL-13 signaling, macrophages undergo M1 (classical) or M2 (alternative) activation, which represent extremes of a continuum in a universe of activation states. Progress has now been made in defining the signaling pathways, transcriptional networks, and epigenetic mechanisms underlying M1-M2 or M2-like polarized activation. Functional skewing of mononuclear phagocytes occurs *in vivo* under physiological conditions (e.g., ontogenesis and pregnancy) and in pathology (allergic and chronic inflammation, tissue repair, infection, and cancer) [54].

Macrophage subpopulations show different types of receptor expression and cytokine/chemokine production [55–59].

Classically activated macrophages, also called M1 cells, are induced by IFN-γ and have a high capacity to present antigen. They produce inflammatory cytokines such as IL-1β, IL-6, IL-12, IL-23, and TNF-α as well as high levels of inducible nitric oxide synthase (iNOS).

In contrast, alternatively activated macrophages, also called M2 cells, are induced by IL-4, which promotes type 2 responses [55].

M2 macrophages are characterized by efficient phagocytic activity, high expression of several receptors such as class A scavenger receptor (CD204), MR, dectin-1, CD209, CD163, production of ornithine and polyamines through the arginase pathway, and an IL-12^{lo}IL-10^{hi}IL-1decoyR^{hi}IL-1RA^{hi} phenotype [54, 56, 60].

Chemokine receptors and ligands are differentially modulated in polarized macrophages. In particular, production of IFN-γ-induced protein of 10 kDa (IP-10/CXCL10) and monokine induced by gamma interferon (MIG/CXCL9) are inhibited by IL-4 and IL-10. However, IL-4 selectively induces eotaxin-2/CCL24, CCL18, and macrophage-derived chemokine (MDC/CCL22) in macrophages, and these effects are inhibited by IFN-γ. Therefore, differential production of chemokines that attract Th1 (CXCL9, CXCL10) and Th2 or T regulatory (Tr) cells (CCL22) integrates M1 and M2 macrophages in circuits of amplification and regulation of polarized T-cell responses [60].

M1 cells are related with chronic inflammation and tumor inhibition, while M2 cells are related with tumor cell growth and metastasis through angiogenesis and tissue remodeling [22]. Allergy is driven by Th2 cells and products and is associated with M2 polarization of macrophages [61–63]. IL-4-inducible chemokines acting on CCR4 (e.g., CCL22) have also been reported to promote skewing of macrophage function [64]. Evidence now indicates that chitin- and arginase-dependent M2 pathways play an active role in the pathogenesis of allergy [65]. Asthma is associated with tissue remodeling, including collagen deposition and goblet cell hyperplasia. IL-4-driven M2 polarization is likely to play a key role as an orchestrator of these processes [66]. Allergy represents a paradigm for IL-4/IL-13-driven type 2 inflammation. However, there is evidence that the inflammasome/IL-1/Th17 pathway can also drive allergic inflammation [67, 68]. Moreover, a Th1-associated cytokine, IL-18, has also been implicated in allergic inflammation [69].

Patients with AD exhibit exaggerated Th2 responses, and initiation of AD lesions is thought to be mediated by means of early skin infiltration of Th2 lymphocytes releasing high levels of IL-4, IL-5, IL-13, and IL-31 [53, 70, 71]. Subsequently, the accumulation of activated monocytes, mature DCs, and eosinophils determines a rise in IL-12 expression and the appearance of a mixed Th2/Th1 cytokine pattern, with reduced IL-4 and IL-13 and the presence of IFN-γ in the chronic phase [70, 71]. It is therefore perhaps not surprising that mixed phenotype macrophages (M2/M1) should be observed in AD which shows a mixed Th2/Th1 phenotype. However, there is no clear investigation in this area, and the exact mechanism of macrophages activation remains elusive. Further investigation should be performed to clarify the role of M2 and M1 in different phases of inflammation in AD.

In addition to the possible role of M2 and M1 macrophages in acute and chronic inflammation of AD, there are several molecules and factors (e.g., histamine, staphylococcal components, cAMP, and FcεRI ligation) which regulate cytokines and chemokines secretion through monocytes and macrophages in AD [72–75].

5.1. Histamine. For instance, histamine influences the profile of proinflammatory and immunoregulatory cytokines produced by blood monocytes, tissue macrophages, and DCs [76, 77]. Histamine induces the production of IL-10 and inhibits that of TNF-α and IL-12 from monocytes. Interestingly, histamine induces TNF-α production from macrophages [78] but not from monocytes or DCs [79, 80]. Although these observations indicate that histamine exerts important immunoregulatory effects, they also illustrate the heterogeneity of the responses of human immune cells to this mediator [79].

The biologic effects of histamine are mediated by activation of 4 distinct receptors (H1, H2, H3, and H4) [72]. In the context of AD, we recently showed that human monocytes express the H4R, and that its stimulation leads to a Ca^{2+}

Table 1: Characteristics of monocytes/macrophages-derived cytokines.

Cytokine	Structure	Size molecular weight	Receptors	Cell targets	Major functions	Disease association
IL-1α, IL-1β	Heterodimer	17 kd	IL-1RI, IL-1RII	T cells, fibroblasts, epithelial and endothelial cells	Induction of Proinflammatory proteins, hematopoiesis, differentiation of Th17 cells	Wide range of autoimmune and inflammatory diseases: AD, RA, IBD, psoriasis
IL-3	Monomer	15 kd	IL-3Rα + βc (CD131)	Erythroid progenitors, Granulocyte macrophages progenitors, CD34+ progenitor cells, basophils, eosinophils	Hematopoietic growth factor, activation of basophils and eosinophils	Role in allergic diseases for example, AD, different types of cancers, lymphocytic and acute myeloid leukemias
IL-6	Homodimer	19–26 kd	IL-6R, (sIL-6R) gp130	Hepatocytes, leukocytes, T cells, B cells, hemopoietic cells	Liver: synthesis of acute phase proteins; leukocytes: trafficking, activation; T cell: differentiation, activation, survival; B cell: differentiation, production of IgG, IgM, IgA hematopoiesis	Autoimmune disease, chronic inflammatory diseases for example, AD, B-cell malignancy, SLE, Castleman's disease, plasmacytoma/multiple myeloma
IL-7	Monomer	25 kd	IL-7R and sIL-7R	B, T, and NK cells	Proliferation of pre-B and pro-B cells (mice), megakaryocytes maturation, VDJ recombinations, naive T-cell survival, synthesis induction of inflammatory mediators in monocytes	Allergy/autoimmunity and psoriasis
IL-8 (CXCL8)	Homodimer	16 kd	CXCR1 and CXCR2	Neutrophils, NK cells, T cells, basophils, eosinophils, endothelial cells	Chemoattractant for neutrophils, NK cells, T cells, basophils, eosinophils; mobilization of hematopoieticstem cells; angiogenesis	Increased levels during inflammatory diseases (e.g., AD, RA, psoriasis, bacterial and viral infections)
IL-10	Homodimer	20.5 kd, predicted size of precursor protein; 18.6 kd, predicted size mature protein, monomer	IL-10R1/IL-10R2 complex	Macrophages, monocytes, T cells, B cells, NK cells, mast cells, DC and granulocytes	Immune suppression	Cancer, autoimmunity, allergy (e.g., AD)

Table 1: Continued.

Cytokine	Structure	Size molecular weight	Receptors	Cell targets	Major functions	Disease association
IL-12 (p35/p40)	Heterodimer	IL-12a p35, 35 kd; IL12b p40, 40 kd	IL-12Rb1 and IL-12Rb2	T cells (Th1 cells), NK cells	Induce Th1-cell differentiation and cytotoxicity	Chronic inflammation (e.g., AD), impaired Th1 response with higher susceptibility to intracellular pathogens, use as anticancer agent
IL-15	Monomer	14-15 kd	IL-15R	T, NK, and NKT cells T-cell activation	Proliferation and activation of NK cells, differentiation of γ/Δ T cells, suppression of IL-2 induced AICD of T cells, homeostasis of CD8+ memory, NK and NKT cells, enhancement of Th2 differentiation and suppression of allergic rhinitis	Autoimmune and inflammatory diseases
IL-16	Homotetramer	56 kd	CD4	T cells, monocytes, macrophages, eosinophils	Chemotaxis, modulation of T-cell response	Increased during various inflammatory and infectious diseases including *atopic eczema*, allergic asthma, Crohn's disease, RA, hepatitis C infection, tuberculosis; inhibits HIV infection
IL-18	Heterodimer	22.3 kd	IL-18R	Variety of cells, T cells, NK cells, macrophages, epithelial cells, chondrocytes	Induction of IFN-γ in presence of IL-12, enhances NK cell cytotoxicity, promoting Th1 or Th2-cell responses depending cytokine milieu	Autoimmune diseases or inflammatory disorders, AD, RA, psoriasis, MS, type I diabetes
IL-19	Monomer	20.5 kd predicted size of precursor; 17 kd, predicted size of mature protein; 35–40 kd, found in transfected cells, glycosylated	IL20Rl/IL-20R2	Keratinocytes	Unknown	Psoriasis

Table 1: Continued.

Cytokine	Structure	Size molecular weight	Receptors	Cell targets	Major functions	Disease association
IL-20	Monomer	20 kd predicted size of precursor; 17.5 kd, predicted size of mature protein	IL-20R1/IL-20R2 and IL-22R1/IL-20R2	Keratinocytes, monocytes	Role in skin biology	Psoriasis, RA, atherosclerosis
IL-23 (p191p40)	Heterodimer	IL-12b p40, 40 kd; IL-23 p19, 19 kd	IL-12Rb1 and IL-23R	T cells (TH17 cells) and macrophages	Stimulate production of proinflammatory IL-17 and promote memory T-cell proliferation	Susceptibility to extracellular pathogens, exacerbate organ specific autoimmune inflammation, chronic inflammatory diseases (psoriasis, AD)
IL-24	Homodimer and monomer	23.8 kd, predicted size of unprocessed precursor; 18 kd, unglycosylated mature protein; 35 kd, observed size of secreted IL-24, glycosylated	IL20R1/IL-20R2 and IL-22R1/IL-20R2	Keratinocytes, cancer cells	Tumor suppression	Melanoma, psoriasis
IL-27 (p281EBI3)	Heterodimer	IL-27a p28, 28 kd; IL-27b EBI3, 25.4 kd	WSX-1 and gp130	T cells, NK cells	Induction of Tbet promoting Th1-cell differentiation, inhibition of Th17-cell response via STAT1	Immune pathology because of uncontrolled inflammatory response: for example, in psoriasis or in epidermal compartment of *patients with eczema*
IL-32	Unknown	14.9–26.6 kd	Unknown	Macrophages, DCs, T cells, PBMCs, monocytes	Induction of TNF-α, IL-8, and IL-6, apoptosis	AD, RA, IBD, autoimmune disease
IL-37	Unknown	17–24 kd	IL-18Ra?	Intracellular mechanism manner and DC	Suppression of proinflammatory cytokines and inhibition of DC activation	RA

Table 1: Continued.

Cytokine	Structure	Size molecular weight	Receptors	Cell targets	Major functions	Disease association
TNF-α	Homotrimer	26 kd transmembrane and a 17 kd secreted form	TNF-R1 and TNF-R2	Both receptors are virtually on all cell types except for the red blood cells, but TNFR1 is more ubiquitous, and TNFR2 is often more abundant on endothelial cells and cells of hematopoietic lineage	Regulation of immune cells: induce fever, apoptotic cell death, (through IL-1 and L-6 production, inhibit tumorigenesis and viral replication, recruiting macrophage and neutrophils to a site of infection	Chronic inflammation (AD, psoriasis, RA, IBD, COPD), Alzheimer's disease, cancer

AD: atopic dermatitis; AICD: activation-induced cell death; COPD: chronic obstructive pulmonary disease; IBD: inflammatory bowel disease; Ig: immunoglobulin; kd: kilo Dalton; NK: natural killer; RA: rheumatoid arthritis; SLE: systemic lupus erythematosus; sIL-6R: soluble interleukin-6 receptor; Th: T helper.

influx and an inhibition of CCL2 production, resulting in a reduction of monocyte recruitment [81].

This could represent a negative feedback mechanism to avoid an overwhelming Th2 environment in case of a persistent histamine release at sites of allergic inflammation and could contribute to the shift from Th2 to Th1 observed in the transition from acute to chronic allergic inflammation such as AD [81].

Furthermore, we showed that histamine downregulates IL-27 production in APCs including monocytes. The downregulation of IL-27 by histamine might be a new mechanism in the pathogenesis of inflammatory skin diseases, in particular, if increased concentrations of histamine are present at sites of inflammation, such as in chronic eczema [82].

5.2. Staphylococcal Exotoxins. Recent findings suggest a role for staphylococcal superantigens in the production of chemokines and cytokines during the development of atopic skin inflammation.

First, superantigen exposure may directly lead to the production of chemokines by T cells, macrophages, and DCs (CCL1 or CCL18). Second, superantigens may induce the release of effector cytokines such as IL-4, IFN-γ, or IL-31 which in turn may upregulate the expression of chemokines such as CCL1, CCL11, CCL17, CCL18, CCL26, CXCL9, or CXCL10. These chemokines are mainly associated with macrophages. Third, IL-31-induced pruritus may be accompanied by skin injury through scratching resulting in the production of primary proinflammatory cytokines such as IL-1α and TNF-α which in turn may amplify chemokine production (e.g., CCL20 or CCL27) [53, 73].

In addition, we found that staphylococcal exotoxins (SEB or α-toxin) significantly upregulates IL-31 receptor A (IL-31RA) expression on monocytes and macrophages. Moreover, IL-31 induces proinflammatory cytokines in monocytes and macrophages following staphylococcal exotoxins stimulation. Such data provide a new link between staphylococcal colonization and the worsening of inflammation via IL-31 in monocyte and macrophages [83].

5.3. Cyclic Adenosine Monophosphate (cAMP). Some studies have shown that leukocytes from patients with AD have increased levels of cAMP-phosphodiesterase activity which results in reduced intracellular cAMP, creating a permissive effect on cell function. This increased activity accounts for subnormal cAMP responses and correlates with increased PGE2 production of monocytes, which inhibits Th1 responses and accentuates IL-4 secretion by Th2 cells [74, 84].

5.4. Fc Epsilon Receptor I (FcεRI) Ligation. FcεRI ligation on monocytes of atopic donors induces indoleamine dioxygenase, which is similar to IL-10 in that it is involved in the control of T-cell responses and the induction of tolerance in the immune system [75].

The expression of FcεRI and FcεRII on monocytes in the peripheral blood is increased in atopic subjects and is significantly higher in patients with extrinsic AD than in patients with intrinsic AD. Recent concepts support the hypothesis that FcεRI-bearing monocytes in the peripheral blood might be the source of subtypes of IgE-bearing DCs in epidermal lesions of patients with AD, which are recruited in the acute phase or during exacerbation of the disease into inflammatory skin by chemokines, cytokines, and other mediators. FcεRI on APCs seems to play a pivotal role in modulating the differentiation [15, 75].

Taken together, these studies provide new insights in contribution of monocytes and macrophages in the complex network of cytokines and chemokines in AD as well as role of these cells in the amplification cycle of atopic skin inflammation.

6. Angiogenesis and Lymphangiogenesis

Angiogenesis and morphological and functional alterations of microvessels are hallmark features of chronic inflammatory disorders, including AD [85].

Vascular endothelial growth factors (VEGFs) are key regulators of blood vessel growth. The VEGF family includes VEGF-A, -B, -C, -D, and placental growth factor. VEGF-A and -B are the most important preangiogenic factors, while VEGF-C and -D primarily regulate lymphangiogenesis. High levels of VEGF-A have been detected in skin tissue of AD patients and correlate with disease activity. The vascular changes in the skin of AD patients appear to be linked to the inflammatory process. Besides human mast cells, basophils, eosinophils, and lymphocytes, macrophages as one of the effector cells of skin inflammation, are major sources of a vast array of angiogenic and lymphangiogenic factors such as VEGFs, angiogenin, and IL-8.

Activated macrophages induce neovascularization and contribute to angiogenesis and lymphangiogenesis in inflammatory diseases. Primary human macrophages express angiogenic (VEGF-A and -B) and lymphangiogenic factors (VEGF-C and -D) [86].

Secretory phospholipases A2 enzymes present in the sites of inflammation enhance the expression and release of VEGF-A and -C in human macrophages [85, 86].

Using a bacterial pathogen-induced model of acute skin inflammation, it has been demonstrated that the lymphangiogenic growth factors (VEGF-A, -C, and -D) secreted from macrophages in inflamed skin tissue seem to be critical not only in lymphatic vessel expansion, but also in antigen clearance and inflammation resolution through enhancement of lymphangiogenesis [87]. The latter observation is interesting because AD patients have increased colonization and superinfection with *Staphylococcus aureus* [88]. There is some evidence that angiogenesis is dysregulated in humans and experimental models of AD.

For instance, Shi et al. [89] investigated the possible link of macrophages recruitment and lymphangiogenesis in Keratin14-IL-4 Transgenic (Tg) mouse model of AD. They demonstrated that the density of VEGF-C-expressing $CD11b^+$ macrophages increases significantly only within the dermis of lesional skin [89].

Their study suggests that $CD11b^+$ macrophages might contribute to neolymphangiogenesis in AD by producing VEGF-C [89].

The possible role of lymphangiogenesis in different phenotypes and phases of AD remains elusive. It is likely that better understanding of altered angiogenesis/lymphangiogenesis in different forms and stages of AD may prove beneficial in the treatment of this common inflammatory skin disorder.

It is possible that specific inhibitors of various mediators (VEGFs) and receptors (VEGFRs, Tie-2, etc.) controlling angiogenesis/lymphangiogenesis may offer novel strategies for dealing with treatment of microvascular changes in inflammatory skin disorders [85]. The possible relevance of angiogenesis/lymphangiogenesis in the pathophysiology and therapy of AD makes the study of vascular remodeling in this disorder a priority for future research.

7. Conclusions and Perspective

Much research effort over the last years has concentrated on the identification of dysregulated genetic and immunologic pathways that could lead to the manifestation of AD. Within this dense network of skin immune cells, APCs including macrophages play an outstanding role and are therefore at the center of focus. Macrophages are an essential component of innate immunity and play a central role in inflammation and host defense. Because of their versatile roles in the pathophysiology of AD, their multifaceted character, and their capacities to both promote and prevent the manifestation of allergic skin inflammation, macrophages represent promising cellular targets for therapeutic approaches in the future.

Abbreviations

AD: Atopic dermatitis
APC: Antigen presenting cells
AMP: Antimicrobial peptide
cAMP: Cyclic adenosine monophosphate
CCL: Chemokine (C-C motif) ligand
CXCL: Chemokine (C-X-C motif) ligand
CCR: Chemokine (C-C motif) receptor
CD: Cluster of differentiation
DC: Dendritic cells
FcεRI: Fc epsilon receptor I
IDEC: Inflammatory dendritic epidermal cell
IFN-γ: Interferon-gamma
IL: Interleukin
IP-10: IFN-γ-induced protein of 10 kd
LC: Langerhans cells
LPS: Lipopolysaccharide
LTA: Lipoteichoic acid
MDC: Macrophages derived chemokine
MIG: Monokine induced by gamma interferon
MR: Mannose receptors
NK: Natural killer cells
NLR: Intracellular nucleotide-binding oligomerization domain receptor
NOD: Nucleotide-binding oligomerization domain
PAMP: Pathogen-associated molecular patterns
PBMC: Peripheral blood mononuclear cells
PRR: Pattern-recognition receptor
S. aureus: *Staphylococcus aureus*
SEB: Staphylococcal enterotoxin B
SNP: Single-nucleotide polymorphism
Th: T helper cells
TLR: Toll-like receptor
TNF: Tumor necrosis factor
VEGF: Vascular endothelial growth factor.

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Review Article

Macrophage Heterogeneity in Respiratory Diseases

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Macrophages are among the most abundant cells in the respiratory tract, and they can have strikingly different phenotypes within this environment. Our knowledge of the different phenotypes and their functions in the lung is sketchy at best, but they appear to be linked to the protection of gas exchange against microbial threats and excessive tissue responses. Phenotypical changes of macrophages within the lung are found in many respiratory diseases including asthma, chronic obstructive pulmonary disease (COPD), and pulmonary fibrosis. This paper will give an overview of what macrophage phenotypes have been described, what their known functions are, what is known about their presence in the different obstructive and restrictive respiratory diseases (asthma, COPD, pulmonary fibrosis), and how they are thought to contribute to the etiology and resolution of these diseases.

1. Introduction

Most tissue macrophages are derived from hematopoietic stem cells and their local expansion within tissues can be due to local proliferation of existing macrophages or due to infiltration of blood-derived monocytes, depending on the circumstances. Traditionally characterized as the first line of defense against foreign invaders, research in the past decade has shown that their role extends to developmental processes and maintenance of tissue homeostasis in many ways [1, 2]. To fulfill these many different roles in tissue, macrophages can adopt a myriad of phenotypes based on signals they receive from their environment. From *in vitro* studies a nomenclature was proposed similar to the Th1/Th2 dichotomy, with M1 macrophages being known as classically activated macrophages induced by interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα) and M2 being known as alternatively activated macrophages induced by interleukin (IL)-4 and IL-13 [3, 4]. The M2 concept already had to expand to M2a, M2b, and M2c to encompass the many different phenotypes labeled alternatively activated, but these *in vitro* concepts have been hard to match to *in situ* tissue macrophages. This is in part caused by a lack of specific markers for the different phenotypes within tissue and by the observation that *in situ* macrophage phenotypes appear as a continuum rather than discrete entities [5, 6].

Macrophages are among the most abundant cells in the respiratory tract and can be broadly divided into two populations depending on their localization: alveolar macrophages (AMs) that line the surface of alveoli and interstitial macrophages (IMs) that reside in the space between alveolar epithelium and vascular endothelium [7]. It has been suggested that AM do not originate directly from blood monocytes, but instead are derived from IMs which therefore serve as an intermediate between blood monocytes and AMs. Compared with AMs, IMs are less efficient in phagocytosing but are better at stimulating T-cell proliferation *in vitro* [8]. In addition, IMs as opposed to AMs, were also found to produce high levels of IL-10 and thereby inhibit DC migration [9]. Although IMs and AMs have distinct functions, they both are among the first to encounter allergens and other threats to the lung homeostasis [8, 10, 11]. They are both capable of quickly dealing with those without perturbing normal gas exchange because they can adopt the most effective phenotypes based on signals from surrounding tissue. These phenotypical changes are also linked to many

respiratory diseases. In both obstructive (asthma, COPD) and restrictive respiratory diseases (pulmonary fibrosis) changes in the number and phenotype of lung macrophages have been found. In this paper we will first briefly discuss the *in vitro* generated phenotypes and then compare this with their role in the pathogenesis of obstructive and restrictive respiratory diseases.

2. M1, M2, and Beyond

2.1. M1 Macrophages. Classically activated or M1 macrophages develop after being exposed to IFNγ and TNFα or lipopolysaccharide (LPS, which induces TNFα production) under the influence of the transcription factor interferon-regulatory factor 5 (IRF5) [12]. They are essential in host defense against intracellular pathogens by generating reactive oxygen species (ROS) and nitric oxide (NO) through upregulated expression of inducible nitric oxide synthetase (iNOS) and amplifying Th1 immune responses by producing proinflammatory cytokines like IL-12, IL-1β, and TNFα (see also Figure 1) [13]. In addition, they show enhanced phagocytosis of microorganisms, antigen-presentation capabilities, and enhanced production and secretion of matrix metalloproteinases (MMPs) such as MMP7 and MMP9 [14–17]. The secretion of MMPs enables macrophage migration during inflammatory responses, but excessive or unregulated production results in tissue damage [5, 17].

2.2. Alternative Activation. Alternatively activated or M2 macrophages were named to indicate that their activation status was distinctly different from the classically activated macrophages. First discovered to be induced by IL-4 and IL-13 [18, 19], this phenotype was soon found to have more siblings, closely resembling each other but distinctly different in function [5, 20]. A variety of different names have been suggested, but for the purpose of this paper we will adopt the names suggested by Mosser and Edwards [5] and Sica and Mantovani [20]. They have suggested alternatively activated or M2 macrophages for the phenotype induced by IL-4/IL-13 and regulatory macrophages or M2-like cells for the phenotype characterized by high IL-10 production that are induced by a variety of stimuli (see also Figure 1).

2.3. M2 Macrophages. M2 macrophages, induced by IL-4/IL-13 under the influence of the transcription factor IRF4 [21], have a role in protection against helminths and are considered wound-healing macrophages because of their association with physiological and pathological tissue remodeling [5, 22]. They are characterized by upregulated expression of mannose receptors and transglutaminase 2 in man and mice [19, 23] and by upregulated expression of arginase-1, chitinase-3-like protein-3 (Chi3l3, also known as Ym1), and resistin-like molecule-α (Relmα, also known as FIZZ1) in mice only (see also Figure 1) [22, 24, 25]. They have poor antigen presenting capabilities and exhibit increased release of iron and clearance of apoptotic cells and extracellular matrix components (efferocytosis) [26–28].

2.4. M2-Like Macrophages. M2-like macrophages also upregulate mannose receptors and in addition produce high levels of IL-10 (see also Figure 1). They are induced by a number of stimuli that need to be combined with a second signal, which is Toll-like receptor (TLR) stimulation. The initial signals include glucocorticosteroids, prostaglandin E2 (PGE2), antibody immune complexes, transforming growth factor beta (TGFβ), and IL-10 itself [5]. They may also be the macrophages that produce TGFβ in addition to IL-10, but this has not been rigorously shown due to the overlap in markers between M2 and M2-like macrophages [29–32].

Transcriptional control of this phenotype is unclear but may involve peroxisome proliferator-activated receptor gamma (PPARγ) and the cAMP-responsive element-binding protein (CREB)-CCAAT/enhancer-binding protein-β (C/EBPβ)-axis [33]. As a result of their high IL-10 production, M2-like macrophages have strong anti-inflammatory activity. This can be beneficial during later stages of immune responses to limit inflammation but may also permit tumor progression when associated with tumors [5, 13]. To date it has been difficult to distinguish genuine M2 macrophages from M2-like macrophages because they share many markers, most notably the mannose receptor. Only IL-10 production would be a reliable marker but is used seldomly to identify M2-like macrophages [5]. The exact differences in tissue distribution and function of these two phenotypes are therefore difficult to establish from the studies published to date. Whenever possible, we will indicate what is known of M2 and M2-like macrophages in the context of respiratory diseases.

3. Macrophages and Asthma

3.1. Asthma. Over the last few decades the prevalence of asthma has rapidly increased, and currently more than half a million people suffer from asthma in The Netherlands (Annual Report 2011 Dutch Lung Fund). More women are affected by this underdiagnosed and undertreated airway disease than men. Asthma is a heterogeneous disorder of the airways, which are chronically inflamed and contract easily in response to nonspecific stimuli. This so-called airway hyperreactivity is accompanied by increased mucus secretion and airway wall remodeling, which leads to symptoms such as wheezing, coughing, and chest tightness [34].

Several distinct forms of asthma have been recognized and can roughly be divided into atopic and the less-studied nonatopic asthma. The majority of asthma patients are atopic, which is a predisposition to mount an immunoglobulin type E (IgE) response. This type is characterized by infiltration of eosinophils in the lungs. In nonatopic asthma there is no evidence of allergen-specific IgE, and this type is characterized by the infiltration of neutrophils in the lungs. A small portion of asthma patients suffer from severe asthma, which includes both atopic and nonatopic characteristics. Severe asthma is defined as being unable to control asthma symptoms despite taking high-dose corticosteroids, also referred to as corticosteroid-resistant asthma [35].

3.2. Pathogenesis of Asthma. Asthma is traditionally considered a T-helper-2- (Th2-) cell driven inflammatory disorder. Activation of a Th2-response is characterized by the release

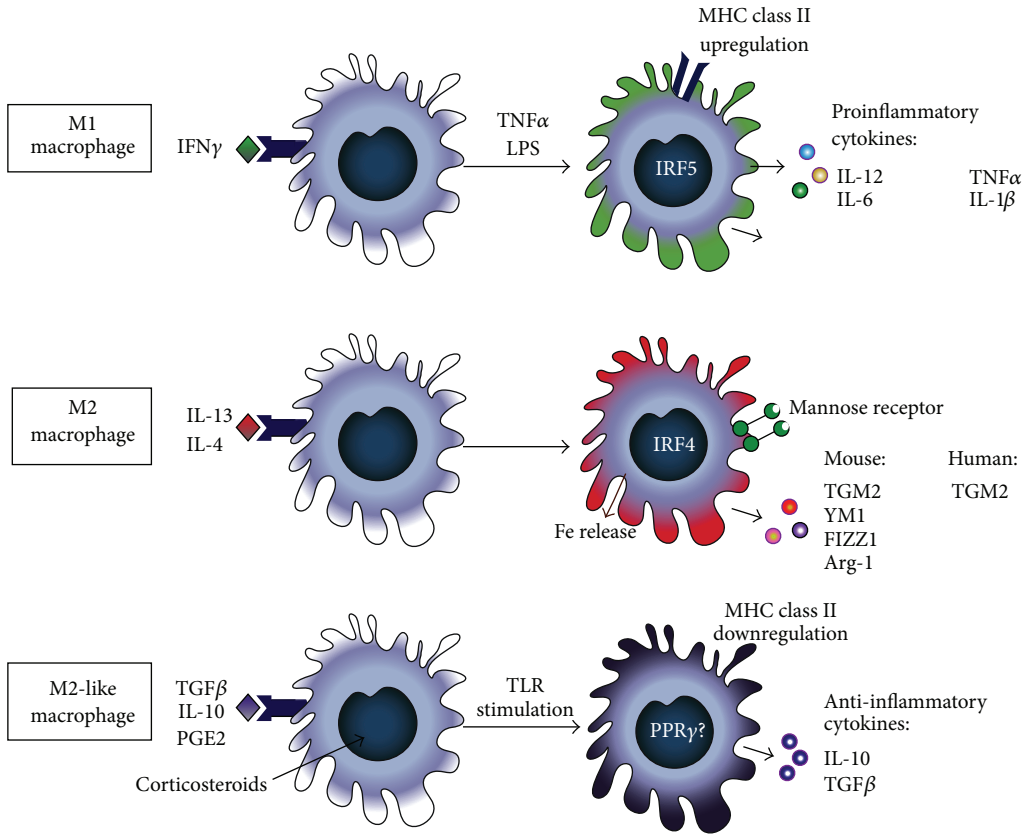

Figure 1: Schematic representation of the three macrophage phenotypes and their characteristics. IFNγ: interferon gamma; TNFα: tumor necrosis factor alpha; LPS: lipopolysaccharide; MHC class II: major histocompatibility complex class II; IL: interleukin; NO: nitric oxide; IRF5: interferon regulatory factor 5; Fe: iron; TGM2: transglutaminase 2; YM1: chitinase-3-like protein-3; FIZZ1/Relmα: resistin-like molecule-α; Arg-1: arginase-1; TGFβ: transforming growth factor beta; TLR: Toll-like receptor; PGE2: prostaglandin E2; PPARγ: peroxisome proliferator-activated receptor gamma.

of the cytokines IL-4, IL-5, IL-9, and IL-13. These Th2-cytokines are responsible for the recruitment of effector cells resulting in eosinophil infiltrates, IgE production, and histamine release among other typical asthma symptoms. The innate immune system is increasingly being recognized as an additional important disease mechanism in asthma [36]. Cells of the innate immune system actively orchestrate adaptive immune responses in asthma [37]. Besides dendritic cells (DC) in the lung taking up allergens and pathogens and presenting those to the adaptive immune system, other cells important for innate immune responses in the lung are macrophages. Their role, however, in asthma has been greatly underestimated, and therefore their contribution to asthma is mostly unexplored [38].

3.3. Macrophage Phenotypes and Asthma. In asthma it appears that effective phenotype switching is impaired and macrophages can actually contribute to the pathogenesis of this disease. The next part will focus on the roles of each known phenotype in the pathogenesis of asthma.

3.4. M1 Macrophages in Asthma. Although the inflammatory process in asthma is dominated by a Th2 inflammation, increasing evidence supports the parallel development and involvement of both M1 and M2 macrophages in this disease. We have recently shown that during the development of house-dust-mite-induced asthma numbers of M1 macrophages are high in a short model as compared to control mice and decrease with longer exposure [39]. Levels of M1 inducers (IFNγ and LPS or TNFα) were found to be significantly higher in asthmatics, especially in those with severe forms of the disease [40–42]. Elevated serum IFNγ correlates with the severity of airway inflammation in atopic asthma, and this cytokine has been linked to mechanisms that induce airway hyperreactivity [43, 44]. In agreement with the findings in human asthma, it was shown that both IFNγ and LPS contribute to airway inflammation and airway hyperreactivity in a mouse model of asthma [45, 46]. TNFα is implicated in many aspects of asthma pathology, including development of airway hyperreactivity and attraction of eosinophils and neutrophils [47, 48].

In both atopic and nonatopic asthmatics, the amount of LPS in house dust has been related to the severity of airway inflammation [49, 50]. Inhalation of pure LPS by asthmatics is associated with bronchoconstriction and a change in airway hyperreactivity [51, 52]. Administration of high doses of LPS into the lungs of allergic mice promotes airway hyperreactivity, neutrophilic inflammation, and expression of

M1 cytokine IL-12. In addition, exposure of asthmatic mice to both IFNγ and LPS induced higher numbers of macrophages in the lungs [40].

M1 macrophages polarize under the influence of the transcription factor IRF5. It was shown that a common *IRF5* gain-of-function haplotype is associated with asthma and the severity of asthmatic symptoms. These associations were more pronounced in nonatopic asthmatics, and it was suggested that IRF5 may only have a profound impact on the pathogenesis and severity of nonatopic asthma and not on atopic asthma [53]. An explanation could be that M1 macrophages are responsible for the recruitment of neutrophils, which are the major effector cells in nonatopic asthma. Neutrophils are also dominant in more severe phenotypes of asthma, and the most commonly used therapy for asthma, corticosteroids, is not effective against neutrophilic inflammation [54]. This is in accordance with recent findings that corticosteroid-resistant asthmatics have increased expression of M1 markers on macrophages in bronchoalveolar lavage fluid (BALF) compared to corticosteroid-sensitive asthmatics, suggesting that M1 macrophages also play a key role in the development of severe corticosteroid-resistant asthma [55].

A few studies have shown that M1 macrophages act preventively in the *onset* of allergic airway inflammation in mice [56, 57] and suppressed DC maturation [58]. This is consistent with the findings of a study that investigated the role of the M1 cytokine IL-12 during the development allergic airway inflammation in mice. They showed that neutralization of IL-12 during the sensitization phase aggravated development of allergic airway inflammation but neutralization of IL-12 during challenges abolished the development of allergic airway inflammation. These data demonstrate a dual role of IL-12: it acts preventive during Th2 sensitization, but it contributes to allergic airway disease during allergen challenges. The effects of IL-12 neutralization were not shown in IFNγ knockout mice, suggesting that IFNγ plays an essential role in the IL-12-induced effect [59].

Thus, both the presence of M1 skewing factors (IFNγ, TNFα, or LPS) and the proinflammatory mediators released by M1 macrophages can contribute to asthma. The data imply that M1 macrophages may be beneficial to prevent allergic sensitization, but in already established disease they promote the development of M2 macrophages and induce corticosteroid resistance. Besides a role in severe asthma, markers of M1 macrophages have also been implicated in nonatopic asthma.

3.5. M2 Macrophages in Asthma. The cytokines IL-4 and IL-13 are abundantly present in the lungs of asthmatics, and it may therefore not come as a surprise that markers expressed by M2 macrophages have been associated with asthma. Elevated levels of chitinase family members have been found in the serum and lungs of patients with asthma, suggesting the presence of increased M2 macrophage numbers [60, 61]. Indeed, we showed that asthmatics have higher percentages of macrophages expressing mannose receptor and transglutaminase 2 in bronchial biopsies than in healthy subjects [23, 62]. In addition Kim et al. showed that severe asthmatics had higher numbers of IL-13-positive M2 macrophages in BALF as compared to healthy controls [63]. Both chitinase levels and the percentage of mannose receptor-positive macrophages also correlated with asthma severity [60, 62]. Higher numbers of M2 macrophages were also found in children undergoing severe exacerbations of asthma [64]. In addition, we have recently shown in several models of house-dust-mite-induced asthma that the number of M2 macrophages positively correlated with the severity of airway inflammation [39]. These clinical and animal model findings demonstrate a correlation between asthma severity and the number of M2 macrophages, but it is unclear whether M2 macrophages actively contribute to the induction and exacerbation of the disease or are just bystanders in allergic airway inflammation responding to the high IL-4 and IL-13 levels.

Credit to the role of M2 macrophages in the exacerbation of the disease was given by adoptive transfer studies. The transfer of *in vitro* differentiated M2 macrophages into the airways of male asthmatic mice aggravated airway inflammation [65]. Another study using IL-4Rα-positive M2 macrophages showed that intraperitoneal injection of these macrophages was sufficient to increase the allergic inflammatory response in the lung [66]. In a different model of fungus-induced asthma, Moreira et al. showed that transfer of M2 macrophages into the lungs of mice enhanced both inflammation and collagen deposition [67] as compared to asthmatic mice not treated with macrophages. Since M2 macrophages and their products have been reported in asthma patients, M2 macrophages may be a target to reduce asthma symptoms. Indeed, the study by Moreira et al. in mice with fungus-induced asthma also showed that treatment with an inhibitor of M2 macrophage generation resulted in lower airway hyperreactivity, mucus cell proliferation, collagen deposition, and M2 numbers as compared to control mice [67]. In support of these results, inhibition of M2-expressed transglutaminase 2 reduced ovalbumin-induced airway hyperreactivity, ovalbumin-specific IgE levels, and infiltration of inflammatory cells in lung tissue [68]. These studies substantiated previous circumstantial evidence concerning a role for M2 macrophages in the pathogenesis of asthma [69, 70].

Unfortunately, the previous studies did not conclusively prove that M2 macrophages play a causative role in the development of allergic airway inflammation. In contrast to what has just been described, Nieuwenhuizen et al. recently demonstrated that M2 macrophages are not necessary for allergic airway disease and may only be a consequence of the elevated Th2 response. They studied the contribution of M2 macrophages to acute, chronic, and house-dust-mite-induced allergic airway inflammation by using mice with abrogated IL-4Rα signaling on macrophages. It was demonstrated that airway hyperreactivity, Th2 responses, mucus hypersecretion, number of eosinophils, and collagen deposition were not significantly affected by decreased development of M2 macrophages. However, the expression of M2 markers was still higher in mice with macrophage-restricted IL-4 receptor-α (IL-4Rα)

deficiency as compared to healthy mice. The presence of these small numbers of M2 macrophages may still have been able to reinforce the Th2 response [71].

To sum up, M2 markers are correlated with severity of allergic airway disease in humans and mice, suggesting that M2 macrophages contribute to the disease. Indeed, elimination of M2 macrophages in established disease by pharmacological interventions remarkably decreased the degree of airway inflammation. However, new data suggest that M2 macrophages are not essential for the development of allergic airway inflammation but only play a bystander role as a consequence of the Th2 response.

3.6. M2-Like Macrophages in Asthma. Reports on the role of M2-like macrophages in asthma are few. These macrophages could play an important role in the resolution of asthma because of their production of IL-10. Interestingly, a lower level of IL-10 production was found in lung macrophages from asthmatics compared to healthy persons [72]. Moreover, macrophages from severe asthmatics produce high levels of IL-6 and IL-8, but IL-10 was undetectable in these cells compared to macrophages from patients with moderate asthma [73].

Studies in mouse models of allergic airway inflammation have investigated the role of IL-10 intensively and found it to be an important mediator in the resolution of airway inflammation [74], but only few studied the production of IL-10 by macrophages. We have just shown that the number of IL-10-positive cells is lower in lungs of mice with house-dust-mite-induced asthma as compared to control mice [39], and recently it was also shown that lung interstitial macrophages produce high levels of IL-10 and prevent airway inflammation in mice [9]. Stimulation of macrophages by ovalbumin uptake and TLR ligands induced increased production of IL-10 by these macrophages, and this resulted in lower levels of IL-5 and ovalbumin-specific IgE and a lower number of eosinophils in a mouse model of asthma [75].

Although evidence for a role of M2-like macrophages in asthma is scarce, these findings suggest a protective effect since active IL-10 production by these cells is low in moderate asthma and absent in severe asthma. In a mouse model of asthma IL-10 was shown to act as an anti-inflammatory. Studies on the resolution of asthma may reveal whether an increased production of IL-10 by these macrophages is involved.

Combining the data available for the different subsets in asthma (see also Figure 2) suggests that M1 macrophages can prevent the induction of asthma but during established disease can cause severe corticosteroid-resistant asthma. M2 macrophages are associated with asthma and their presence correlates with more severe disease. However, it is still a matter of debate whether they genuinely contribute to asthma pathogenesis or are just innocent bystanders of the inflammation. M2-like macrophages seem to be beneficial to the resolution of asthma through production of IL-10 but are not present or not functional in asthma, and therefore allergic inflammation can progress.

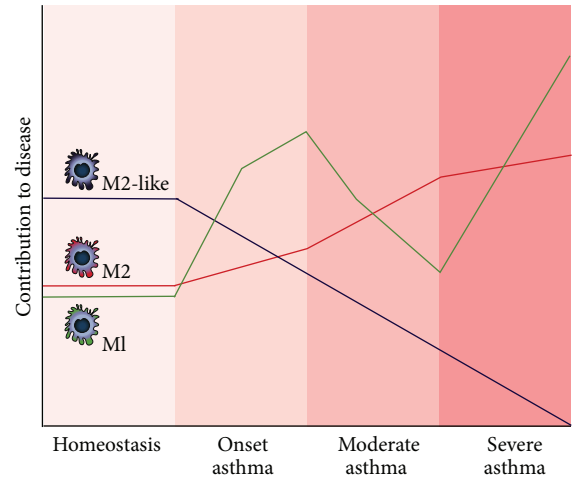

Figure 2: Schematic representation of the presence of M1, M2, and M2-like macrophages in lung tissue during homeostatic conditions, induction of asthma, and during moderate and severe asthma.

4. Macrophages and Chronic Obstructive Pulmonary Disease (COPD)

4.1. COPD. COPD is one of the most common respiratory diseases and affects around 320,000 people in The Netherlands (Annual Report 2011 Dutch Lung Fund). It is projected to be the fourth leading cause of death worldwide by 2030 and places a huge economic burden on society [76]. COPD is caused by lung inflammation due to inhalation of noxious gasses and particles: in the Western World most commonly from cigarette smoking and in developing countries from indoor biomass cooking and heating [77]. The disease is characterized by airflow limitation that is not fully reversible, which is caused by a combination of obstructive bronchiolitis (also known as chronic bronchitis) and destruction of alveoli resulting in airspace enlargement (also known as emphysema) [78]. The relative contributions of chronic bronchitis and emphysema to the COPD phenotype can vary from person to person.

4.2. Pathogenesis of COPD. Exposure to smoke and particles leads to an exaggerated chronic inflammation in lungs of people susceptible to the development of COPD. Excess mucus production and progressive narrowing of the respiratory bronchioles characterize chronic bronchitis. The mucosa, submucosa, and glandular tissue become infiltrated with inflammatory cells and the walls of the respiratory bronchioles become thickened because of edema and fibrosis [79]. Chronic mucus hypersecretion is induced by goblet cell hyperplasia and hypertrophy of submucosal glands [80], which further contributes to occlusion of small airways. This progressive narrowing leads to obliteration or even complete disappearance of respiratory bronchioles. Not much is known about the role of macrophages in this part of the disease, but pigmented macrophages were found to cluster around small airways and these were associated with peribronchiolar fibrosis [81].

The alveolar destruction that characterizes emphysema is the result of infiltration of inflammatory cells with a prominent role for macrophages. Both neutrophils and macrophages are being recruited to the lung because smoke/particle exposure injures epithelial cells that subsequently release cytokines and chemokines to recruit them [82, 83]. They have been postulated to be the main effector cells contributing to the excess tissue damage seen in emphysema because of their ability to produce proteolytic MMPs like neutrophil elastase and macrophage elastase (MMP12) [83, 84]. Increased numbers of macrophages and neutrophils have been found in airways and lung parenchyma of patients with COPD [85–87]. However, only the number of parenchymal alveolar macrophages was directly proportional to the severity of lung destruction in emphysematous lung tissue from COPD patients [88]. Animal studies confirmed the dominant role for macrophages, because deletion of neutrophils in smoke-exposed rats did not prevent cigarette smoke-induced emphysema, whereas deletion of macrophages did [89]. In addition, mice deficient in MMP12 (mainly produced by macrophages) were completely protected from cigarette-smoke-induced emphysema even though they could still produce neutrophil elastase [90]. Similarly, inhibiting MMP12 reduced smoke-induced airway inflammation in mice [91].

4.3. Macrophage Phenotypes and COPD. The role of the different macrophage phenotypes in COPD is the topic of quite a few studies recently and the subject of much debate as the results have been somewhat counterintuitive. Based on studies in mice and results from patient studies, M1 polarization is expected to play an important role in the pathogenesis of COPD. However, the results of other studies have questioned this view, and this is nicely illustrated by studies from Shaykhiev et al. and Hodge et al. [92, 93]. The first ones recently studied the transcriptome of alveolar macrophages from healthy smokers and nonsmokers and compared them to alveolar macrophages from COPD smokers [92]. Their results showed a mixed phenotype for alveolar macrophages after smoking with downregulation of M1 genes and partial upregulation of M2 genes, which was progressively worse in COPD. Hodge et al. showed a mixed phenotype in alveolar macrophages of smoking COPD patients with some M1 (MHC II expression) and M2 (efferocytosis) markers going down and some going up (proinflammatory cytokine production and DC-SIGN expression) [93]. In the next part we will touch upon this debate as we discuss the separate phenotypes in the pathogenesis of COPD.

4.4. M1 Macrophages in COPD. Several lines of evidence support not only a role for M1 macrophages but also a role for dysregulated M1 macrophages in the development of COPD. First of all, exposure to compounds in smoke appears to induce M1 polarization of macrophages. Smoking is the most important risk factor for COPD and cigarette smoke contains many thousands of compounds, including LPS that can activate macrophages in the lung [94]. Indeed, increased expression of iNOS in alveolar macrophages was found in COPD patients [95–97], indicating a polarization towards an M1 phenotype. Upregulation of iNOS increases ROS and NO production and can then cause oxidative stress. Oxidative stress has been shown to be an important contributor to the pathogenesis of COPD [98]. Smoking itself of course causes oxidative stress, and increased iNOS activity through M1 polarization can add to this stress [99–101].

Furthermore, many studies have shown that smoke exposure enhances the release of the M1 proinflammatory cytokines IL-1β, IL-6, IL-8, and TNFα [102–107]. M1-derived cytokines also play a role in the pathogenesis of COPD. IL-1β, IL-6, IL-8, and TNFα have all been found to be elevated in COPD [108–118] and in experimental settings have been found to contribute to the development of persistent airway inflammation, emphysema, and mucus production [102, 119–124]. TNFα was found to drive most of the emphysema development in mice after smoking because mice lacking receptors for TNFα only developed mild emphysema [124]. In addition, mice overexpressing TNFα in lung tissue develop chronic inflammation and emphysema [119, 125, 126]. However, in humans antibodies against TNFα seem to be ineffective in COPD, questioning the relevance of this cytokine for human COPD [127]. In addition to TNFα, M1 cytokine IL-1β was also found to play a role [102, 120]. Overexpression of IL-1β in lung caused lung inflammation, emphysema, mucus metaplasia, and airway fibrosis in mice [121]. Taken together these data suggest cytokines produced by M1 macrophages at least play a role in the pathogenesis of COPD.

Another important M1-related cytokine with a role in COPD is IFNγ. It is produced by CD8+ T cells that infiltrate the lungs in COPD [88, 128, 129] and can cause M1 polarization. Inducible overexpression of IFNγ in lungs of mice caused emphysema with alterations in the balance of MMPs and antiproteases [53]. However, in human alveolar macrophages from smokers reduced expression of IFNγ receptors and reduced IFNγ signaling were found, suggesting M1 polarization may be impaired after smoking. This of course is in line with the above-cited finding by Shaykhiev et al. that M1 genes are downregulated in alveolar macrophages of healthy smokers and smoking COPD patients as compared to nonsmokers [92].

M1 macrophages have also been found to produce MMP9, presumably to enable macrophage migration during inflammatory responses [5, 17]. MMP9 is associated with the breakdown of extracellular matrix in COPD as macrophages from patients with COPD have a significantly higher production of MMP9 as compared to control macrophages [130]. In addition, overexpression of human MMP9 in mouse macrophages induced emphysema and loss of alveolar elastin pointing at a role for M1 macrophages in COPD development [131].

Finally, an important property of M1 macrophages that appears to be dysregulated is phagocytosis of microorganisms. M1 macrophages are geared towards killing and disposal of microbial threats and phagocytosis of microorganisms is part of that function [14, 15]. COPD is often exacerbated by infections [132], and there is accumulating evidence that reduced macrophage phagocytosis in COPD may be responsible for the persistence of microorganisms in the lungs [133–135]. This dysfunction of phagocytosis is not restricted to

microorganisms but also appears to be present for M2-related phagocytic functions such as efferocytosis and mannose receptor-mediated uptake [136, 137]. This overall inhibition of phagocytosis irrespective of macrophage phenotype was further confirmed by the later study of Hodge et al. that has already been mentioned before [93].

Taken together, the available data suggest that a dysregulated M1 response plays a role in COPD rather than an increased number of M1 macrophages. Some aspects of the M1 activation signature are upregulated in COPD (ROS generation, proinflammatory cytokines, production of MMP9), but some aspects are also downregulated (phagocytosis, IFNγ responsiveness).

4.5. M2 Macrophages in COPD. Overexpression of prototypical M1-inducer IFNγ may be able to induce emphysema, but so does overexpression of prototypical M2 induced IL-13. Zheng et al. showed that mice overexpressing IL-13 in lung tissue caused lung pathology mirroring human COPD with macrophage- and lymphocyte-rich inflammation, emphysema, and mucus metaplasia [138]. Unfortunately, macrophages were not further characterized in this study, so it is not known if IL-13 overexpression also induced more alternative activation of macrophages. Further evidence for a role for M2 macrophages came from a study by Kim et al. who showed that viral infections could induce an IL-13-producing M2 phenotype through interactions with natural killer T cells leading to chronic airway inflammation [63]. They also showed higher numbers of IL-13-positive M2 macrophages in lung tissue of COPD patients.

In mice, M2 macrophages produce large amounts of chitinases like Ym1 and Ym2 [139]. Whether their human counterparts are also induced by alternative activation is unclear, but another member of this family, stabilin-1 interacting chitinase-like protein (SI-CLP), has been found upregulated in M2 macrophages [140]. Whether or not pointing at alternative activation, many members of the chitinase family associate with COPD. Chitotriosidase levels, for instance, were increased in bronchoalveolar lavage of smokers with COPD and they also had more chitotriosidase-positive cells in bronchial biopsies and an elevated proportion of alveolar macrophages expressing chitotriosidase as compared to smokers without COPD or never smokers [141]. Furthermore, macrophage chitinase-1 was selectively increased in a subset of patients with severe COPD [142], and serum concentrations of YKL-40 were significantly higher in smokers with COPD as compared to nonsmokers or smokers without COPD and correlated negatively with lung function [143–145]. Interestingly, YKL-40 also stimulated the production of proinflammatory cytokines and MMP9 by macrophages from COPD patients, suggesting YKL-40 itself actually induces more of an M1 phenotype [145].

Data from studies investigating MMPs indicate a possible role for M2 macrophages. As mentioned above MMP12 plays an important role in mouse emphysema [90, 91], and MMP12 was found specifically induced in IL-4-stimulated M2 macrophages [146]. Furthermore, Woodruff et al. showed increased M2 polarization of alveolar macrophages in smokers using MMP12 as a marker for alternative activation [147], and many others showed that smoke induces MMP12 in macrophages [148–154]. Interestingly, MMP12 production by macrophages was also found to be necessary to terminate both neutrophil and macrophage influx at the end of an inflammatory response and may therefore be an instrument of M2 macrophages to dampen inflammation to be able to start remodeling of damaged tissue [155]. How that ties in with the potential proemphysematous role of M2 macrophages remains an open question.

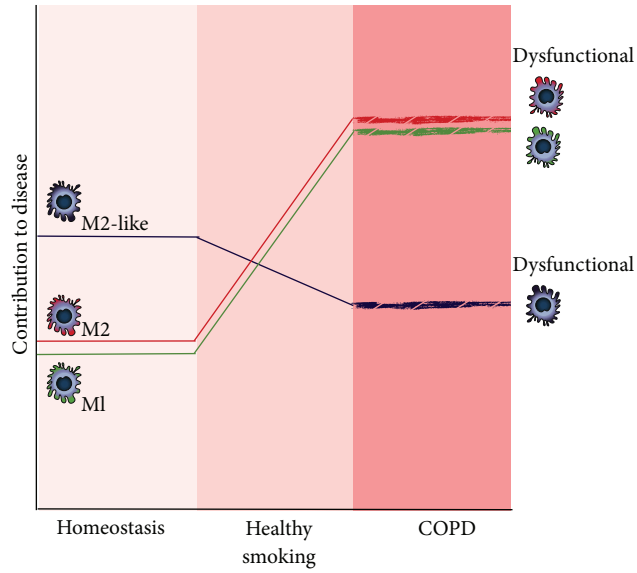

Figure 3: Schematic representation of the presence of M1, M2, and M2-like macrophages in lung tissue during homeostatic conditions, during healthy smoking, and in COPD. Please note the dysfunctional state of macrophages during COPD.

Summarizing, there is some evidence for a role of M2 activation in COPD, and this evidence points at a role contributing to the development of COPD. The data by Hodge et al. suggest that, similar to dysfunctional M1 activation, M2 activation is also dysregulated with reduced efferocytosis but increased expression of M2 marker DC-SIGN [93].

4.6. M2-Like Macrophages in COPD. No attempts have been made to distinguish the roles of M2 and M2-like macrophages in COPD. Two studies reported IL-10 in the context of COPD. A study by Hackett et al. showed diminished IL-10 production in lung tissue of COPD patients after LPS stimulation as compared to lung tissue of patients with normal lung function [156]. Takanashi et al. demonstrated that the level of IL-10 and the number of IL-10-positive macrophages in sputum from COPD patients and healthy smokers was decreased as compared to healthy nonsmokers [157]. This would suggest that M2-like macrophages are impaired in smoking and COPD and therefore cannot suppress the ongoing inflammation induced by smoke.

Combining the data available for M1, M2, and M2-like macrophages (see also Figure 3), it appears COPD is a disease of dysfunctional macrophages rather than a disease of

one particular polarization state. Macrophages in COPD are promoting ongoing inflammation and tissue damage but are unable to effectively dampen inflammation because they have lost the ability to phagocytose microorganisms and apoptotic bodies and produce anti-inflammatory cytokines like IL-10.

5. Macrophages and Pulmonary Fibrosis

5.1. Pulmonary Fibrosis. Pulmonary fibrosis is a disease that encompasses a collection of restrictive pulmonary disorders characterized by progressive and irreversible destruction of lung architecture by excessive deposition of extracellular matrix (ECM) [158]. While ECM formation usually functions as an essential process of tissue healing after lung injury, continuous damage may result in abnormal wound repair and progress to fibrosis. Fibrosis of the interstitium ultimately leads to organ malfunction because of the disturbed architecture of the lung, causing impaired gas exchange and eventually death from respiratory failure [158]. In some cases, fibrotic lesions remain localized to a limited area of the lung because the initial trigger is removed, for example after tuberculosis or a fungal infection, while in others such as in sarcoidosis and idiopathic pulmonary fibrosis (IPF) the fibrotic process continues to progress throughout the lungs in a diffuse manner [159].

IPF is the most common and most dangerous of the fibrotic lung diseases. The chronic and slowly progressing character of the disease together with an unknown aetiology makes it a difficult disease to diagnose and treat. The incidence of IPF appears to be increasing and is currently estimated at 7–16 cases per 100,000 persons [160]. Patients diagnosed with IPF have a poor life expectancy with a median survival of 2–5 years [161]. Currently there are no effective therapies available for these patients, as no therapy has yet been proven to cure or even halt the progression of fibrosis [159].

5.2. Pathogenesis of Pulmonary Fibrosis. To describe the pathogenesis of pulmonary fibrosis and to be able to unravel the complex interactions of macrophages, tissue repair after injury can be divided into four different stages: the clotting phase for emergency tissue repair, then the inflammatory phase to fight the inciting agent, followed by formation of scar tissue in the fibrotic phase for more permanent repair, and eventually resolution of scar tissue and restoration of tissue homeostasis in the resolution phase. During fibrosis some or all of these stages are dysregulated as will be discussed below.

Pulmonary fibrosis is thought to be the result of repetitive injury to the epithelial cell layer lining the alveoli. This damage initiates a blood coagulation cascade to prevent severe blood loss and to maintain some sort of homeostasis. This includes platelet accumulation and production of fibrin by epithelial cells, which is essential for fibrin-containing clot formation [162]. To restore the function of damaged tissue, plasminogen activator (PA) eventually breaks down this fibrin matrix again. In pulmonary fibrosis, changes in both the coagulation cascade itself and the resolution of the wound-healing clot can affect the disease. Impaired fibrin degradation for instance has been shown to worsen epithelial cell survival [163]. Impaired resolution of clots can be caused by either the absence of PA [164] or by increased production of PA inhibitors PAI-1 or PAI-2 [165].

Cell damage furthermore triggers an inflammatory reaction in lung tissue. It has been difficult to investigate the role of the former and this phase in fibrosis because patients usually present with end-stage disease. Nevertheless, the inflammatory response has been extensively studied in LPS-induced inflammation in humans (reviewed by Rossol et al. [166]). It was shown that epithelial cell damage induces the release of several cytokines and chemokines that triggers an influx of neutrophils, closely followed by monocytes to fight the inciting agent [167]. Epithelial cells also release growth factors like TGFβ, TNFα, and epidermal growth factor alfa (EGFα) that stimulate tissue healing by activating fibroblasts, which are the main producers of collagen and other ECM proteins [168, 169].

Control of the inflammatory event, however, is essential for a proper wound healing process [169]. Dysregulation of the inflammatory phase with a prominent role for M1 macrophages has long been thought to be important to the process of fibrosis. The fact that anti-inflammatory drugs such as corticosteroids have no therapeutic effects in patients with pulmonary fibrosis has made this assumption unlikely [170]. Now the new prevailing hypothesis is that pulmonary fibrosis probably develops when the fibrotic phase and/or resolution phase become dysregulated [171].

To progress from the inflammatory phase to the next phase of tissue repair, inflammation needs to be dampened. The release of IL-10 and TGFβ dampens inflammation and promotes ECM production by myofibroblasts [172]. Under the influence of TGFβ and PDGF produced by damaged epithelial cells and platelets, fibroblasts differentiate into myofibroblasts, proliferate, and produce ECM proteins [173]. Furthermore, they start producing their own TGFβ to maintain tissue healing [174]. In pulmonary fibrosis this phase is probably dysregulated as increased numbers of myofibroblasts and increased production of ECM are found in fibrotic lungs. Increased numbers of M2 macrophages are also associated with this phase, and these macrophages are therefore suggested to play an important role in the development of fibrosis [169].

Eventually repair of the epithelial cell barrier and removal of excess ECM are essential to recover normal lung function. To overcome the loss of alveolar epithelial type I cells (AEC I), alveolar epithelial type II cells (AEC II) become hyperplastic and provisionally restore the epithelial cell layer along with the ECM produced by myofibroblasts [168]. Normally these type II cells would revert back to AEC I and homeostasis is restored. However, when injury is repetitive this does not seem to occur; ECM is produced continuously and AEC II continue to proliferate without reverting back to AEC I. In a proper tissue healing response, the excess of ECM products is removed to gain full function of the lungs again. Macrophages are important cells in degrading and taking up ECM components. In order to do so they produce MMPs and their inhibitors (tissue inhibitors of metalloproteinases, TIMPs). A balance between the activities of MMPs and

TIMPs is important to maintain tissue homeostasis [175]. Levels of both MMPs and TIMPs are elevated in patients and mouse models of pulmonary fibrosis [176], but their balance is clearly disrupted as the net result is an excess of ECM in those lungs.

5.3. Macrophages in Pulmonary Fibrosis. Macrophages play an important role in the pathogenesis of lung fibrosis, but their role is complex. They are involved in many of the dysregulated tissue healing responses in fibrosis, and they can also adopt many phenotypes. This complexes studies into their role in fibrosis tremendously. In the next part we will discuss what is known about the contribution of each macrophage phenotype to each stage of fibrosis.

5.4. M1 Macrophages in Pulmonary Fibrosis. We have found no studies reporting on the presence of M1 macrophages in pulmonary fibrosis except for one study by Nagai et al. showing that folate-receptor-beta- (FRβ-) positive macrophages were higher in patients with IPF as compared to controls [177]. These macrophages have previously been shown to produce TNFα and oxygen radicals and are therefore very likely M1 macrophages [178].

Several lines of evidence suggest that M1 macrophages may play a role in both the inflammatory phase as well as the resolution phase of pulmonary fibrosis. As a reaction towards epithelial cell damage, monocytes are recruited to the site of inflammation and differentiate into M1 macrophages under the influence of proinflammatory cytokines. Once activated, M1 macrophages themselves produce TNFα, IL-1β, and oxygen radicals to kill and phagocytose microbes to fight an infection or remove an exogenous agent [179]. Many studies indicate that these proinflammatory cytokines and oxygen radicals are associated with fibrosis development [180–188]. In the study by Nagai et al. ablation of the FRβ-expressing M1 macrophages during the inflammatory phase of bleomycin-induced fibrosis abrogated fibrosis development [177]. However, the importance of the contribution of inflammation to established fibrosis has been challenged because anti-inflammatory drugs such as corticosteroids have no therapeutic effects in patients with pulmonary fibrosis [170]. This view was confirmed by a study from Gibbons et al. They studied newly recruited inflammatory macrophages in a mouse model of bleomycin-induced lung fibrosis and showed that depletion of tissue-resident macrophages and/or circulating inflammatory monocytes during the inflammatory phase did not affect the onset or degree of fibrosis that developed after this inflammatory phase [189]. Another study pointed out that the M1 cytokine TNFα has beneficial effects on alveolar epithelial cell recovery and therefore also contributes to resolution [190].

In the resolution phase, macrophages are involved in the degradation of excess ECM and the uptake of matrix components [189, 191]. Depletion of macrophages during this recovery phase impaired the resolution of fibrosis by slowing down the degradation of ECM [189]. It is unclear what type of macrophages is responsible for degradation of ECM, but a case can be made for M1 macrophages as these have been shown to produce several MMPs including MMP7 and MMP9. Levels of MMP9 have been found to be increased in lungs of IPF patients and this may reflect a failing attempt of the lungs to remove excess ECM and may be caused by a simultaneous increase of the inhibitor TIMP-1 [192–194].

Macrophages are also important in the subsequent removal of ECM components through endocytosis-mediated mechanisms. Again it is unclear if this is restricted to one particular phenotype, but the receptors involved would suggest more of an M2 phenotype, and this will therefore be discussed in the next part on M2 macrophages.

In summary, M1 macrophages are important in the inflammatory phase, but their presence does not appear to affect the subsequent fibrotic phase. During resolution of scar tissue, macrophages are indispensable for degradation of ECM. This may be related to an M1 phenotype, and it may therefore be beneficial to stimulate recruitment of M1 macrophages to reverse fibrosis.

5.5. M2 Macrophages in Pulmonary Fibrosis. There is a great deal of evidence that Th2 responses are important in the development of fibrosis, and it appears that IL-13 is the predominant cytokine in the profibrotic responses [195–202]. Levels of IL-13 are higher in patients with pulmonary fibrosis as compared to controls, and macrophages isolated from these fibrotic lungs produce more IL-13 than macrophages from control lungs [203]. It therefore comes as no surprise that M2 macrophages are associated with pulmonary fibrosis, although we could not find publications directly showing numbers of M2 macrophages are increased in lung tissue of patients with pulmonary fibrosis. We did find one study showing higher numbers of M2 macrophages in BALF of IPF patients as compared to controls and two studies showing higher numbers of insulin-like growth factor-I (IGF-I)-positive and PDGF-positive interstitial macrophages in lung tissue of IPF patients as compared to controls [204–206]. Both these markers are important profibrotic mediators, and a recent study by Chen et al. showed that expression of IGF-I colocalized with arginase-1 and not with IL-10 expression in macrophages suggesting genuine M2 macrophages express IGF-I and not the M2-like subset [207]. This was a study in mice; it therefore remains to be investigated whether this is also true in humans.

Markers found on or produced by M2 macrophages have also been found to be increased in pulmonary fibrosis. Levels of galectin-3, a carbohydrate-binding lectin that is necessary for alternative activation [208], were higher in BALF of IPF patients as compared to control patients [209]. Furthermore, macrophages from IPF patients produced more of the human M2 marker CCL18 than control macrophages, and this correlated negatively with pulmonary function test parameters [210]. IPF patients were also found to have higher serum and pulmonary levels of chitinase-like protein YKL-40 as compared to controls, although it is still unclear whether chitinases are true markers of alternative activation in human macrophages [211]. This is also the case for arginase-1, which is a marker of M2 macrophages in mice but its specificity in humans is debated [22]. Nevertheless, lung tissue from IPF patients had higher expression of arginase-1 in macrophages

than normal lung tissue [212]. Lastly, circulating monocytes from systemic sclerosis patients with pulmonary fibrosis showed enhanced profibrotic phenotype by increased expression of CD163, a marker of alternative activation in humans [213].

Experimental models of pulmonary fibrosis have revealed more about the role of M2 macrophages in fibrosis of the lung. Depletion of macrophages during the fibrotic phase of lung fibrosis reduced the deposition of ECM in this organ [189]. To confirm a role for M2 macrophages, levels of Ym1 and arginase-1 were measured before and after macrophage depletion. Both markers showed decreased expression in the lungs after removal of macrophages. The M1 marker iNOS did not show a reduction in expression, indicating that M2 macrophages are predominantly responsible for the development of fibrosis. Furthermore, M2 marker MMP12 was shown to be essential in the development of fibrosis induced by excessive activation of Fas [214] and in a model of IL-13 dependent fibrosis [215].

There is some evidence or how M2 macrophages would contributes to the fibrosis development. The aforementioned production of IGF-I and PDGF contribute to proliferation of fibroblasts and their transformation to ECM-producing myofibroblasts [173]. Furthermore, FIZZ1 (also known as resistin-like molecule alpha) was found to increase ECM production in fibroblasts [216], but a recent paper by Pesce et al. showed that FIZZ1 actually ameliorated fibrosis development by negatively regulating Th2-dependent responses [217]. This contradictory finding highlights other new findings that also suggest that M2 macrophages could be antifibrotic. A mechanistic study in a model of Schistosoma-induced liver fibrosis with specific deletion of the IL-4Rα on myeloid cells preventing alternative activation of macrophages showed that M2 macrophages are not required for fibrosis development [218]. In addition, related studies with mice lacking arginase-1 in M2 macrophages showed that the arginase-1-expressing M2 macrophages were required for suppression and resolution of fibrosis [217]. This correlates well with findings that uptake of ECM components appears to be mediated by M2 macrophages. Uptake of these components is mediated by different mannose receptors [27] and by glycoprotein milk fat globule epidermal growth factor 8 (Mfge8) [219]. Mannose receptors of course are a known M2 marker, and for Mfge8 this is unclear. Both mannose receptor 2 and Mfge8 were shown to attenuate fibrosis in different models [219, 220].

To summarize, M2 macrophages are firmly associated with fibrosis development, but new evidence suggests they may actually contribute to resolution of fibrosis. Their presence during fibrosis may be explained as a failing attempt to clear the excess ECM. The conflicting roles described in the literature may be the result of difficulties separating the effects of M2 and M2-like macrophages simply because these two subsets are difficult to distinguish. M2-like macrophages may be a more likely candidate for the promotion of fibrosis as will be discussed below.

5.6. M2-Like Macrophages in Pulmonary Fibrosis. The specific role of M2-like macrophages has not been investigated in lung fibrosis yet, but M2-like macrophages may be important during the transition from inflammation towards tissue healing. The signature marker of M2-like macrophages is IL-10, which is the canonical anti-inflammatory cytokine with profibrotic actions. Elevated levels of IL-10 and enhanced production of IL-10 by alveolar macrophages have been reported in several fibrotic diseases, including IPF [221–223] and in systemic sclerosis patients with interstitial lung disease [213]. Its anti-inflammatory actions in lung are illustrated by a study from Armstrong et al., showing that IL-10 inhibited TNFα production by alveolar macrophages after LPS stimulation [224]. In addition, several studies in mice using the model of bleomycin-induced fibrosis suggest that IL-10 attenuates bleomycin-induced inflammation and can thereby attenuate fibrosis development [182, 225, 226]. However, overexpression of IL-10 in lungs of mice was found to be profibrotic [227]. Sun et al. found that inducible IL-10 overexpression in Clara cells induced fibrosis by fibrocyte recruitment and activation of macrophages towards an M2 phenotype. The increased levels of IL-10 found in lungs of IPF patients may therefore contribute to the fibrotic process. The production of the profibrotic, anti-inflammatory cytokine TGFβ would also fit with this role of dampening inflammation and promoting tissue repair by this subset of macrophages. Whether TGFβ production is restricted to the IL-10-producing M2-like macrophage subtype remains to be investigated.

In summary, M2-like macrophages are likely candidates for promotion of fibrosis. They may be recruited or induced by damage to the epithelium to dampen inflammation and start repair. In the event of ongoing damage they are continually induced or recruited and may contribute to fibrosis by overexpression of IL-10. Since corticosteroids are also capable of inducing M2-like macrophages, this would explain why these drugs are not effective against fibrosis and may even be disadvantageous. This is illustrated by our finding that when corticosteroids are specifically delivered to liver macrophages in a model of liver fibrosis, fibrosis actually becomes worse [228].

Overall, current data on the role of macrophages in the development of pulmonary fibrosis show that macrophages are important cells in the pathogenesis of this disease (see also Figure 4). M1 macrophages are important in the inflammatory phase and may also be important for resolution of the disease, although this hypothesis needs testing. M2 and M2-like macrophages are highly associated with fibrogenesis. However, new data suggest that M2 macrophages may actually protect against development of fibrosis while M2-like macrophages contribute to fibrosis. Therefore, key to understanding how these two phenotypes contribute to pulmonary fibrosis are studies differentiating between M2 and M2-like macrophages.

6. Conclusion

The literature on lung macrophages summarized in this paper clearly shows that macrophages are important in maintaining tissue homeostasis in the lung. Through their ability to change phenotypes they are able to regulate responses to

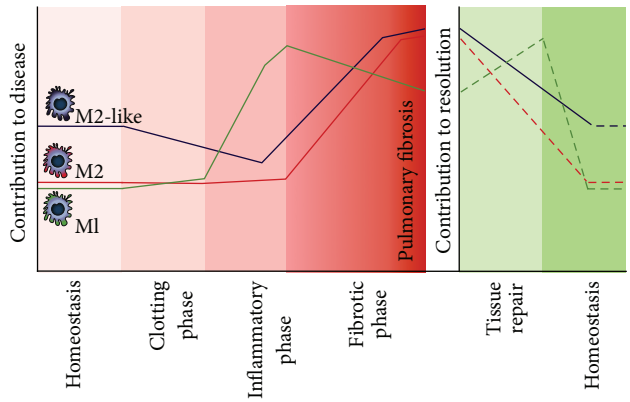

Figure 4: Schematic representation of the presence of M1, M2, and M2-like macrophages in lung tissue during homeostatic conditions and after injury to the lung. Normally after lung injury a process of tissue repair is initiated with four distinct phases leading to homeostatic conditions again. In lung fibrosis this normal tissue repair response is dysregulated leading to deposition of excess extracellular matrix and little resolution of scar tissue.

homeostatic threats without impairing the functionality of the organ. The available literature also shows that when phenotype switching becomes dysfunctional or when some aspects of a particular phenotype become dysfunctional, pathologies develop. However, data on the distribution of macrophage subsets in healthy lung tissue and during disease is sorely lacking for humans as well as experimental models of respiratory diseases.

In general, asthma, COPD, and pulmonary fibrosis are diseases characterized by changes in macrophage subsets in the lung (M1, M2, and M2-like). It seems likely that changes in the interactions between the different subsets, that is, the balance, and changes in their function are a cause for disease, rather than the presence of one particular subset. The next challenge will be to specifically improve a particular function of a subset *in vivo* or specifically change a phenotype as a novel therapeutic approach for obstructive and restrictive respiratory diseases.

Conflict of Interests

The authors have no conflict of interests to declare.

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Research Article

Characterization of Macrophage Phenotypes in Three Murine Models of House-Dust-Mite-Induced Asthma

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In asthma, an important role for innate immunity is increasingly being recognized. Key innate immune cells in the lungs are macrophages. Depending on the signals they receive, macrophages can at least have an M1, M2, or M2-like phenotype. It is unknown how these macrophage phenotypes behave with regard to (the severity of) asthma. We have quantified the phenotypes in three models of house dust mite (HDM-)induced asthma (14, 21, and 24 days). M1, M2, and M2-like phenotypes were identified by interferon regulatory factor 5 (IRF5), YM1, and IL-10, respectively. We found higher percentages of eosinophils in HDM-exposed mice compared to control but no differences between HDM models. T cell numbers were higher after HDM exposure and were the highest in the 24-day HDM protocol. Higher numbers of M2 macrophages after HDM correlated with higher eosinophil numbers. In mice with less severe asthma, M1 macrophage numbers were higher and correlated negatively with M2 macrophages numbers. Lower numbers of M2-like macrophages were found after HDM exposure and these correlated negatively with M2 macrophages. The balance between macrophage phenotypes changes as the severity of allergic airway inflammation increases. Influencing this imbalanced relationship could be a novel approach to treat asthma.

1. Introduction

Asthma is characterized by irreversible obstruction and chronic inflammation of the airways, and is traditionally considered a T helper 2 (Th2-)cell driven inflammatory disorder [1]. However, an important role for the innate immune system in addition to the adaptive immune system is increasingly being recognized in asthma [2].

Macrophages are key cells in innate immune responses in the lung: they are among the most abundant cells and one of the first to encounter allergens and other threats to homeostasis. They also have the plasticity to quickly deal with those without endangering normal gas exchange. Depending on the signals received, macrophages can be pro- or anti-inflammatory, immunogenic or tolerogenic, and destroying or repairing tissue. Each characteristic may belong to a different macrophage phenotype with distinct functions [3, 4].

Tumor necrosis factor α (TNFα) and interferon γ (IFNγ) induce, under the influence of the transcription factor interferon-regulatory factor 5 (IRF5), a phenotype of M1 macrophages with increased microbicidal and/or tumoricidal activities [4, 5]. Exposure to IL-4 or IL-13 results in a population of M2 macrophages that is involved in anti-parasite and tissue repair responses [6, 7]. In mice, these cells are recognized by high production of chitinase and chitinase-like molecules such as YM1 [7, 8]. A close sibling of M2 macrophages is the M2-like macrophage phenotype. These macrophages can be induced by a variety of stimuli including exposure to a TLR-ligand in the presence of IL-10 or many more compounds. The main characteristic of the subtly

different M2-like population is the production of IL-10. Since IL-10 is a potent anti-inflammatory cytokine, these M2-like macrophages are effective inhibitors of inflammation [4].

Despite the broad the spectrum of macrophage activation, the role of macrophages in asthma has scarcely been studied [9]. From what is known, all three macrophage phenotypes have been implicated in the development of murine and human asthma [10–12]. In mice, depending on the protocol used, asthma phenotypes can greatly differ [13, 14]. We aimed to investigate the distribution of the three main macrophages phenotypes in three different models of HDM-induced asthma and also included the effects of sex on asthma development. First, we show the general differences in airway inflammation in the three HDM models and next we study the distribution of the macrophage phenotypes with regard to severity of allergic airway inflammation.

2. Materials and Methods

2.1. Animals. Male and female BALB/c mice (aged 6–8 weeks) were obtained from Harlan (Horst, The Netherlands). The mice were fed *ad libitum* with standard food and water and were held under specific pathogen-free conditions in groups of 4 mice per cage. The animal procedures, approved by the Institutional Animal Care and Use Committee of the University of Groningen (application number 5318), were performed under strict governmental and international guidelines.

2.2. House Dust Mite (HDM)-Induced Airway Inflammation Models. Male ($n = 4$ per model) and female mice ($n = 4$ per model) were anaesthetized with isoflurane and exposed intranasally to whole body HDM extract (*Dermatophagoides pteronyssinus*, Greer laboratories, Lenoir, USA) in 40 μL phosphate-buffered saline (PBS) according to three different protocols. Control animals ($n = 8$) were exposed to 40 μL PBS according the 21-day protocol described.

Mice of the first model ($n = 8$) received 100 μg HDM extract intranasally on day 0, were subsequently exposed to 10 μg HDM on day 7–11 according to the protocol of Hammad et al. and were sacrificed on day 14 (abbreviated as 14-day HDM) [15]. In the second model, according to Gregory et al. [16], mice ($n = 8$) were exposed to 25 μg HDM extract three times a week during three weeks and were sacrificed on day 21 (abbreviated as 21-day HDM). For the last model ($n = 7$, due to illness one female was excluded from the study), mice were intranasally exposed to 100 μg HDM on days 0, 7, 14 and 21 according to the protocol of Arora et al. and were sacrificed on day 24 (abbreviated as 24-day HDM) [17].

During sacrifice lungs were lavaged three times with 1 mL cold PBS to determine the number of eosinophils and YM1 levels. Then, the left lung lobe was collected to isolate lung cells from digested lung for flow cytometry and the right lung was inflated with 0.5 mL 50% Tissue-Tek O.C.T. compound (Sakura, Finetek Europe B.V., Zoeterwoude, The Netherlands) in PBS and snap frozen/formalin-fixed for histological analyses. Serum was collected for analysis of HDM-specific IgE levels. Figure 1 shows an overview of the experimental designs.

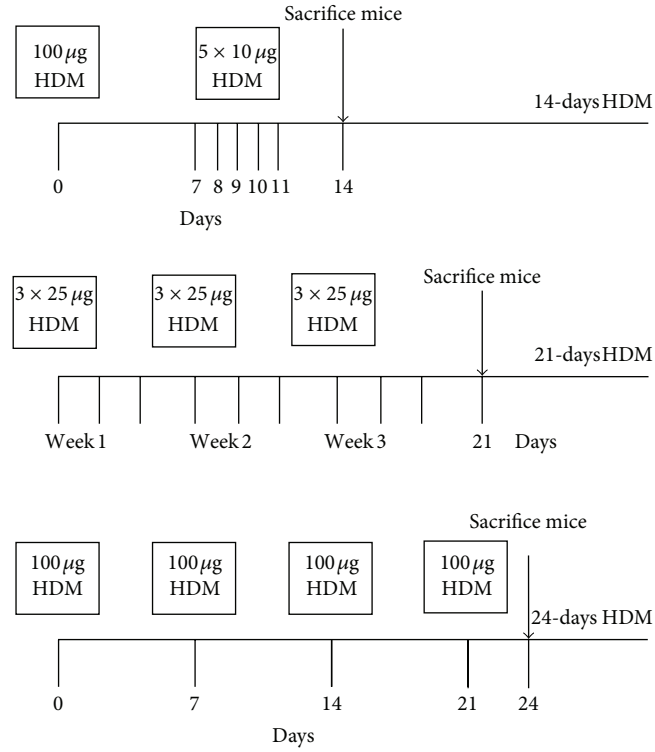

Figure 1: Experimental design of the study: three models of HDM-induced allergic inflammation. HDM, house dust mite.

2.3. HDM-Specific IgE. Serum levels of HDM-specific IgE were measured by ELISA as described previously [18]. Arbitrary ELISA units of HDM-specific IgE titers were calculated as relative values to the optical density of pooled sera from HDM-exposed mice.

2.4. Bronchoalveolar Lavage Fluid (BALF). BALF was collected and total numbers of cells were determined using a Casy cell counter (Roche Innovatis AG, Reutlingen, Germany). After centrifugation at 300 ×g for 10 minutes, BALF supernatants were stored at −80°C for further analysis (YM1 ELISA) and the cells were resuspended in RPMI medium (BioWhittaker Europe, Verviers, Belgium) for preparation of cytospots. Approximately 50,000 cells were spotted onto slides using a cytospin 3 (Thermo Shandon, Waltham, MA, USA) at 450 rpm for 5 minutes. To determine the percentage of eosinophils in the cytospots, a Giemsa staining (Sigma-Aldrich, Zwijndrecht, The Netherlands) was performed and the number of eosinophils was counted in a total of 300 cells.

The level of mECF-L (YM1) in BALF supernatants was determined by an ELISA kit according to the manufacturer's instructions (R&D Systems, Oxon, UK).

2.5. Lung Digestion. After bronchoalveolar lavage, the left lung was minced and incubated in RPMI medium supplemented with 10% fetal calf serum (both Lonza, Verviers,

Belgium), 10 μg/mL DNAse I (grade II from bovine pancreas, Roche Applied Science, Almere, Netherlands), and 0.7 mg/mL collagenase A (Sigma-Aldrich) for 45 minutes at 37°C in a shaking water bath. The digested lung tissue was passed through a 70 μm nylon strainer (BD Biosciences, Breda, Netherlands) to obtain single cell suspensions. Incubation with 10 times diluted Pharmlyse (BD Biosciences, Breda, The Netherlands) was performed to lyze contaminating erythrocytes. Cells were centrifuged through 70 μm strainer caps and counted using a Casy cell counter (Roche Innovatis AG). Cells were subsequently used for flow cytometry.

2.6. Flow Cytometric Analysis. The single lung cell suspensions were stained for T-cell subsets using antibodies for flow cytometry. Frequencies of effector T cells (CD3+CD4+CD25+Foxp3−) and regulatory T cells (CD3+CD4+CD25+Foxp3+) were examined using αCD3-APC/Cy7 (Biolegend, Fell, Germany), αCD4-PE/Cy7 (Biolegend), αCD25-PE (Biolegend), and αFoxp3-APC (eBioscience, Vienna, Austria). An appropriate isotype control was used for the Foxp3 staining (rat IgG2ak-APC, eBioscience).

Approximately 10^6 cells were incubated with the appropriate antibody mix including 1% normal mouse serum for 30 minutes on ice, protected from light. After washing the cells with PBS supplemented with 2% FCS and 5 mM EDTA, the cells were fixed and permeabilized for 30 minutes using a fixation and permeabilization buffer (eBioscience). Then cells were washed with permeabilization buffer and incubated with anti-Foxp3 including 1% normal mouse serum for 30 minutes. Subsequently, the cells were washed with permeabilization buffer, resuspended in FACS lysing solution (BD Biosciences) and kept in the dark on ice until flow cytometric analysis. The fluorescent staining of the cells was measured on a LSR-II flow cytometer (BD Biosciences) and data were analyzed using FlowJo Software (Tree Star, Ashland, USA).

2.7. Histology. Sections of 4 μm were cut from the frozen part of the right lung and stained for all macrophages (rat αCD68, Serotec, Oxford, UK). The numbers of M2 macrophages were determined in frozen sections by staining for YM1 (goat α-mECF-L, R&D Systems, Oxon, UK) using standard immunohistochemical procedures. CD68 and YM1 were visualized with 3-amino-9-ethylcarbazole (AEC, Sigma Aldrich, Zwijndrecht, The Netherlands).

The formalin-fixed part of the right lung was embedded in paraffinthen sections of 3 μm were cut. To identify the M1 macrophages in tissue sections, antigen retrieval was performed by overnight incubation in Tris-HCL buffer pH 9.0 at 80°C and then sections were stained for IRF5 (rabbit α-IRF5, ProteinTech Europe, Manchester UK) using standard immunohistochemical procedures. To determine the number of IL-10 producing cells, antigen retrieval was performed by boiling the sections in citrate buffer pH 6.0 for 10 minutes. The sections were pretreated with 1% bovine serum albumin (Sigma Aldrich) and 5% milk powder in PBS for 30 minutes and incubated with rabbit α-IL-10 overnight (Hycult Biotech, Uden, The Netherlands). IRF5 and IL-10 were both visualized with ImmPACT NovaRED kit (Vector, Burlingame, CA, USA).

Positive cells were quantified by manual counting in parenchymal lung tissue (thus excluding large airways, vessels, and infiltrates, magnification 200–400x) and the total tissue area was quantified by morphometric analysis using ImageScope analysis software (Aperio, Vista, CA, USA). The numbers of cells were expressed per mm^2 of tissue.

2.8. Statistical Analysis. To determine if the data were normally distributed a Kolmogorov-Smirnov test was used. If data sets were not normally distributed, appropriate transformations were performed. The differences between the models were tested using one-way analysis of variance (ANOVA) with Tukey's post-hoc test for multiple comparisons and sex differences were tested with the Student's t test. Pearson correlation coefficients were calculated to analyze the correlation between the inflammation parameters and macrophages phenotypes, and correlations within macrophage phenotypes (GraphPad Software, La Jolla, CA, USA). Differences were considered significant when $P < 0.05$, and $P < 0.10$ was considered a statistical trend.

3. Results

3.1. HDM Exposure Induces Allergic Airway Inflammation. To test whether exposure to HDM, according to three different protocols, induced allergic airway inflammation differently, we studied a number of general inflammation parameters.

Higher percentages of eosinophils in BALF were found in all three HDM-exposed groups as compared to control mice (Figure 2(a)). No differences in percentage of eosinophils in BALF were observed between the three HDM protocols. HDM-specific IgE levels in serum were not affected by the different HDM exposures, only a trend of higher levels was found in the group that was exposed to HDM in the 21-day protocol. In all protocols of HDM exposure, HDM-specific IgE levels were very low measuring just above the limit of detection in the calibration curve (Figure 2(b)).

The higher airway inflammation in the three HDM-exposed groups was accompanied by higher percentages of effector T cells in lung tissue as compared to the control group (Figure 3(a)). The 24-day protocol showed a higher percentage of effector T cells in lungs than the 14- and 21-day protocol. After HDM exposure the percentage of regulatory T cells was also higher in all three protocols as compared to control mice (Figure 3(b)). The 24-day HDM protocol induced higher percentages of regulatory T cells in lungs compared to the 14-day protocol. The ratio of effector T cells to regulatory T cells was higher in the 24-day HDM protocol as compared to the control group and the other two HDM protocols (Figure 3(c)).

In females, HDM exposure induced more eosinophilia ($P < 0.01$), effector T cells ($P < 0.05$), regulatory T cells ($P < 0.05$), and higher levels of HDM-specific IgE ($P < 0.05$) than in males.

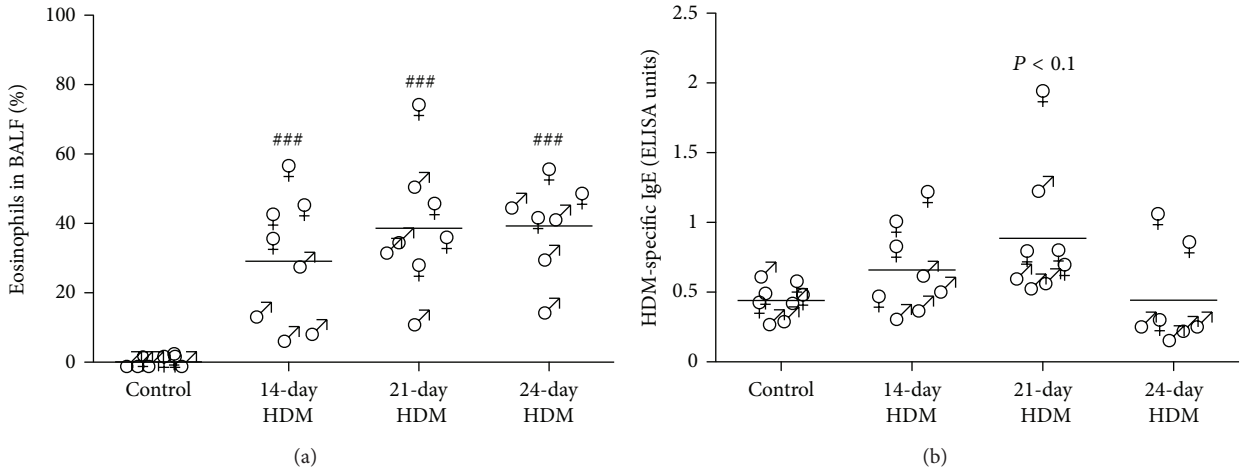

Figure 2: (a) HDM exposure induced higher percentages of eosinophils in BALF of males (♂) and females (♀) as compared to control, but no differences were found between the HDM protocols. Combining all models, higher percentages of eosinophils after HDM exposure were found in females as compared to males ($P < 0.01$). (b) The 21-day protocol increased HDM-specific IgE in serum of male and female mice as compared to control (trend, $P < 0.1$). Again combining all models, HDM exposure induced higher levels of HDM-specific IgE in females than in males ($P < 0.05$). $^{###}P < 0.001$ compared to control.

3.2. HDM Exposure Induces M1 and M2 Macrophages but Inhibits M2-Like Macrophages. To study the presence of macrophage phenotypes after HDM exposure according to the three different protocols, we stained lung tissue for markers of total macrophages (CD68), M1 macrophages (IRF5), M2 macrophages (YM1), and M2-like macrophages (IL-10) and counted positive cells in parenchymal tissue.

HDM-exposed mice had more CD68-positive cells in lung tissue as compared to control mice (Figure 4(a)). No differences in CD68-positive numbers were observed between the HDM protocols. Compared to control mice, IRF5-positive cell numbers were higher in 14- and 21-day protocol, but not in mice exposed to HDM according to the 24-day protocol (Figure 4(b)). Between the HDM models, lower IRF5-positive numbers were found in lungs of mice that were exposed to HDM according to the 24-day protocol as compared to the mice of the 14-day HDM protocol.

For YM1, all HDM protocols induced more YM1-positive cells as compared to control (Figure 4(c)). However, mice that were exposed in the 24-day HDM protocol had higher numbers of YM1-positive cells in lung tissue than the mice of the 14-day HDM protocol. YM1 levels in BALF were elevated in all HDM models as compared to control, but no differences were found between the models (Figure 5). Interestingly, HDM exposure resulted in significantly lower numbers of IL-10-positive cells in all three protocols compared to the control-treated group (Figure 4(d)). There were no differences observed in IL-10-positive cell numbers between the three HDM protocols.

HDM-exposed females had more CD68-positive cells ($P < 0.05$), YM1-positive cells ($P < 0.01$), and higher levels of BALF YM1 ($P < 0.05$) than males, whereas no differences were found in IRF5- and IL-10-positve cells numbers between the two sexes.

3.3. M2 Macrophages Positively Correlate with Parameters of Airway Inflammation. To assess how severity of airway inflammation is reflected by the presence of the three main macrophage phenotypes, we correlated parameters of allergic airway inflammation with the different macrophage phenotypes in HDM-exposed mice (Table 1).

Numbers of CD68-positive cells correlated positively with the percentage of eosinophils in BALF ($r = 0.58$), effector T cells (trend, $r = 0.37$) and regulatory T cells ($r = 0.42$) in lungs of HDM-exposed mice, indicating that more severe disease was accompanied by more macrophages. Most of these macrophages appear to be YM1-positive as only YM1-positive cell numbers correlated significantly with the percentage of eosinophils in BALF ($r = 0.48$, Figure 6) and the percentage of regulatory T cells ($r = 0.51$) in lung tissue. No differences were found between males and females.

3.4. M2 Macrophages Negatively Correlate with IRF5-Positive and IL-10-Positive Cells. To study the relationship between the different macrophage phenotypes in allergic airway inflammation, correlations were made between YM1-postive, IRF5-positive, IL-10-positive, and CD68-positive cells in lung tissue of all HDM-exposed mice (Table 2).

Numbers of IRF5-positive cells negatively correlated with cells positive for CD68 (trend, $r = -0.40$) and YM1 in lung tissue ($r = -0.70$, Figure 7(a)). YM1-positive cell numbers correlated negatively with numbers of IL-10-positve cells ($r = -0.48$, Figure 7(b)) and positively with numbers of CD68-positive cells in lung tissue ($r = 0.66$). No differences were found between males and females.

4. Discussion

Our study has shown that the balance between macrophage phenotype changes as the severity of allergic inflammation increases. Higher numbers of M2 macrophages in

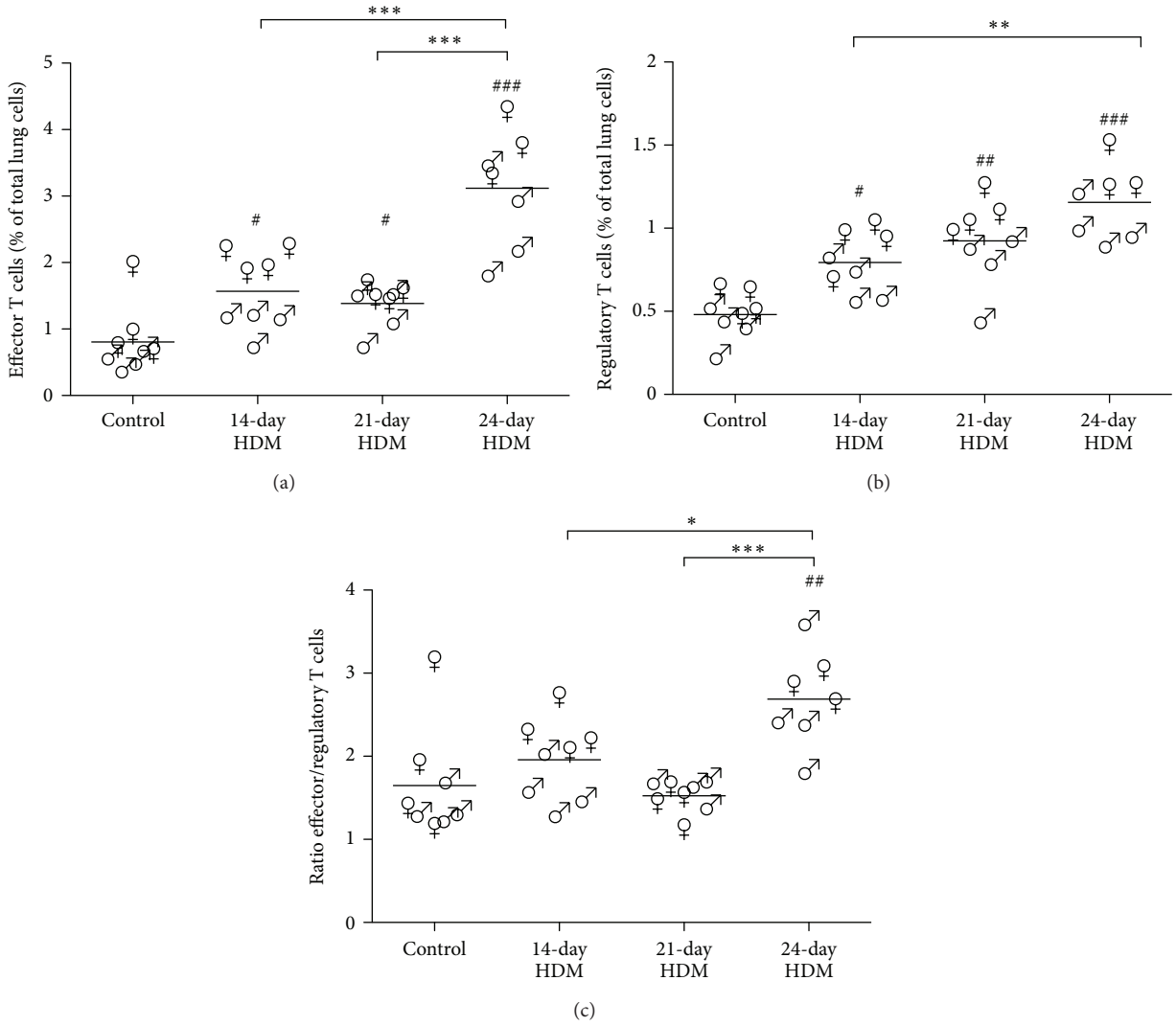

Figure 3: (a) HDM exposure induced higher percentages of effector T cells in lung tissue of males (♂) and females (♀) as compared to control. Higher percentages of effector T cells were found in the 24-day protocol as compared to the 14- and 21-day protocol of HDM exposure. Combining all models, HDM exposure induced higher percentages of effector T cells in females than in males ($P < 0.05$). (b) HDM exposure induced higher percentages of regulatory T cells in lung tissue of males and females as compared to control. Higher percentages of regulatory T cells were found in the 24-day protocol as compared to the 14-day protocol of HDM exposure. Combining all models, HDM exposure induced higher percentages of regulatory T cells in females than in males ($P < 0.05$). (c) The 24-day protocol had higher ratios of effector T cells to regulatory T cells in lung tissue of males and females as compared to control and the 14- and 21-day protocols of HDM exposure. Combining all models, no differences were found between males and females. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ compared to control. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

HDM-exposed mice correlated with higher percentages of eosinophils in BALF. At the same time lower numbers of M1 macrophages and M2-like macrophages were found in the mice with more severe inflammation and these therefore correlated negatively with M2 macrophages. In addition, we have confirmed again that females have more pronounced airway inflammation with higher numbers of M2 macrophages as compared to males [19].

The models we used for our study were short-term exposure to HDM and they give us much information about the distribution of the different macrophage phenotypes during induction of asthma. In these models we found that longer exposure to HDM did not induce more severe eosinophilic inflammation but it did lead to higher numbers of M2 macrophages and higher percentages of effector and regulatory T cells in lungs of mice. The fact that we found no differences in eosinophils between the models is probably due to the large variation within the groups. However, when analyzing all HDM-exposed mice separately, eosinophils correlated positively with total macrophages and M2 macrophages, confirming our previous findings in humans that M2 macrophages increase with increasing asthma severity [20]. Another important parameter of allergic airway inflammation, serum HDM-specific IgE, could

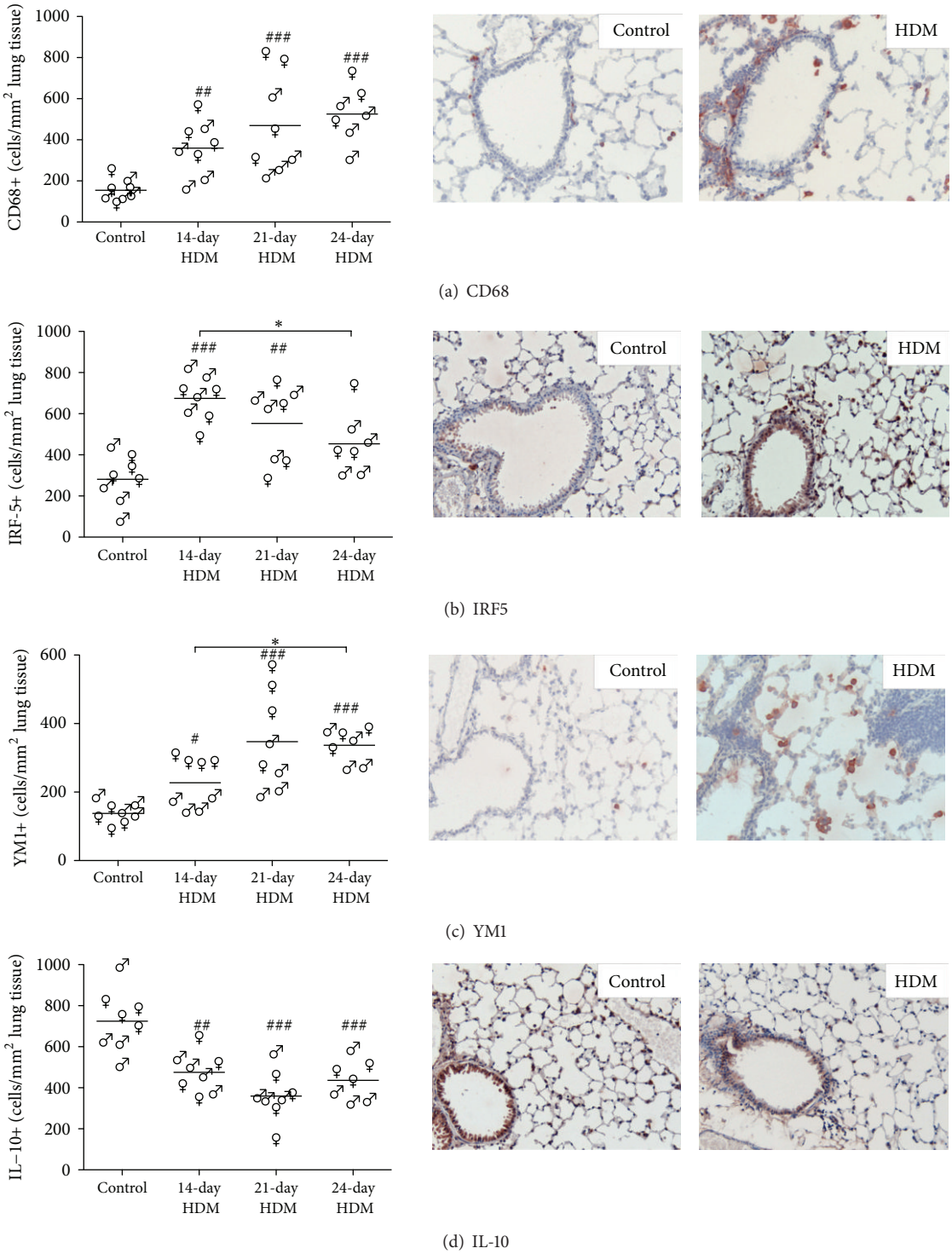

Figure 4: (a) HDM-exposed male (♂) and female (♀) mice had more CD68-positive cells in lung tissue as compared to control, but no differences were found between the HDM protocols. Combining all models, HDM exposure induced higher numbers of CD68-positive cells in females than in males ($P < 0.05$). (b) HDM-exposed male and female mice had more IRF5-positive cells in lung tissue as compared to control, but no differences were found between males and females when combining all models. The 14-day HDM protocol induced higher numbers of IRF5-positive cells as compared to the 24-day HDM protocol. (c) HDM-exposed male and female mice had more YM1-positive cells in lung tissue as compared to control, with higher numbers of YM1-positive cells in females than in males ($P < 0.01$). The 24-day HDM protocol induced higher numbers of YM1-positive cells as compared to the 14-day HDM protocol. (d) HDM-exposed male and female mice had lower numbers of IL-10-positive cells in lung tissue as compared to control, but no differences were found between the HDM protocols and between the sexes. The middle and right-hand panels are representative photos of control and HDM-exposed mice for all four stainings (magnification 200x). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ compared to control. $^{*}P < 0.05$.

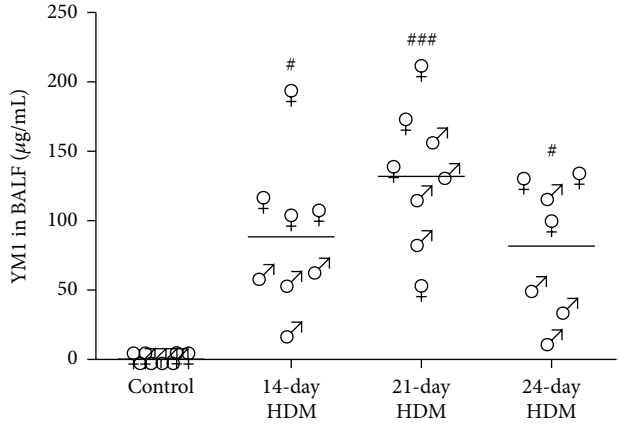

Figure 5: HDM exposure induced higher levels ofYM1 in bronchoalveolar lavage fluid (BALF) of male (♂) and female (♀) mice as compared to control, but no differences were found between the HDM protocols. Combining all models, HDM exposure induced higher levels of YM1 in BALF in females than in males ($P < 0.05$). $^{\#}P = 0.05$ and $^{\#\#\#}P < 0.001$ compared to control.

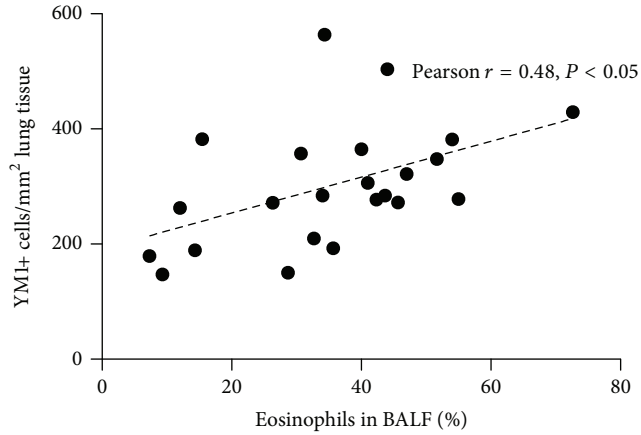

Figure 6: YM1-positive cell numbers (cells/mm^2 lung tissue) correlated with percentage of eosinophils in BALF of male and female mice exposed to HDM according to three different protocols. No differences were found between males and females when combining all models.

Table 1: Correlations between macrophage phenotype markers and parameters of allergic airway inflammation.

	CD68+ cells	IRF5+ cells	YM1+ cells	IL-10+ cells
Eosinophils	0.58^{**}	−0.26	0.48^{*}	0.01
HDM-specific IgE	−0.06	−0.18	0.33	−0.13
Effector T cells	$0.37^{\#}$	−0.30	0.29	0.05
Regulatory T cells	0.42^{*}	−0.20	0.51^{*}	−0.21

Values are correlations coefficients (Pearson correlation). $^{\#}P < 0.1$, $^{*}P < 0.05$ and $^{**}P < 0.01$.

Table 2: Correlations between macrophage phenotype markers.

	CD68+ cells	IRF5+ cells	YM1+ cells	IL-10+ cells
CD68+ cells		$-0.40^{\#}$	0.66^{**}	−0.17
IRF5+ cells			-0.70^{**}	0.20
YM1+ cells				-0.48^{*}

Values are correlations coefficients (Pearson correlation). $^{\#}P < 0.1$, $^{*}P < 0.05$ and $^{**}P < 0.01$.

barely be detected probably because the duration of the models was too short. It takes around 3 weeks for naive B cells to mature to plasma cells and switch from IgM production to IgE after first contact with an antigen [21]. Our models lasted 24 days at the most and we therefore sacrificed our animals before a full-blown IgE response could develop. Also, studies from other groups using these models did not show HDM-specific IgE in serum [15–17]. To investigate how macrophage phenotypes are distributed during and contribute to more chronic disease, longer models of HDM-exposure need to be used.

We sought to phenotype the distinct macrophage subsets in lung tissue using markers that could distinguish each phenotype. We identified M2 macrophages using expression of YM1, which is unique for this phenotype in the lung. Previous studies found that only macrophages express YM1 and the staining is not complicated by other cells staining positive [22–24]. A unique marker for identifying M1 macrophages in lung tissue is, however, more difficult to find. To our knowledge selective surface markers for tissue are not available and therefore the production of IL-12 and oxygen radicals have been used in several mouse studies [25, 26]. In asthma, oxygen radicals cannot be used to stain for M1 macrophages because these are also copiously produced by eosinophils that are present in great numbers [27]. Recently, IRF5 was found to be the transcription factor controlling M1 differentiation. It is highly expressed in M1 macrophages while it suppresses the M2 phenotype [5]. Our study was the first to use IRF5 as an M1 marker on lung tissue and it is a fairly selective marker. Bronchial epithelial cells and incoming leukocytes in infiltrates also stain positive for IRF5 but these could be excluded from the quantification of parenchymal tissue based on localization. A double staining with CD68 to make sure only macrophages are included in the quantification is unfortunately at present not possible due to technical incompatibilities. In previous studies, M2-like macrophages are often not distinguished from M2 macrophages because they share many markers, including mannose receptors. The production of the immunosuppressive cytokine IL-10 is the most important and reliable characteristic of M2-like macrophages and can be used to identify these cells with [4]. Similar to IRF5, bronchial epithelial cells and some cells in infiltrates are also expressing IL-10 but these can easily be excluded from the quantification of parenchymal tissue. To exclude other IL-10-producing cells from the analysis a double staining with CD68 is needed, but at present also not possible. These limitations in the stainings for IRF5 and IL-10 may also explain why the total number of YM1-, IRF5- and IL-10-positive cells is higher than the number of CD68-positive cells.

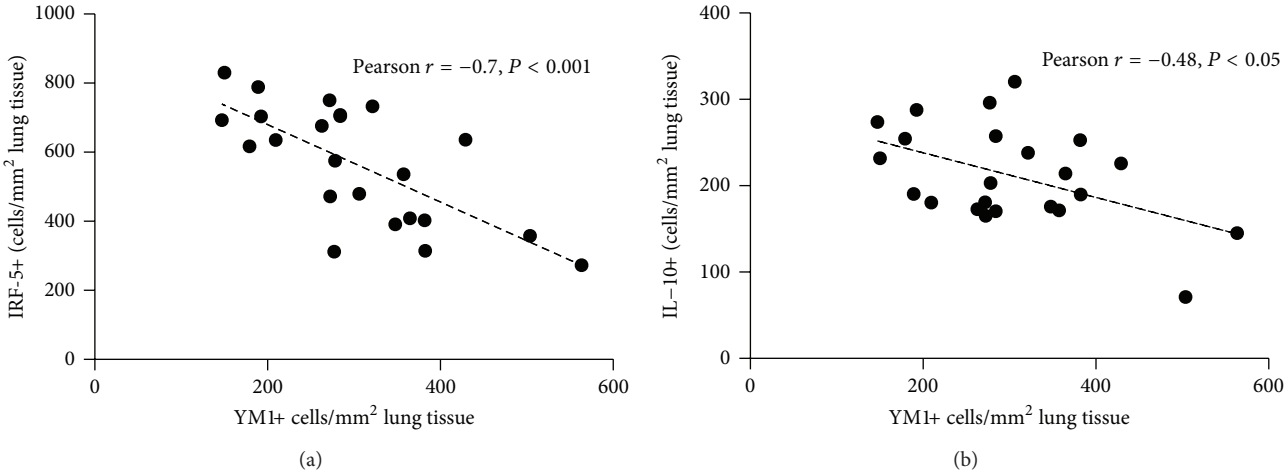

Figure 7: (a) YM1-positive cell numbers correlated with IRF5-positive cell numbers in lung tissue of male and female mice exposed to HDM according to 3 different protocols. (b) YM1-positive cells numbers correlated with IL-10-positive cell numbers in lung tissue of male and female mice exposed to HDM according to three different protocols. No differences were found between males and females when combining all models.

Higher numbers of M2 macrophages were found in lungs of HDM-exposed mice, which correlates with previous studies [19, 20, 28]. Interestingly, numbers of M2 macrophages and the levels of YM1 in BALF correlated strongly with eosinophils in BALF. YM1 is also known as eosinophil chemotactic factor (ECF-L) [29], but it was suggested that YM1 only has weak chemotactic properties for eosinophils [24, 30]. The chemotactic strength of YM1 is still debated and our findings and those of others do suggest otherwise [23, 31]. The number of M2 macrophages also showed a positive correlation with regulatory T cells. In an interesting study by Tiemessen et al., regulatory T cells were shown to promote the induction of M2 macrophages to help with trying to maintain tissue homeostasis and preventing too much tissue damage of the inflammatory response [32]. Our data could be explained along these lines with incoming regulatory T cells inducing M2 macrophages in an attempt to restrict inflammation and tissue damage induced by HDM. The M2 macrophages would then be the result of inflammation and be beneficial instead of contributing to allergic airway inflammation, which is still an ongoing debate [19, 33, 34].

Asthma is dominated by a Th2-driven inflammation, but evidence shows that M1 macrophages are also present in this disease. In two interesting studies, IFNγ-stimulated macrophages were shown to prevent the onset of allergic airway inflammation [35, 36]. Our findings with higher numbers of M1 macrophages in the shorter protocols and in less severe diseases suggest that these macrophages are induced as a counterregulatory mechanism to dampen inflammation. However, higher numbers of M1 macrophages in asthma have also been shown in severe asthma and markers of M1 macrophages correlate with asthma severity, suggesting that M1 macrophages play a role in severe asthma as well [37–39]. This appears not to be the case for our results because we find higher numbers of M1 macrophages at the onset of allergic airway inflammation when numbers of effector T cells are lower, and lower numbers of M1 macrophages in the 24-day HDM protocol when effector T-cell numbers are higher. M1 macrophages appear to have a beneficial role in preventing allergic sensitization, but in already established disease they promote the development of a severe phenotype. A similar double role has been reported for the M1 cytokine IL-12 in allergic airway inflammation [40]. Since our HDM models are short and focus on the onset of the disease, we do not have information on the presence of M1 macrophages in established, more severe disease.

Since M2-like macrophages have anti-inflammatory functions, we were interested in the presence of this macrophage phenotype in HDM-induced asthma. Others have reported that interstitial macrophages are the IL-10-producing macrophages and treatment of sensitized mice with these macrophages prevented the development of allergic airway inflammation [41]. Our observation of lower numbers of M2-like macrophages in asthma as compared to control is in line with these and previous findings in human asthmatics [42, 43]. Interestingly, it was shown that IL-10 production in severe asthmatics is even lower as compared to moderate asthmatics and that treatment with corticosteroids induces IL-10 production by macrophages [43, 44]. This suggests that specific stimulation of macrophages to polarize to M2-like macrophages that produce IL-10 may reduce asthma symptoms.

Women suffer from more severe asthma than men and this phenomenon was also found in mouse models of asthma [45, 46]. A role for sex hormones has been suggested, but the underlying mechanisms are unknown [47]. In accordance with the previous findings, we show that HDM-exposed female mice had more pronounced airway inflammation than male mice. In macrophage phenotypes, we only found a difference in the M2 macrophages. Since we and others found

a correlation between M2 macrophages and asthma severity, it may suggest that this phenotype could play a role in the increased airway inflammation in females [19].

5. Conclusion

Taken together, our data suggest that during the development of allergic airway inflammation M1 and M2 macrophages are induced or recruited, whereas M2-like macrophages are prevented from moving in or inhibited. As HDM-induced inflammation progresses, M1 macrophages are diminished in favor of M2 macrophages possibly under the influence of regulatory T cells that try to restrict inflammation. Their work may be hampered by the fact that IL-10-producing M2-like macrophages do not develop in HDM-induced inflammation and the inflammation can progress. Influencing this imbalanced relationship by therapeutic macrophage targeting could be a novel way to treat asthma.

Conflict of Interests

The authors have no conflict of interests to disclose.

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Research Article

Palmitoylethanolamide Is a Disease-Modifying Agent in Peripheral Neuropathy: Pain Relief and Neuroprotection Share a PPAR-Alpha-Mediated Mechanism

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Neuropathic syndromes which are evoked by lesions to the peripheral or central nervous system are extremely difficult to treat, and available drugs rarely joint an antihyperalgesic with a neurorestorative effect. *N*-Palmitoylethanolamine (PEA) exerts antinociceptive effects in several animal models and inhibits peripheral inflammation in rodents. Aimed to evaluate the antineuropathic properties of PEA, a damage of the sciatic nerve was induced in mice by chronic constriction injury (CCI) and a subcutaneous daily treatment with 30 mg kg^{-1} PEA was performed. On the day 14, PEA prevented pain threshold alterations. Histological studies highlighted that CCI induced oedema and an important infiltrate of CD86 positive cells in the sciatic nerve. Moreover, osmicated preparations revealed a decrease in axon diameter and myelin thickness. Repeated treatments with PEA reduced the presence of oedema and macrophage infiltrate, and a significant higher myelin sheath, axonal diameter, and a number of fibers were observable. In PPAR-α null mice PEA treatment failed to induce pain relief as well as to rescue the peripheral nerve from inflammation and structural derangement. These results strongly suggest that PEA, via a PPAR-α-mediated mechanism, can directly intervene in the nervous tissue alterations responsible for pain, starting to prevent macrophage infiltration.

1. Introduction

Neuropathic pain may originate from several different causes. Mechanical peripheral neuropathies are the consequence of local or extrinsic compression phenomena or impingement by an anatomic neighbor causing a localized entrapment. Traumatic neuropathies are the result of either closed injuries or open injuries to peripheral nerves [1, 2]. Both of these categories are characterized by an important inflammatory component that plays a central role in the pathogenesis of neuropathic pain. Inflammatory cells (e.g., macrophages), the production of molecules that mediate inflammation (cytokines), and the production of nervous growth factors are involved [3, 4]. In animal models it has been demonstrated that peripheral nerve injuries induce a profound local inflammatory response that involves T cells and macrophages [5]. In particular, in the neuropathic pain model induced by chronic constriction injury (CCI) an important macrophage infiltrate has been described in the damaged sciatic nerve [5–7] and in the dorsal root ganglia [8, 9]. The inflammatory response paralleled with nervous tissue alterations and pain [7, 10].

N-Palmitoylethanolamine (PEA), the endogenous amide between palmitic acid and ethanolamine, belongs to the family of fatty acid ethanolamides (FAEs), a class of lipid mediators. PEA exerts antinociceptive effects in several animal models [11, 12], prevents neurotoxicity and neurodegeneration [13, 14], and inhibits peripheral inflammation and mast cell degranulation [15]. Endogenous and exogenous PEA can modulate macrophage response [16, 17].

Anti-inflammatory effects of PEA have been associated with peroxisome proliferator-activated receptor-(PPAR-)α activation [18], a nuclear receptor fundamental in the control

of inflammatory responses, and expressed in various cells of the immune system [19, 20]. PEA does not elicit anti-inflammatory effects in mutant PPAR-α null mice (PPAR-$\alpha^{-/-}$). Indeed, when assessed in either the carrageen hindpaw or phorbol ester ear pinna tests, PEA reduced inflammation in wild-type, but not in PPAR-$\alpha^{-/-}$, mice [18]. On the other hand, LoVerme and collegues [12] demonstrated the pivotal role of PPAR-α in the PEA pharmacodynamic mechanism to relieve pain.

In a mouse peripheral neuropathy model (CCI) we evaluated the effects of repeated PEA treatments on the sciatic nerve lesions responsible for neuropathic pain. Aimed to highlight the role of PPAR-α in PEA-evoked neurorestoration during neuropathy, a morphological study has been performed in both wild-type and PPAR-α null mice.

2. Materials and Methods

2.1. Animals. All procedures met the European guidelines for the care and use of laboratory animals (86/609/ECC and 2010/63/UE), and those of the Italian Ministry of Health (DL 116/92). Male wild-type (WT) and PPAR-$\alpha^{-/-}$ (KO) (B6.129S4-SvJae-Pparatm1Gonz) mice, previously backcrossed to C57BL6 mice for 10 generations, were bred in our animal facility, where a colony was established and maintained by heterozygous crossing. Mice were genotyped as described on the supplier webpage (http://jaxmice.jax.org/), with minor modifications. DNA was extracted from tails using the RedExtract kit (Sigma-Aldrich, Milan, Italy). All animals were maintained on a 12 h light/12 h dark cycle with free access to water and standard laboratory chow.

2.2. Chemicals. PEA was from Tocris (Bristol, UK); it was dissolved in PEG and Tween 80 2 : 1 (Sigma-Aldrich) and kept overnight under gentle agitation with a microstirring bar. Before injection, sterile saline was added so that the final concentrations of PEG and Tween 80 were 20 and 10% v/v, respectively. Drug was injected subcutaneously (s.c.) in a dose of 30 mg kg^{-1}—0.3 mL mouse—for consecutively 14 days from the day after surgery.

2.3. CCI Model. The sciatic nerve of 5-6-week-old WT and KO mice were surgically ligated as described [21]. In brief, the animals were anesthetized with ketamine (100 mg kg^{-1} i.p) and xylazine (10 mg kg^{-1} i.p.), the left thigh was shaved and scrubbed with Betadine, and a small incision (2 cm in length) was made in the middle of the left thigh to expose the sciatic nerve. The nerve was loosely ligated at two distinct sites (spaced at a 2 mm interval) around the entire diameter of the nerve using silk sutures (7-0). Behavioral tests were performed on the day 14 after surgery.

2.4. Mechanical Hyperalgesia. We measured mechanical hyperalgesia using a Randall-Selitto analgesimeter for mouse (Ugo Basile, Varese, Italy). Latencies of paw withdrawal to a calibrated pressure were assessed on ipsilateral (ligated) paws on day 14 following ligatures. Cut-off force was set at 60 g.

2.5. Mechanical Allodynia. To assess for changes in sensation or in the development of mechanical allodynia, sensitivity to tactile stimulation was measured using the Dynamic Plantar Aesthesiometer (DPA, Ugo Basile, Italy). Animals were placed in a chamber with a mesh metal floor covered by a plastic dome that enabled the animal to walk freely but not to jump. The mechanical stimulus was then delivered in the mid-plantar skin of the hind paw. The cutoff was fixed at 5 g. Each paw was tested twice per session. This test did not require any special pretraining, just an acclimation period to the environment and testing procedure. Testing was performed on ipsilateral (ligated) paw on day 14 after ligation. Cutoff force was set at 5 g.

2.6. Tissue Explants. After the algesic test, animals were sacrificed and the ipsilateral sciatic nerves, 1 cm proximal and distal to the ligation, were explanted; the portion containing the ligature was eliminated. Contralateral nerves were also dissected, and an equivalent portion was collected. Spinal cord was removed, and lumbar section was immediately frozen in liquid nitrogen.

2.7. Osmic Acid Staining. The sciatic nerve was stored in a 4% glutaraldehyde solution. The tissue samples were osmicated in 1% solution of osmium tetroxide for 2 h under constant agitation. Before and after osmication, the tissue was repeatedly rinsed in 0.1 M sodium cacodylate at pH 7.4. After gradual dehydration in ethanol, the osmicated nerve samples were embedded in paraffin (Diapath, Milan, Italy). Transverse 5 μm sections were cut on a Reichert microtome (Leica, Rijswijk, The Netherlands), mounted with Canada balsam, and observed under a light microscopy.

2.8. Azan-Mallory Stain. After the sacrifice, the sciatic nerve was fixed in situ using 4% formalin in phosphate buffer (pH 7.4), nerves were fixed in 4% buffered neutral formalin solution, and then the nerve was embedded in paraffin. Finally 5 μm sections were stained with Azan-Mallory for light microscopy studies and were graded for oedema and infiltrate [22]. The sections were semiquantified by an arbitrary scale starting from 1, mild infiltrate and oedema, up to 10, severe infiltrate and widespread oedema. The procedure was carried out by an independent researcher who was masked to the experiment.

2.9. Morphometry. Morphometry was performed on cross sections of osmium-fixed sciatic nerves 10 mm starting from the level of injury or at the corresponding level in uninjured control nerves. The 10 mm cross section corresponded to a level distal or proximal to the injury. Counts and measurements were carried out using ImageJ analysis software.

The first step of morphometric analysis of the sciatic nerve consisted of identifying and capturing the entire fascicle image, followed by measurement of the fascicle perimeter and area by contouring its internal epineural (magnification: objective 20x). The next step consisted of capturing sequential inner areas of the fascicle (magnification: objective 100x). The high-magnification micrographs were randomly selected

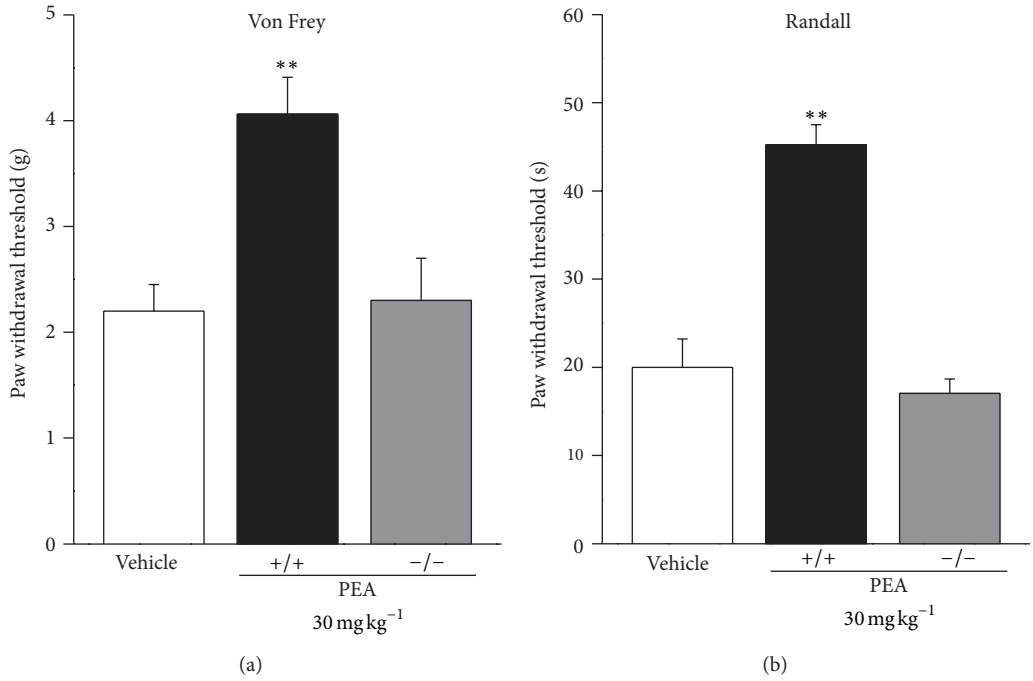

Figure 1: Chronic treatment effects on pain behaviour. Comparison of PEA-effects in PPAR-$\alpha^{+/+}$ and $^{-/-}$ mice. (a) Response to a mechanical noxious stimulus on the ipsilateral paw 14 days after CCI evaluated by Randall-Selitto test. (b) Response to a mechanical nonnoxious stimulus evaluated by Von Frey test. PEA (30 mg kg^{-1}) was daily s.c. administered for 14 days starting from the day of operation. Each value represents the mean of 2 experiments with 12 mice per group. $^{**}P < 0.01$ versus vehicle-treated mice.

in nonoverlapping areas to cover 50–75% of the total cross-sectional area of the nerve. The random selected histological images were converted into binary (black and white) images and cleaned of any blood vessels, degenerated nerve fibers, and artifacts, and the following parameters were then measured: total number and density of nerve fibers, axon diameter, and myelin sheath area. The number of small fibers, defined as fibers $< 4\,\mu$m, and large fibers, defined as fibers $\geq 4\,\mu$m, was calculated, and the myelin thickness was determined from the difference in perimeter between the fiber and the axon. The procedure was carried out by an independent researcher who was masked to the experiment.

2.10. Immunohistochemistry. Sections of 5 μm were deparaffinized, dehydrated, and submitted to antigen retrieval. Sections were then blocked by incubation with 3% normal goat serum for 20 min. Macrophages were detected using anti-CD86 antibody. The slides were then incubated with the primary antibodies for 18 h at 4°C. After washing in TBS, the sections were treated with the secondary antibody conjugated to Alexa Fluor 488 (1 : 1000, Invitrogen, Milan, USA) for 1 h at room temperature. Nuclei were counterstained with DAPI. Quantitative analysis of CD86 positive cells was performed collecting at least three independent fields through a 20 × 0.5 NA objective, and positive cells were counted using the "cell counter" plugin of ImageJ.

2.11. Western Blot Analysis. The lumbar portion of the spinal was homogenized in lysis buffer containing 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, Complete Protease Inhibitor (Roche), and the homogenate was incubated on ice for 30 minutes. Then, the suspension was sonicated on ice using three 10-second bursts at high intensity with a 10-second cooling period between each burst. After centrifugation (13000 ×g for 15 minutes at 4°C) aliquots containing 20 μg total protein underwent to western blot analysis using a mouse anti-COX2 antiserum (1 : 1000; Cell Signalling, USA). Densitometric analysis was performed using the "ImageJ" analysis software, and results were normalized to β-actin immunoreactivity (1 : 1000 rabbit antiserum, SantaCruz Biotechnology) as internal control.

2.12. Statistical Analysis. For behavioral experiments, results were expressed as the mean ± SEM, and analyses were conducted using Statistica (Statsoft, Tulsa, OK, USA).

Histological, morphometric, and immunohistochemical analyses were performed on 5 mice per group, evaluating 6 sciatic nerve sections for each animal. Comparison, were carried out using Mann-Whitney nonparametric tests. In all cases, the investigator was blind to the experimental status of each animal. Slides from control and experimental groups were labeled with numbers so that the person performing the image analysis was blinded as to the experimental group. In addition, all images were captured and analyzed by an investigator other than the one who performed measures to avoid possible bias. Western blot analysis were performed on 5 mice per group; comparisons were carried out using Bonferroni posttest. Data were analyzed using the "Origin 7.5" software. Differences were considered significant if $P < 0.05$ or otherwise differently reported.

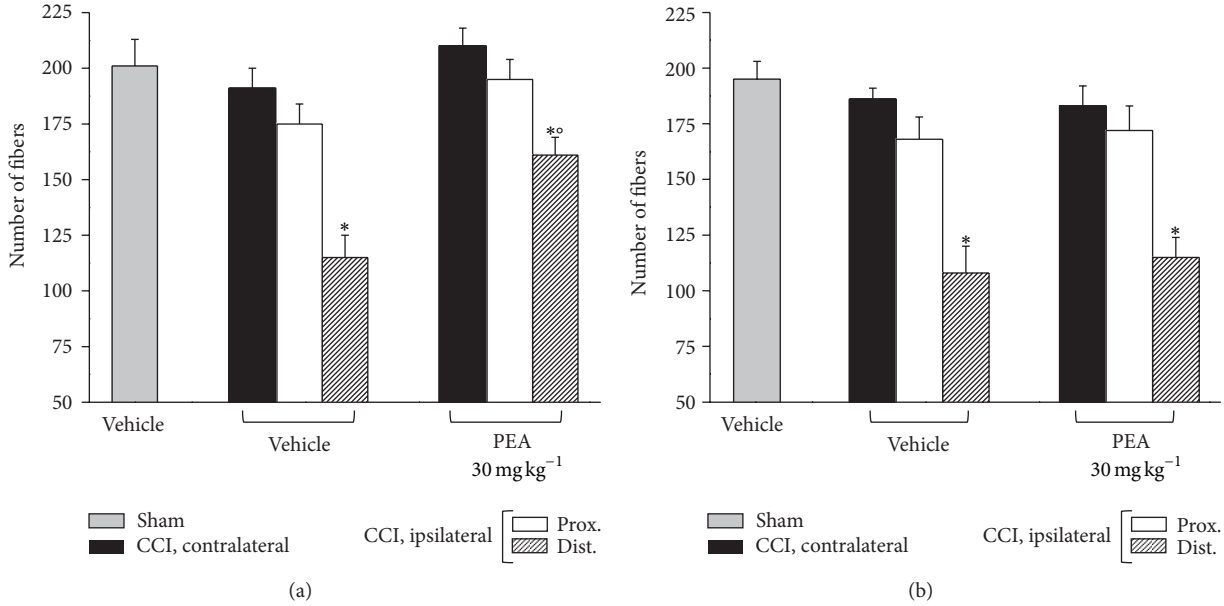

Figure 2: Morphometry: number of fibers. 5 μm nerve sections of osmium-fixed tissues were analyzed. The distal and the proximal tract of the ipsilateral ligated nerve (CCI) was compared with the contralateral and with the sciatic nerve of sham-operated animals (sham). Effect of repeated PEA administrations (30 mg kg^{-1} s.c. for 14 days) on the number of nervous fibers in respect to vehicle-treated CCI animals and vehicle-treated sham animals. (a) PPAR-$\alpha^{+/+}$ mice versus (b) knock-out animals. Quantitative analysis was performed evaluating 5 animals for each group. $^{*}P < 0.05$ was considered as significantly different from sham, vehicle-treated mice. $^{\circ}P < 0.05$ was considered as significantly different from CCI, vehicle-treated mice.

3. Results

PEA, (30 mg kg^{-1}) s.c. daily administered starting on the day of operation, prevented pain threshold alterations elicited by CCI (Figure 1). In wild-type animals 14 days after injury, PEA reduced the hypersensitivity to a mechanical noxious stimulus (Randall-Selitto test; Figure 1(a)) as well as the hypersensitivity to a nonnoxious mechanical stimulus (Figure 1(b)). PEA efficacy against CCI-evoked pain was lacked in PPAR-α knock-out (−/−) mice (Figures 1(a) and 1(b)).

On the 14th day after injury, a morphological evaluation of the sciatic nerves was performed both on the proximal and distal parts from the ligation. 5 μm sections of paraffin-embedded nerve were stained by the Azan-Mallory procedure revealing that CCI was able to induce a stronger alteration in the distal portion of the ipsilateral nerve. The histological analysis highlighted an extensive demyelination, myelin abnormalities like characteristic aggregate, generally termed "ovoids", pathognomonic of myelin degeneration (Figure 7, arrows), and an axonal damage. As shown in Figure 2 the number of fibers was significantly reduced, particularly in the distal part of the nerve of both wild-type (+/+; graph (a)) and PPAR-α null (−/−; graph (b)) animals.

Morphometric evaluation of osmium-fixed tissues allowed the characterization of the alteration of the myelin sheet thickness and axonal diameter, as well as the discrimination of large against small fibers (stratified by diameter in >6 μm for large and <6 μm for small). CCI was able to decrease the myelin thickness of large and small fibers in the distal portion of the ipsilateral nerve in respect to the sham both in wild-type and in knock-out mice (Figures 3(a) and 3(b), large fibers; Figures 3(c) and 3(d), small fibers).

In regard to axon diameter, a time-dependent decrease was revealed for all the fibers, particularly the small type, both in the distal and in the proximal portions of the ipsilateral nerve; morphometry revealed a similar profile in PPAR-$\alpha^{+/+}$ (Figures 4(a) and 4(c)) and PPAR-$\alpha^{-/-}$ (Figures 4(b) and 4(d)) animals. In wild-type mice repeated PEA administrations, 30 mg kg^{-1} for 14 days, were able to preserve the nerve morphology. Nerve sections of PEA-treated mice showed a higher number of fibers in respect to the saline-treated groups (Figure 2(a)); the myelin thickness in the distal portions of the nerve was decreased to a lesser extent (Figures 3(a) and 3(c)); the axon diameter was protected in the PEA group both in the proximal and in the distal nerve parts, even in the small fibers (Figures 4(a) and 4(c)). PEA was completely ineffective in PPAR-α null mice in preventing sciatic nerve alterations evaluated as number of fibers (Figure 2(b)), myelin thickness (Figures 3(b) and 4(d)) and axon diameters (Figures 4(b) and 4(d)).

Azan-Mallory staining revealed an abundant inflammatory infiltrate in the ligated nerve. Figure 5 shows the infiltrate evaluation 14 days after ligation: inflammatory cells were present in the proximal and, at higher level, in the distal parts of both PPAR-$\alpha^{+/+}$ (graph (a)) and PPAR-$\alpha^{-/-}$ (graph (b)) mice. 30 mg kg^{-1} PEA significantly prevented the cellular infiltrate number in wild-type (Figure 5(a)) but not in KO animals (Figure 5(b)).

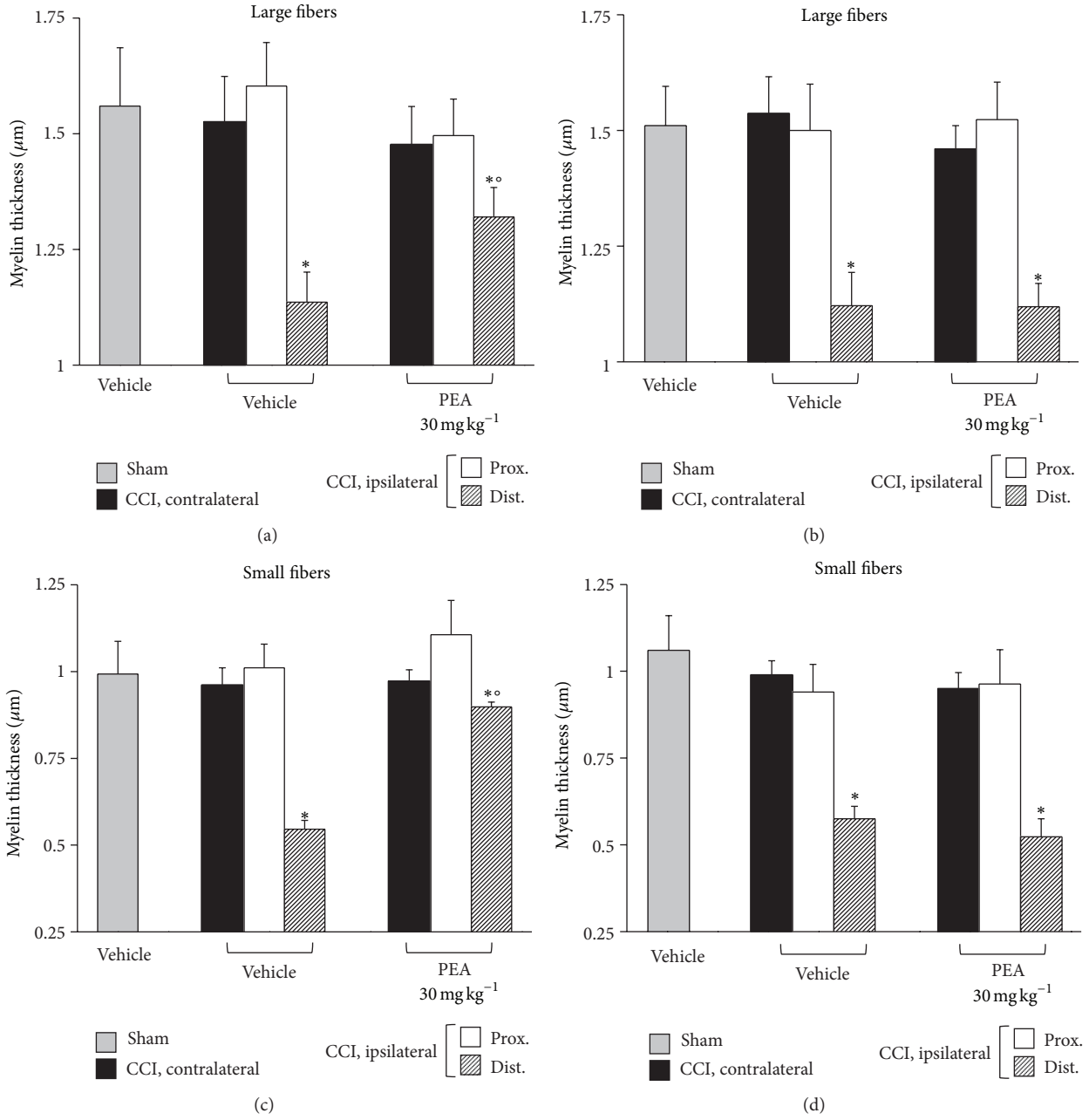

Figure 3: Morphometry: myelin thickness. Nerve sections (5 μm) of osmium-fixed tissues were analyzed. The distal and the proximal tract of the ipsilateral ligated nerve (CCI) was compared with the contralateral and with the sciatic nerve of sham-operated animals (sham). Myelin thickness of large and small fibers of PEA-treated (30 mg kg^{-1} s.c. for 14 days) PPAR-$\alpha^{+/+}$ ((a) and (c)) and PPAR-$\alpha^{-/-}$ ((b) and (d)) mice in respect to saline-treated CCI and saline-treated sham animals. Quantitative analysis was performed evaluating 5 animals for each group. $^{*}P < 0.05$ was considered as significantly different from sham, vehicle-treated mice. $^{\circ}P < 0.05$ was considered as significantly different from CCI, vehicle-treated mice.

Moreover, both osmium fixed and Azan-Mallory-stained sections (Figure 7) allowed the observation of a massive presence of oedema among the fibers of CCI animals. Figure 6 show the quantitative oedema evaluation 14 days after operation: the alteration was more evident in the distal portion than in the proximal one without revealable differences due to knock down PPAR-α gene. PEA (30 mg kg^{-1} s.c. for 14 days) was able to prevent the oedema induction of about 50% in CCI wild-type mice (Figure 6(a)). No oedema protective effects were observable in PEA-treated PPAR-$\alpha^{-/-}$ animals in respect to vehicle (Figure 6(b)).

The immune inflammatory cells evaluated were diffusely distributed throughout the nerve tissue in all samples of the CCI mice, whereas a mild CD86 positive reaction

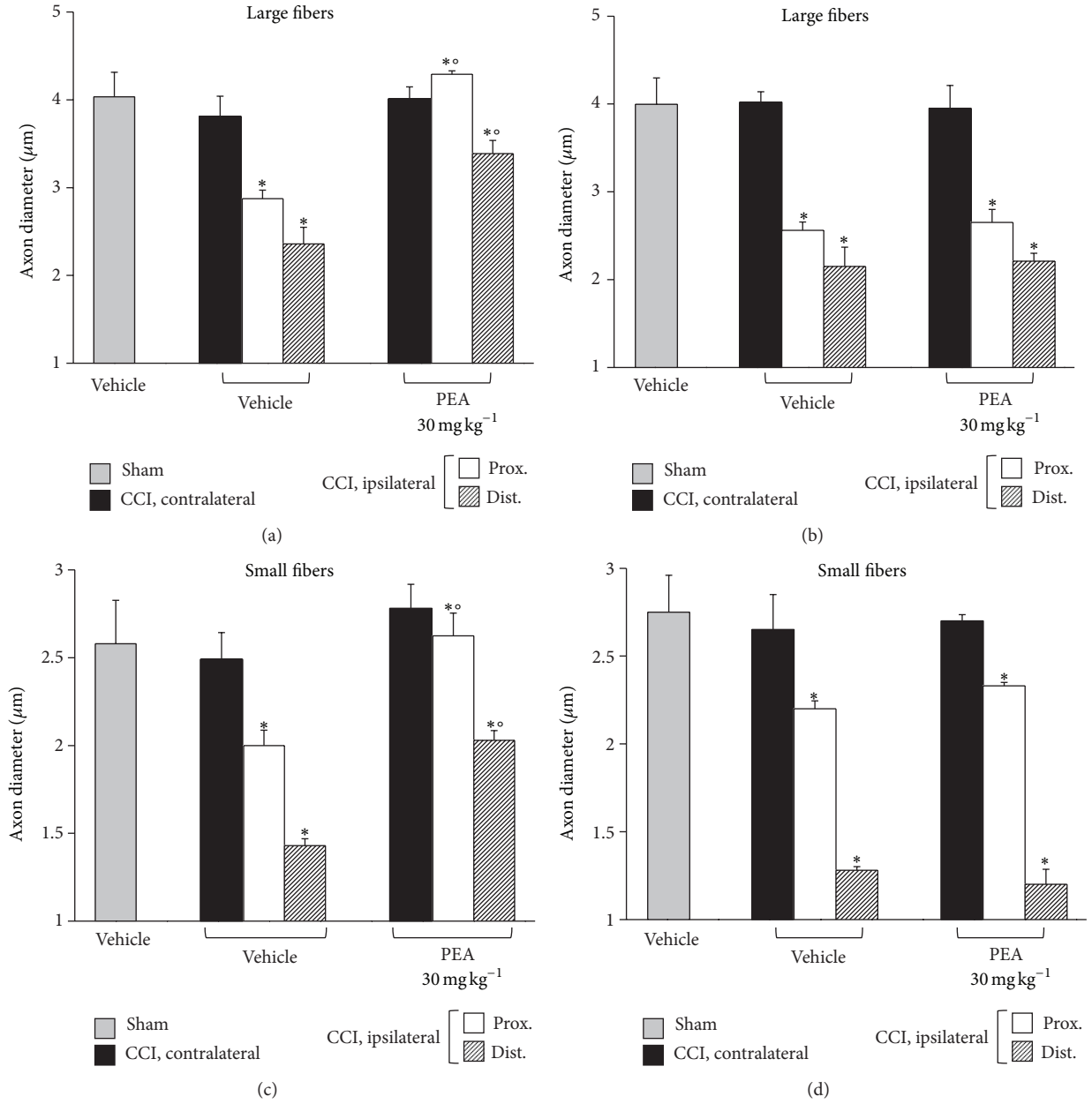

Figure 4: Morphometry: axon diameters. Nerve sections (5 μm) of osmium-fixed tissues were analyzed. The distal and the proximal tract of the ipsilateral ligated nerve (CCI) was compared with the contralateral and with the sciatic nerve of sham-operated animals (sham). Axon diameters of large and small fibers of PEA-treated (30 mg kg^{-1} s.c. for 14 days) wild-type ((a) and (c)) and PPAR-α null ((b) and (d)) mice in respect to saline-treated CCI and saline-treated sham animals. Quantitative analysis was performed evaluating 5 animals for each group. $^{*}P < 0.05$ was considered as significantly different from sham, vehicle-treated mice. $^{\circ}P < 0.05$ was considered as significantly different from CCI, vehicle-treated mice.

was detectable in CCI mice administered with PEA as well as in sham-operated animals. PPAR-$\alpha^{-/-}$ mice showed a persistence of macrophage infiltrate also in the nerve of PEA-treated animals (Figures 8 and 9).

CCI-dependent inflammatory response in the central nervous system was evaluated measuring COX2 levels. As shown in Figure 10, in wild-type mice PEA was able to significantly prevent COX2 increase induced by nerve ligation. This anti-inflammatory effect was lost in PPAR-$\alpha^{-/-}$ animals.

4. Discussion

Pharmacological treatment of peripheral neuropathy is actually restricted to symptomatic drugs that are only partially able to control pain perception [23]. Antidepressants, antincovulsants, and opioids cannot intervene in nervous tissue alterations that act as a base of neuropathic pain. The present results describe the direct protective effect of repeated PEA treatment on lesioned peripheral nerves.

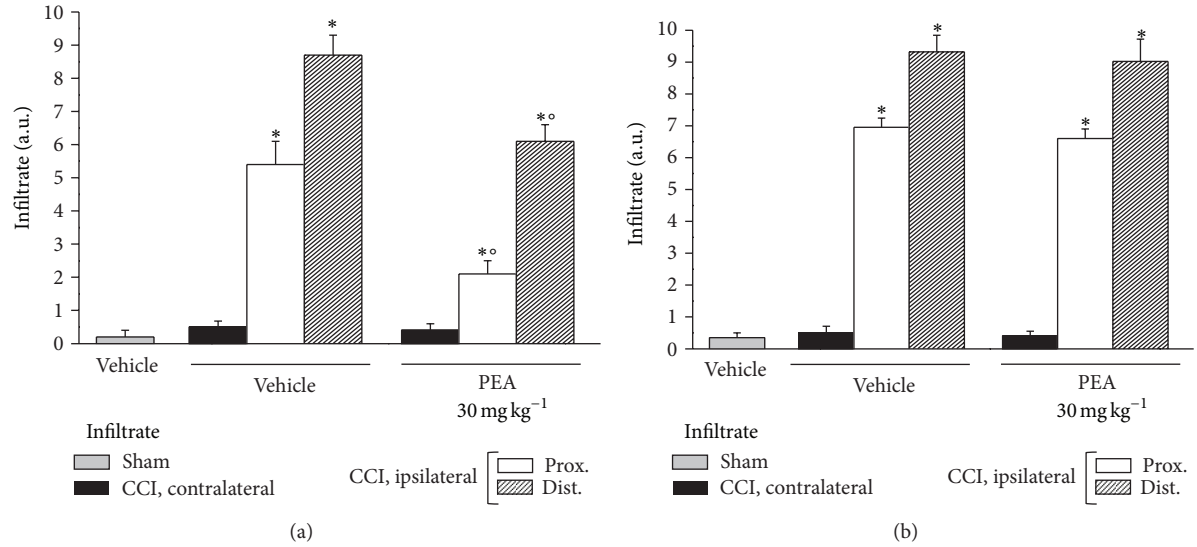

Figure 5: Infiltrate evaluation. On the 14th day after CCI, 5 μm nerve sections of formalin-fixed tissues were analyzed by Azan-Mallory stain, and the presence of inflammatory infiltrate was evaluated and quantified by an arbitrary scale starting from 1, mild infiltrate, up to 10, severe infiltrate. Effect of PEA repeated treatments (30 mg kg^{-1} s.c. daily) was observed in (a) PPAR-$\alpha^{+/+}$ and in (b) $^{-/-}$ mice where the distal and the proximal tract of the ipsilateral ligated nerve of CCI was compared with the contralateral and with the sciatic nerve of sham-operated animals. Quantitative analysis was performed evaluating 5 animals for each group. $^{*}P < 0.05$ was considered as significantly different from sham, vehicle-treated mice. $^{\circ}P < 0.05$ was considered as significantly different from CCI, vehicle-treated mice.

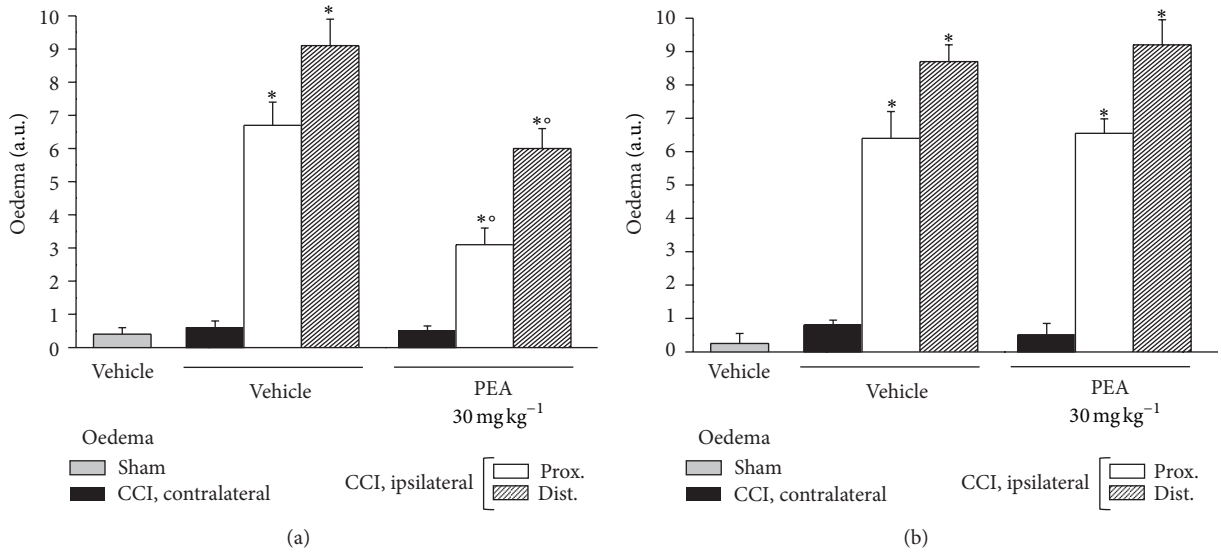

Figure 6: Oedema evaluation. On the 14th day after CCI, 5 μm nerve sections of formalin-fixed tissues were analyzed by Azan-Mallory stain and the presence of oedema infiltrate was evaluated and quantified by an arbitrary scale starting from 1, mild oedema, up to 10, widespread oedema. Effect of PEA repeated treatments (30 mg kg^{-1} s.c. daily) was observed in (a) PPAR-$\alpha^{+/+}$ and in (b) $^{-/-}$ mice where the distal and proximal tract of the ipsilateral ligated nerve of CCI was compared with the contralateral and with the sciatic nerve of sham-operated animals. Quantitative analysis was performed evaluating 5 animals for each group. $^{*}P < 0.05$ was considered as significantly different from sham, vehicle-treated mice. $^{\circ}P < 0.05$ was considered as significantly different from CCI, vehicle-treated mice.

CCI induces morphometric alterations of the sciatic nerve that dramatically affect the portion distal from the injury and that are also able to induce severe proximal impairment. At the same dose active in pain relief, PEA prevents the reduction of myelin sheet thickness and axonal diameter and improves a characteristic degeneration of myelin as highlighted by Azan-Mallory staining. According to previous results [7, 24, 25] CCI-mediated nerve architecture derangement is accompanied by a profound local inflammatory reaction which includes oedema, infiltration of hematogenous immune cells, and induction of various soluble factors like cytokines and chemokines. In particular, the present results

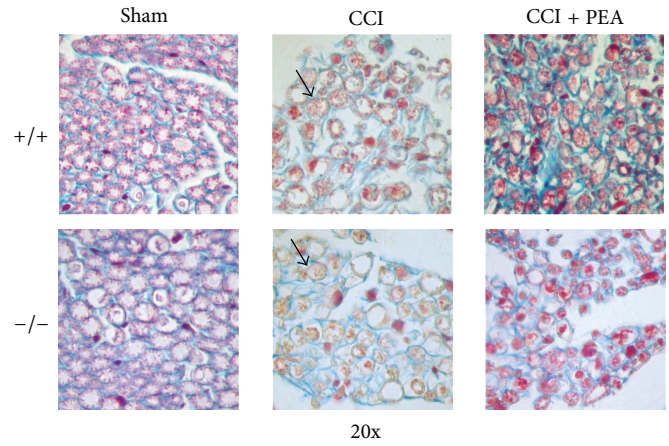

Figure 7: Light micrographs from 5 μm transverse sections of mouse sciatic nerve stained by Azan-Mallory, comparison between PPAR-$\alpha^{+/+}$ and PPAR-$\alpha^{-/-}$ animals; 14th day. Sham: section of sciatic nerve from sham animals; CCI + vehicle: distal part of the sciatic nerve of injured vehicle-treated animals; CCI + PEA distal part of the sciatic nerve of CCI mice s.c. treated with 30 mg kg^{-1} PEA daily for 14 days starting from the surgery. Ovoids are evidenced by arrows. Original magnification 20x.

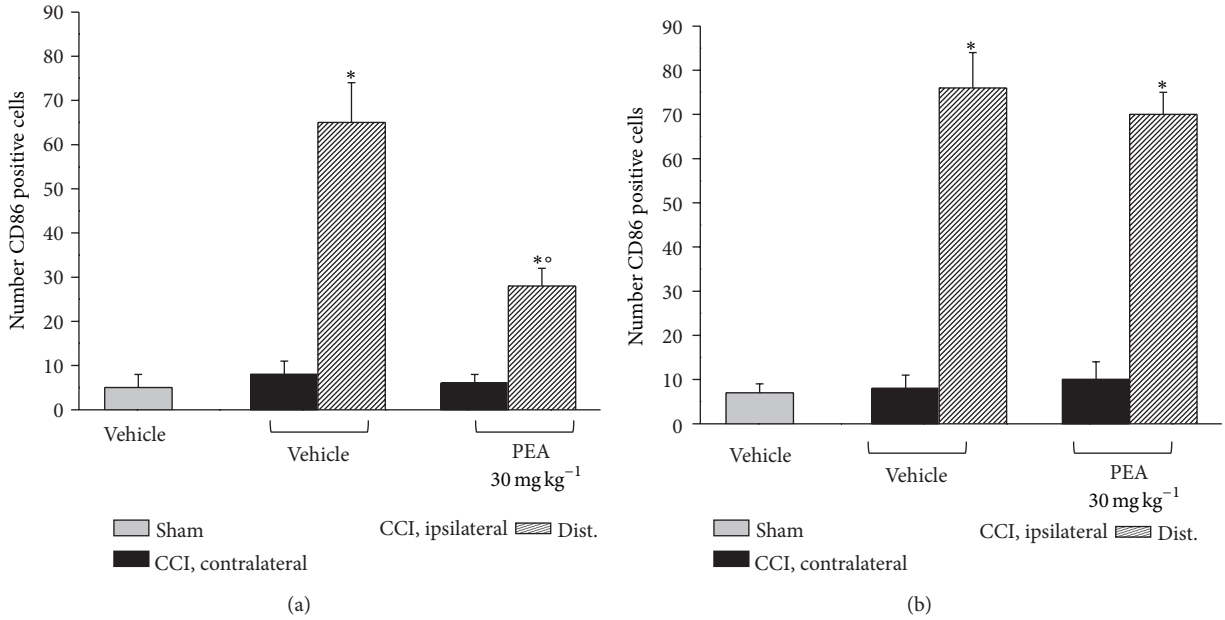

Figure 8: CD86 positive cells evaluation in sciatic nerve. 14 days after CCI, 5 μm sections of the formalin-fixed distal part of the sciatic nerve underwent immunohistochemical staining for CD86. Effect of PEA repeated treatments (30 mg kg^{-1} s.c. daily) was evaluated in (a) PPAR-$\alpha^{+/+}$ and in (b) PPAR-$\alpha^{-/-}$ mice, and quantitative analysis was performed evaluating 5 animals for each group. $^{*}P < 0.05$ was considered as significantly different from sham, vehicle-treated mice. $^{\circ}P < 0.05$ was considered as significantly different from CCI, vehicle-treated mice.

describe a characteristic infiltrate of CD86 positive cells. CD86 is a phenotypic marker of the "classically activated" M1 macrophages stimulated by proinflammatory cytokines, as IFNγ, or by lipopolysaccharide and typically recruited after nervous system trauma [26]. M1 macrophages produce high levels of oxidative metabolites (e.g., nitric oxide and superoxide) and proinflammatory cytokines that are essential for host defense and tumor cell killing but that also cause collateral tissue damage [27]. The treatment with PEA attenuates the degree of peripheral inflammation, reducing oedema and macrophage infiltration allowing for hypothesizing a synergism between the anti-inflammatory and the neuroprotective mechanisms of PEA. On the other hand, an inflammation control mediated by PEA is highlighted also in the spinal cord. According to previous data CCI induces a COX-2 increase in locations of the central nervous system consistent with the neuroanatomical substrates of spinal nociception [28]. COX-2 produces prostaglandins that contribute to the development and maintenance of spinal hyperexcitability after peripheral nerve injury [28, 29]. Reducing COX-2 levels,

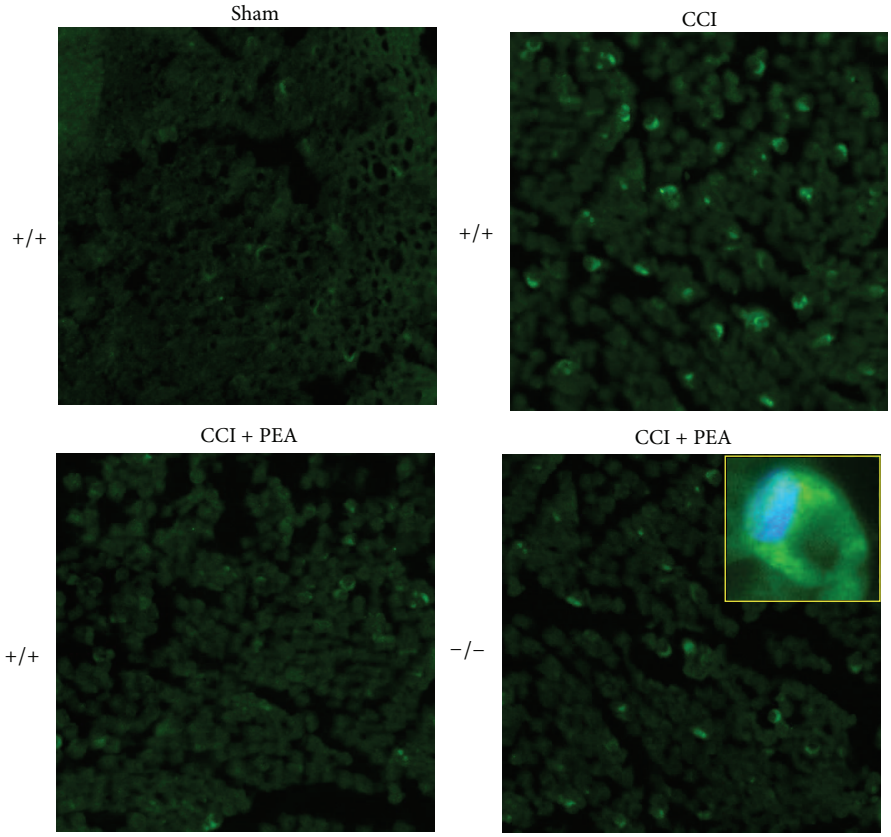

Figure 9: CD86 positive cells evaluation in sciatic nerve. 14 days after CCI, 5 μm sections of the formalin-fixed distal part of the sciatic nerve underwent immunohistochemical staining for CD86. Effect of PEA repeated treatments (30 mg kg^{-1} s.c. daily) was evaluated in PPAR-$\alpha^{+/+}$ and in $^{-/-}$ mice, and representative images are showed, and a detailed image is shown in the insert. Original magnification 20x.

PEA seems able to intervene also in these central mechanisms of pain chronicization. To note that a direct inhibition of pro-inflammatory cytokines, using a TNF-α antibody, for instance, attenuates pain-related behavior but has no effect on nerve regeneration [30].

PEA is a naturally occurring amide between palmitic acid and ethanolamine it is a lipid messenger known to mimic several endocannabinoid-driven actions even though PEA does not bind CB1, CB2, and abn-CBD receptors [31]. So far, numerous actions of PEA on immune cells such as inhibition of mast cell degranulation, attenuation of leukocyte extravasation, and modulation of cytokine release from macrophages have been described [16, 32]. Anti-inflammatory effects of PEA have been associated with peroxisome proliferator-activated receptor (PPAR)-alpha activation [18]. PPAR-α, well known for its role in lipid metabolism, controls transcriptional programs involved in the development of inflammation through mechanisms that include direct interactions with the proinflammatory transcription factors, NF-κB and AP1, and modulation of IkB function [33]. Pharmacological studies have demonstrated that PPAR-α agonists are therapeutically effective in rodent models of inflammatory and autoimmune diseases [34]. Our results show that in a neuropathic pain model the PPAR-α genetic ablation determines a loss of PEA effectiveness in reducing oedema prevention and CD-86 positive infiltrating cells. On the other hand, the recruitment of reactive inflammatory cells and subsequent proinflammatory cascades offers a prime target for neuroprotective agents. Agonists of PPAR-α such as fenofibrate and Wy-14643 protect against cerebral injury by antioxidant and anti-inflammatory mechanisms and reduce the incidence of stroke in mice [35, 36]. Using a spinal cord injury model, Genovese et al. [37] demonstrated that dexamethasone utilizes PPAR-α to reduce inflammation and tissue injury in a rat model of spinal cord trauma. On the contrary, the PPAR-α agonist gemfibrozil does not promote tissue preservation and behavioral recovery after spinal contusion injury in mice [38]. Our study shows the obligatory role of PPAR-α for the neuroprotective effect of PEA in peripheral neuropathy. In the sciatic nerve of CCI mice PEA exerts a widespread protective effect on both myelin, and axons throughout a PPAR-α-mediated mechanism, since PEA treatment fails to rescue nerve tissue in PPAR-α knock-out animals. The neuroprotective PEA properties were suggested by Skaper et al. [39] since dose dependently PEA protected cerebellar granule cells from glutamate toxicity in neuronal single cell cultures and prevented histamine-induced cell death in hippocampal cultures. These effects were elicited without involvement of CB receptors. More recently Koch et al. [40] described the protective effect of PEA on dentate gyrus granule cells

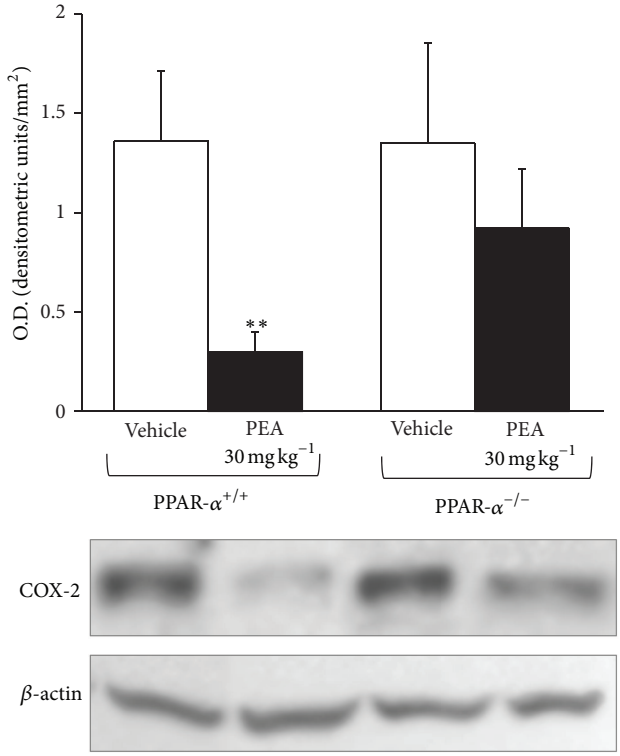

Figure 10: COX-2 expression levels in spinal cord. On the 14th day the spinal cord of PPAR-$\alpha^{+/+}$ and PPAR-$\alpha^{-/-}$ mice was analyzed by western blot. Upper panel shows the densitometric quantification of COX-2 levels in CCI vehicle-treated animal in comparison with CCI mice repetitively treated with PEA (30 mg kg^{-1} s.c. daily). Values were normalized to β-actin immunoreactivity. Data are expressed as the mean ± SEM of triplicate determinations performed on 5 animals for each group. $^{**}P < 0.01$ was considered as significantly different from vehicle (+/+).

in excitotoxically lesioned organotypic hippocampal slice cultures; the specific PPAR-α antagonist GW6471 blocked these effects. PEA exerts neuroprotective activities in neurodegenerative diseases. In a mouse model of Alzheimer's and Parkinson's diseases, PEA reduced oxidative and apoptotic damages and improve behavioral dysfunctions by a PPAR-α-mediated mechanism [14, 41]. In a cellular model, PEA was able to blunt β-amyloid-induced astrocyte activation and, subsequently, to improve neuronal survival through selective PPAR-α activation [42].

In neuropathic conditions PPAR-α seems to join the anti-hyperalgesic, anti-inflammatory, and neuroprotective effects of PEA. On the other hand the inflammatory response to a damage is crucial for both pain sensation and tissue alterations; the importance of inflammatory mediators is well demonstrated in the pathogenesis of neuropathic pain, where infiltrating macrophages and Schwann cells may be involved in the modulation of these mediators in response to nerve injury [43].

Starting from the relevance of the PPAR-α in PEA anti-neuropathic properties, the misunderstood role of peroxisome is intriguing. Peroxisomes fulfill multiple tasks in metabolism and adapt contents and functions according to cell type, age, and organism. Among the metabolic reactions that take place in peroxisomes, oxygen metabolism, β-oxidation of a number of carboxylates that cannot be handled by mitochondria, α-oxidation of 3-methyl-branched chain and 2-hydroxy fatty acids, ether lipid synthesis, and detoxification of glyoxylate are the most important [44, 45]. Patients with peroxisomal dysfunction present with severe and diverse neurological anomalies, including neuronal migration defects, dysmyelination and inflammatory demyelination, macrophage infiltration, and axon damage, proving that these organelles are indispensible for the normal development and maintenance of the nervous system [45–47]. Thereafter, the peroxisome stimulation could be a broad spectrum approach to prevent nervous tissue damage, and a PEA, PPAR-α-mediated, increase in peroxisome number and/or functionality could be also hypothesized. A preclinical study [48] showed that PEA-mediated reduction of spinal cord damage was paralleled by an induction of PPAR-α expression and an up-regulation of potential PPAR-α target genes, but a clear relationship between PPAR-α activation and peroxisome boosting is still lacking.

5. Conclusions

The present results demonstrate the neuroprotective properties of PEA in a preclinical model of neuropathic pain. Anti-hyperalgesic and neuroprotective properties are related to the anti-inflammatory effect of PEA and its ability to prevent

macrophage infiltration in the nerve. PPAR-α stimulation is the common pharmacodynamic code.

Acknowledgments

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Research Article

Long Course Hyperbaric Oxygen Stimulates Neurogenesis and Attenuates Inflammation after Ischemic Stroke

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Several studies have provided evidence with regard to the neuroprotection benefits of hyperbaric oxygen (HBO) therapy in cases of stroke, and HBO also promotes bone marrow stem cells (BMSCs) proliferation and mobilization. This study investigates the influence of HBO therapy on the migration of BMSCs, neurogenesis, gliosis, and inflammation after stroke. Rats that sustained transient middle cerebral artery occlusion (MCAO) were treated with HBO three weeks or two days. The results were examined using a behavior test (modified neurological severity score, mNSS) and immunostaining to evaluate the effects of HBO therapy on migration of BMSCs, neurogenesis, and gliosis, and expression of neurotrophic factors was also evaluated. There was a lower mNSS score in the three-week HBO group when compared with the two-day HBO group. Mobilization of BMSCs to an ischemic area was more improved in long course HBO treatments, suggesting the duration of therapy is crucial for promoting the homing of BMSCs to ischemic brain by HBO therapies. HBO also can stimulate expression of trophic factors and improve neurogenesis and gliosis. These effects may help in neuronal repair after ischemic stroke, and increasing the course of HBO therapy might enhance therapeutic effects on ischemic stroke.

1. Introduction

Ischemic stroke is characterized by the interruption of blood flow and oxygen to brain tissues [1]. During focal ischemia, tissue surrounding the ischemic core is called penumbra, which is still viable and is a possible target to be rescued [2]. The only effective treatment approved in clinical practice at present is early thrombolytic therapy and reperfusion. However, most patients with ischemic stroke failed to receive proper management in time. Stroke remains an important cause of death and disability for humans and stroke therapy remains an important health issue today.

Hyperbaric oxygen (HBO) has been used as a primary or adjunctive stroke therapy over years. Mechanism of the neuroprotection of HBO treatment after ischemia was thought to be mediated by improving oxygen supply [3]. A good body of evidence suggests that HBO treatment is neuroprotective. HBO treatment can decrease infarction volume on MRI examination and improve neurological outcome [4]. Hyperbaric oxygen was also found to decrease ischemia-reperfusion injury induced by neutrophil [5]. Researchers have also demonstrated that exposure to HBO will cause rapid mobilization of bone marrow stem cells in humans, and the number of bone marrow stem cells (BMSCs) remains elevated in peripheral blood during the course of HBO treatments [6].

BMSCs transplantation in rats has been shown to improve outcome of various neuronal diseases, such as ischemic stroke [7], spinal cord injury [8], and traumatic brain injury [9]. However, the "homing" of BMSCs is very important in regenerative therapy. In this study, we tested the hypothesis that HBO could promote the mobilization and migration of

BMSCs to ischemic brain, attenuating ischemic injury, and improving functional recovery. We evaluated the therapeutic effects of HBO on transient middle cerebral artery occlusion (MCAO). Further we tested the hypothesis that BMSCs would improve neurogenesis, gliosis, neurotrophic factor (brain-derived neurotrophic factor, BDNF; nerve growth factor, NGF; and glial-derived neurotrophic factor, GDNF) level, and the expression of MPO (presenting neutrophil activity).

2. Materials and Methods

2.1. Animal. Adult male Sprague-Dawley rats weighing 270 g to 320 g were used in these experiments. They were on a 12-hour light/dark cycle and allowed free access to food and water. All experimental procedures followed the instructions of the Taiwan National Science Council and were approved by the National Cheng Kung University Animal Care and Use Committee with every effort being made to minimize discomfort during the surgery and recovery period. After MCAO insult or Sham procedure, the rats were subjected to hyperbaric oxygen (HBO) therapy or the normal oxygen condition according to group destination. The rats were randomly assigned to one of four groups: (1) Sham group: rats received surgical procedure but without MCAO; (2) nontreatment group: rats sustained MCAO but without HBO treatments; (3) HBO2ds: rats sustained MCAO and received two days HBO treatment course, sacrificed on day 21; (4) HBO3wks: rats sustained MCAO, and received 15-days HBO (5 days/week) course, sacrificed on day 21. The flow charts are represented in Figure 1. Food and water were freely available ad libitum throughout the experimental course.

2.2. Middle Cerebral Artery Occlusion. Rats were anesthetized with intraperitoneal (ip) injection of ketamine (2 mg/100 g). Transient middle cerebral artery occlusion [10–12] was induced using the method of intraluminal vascular occlusion. Temperature was continuously monitored with a rectal probe and maintained at 37.0°C with a thermostatically controlled heating pad. Briefly, the right common carotid artery, external carotid artery (ECA), and internal carotid artery (ICA) were exposed. A 4–0 monofilament nylon suture, with its tip rounded by heating near a flame, was advanced from the ECA into the lumen of the ICA until it blocked the origin of the MCA. One hour after MCAO, reperfusion was achieved by withdrawal of the suture until the tip cleared the lumen of the ECA.

2.3. HBO Therapy. HBO sessions were conducted as the following procedures. Each rat was put into HBO chamber after reperfusion, then was treated with 100% oxygen at 253 kPa (2.5 atm) for 90 mins. The chamber filled with pure oxygen (100%) was pressurized to 253 kPa at a rate of 51 kPa/min for 90 mins and was terminated at the decompression rate of 20 kPa/min.

2.4. Assessing Cerebral Infarction and Functional Outcome. Functional outcome was evaluated using modified neurological severity score (mNSS) [13]. The rats were evaluated by several tests, such as raising rat by tail, placing rat on floor, and beam balance walking, then all test scores were added into the mNSS score (as shown in Table 1). The mNSS evaluations were performed before MCAO and at 1, 7, 14, and 21 days after MCAO. In addition, the triphenyltetrazolium chloride (TTC) staining [14] was used to check the brain infarction size. The colorless TTC is reduced to a red formazan product by dehydrogenases, which are most abundant in mitochondria [15]. Rats were sacrificed at day 3 and day 21. Due to TTC staining is a function test of dehydrogenase enzyme activity and is usually used for early histochemical diagnosis of infraction [16]. Therefore, rats were sacrificed at day 3 to check infraction change by TTC. After documenting mNSS score till day 21, rats were all sacrificed on day 21, then to do immunochemical staining. Under deep anesthesia (Sodium pentobarbital, 100 mg/kg, ip) rats were perfused intracardially with saline. The brain tissue was then removed, immersed in cold saline for 5 min, and sliced into 2.0 mm sections. The brain slices were incubated in 2% TTC dissolved in Phosphate buffered saline (PBS) for 30 min at 37°C and then transferred to 5% formaldehyde solution for fixation.

2.5. BrdU Labeling. BrdU (Roche), a thymidine analogue that is incorporated into the DNA of dividing cells during S phase, was used for mitotic labeling. The labeling protocol followed those previously described [17]. A cumulative labeling method was used to examine the population of proliferative cells, with the rat receiving daily intraperitoneal injections of 50 mg/kg BrdU for 15 consecutive days, starting on the first day of HBO. The $BrdU^+$ cells in both hemispheres of the hippocampus and cortex were digitally counted with the use of a 20x objective lens with a laser scanning confocal microscope (LSM510; Carl Zeiss MicroImaging, Inc.) via a computer imaging analysis system (Imaging Research). For each animal, 40 coronal sections (each 12 μm thick) throughout the hippocampus and cortex were analyzed. The image analysis was also used to examine the distributions of $BrdU^+$ cells with Neu-N and GFAP.

2.6. Immunohistochemistry. Animals were allowed to survive for 21 days after MCAO, and at that time animals were sacrificed with urethane (1.5 g/kg, IP). Rat brains were fixed by transcardial perfusion with saline, followed by perfusion and immersion in 4% paraformaldehyde. The brains were removed and then immersed in PBS with 15% and 30% sucrose overnight. The indirect lesion area (the intact area of the ipsilateral hemisphere) was subtracted from the area of the contralateral hemisphere and was calculated. The lesion volume is presented as a volume percentage of the lesion compared with the contralateral hemisphere.

Single or double immunofluorescence staining was used to identify cells derived from BMSCs. For staining, adjacent 12 μm thick sections were consecutively (1) 2 mol/L HCl-incubated for 30 minutes, (2) rinsed with 0.1 mol/L boric acid (pH 8.5) at room temperature for 10 minutes, (3) incubated overnight with primary antibodies in PBS containing 0.5% normal donkey serum at 4°C, and (4) incubated at room temperature for 1 hour with secondary antibodies.

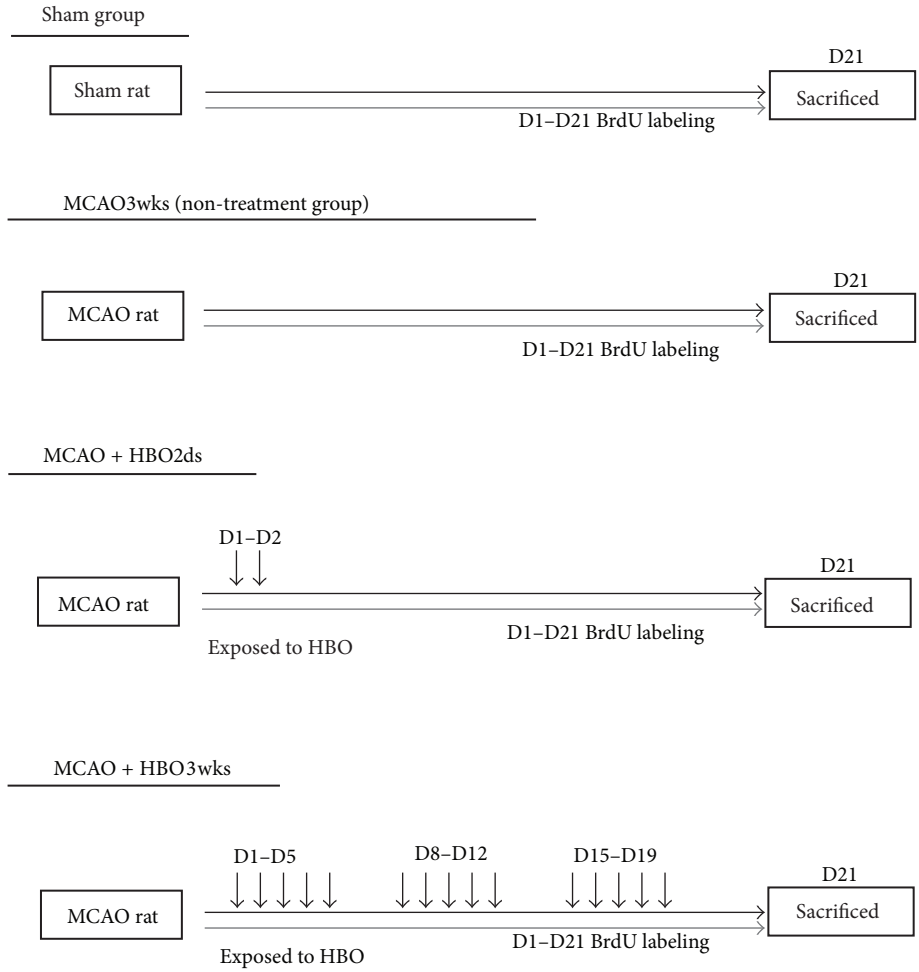


Figure 1: Demonstration of HBO treatment protocol in different groups.

The antibodies were, sequentially, rat monoclonal anti-BrdU (abcam, 1 : 200), rat monoclonal anti-CD34 (Biolegend, 1 : 200), mouse monoclonal anti-NeuN (Millipore, 1 : 200), rabbit polyclonal anti-GFAP (Millipore, 1 : 1000), rabbit polyclone anti-Myeloperoxidase (abcam, 1 : 100), DAPI (Sigma, 1 : 1000), Alexa Flour 488-conjugated goat anti-mouse antibodies, Alexa Flour 488-conjugated donkey anti-rabbit antibodies, Alexa Flour 488-conjugated donkey anti-mouse antibodies, and the Alexa Flour 594-conjugated donkey anti-rat antibodies (Invitrogen). The sections without BrdU were then incubated with DAPI and coverslipped the mounting medium (fluorescent mounting medium; Dako).

2.7. Laser Scanning Confocal Microscopy and Cytometry. Colocalization of BrdU with neuronal marker was conducted by laser scanning confocal microscopy (LSCM) with the use of a Bio-Rad MRC 1024 (argon and krypton) laser scanning confocal imaging system mounted onto a Zeiss microscope (Bio-Rad). For immunofluorescence double-labeled coronal sections, green (FITC for NeuN and GFAP) and red cyanine-5.18 (Cy5 for BrdU) fluorochromes on the sections were excited by a laser beam at 488 nm and 647 nm; emissions were sequentially acquired with 2 separate photomultiplier tubes through 522 nm and 680 nm emission filters, respectively. Interested cells were counted with tissue cytometry using TissueQuest software.

2.8. Reverse Transcription PCR. Hippocampi from Sham, MCAO, HBO2ds, and HBO3wks were dissected. Total RNAs were isolated using TRIzol reagent (Invitrogen) according to manufacturer's instructions. Reverse transcription of equal amounts of total RNA were carried out using Superscript II First-Strand Synthesis kit (Invitrogen) according to the manufacturer's instructions. Obtained cDNA were amplified using the following primers: for BDNF, 5′-CAGTGGACATGTCCGGTGGGACGGTC-3′ and 3′-TTC-TTGGCAACGGCAACAAACCACAAC-5′; for GDNF, 5′-AGGGGCAAAAATCGGGGGTG-3′ and 3′-GCATGC-ATCCACGACTCGGA-5′; and for GAPDH, 5′-GACCCC-TTCATTGACCTCAAC-3′ and 3′-TCTTACTCCTTGGAG-GCCATG-5′.

2.9. Statistical Analysis. Results are expressed as the mean ± SE for three or more independent experiments. To compare data, we used the ANOVA test. A value of $P < 0.05$ was considered to be statistically significant.

Table 1: Detail description of the items forming the modified neurological severity score (mNSS).

Motor tests	
Raising rat by tail (normal = 0, maximum = 3)	(3)
Flexion of forelimb	1
Flexion of hindlimb	1
Head moved >10 degree limb vertical axis within 30 s	1
Placing rat on floor (normal = 0, maximum = 3)	(3)
Normal walk	0
Inability to walk straight	1
Circling toward the paretic side	2
Falling down to paretic side	3
Sensory tests (normal = 0, maximum = 2)	(2)
Placing test (visual and tactile test)	1
Proprioceptive test (deep sensation, pushing paw against table edge to stimulate limb muscles)	1
Beam balance tests (normal = 0, maximum = 6)	(6)
Balance with steady posture	0
Grasps side of beam	1
Huging beam and 1 limb falling down from beam	2
Huging beam and 2 limbs falling down from beam, or spins on beam (>60 s)	3
Attempting to balance on beam but falling off (>40 s)	4
Attempting to balance on beam but falling off (>20 s)	5
Falling off; no attempt to balance or hang on the beam (<20 s)	6
Reflex absence and abnormal movements (normal = 0, maximum = 4)	(4)
Pinna reflex (head shaken when auditory meatus is touched)	1
Corneal reflex (eyes blink when cornea is lightly touched with cotton)	1
Startle reflex (motor response to brief noise from clapping hands)	1
Seizures, myoclonus, myodystony	1
Maximum points	(18)

One point is given for an absent reflex tested or for the animal's inability to perform a task: 1–6 mild injury, 7–12 moderate injury, and 13–18 severe injury

3. Results

3.1. HBO Improved Functional Outcome and Decreased Infarction Size. HBO therapy outcomes in rats were evaluated using the modified neurological severity score (mNSS). A lower score indicated rats had less neurological defects from MCAO, presented with more improved outcome by HBO therapy. Behavior tests showed rats had significantly improved functional outcome when receiving longer repetitive HBO therapy ($P < 0.001$). Despite there only being two days of HBO therapy in the HBO2ds group, rats still showed functional improvement in the following days, with expressing declining curve of mNSS, until day 14 ($P < 0.01$). And the declining curve of mNSS in the HBO3wks group became more obvious after day 14 ($P < 0.01$) (Figure 2(a)). TTC staining showed the ischemic area on the rat's brain tissue. More white color change over brain tissue was found in the MCAO group, which was correlated with more ischemic injury, compared with other groups (Figure 2(b)). Behavior tests were evaluated with mNSS: MCAO3wks, rats sustained MCAO, but without HBO therapy; HBO2ds, rats sustained MCAO, but with two days HBO therapy; HBO3wks, rats sustained MCAO, but with repetitive HBO therapy for three weeks. The infarcted area showed TTC staining (white color) was prominent in the MCAO group, but decreased in HBO treated groups. This indicated that HBO might attenuate cerebral ischemic injury in rats (Figure 2).

3.2. HBO Improved BMSCs Migration to Brain. CD34-DAPI double staining showed the presentation of BMSCs in brain tissue. The number of CD34-DAPI double staining cells was higher in the HBO3wks group, as compared with the shame group, MCAO3wks group, and HBO2ds group (Figure 3(a)). This indicated that rats would recruit BMSCs to brain after acute stroke injury, without HBO therapy. However, the recruited amount of BMSCs was not enough. HBO therapy promotes migration of BMSCs to brain after focal ischemic injury. Longer duration and repetitive HBO would enhance increased BMSCs migration (Figures 3(b) and 3(c)). Migrated BMSC, presenting with double staining of CD34 and DAPI, were found in the MCAO3wks group, HBO2ds group, and

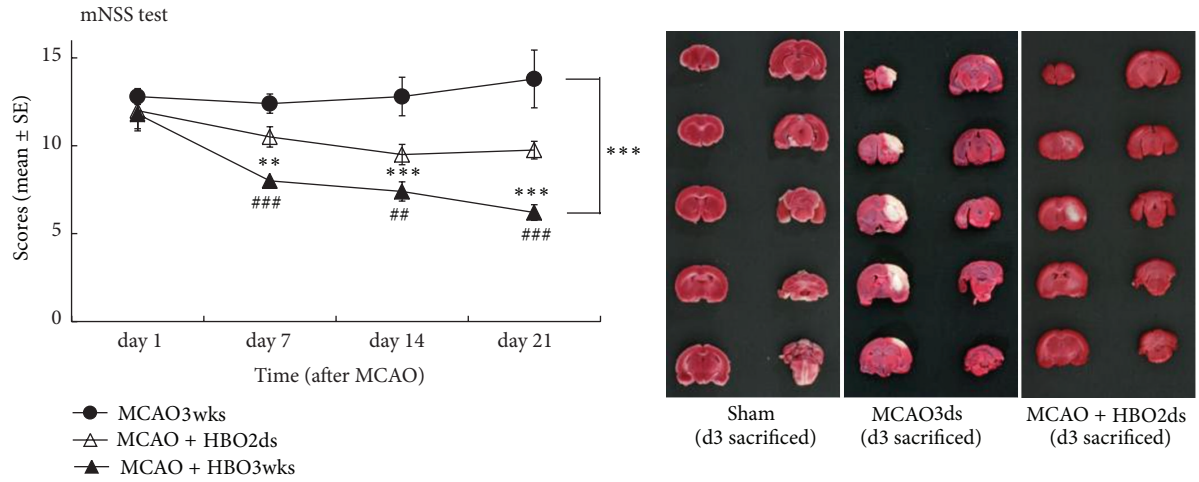


Figure 2: Long course HBO improved functional outcome and decreased infarction size. (a) Behavior tests showed HBO significantly improved function outcome with dose-dependent effect. The mNSS in groups received HBO therapies is significantly less than that in control group ($^{***}P < 0.001$). The declining curve of mNSS in the HBO3wks group became more obviously after day 14. (b) Infarcted area shown on TTC staining (white color) was prominent in the MCAO group but decreased in HBO treated groups.

HBO3wks group. And there were increased numbers of purple cells (double staining with CD34, red color, and DAPI, blue color) in the HBO3wks group. Representative images of tissue cytometry using TissueQuest software are shown in Figure 3(b). CD-34 positive cells and DAPI positive cells were counted and the signal intensity was quantified. The number of double staining cells with CD34 and DAPI was 14.16% in the Sham group, 17.09% in the MCAO3wks group, 21.99% in the HBO2ds group, and 39.4% in the HBO3wks group, respectively. The amount of double positive cells with CD34 and DAPI in the ischemic boundary was recorded as the percentage of CD34 positive cells in all cells. Data were presented as mean± standard error of the mean (SEM). The difference was significant as compared with MCAO3wks group and HBO3wks group ($P < 0.05$). The difference was even more significant as compared with Sham group and HBO3wks group ($P < 0.01$).

3.3. HBO Increased MCAO-Induced Neurogenesis. BrdU and Neu-N double staining cells showed the number of new proliferation of neuronal cells in the perilesioned cortex and hippocampus (Figures 4(a) and 4(b)). The BrdU positive cells presented with a red color and the Neu-N positive cells were presenting with a green color. The double staining of BrdU and NeuN cells would present with a yellow color. There were more yellow-colored cells (indicating new proliferated neuronal cells) found in the HBO2ds group and HBO3wks group. However, the number of double staining cells (yellow color) was higher in the HBO3wks group. This feature indicated HBO improved neurogenesis, but longer and repetitive HBO course would induce a greater degree of neurogenesis.

BrdU-NeuN double staining showed that there were significantly more newly formed neurons (cells with yellow color) in the ischemic boundary area of hippocampus (Figure 4(a)) and perilesioned cortex (Figure 4(b)). There were more BrdU and NeuN double staining cells in the HBO3wk group. Figure 4(c) showed the representative image of tissue cytometry using TissueQuest software. BrdU positive cells and Neu-N positive cells were counted and the signal intensity was quantified. The number of double staining cells with BrdU and Neu-N was 10.43% in the Sham group, 15.63% in MCAO3wks group, 22.81% in HBO2ds group, and 34.95% in HBO3wks group, individually. The amount of double staining positive cells with BrdU and Neu-N in the ischemic boundary was recorded as the percentage of BrdU positive cells in all cells. Data were presented as mean± standard error of the mean (SEM). The difference was significant, as compared with the MCAO3wks group and HBO3wks group ($P < 0.01$). There was still a difference between the HBO2ds group and HBO3wks group ($P < 0.05$). The difference was more significant, as compared with the Sham group and HBO3wks group ($P < 0.001$).

3.4. HBO Increased MCAO-Induced Gliosis. BrdU and GFAP double staining cells showed the number of new proliferations of glial cells in the dentate gyrus and perilesioned cortex (Figures 5(a) and 5(b)). The BrdU positive cells were presented with red color and the GFAP positive cells were presented as a green color. The double staining of BrdU and GFAP cells would present as a yellow color. There were yellow-colored cells (indicating new proliferated glial cells) found in the MCAO3wks group (24.61%), HBO2ds group (22%), and HBO3wks (30.93%) group. However, the number of double staining cells (yellow color) seemed higher in the HBO3wks group. This suggests that longer and repetitive HBO course would induce a greater degree of gliosis. Demonstrated figures of BrdU-GFAP double staining cells showed that there were more newly forming or reactive glia in

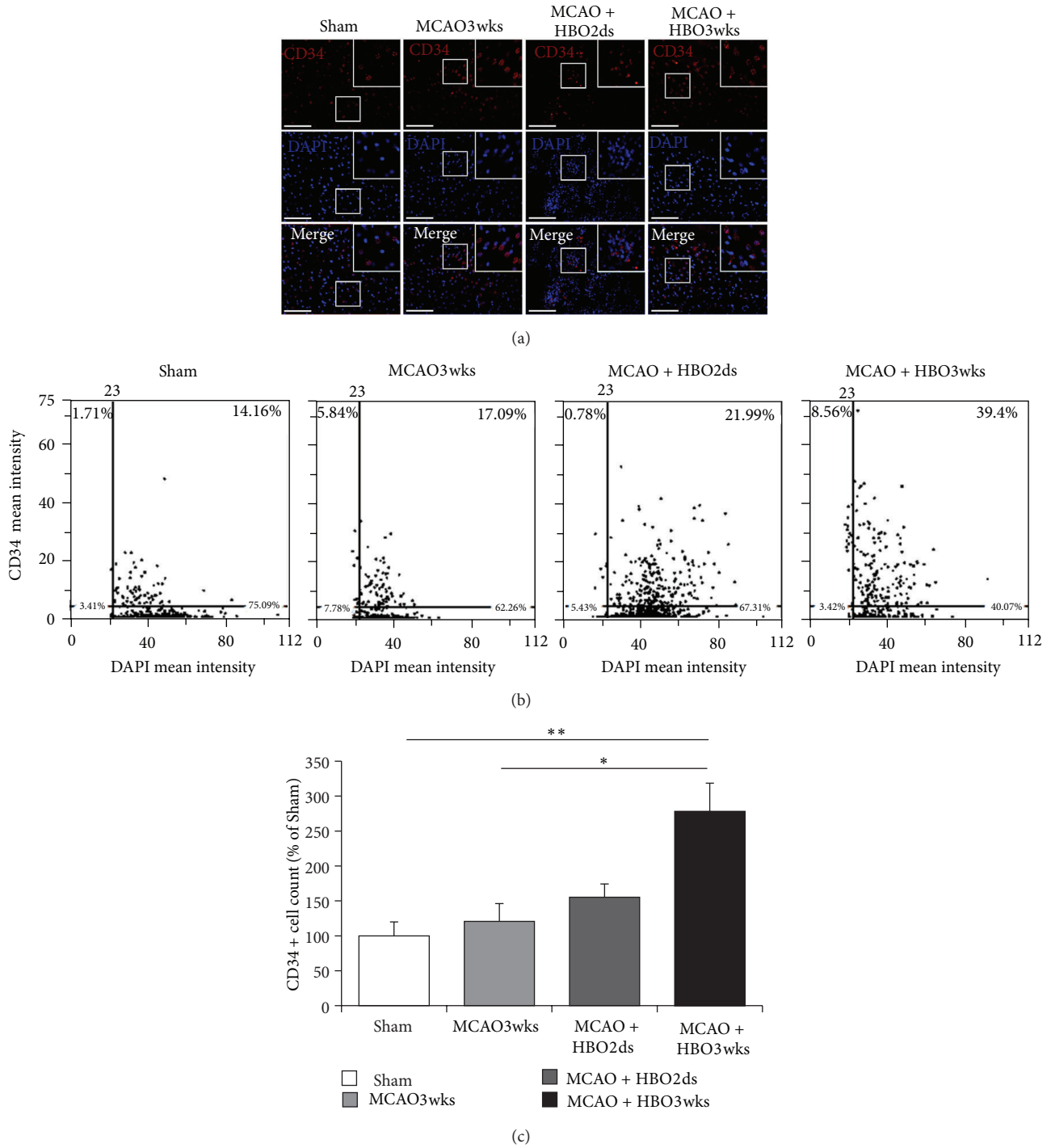
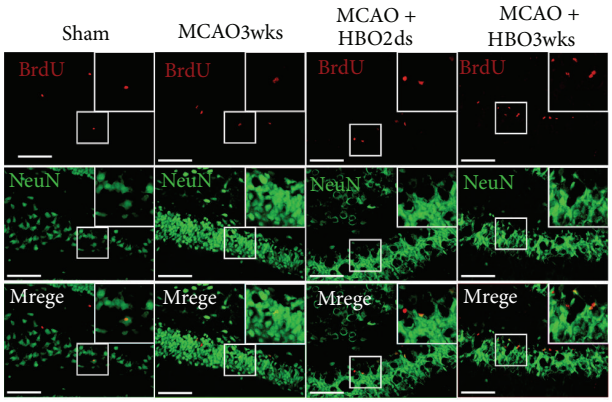
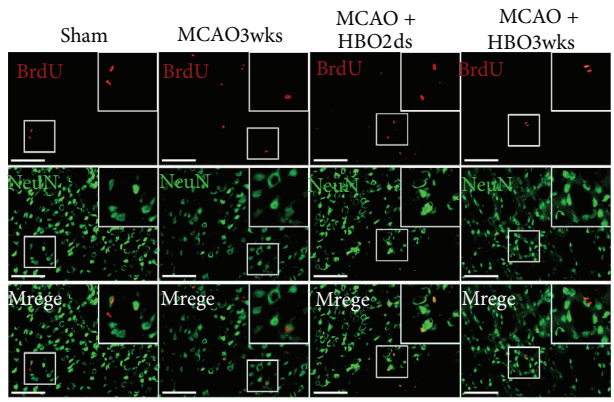


Figure 3: Long course HBO improved BMSCs migration to brain. (a) Demonstration of CD34-DAPI double staining showed presentation of BMSCs after brain ischemia. (b) Representative image of tissue cytometry using TissueQuest software. CD-34 positive cells and DAPI positive cells were counted and the signal intensity was quantified. (c) The amount of double positive cells with CD34 and DAPI in the ischemic boundary was recorded as the percentage of CD34 positive cells in all cells. The difference was significant, as compared with MCAO3wks group and HBO3wks group ($^{*}P < 0.05$). The difference was more significant, as compared with Sham group and HBO3wks group ($^{**}P < 0.01$).

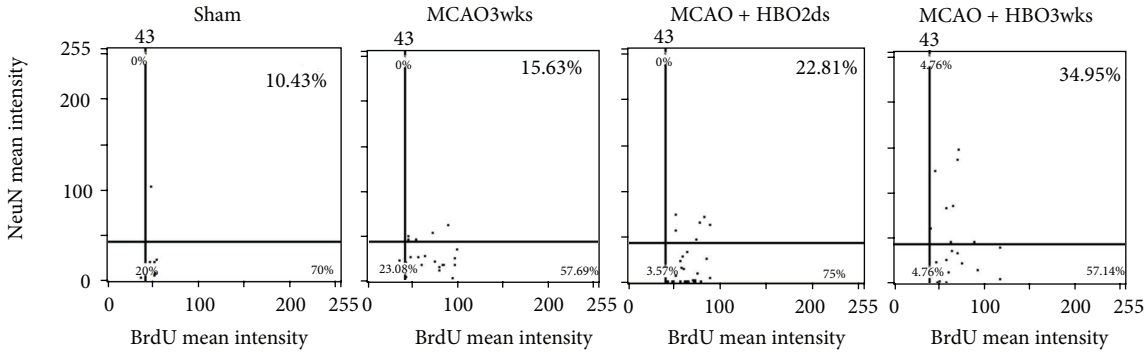
the ischemic boundary area (dentate gyrus (Figure 5(a)) and cortex (Figure 5(b))). And there were more double staining cells (yellow colored cells) in the HBO3wks group. The amount of BrdU and GFAP double staining positive cells in the ischemic boundary was recorded as the percentage of BrdU positive cells in all cells. Data were presented as mean ± standard error of the mean (SEM). Despite there being more double staining cells found in the HBO3wks group, there was



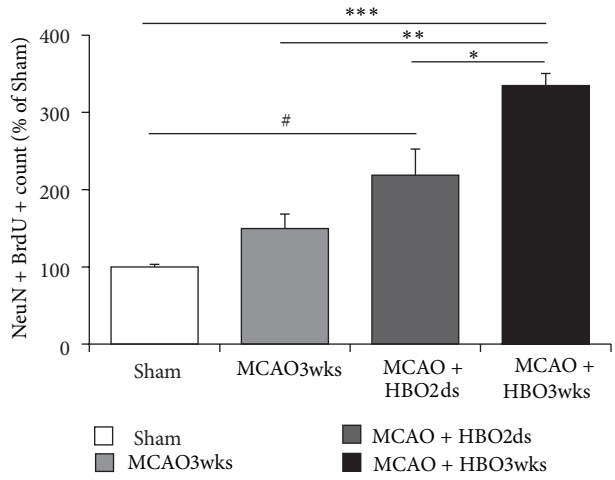
(a) BrdU/NeuN in hippocampus



(b) BrdU/NeuN in cortex



(c)



(d)

Figure 4: Long course HBO increased neurogenesis. ((a) and (b)) BrdU-NeuN double staining showed that there were significantly more newly forming neurons in the ischemic boundary area (perilesioned cortex (Figure 4(b)) and hippocampus (Figure 4(a))) of HBO3wks rats than HBO2ds rats. (c) Representative images of tissue cytometry using TissueQuest software. BrdU-NeuN positive cells were counted and the signal intensity was quantified. The intensity of co-staining with BrdU and Neu-N was more prominent in HBO3wks group. The difference was significant, as compared with MCAO3wks group and HBO3wks group ($^{**}P < 0.01$). There was still significant difference between HBO2ds group and HBO3wks group ($^{*}P < 0.05$). The difference was even more significant as compared with Sham group and HBO3wks group ($^{***}P < 0.001$).

no statistical difference with regards to the HBO3wks group, as compared with other groups.

3.5. HBO Reduced MCAO-Induced Inflammation in the Acute Phase. Acute inflammation in the acute phase of cerebral infarction would recruit neutrophils, depositing in the vessels of ischemic boundary area of brain, and caused further brain tissue injury. MPO expression was evaluated for degree of acute inflammation and MPO staining showed the presentation of MPO in penumbra striatum (Figure 6(a)). There was found with decreased MPO expression in the HBO2ds group (8.24%) and HBO3wks group (2.15%), compared with MCAO3wks group (27.2%) (Figures 6(b) and 6(c)). It indicated that HBO treatment would reduce MPO expression, representing with attenuating acute inflammation. Longer and repetitive HBO therapy seemed to have a greater effect in attenuating inflammation.

3.6. HBO Increased MCAO-Induced Neurotrophic Factor Level. Expression of neurotrophic factor, including BDNF and GDNF, was evaluated by using reverse transcription PCR (RT-PCR). Neurotrophic factors might provide paracrine effect to cells nearby and promote cell proliferation. The mRNA expression of BDNF and GDNF showed that there were BDNF and GDNF production in the MCAO rats and MCAO rats treated with HBO. However, higher expression of BDNF and GDNF was found in the HBO3wks group (Figure 7(a)). There was more BDNF and GDNF production in the HBO3wks group (individually increasing 3.32- and 2.02-fold than Sham), compared with HBO2ds group (individually increasing 1.43- and 1.43-fold than Sham) and MCAO3wks group (individually increasing 0.94- and 1.02-fold than Sham) (Figures 7(b) and 7(c)). The results indicated that longer and repetitive HBO therapy promoted more neurotrophic factor production, including BDNF and GDNF.

4. Discussion

Stroke therapy is an important topic because strokes may result in death or disability, change patients daily life quality, and significantly increase social-economic costs. Ischemic stroke is characterized by cerebral artery occlusion, causing regional cerebral flow reduction or interruption. Previous study showed that hyperbaric oxygen treatment could attenuate ischemic injuries in adult rats [18].

HBO therapy enhanced brain tissue oxygenation during treatment. However, after the termination of HBO treatment and returning to normal oxygenation, the body was temporarily in a relative hypoxic status. This hyperoxia and then turning with relative hypoxia, naming oxygen cycling, would lead to hypoxia inducing factor-1α (HIF-1α) production [19, 20]. The preconditional hypoxia would decrease ischemic stroke related injury [21]. Recent studies also showed oxygen cycling helped in traumatic brain injury [19] and stem cell therapy for myocardial infarction [22]. The above studies suggested that oxygen cycling may attenuate ischemic injuries.

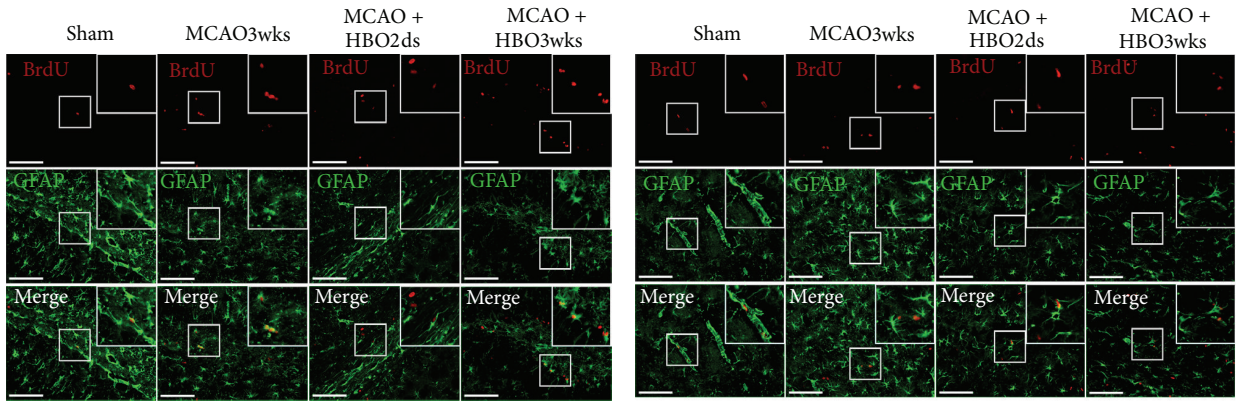
In our study, 3 weeks of oxygen cycling treatment course for rats with MCAO had better functional outcome compared with rats treated with two-day hyperbaric oxygen treatment course. It presented with lower mNSS, decreased infarction size on brain slice, and correlated with decreased area of TTC staining. There were several beneficial effects in HBO treated stroke, including with reduction of blood-brain barrier permeability, brain edema [22], and attenuation of inflammation [23]. HBO may also attenuate hydroxyl radical production and glutamate release and therefore decrease brain damage [24].

Despite hyperbaric oxygen might cause oxidative stress, activate reactive oxygen species (ROS) (meaning production of reactive oxygen free radical), was thought harmful to brain. Oxygen-activated reactive oxygen species (ROS) has been shown to activate nitric oxide synthase (NOS) [25], promoting NO generation. NO was well known as a potent vasodilator and was crucial in attenuating platelet aggregation, superoxide production, and modulating microvascular permeability [22, 26–28]. Previous study found HBO increases nitric oxide levels in perivascular tissues via stimulation of nitric oxide synthase (NOS) [29]. Furthermore, HBO also stimulate NOS in bone marrow. Bone marrow stem cells will be more proliferated, more easily moving out of bone marrow (mobilization), and homing to ischemic lesions (migration) after HBO treatment [6].

HBO could stimulate vasculogenic stem cell mobilization from bone marrow of diabetics and more cells are recruited to skin wounds to help healing [30, 31]. And in traumatic brain injury, HBO might stimulate neurogenesis. Bone marrow stem cells might be recruited to brain after injury [19]. Despite previous studies about HBO treatment in cerebral infarction in rats showing that bone marrow stem cells were recruited to ischemic brain, most of these studies used short HBO cycling courses. In our study, three weeks of oxygen cycling treatment course induced more BMSCs mobilization to the brain than a two days course. The CD34+ cells were found in ischemic boundary area. This is corresponding with migration of BMSCs to the ischemic brain tissue [32, 33]. This data suggested the dose dependent effect of HBO treatment on homing of BMSC to ischemic brain tissue. Under HBO therapy, BMSCs were more prominent in the group 3wks, than the group 2ds. This data showed that repetitive HBO therapy activated more BMSCs mobilization from bone marrow, then migration to the brain.

In this study, repetitive and longer HBO treatment increased more BMSCs mobilization to the ischemic brain. It was known that BMSCs traffic to the ischemic tissues was relaed to the interaction with stromal-cell-derived factor-1α (SDF-1α) and chemokine receptor 4 (CXCR4) [34, 35]. In the rats with ischemic stroke, SDF-1 level is significantly increased in the injured hemisphere compared to the uninjured hemisphere [36]. In ischemic stroke patients, It was also found with increased SDF-1α [37]. The secretion of SDF-1α may act as a chemoattractant to facilitate the homing of circulating CXCR4 positive cells (such as BMSCs) [38] and then help injured tissue with cell repairment. Therefore, further studies about HBO, SDF-1α, and CXCR4 are still needed.

The homing by BMSCs is very important in regenerative therapy, not only in stroke treatment, but also in other



(a) BrdU/GFAP in hippocampus

(b) BrdU/GFAP in cortex

Figure 5: Long course HBO increased gliosis. ((a) and (b)) Demonstration of double staining showed that there were significantly more newly forming or reactive glia in the ischemic boundary area (dentate gyrus (a) and cortex (b)) of HBO3wks rats than HBO2ds rats. The intensity of co-staining with BrdU and Neu-N was more prominent in HBO3wks group.

CNS diseases. BMSCs transplantation in rats were found to attenuate neuronal injury in ischemic stroke [7], spinal cord injury [8], and traumatic brain injury [9]. In the degenerative CNS disease such as Alzheimer's disease (AD), subcutaneous injection with G-CSF would increase BMSCs mobilization to brain to rescue the lesions [39]. In this study, we provided another aspect for HBO treated stroke in the point of view of BMSCs rescue. The homing of BMSCs to the ischemic boundary of rat brain, stimulated by HBO, would promote neural plasticity, stimulate production of growth factor or cytokine, and attenuate inflammation [40].

BrdU-labeled cells with expressing Neu-N (unique to neuron and axon) were present in HBO treated MCAO rats. The expressing level was more prominent in the group of HBO3wks, compared with the group of HBO2ds. This indicated that longer oxygen cycling had better protection of ischemic rats, far from neural loss, by increasing neural cell numbers. Furthermore, those migrated BMSCs, more promoted by HBO, might have cell transdifferentiation [41] or help axon remodeling [42]; they might also stimulate mRNA transcription of growth factor and cytokine, which would activate neural stem cells (already housing in the dentate gyrus and subventricular zone) [43, 44] to proliferate and migrate.

In the study, BrdU-labeled cells with expressing GFAP (fairly to glial lineage) were present in HBO treated MCAO rats. The expressing level was more prominent in the group of HBO3wks, compared with the group of HBO2ds. This indicated that there was more degree of gliosis in the group of HBO3wks. Recent studies have emphasized the dual effects of gliosis (both detrimental and beneficial in neuroprotection and functional recovery) [45]. Time and signaling molecule for gliosis were referring to its final fate [46]. Corresponding to final functional improvement in rats, we presumed that longer oxygen cycling might help overcoming detrimental effect of gliosis and donating its beneficial effect, by regulating inflammation and influencing trophic factor production.

In this study, neurotrophic factors such as BDNF, NGF, and GDNF were evaluated. Under oxygen cycling, the level of BDNF, NGF, and GDNF were increased compared with control group. And there was more BDNF and NGF production in the group HBO3wks, compared to the group HBO2ds. A concept of "entire protection of neurovascular unit" in cerebral infarction treatment has been proposed, which suggested the protection of neurons alone might not work and emphasized the interaction between neurons, glia, and the cerebral endothelium [47]. Cerebral endothelium was the major source of BDNF. Brain-derived endothelial medicated paracrine and autocrine via BDNF and NGF [48]. More BDNF production indicated that cerebral endothelium was with less damage and getting recovery [12] and perhaps contributed to increased cell numbers from transdifferentiation of migrated BMSCs or secretion of trophic factor by grafted BMSCs [49].

Inflammation is a defense response against the insults that limit injury and remove noxious agents [50]. Ischemic stroke is associated with acute and prolonged inflammatory process characterized by the activation of resident glial cells, production of inflammatory cytokine, and leukocyte and monocyte infiltration in the brain [51]. Neutrophil recruitment may contribute to microvascular occlusion, releasing proinflammatory cytokine, and then further cause damage. In this point of view, MPO activity, presenting neutrophil activity, was evaluated in this study. The result showed that there was lower MPO activity in the group HBO3wks. It indicated longer oxygen cycling would attenuate inflammation. The result was also correspondent with previous study, which showed that HBO treatment might reduce local MPO activity, neutrophils infiltration, and infarction volume and thus enhance functional outcome for rats with focal ischemia [19, 51].

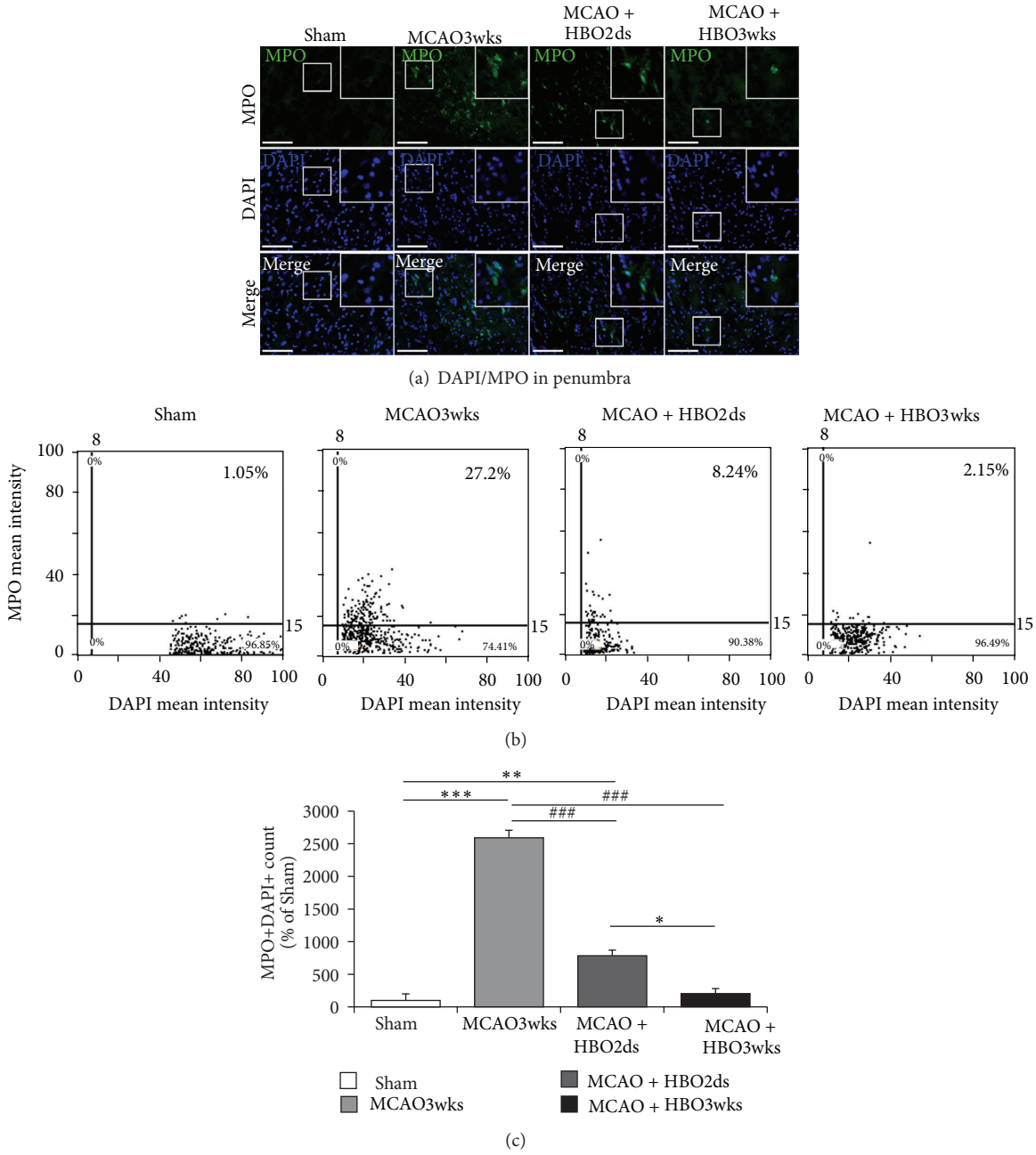


Figure 6: Long course HBO reduced inflammation. (a) MPO staining in penumbra striatum showed there was lower MPO expression in HBO2ds and HBO3wks group. (b) Representative images of tissue cytometry using TissueQuest software. MPO positive cells and DAPI positive cells were counted and the signal intensities were quantified. (c) The amount of double positive cells with MPO and DAPI in the ischemic boundary was recorded as the percentage of MPO positive cells in all cells. The difference was significant, as compared with HBO2ds group and HBO3wks group ($^{*}P < 0.05$). The difference was more significant, as compared with MCAO3wks group and HBO3wks group ($^{***}P < 0.001$).

5. Conclusion

HBO therapy might attenuate inflammation in rat with ischemic stroke. It might promote more BMSCs production, mobilization, and migration to ischemic brain. Those BMSCs stimulated neurogenesis and gliosis. This longer oxygen cycling (treated stroke by HBO for repetitive schedules for 3 weeks) therapy orchestrated gliosis and trophic factor production (BDNF, NGF, and GDNF) and decreased harmful effects by neutrophils in the early phase.

Authors' Contribution

C.-P. Chang and L.-C. Wang equally contributed to the work.

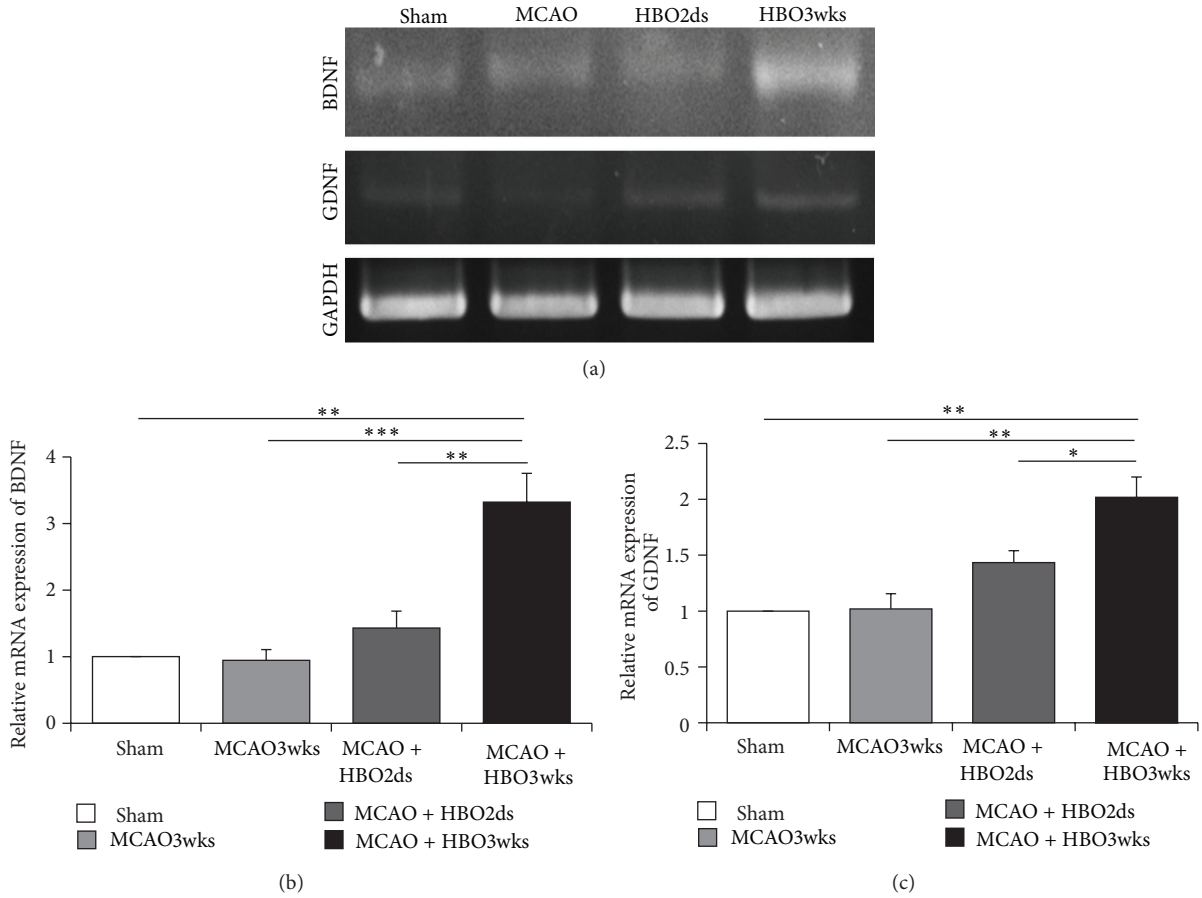


Figure 7: Long course HBO increased neurotrophic factor level. (a) The mRNA levels of BDNF and GDNF showed that mRNA expression levels were increased in HBO treatment group. ((b) and (c)) Expression levels of BDNF and GDNF were significantly increased in the HBO3wks group ($P < 0.05$).

Conflict of Interests

The authors report no conflict of interests related to this study or the findings specified in this paper.

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Review Article

Remarkable Role of Indoleamine 2,3-Dioxygenase and Tryptophan Metabolites in Infectious Diseases: Potential Role in Macrophage-Mediated Inflammatory Diseases

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Indoleamine 2,3-dioxygenase 1 (IDO1), the L-tryptophan-degrading enzyme, plays a key role in the immunomodulatory effects on several types of immune cells. Originally known for its regulatory function during pregnancy and chronic inflammation in tumorigenesis, the activity of IDO1 seems to modify the inflammatory state of infectious diseases. The pathophysiologic activity of L-tryptophan metabolites, kynurenines, is well recognized. Therefore, an understanding of the regulation of IDO1 and the subsequent biochemical reactions is essential for the design of therapeutic strategies in certain immune diseases. In this paper, current knowledge about the role of IDO1 and its metabolites during various infectious diseases is presented. Particularly, the regulation of type I interferons (IFNs) production via IDO1 in virus infection is discussed. This paper offers insights into new therapeutic strategies in the modulation of viral infection and several immune-related disorders.

1. Introduction

Inflammation is the physiological response of the body to harmful stimuli, such as injury, pathogens, damaged cells, or irritants. Inflammatory response can be either acute or chronic, which leads to pathology. The major function of innate immune cells is identification and recognition of the injurious and/or foreign substances causing the defense response. Macrophages are actively involved in all phases of inflammation, and their role as effector and regulatory cells is now widely recognized. Another interesting and important role of macrophages is their high level of specialization and tissue specificity. While all tissue-bound macrophages differentiate from circulating monocytes, they acquire distinct characteristics and functions locally due to their response profiles. One of the major factors for this diversity is the complexity of microbial load as well as tissue architecture. Thus, it is not a surprise that some of the most sophisticated interactions between the host and parasites also dictate the most evolved phenotypic characteristics of the macrophage.

Indoleamine 2,3-dioxygenase 1 (IDO1) has been identified as an enzyme endowed with powerful immunomodulatory effects, resulting from its enzymatic activity that leads to catabolism of the essential amino acid L-tryptophan (L-TRP) [1, 2]. This enzyme is expressed in epithelial cells, macrophages, and dendritic cells (DCs) induced by proinflammatory cytokines [3–5]. The initial observation suggesting the immunoregulatory role of IDO1 dates back to the finding that its inhibition by 1-methyl-DL-tryptophan (1-MT) during pregnancy would cause rejection of semiallogeneic, but not syngeneic, fetuses in mice [6]. A second observation expanding upon that initial finding was that IDO1 mediates a bidirectional flow of information between cytotoxic-T-lymphocyte-associated-antigen-4- (CTLA-4-) expressing T cells and accessory cells of the immune system; IDO1 activation in antigen-presenting cells (APCs) by CTLA-4

ligation of CD80/CD86 "counterreceptors" on those cells represents an important effector pathway for regulatory T (Treg) cells, to induce and maintain peripheral tolerance [7, 8]. Third, it was later found that, in T cells, the general control nonderepressing-2 (GCN2) protein kinase, with a putative binding site for free acyl-transfer RNAs (tRNAs), acts as a molecular sensor for intracellular TRP, participating in the integrated stress response (ISR) pathway, which controls cell growth and differentiation [9]. Finally, IDO1 was found to possess signaling activity in DCs, which are stably turned into regulatory DCs by its activation. Thus, IDO1 may contribute to long-term immune homeostasis and immune-related functions not only in pregnancy, but also in infectious, allergic, autoimmune, and chronic inflammatory diseases, as well as in transplantation and immune-escaping tumoral mechanisms [7, 10–12]. The aim of this paper is to summarize the current knowledge about the physiological role of IDO1 following certain immune-related disorders. Further, new therapeutic targets via regulation of IDO1 are discussed against macrophage-related inflammatory diseases.

2. Tryptophan and Its Degradation Pathways

TRP is an essential starting point of two biochemical pathways: (1) the enzyme tryptophan 5-hydroxylase converts TRP into 5-hydroxytryptophan, which is subsequently decarboxylated to 5-hydroxytryptamine (5-HT, serotonin), an essential neurotransmitter, and (2) two atoms of oxygen are inserted into TRP to form N-formylkynurenine, the first and rate-limiting step in the kynurenine (KYN) pathway (Figure 1). It is estimated that only 1% of dietary TRP can be converted into 5-HT [13]. The remaining 99% of TRP is metabolized via the KYN pathway. TRP is catalyzed by three different enzymes: tryptophan 2,3-dioxygenase (TDO), IDO1, and IDO2. While the expression and function of IDO2 have been well explored in the mouse model, there is a lack of knowledge about its expression and functional significance in human tissue. Human IDO1 and IDO2 seem to have different kinetic parameters and inhibition profiles. The Km for L-TRP of human IDO2 protein is approximately 500–1000-fold higher than that of mammalian IDO1 enzymes [14], and IDO2 is especially inhibited by 1-methyl-D-tryptophan (1-D-MT) [15, 16]. In contrast to both IDOs, TDO is a highly substrate-specific dioxygenase and deoxygenates only L-TRP and some TRP derivatives. The expression of TDO is normally restricted to mammalian liver cells where it is believed to regulate systemic TRP concentrations [17]. Although TDO has been identified in the brain and epididymis of some species recently [18], it has been found that TDO is expressed in human malignant glioma cells of the brain [19]. On the other hand, IDO1 is expressed in a broad variety of mammalian cells related to immune function, such as activated macrophages and DCs. IDO1 is induced by proinflammatory cytokines such as tumor necrosis-α (TNF-α) and IFN-γ [20]. The enzymatic activity of IDO1 is restricted to specific tissues, including lungs, cecum, colon, and epididymis [21]. In addition, Takikawa et al. found that immune activation, such as an endotoxin, lipopolysaccharide (LPS) injection, could induce IDO1 enzyme activity only in specific tissue; however, this local induction of TRP metabolism resulted in a threefold increase in KYN concentrations in serum [22]. Thus, these findings have suggested that TRP metabolism can be initiated in local tissues, whereas KYN may diffuse into the bloodstream. Therefore, increased KYN concentration in the serum can indicate increased TRP metabolism taking place in a specific tissue. While TDO and IDO1 alter local and systemic TRP concentrations and initiate the production of neuroactive and immunoregulatory TRP metabolites; the known immunologic function of TRP degradation is largely dependent on IDO1. In addition, the biological function of IDO2 is still unclear and needs clarification. Therefore, in this paper, we describe IDO1 and immune regulation, unless specifically noted.

3. Signal Pathways related to IDO1

IDO1 is induced by IFN-γ-mediated effects of the signal transducer and activator of transcription 1α (STAT1α) and interferon regulatory factor-1 (IRF-1). The IDO1 gene has two interferon-stimulated response elements (ISREs) and IFN-γ-activated site (GAS) element sequences in the 5′-flanking region [23–25]. IDO1 induction is also mediated by an IFN-γ-independent mechanism under certain circumstances [26–28]. Fujigaki et al. demonstrated that IDO1 induction by LPS is not mediated by STAT1α or IRF-1 binding activities that induce IDO1 transcriptional activity by IFN-γ in many cells [28]. LPS stimulation of human monocytes and macrophages activates several intracellular signaling pathways, including the IkappaB kinase-nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) pathways. These pathways, in turn, activate a variety of transcription factors that include NF-κB and activator protein-1 (AP-1). A part of the induction of IDO1 by LPS is mediated by a signal from NF-κB or p38-MAPK pathways. A homology search of the 5′-flanking region of the IDO1 gene shows consensus sequences for transcriptional factors such as AP-1, NF-κB, and NF-IL-6, which are activated by LPS and other proinflammatory cytokines: TNF-α, IL-6, and IL-1β. Therefore, the IDO1 gene could be upregulated by LPS or these cytokines in a synergistic manner.

Posttranslational modifications (PTMs) of proteins perform crucial roles in the biological regulation of cells. PTMs provide a dynamic mechanism for regulating protein function and potentially change physical or chemical properties, activity, localization, or stability of proteins [29, 30]. Our group demonstrated for the first time that IDO1 activity is regulated by PTMs [31]. Peroxynitrite, a nitric-oxide-(NO-) derived reactive species, inhibits IDO1 activity via the nitration of tyrosine residues in IDO1 protein. This inhibition occurs at the posttranslational level because peroxynitrite inhibits IDO1 enzyme activity without affecting the expression level of the IDO1 protein. Activated macrophages can simultaneously generate large fluxes of NO and superoxide anions [32, 33], which rapidly combine to produce the far more reactive peroxynitrite anions. Peroxynitrite is considered to be produced by inflammatory cells to defend against infectious pathogens, such as parasites, viruses, and bacteria [34–36]. Thus, an understanding of protein

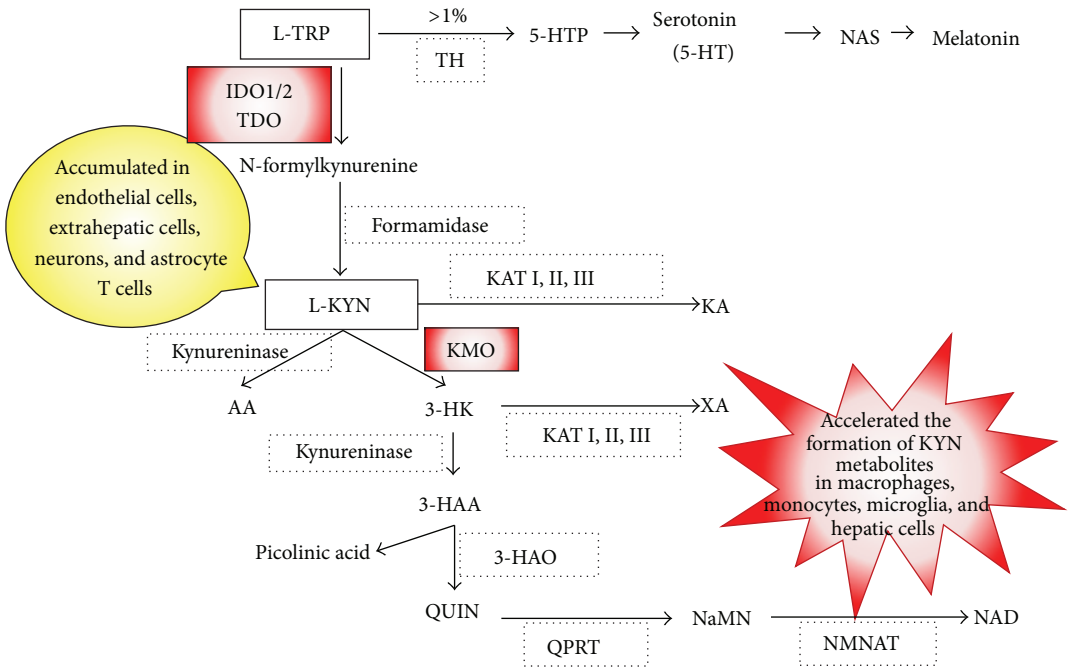

Figure 1: Schematic overview of the kynurenine pathway. It is estimated that only 1% of dietary tryptophan (TRP) can be converted into serotonin (5-HT). The remaining 99% of TRP is metabolized via the kynurenine (KYN) pathway. Tryptophan hydroxylase (TH), 5-hydroxy TRP (5-HTP), N-acetylserotonin (NAS), indoleamine 2,3-dioxygenase 1 and 2 (IDO1/2), tryptophan 2,3-dioxygenase (TDO), kynurenine 3-monooxygenase (KMO), kynurenine aminotransferase (KAT I, II, III), kynurenic acid (KA), anthranilic acid (AA), 3-hydroxykynurenine (3-HK), xanthurenic acid (XA), 3-hydroxyanthranilic acid (3-HAA), 3-hydroxyanthranilic acid oxidase (3-HAO), quinolinic acid (QUIN), quinolinic-acid phosphoribosyl transferase (QPRT), nicotinic acid mononucleotide (NaMN), nicotinamide mononucleotide adenylyltransferase (NMNAT), nicotinamide adenine dinucleotide (NAD).

nitration and PTMs on IDO1 will provide insight into the pathogenic mechanisms of inflammatory diseases related to macrophages and into novel therapeutic strategies for limiting tissue inflammatory injury.

4. Immune Regulation by IDO1

IDO1 was first isolated from rabbit intestine in 1967 [37], and it became rapidly clear that its induction serves the mechanism of antimicrobial resistance. Infection by bacteria, parasites, or viruses induces a strong IFN-γ-dependent inflammatory response. IFN-γ-induced IDO1 degrades TRP, and the depletion of TRP results in the regulation of intracellular pathogens [38–42]. On the other hand, Munn et al. provided evidence for a much broader immunoregulatory significance of TRP degradation by IDO1. They demonstrated that tolerance to allogeneic fetuses is regulated by IDO1-expressing cells in the mice placenta [1]. They and others also showed that a marked increase in IDO1 suppresses immune responses by locally depleting TRP and hence preventing T-lymphocyte proliferation using the IDO1 inhibitor, 1-MT [43–45]. These previous studies clearly showed that TRP degradation by IDO1 substantially contributes to immunoregulation, and therefore IDO1 has been considered as a strong immunoregulatory factor.

As shown in Figure 2, IDO1 is predominantly expressed in APCs of the immune system—the DCs, the monocytes, and the macrophages (Figure 2(a)) [46, 47]. IDO1 can be introduced by soluble cytokines such as IFN-γ, type I IFNs, transforming growth factor-β (TGF-β), TNF-α, or Toll-like receptors (TLRs) ligands such as LPS [27]. In addition, KYN and 3-hydroxykynurenine (3-HK) could be also involved in exacerbation of TRP starvation in T cells. Kaper et al. have proposed the existence of a positive feedback between IDO1-mediated TRP metabolism in DCs and KYN-induced TRP depletion in CD98-expressing T cells [48]. CD98 is expressed on astrocytes and activated T cells. T cells are sensitive to low levels of TRP and TRP metabolites *in vitro*. TRP deficiency specifically activates the GCN2 kinase in murine and human T cells, which leads to a halt in the G2 phase of T-cell division and T-cell suppression (Figure 2(b)) [49]. Moreover, a specific combination of TRP metabolites can inhibit anti-CD3 antibody-induced T-cell proliferation and induce T-cell apoptosis *in vitro* [50, 51]. The combination of low TRP concentrations and specific TRP metabolites leads to the generation of Tregs from naïve T cells *in vitro* [52, 53]. Tregs inhibit the activation, differentiation, and survival of effector T cells through the induction of IDO1 in APCs by ligation of inhibitory ligands and cytokines from Tregs [54].

It is possibly the selective pressure by Tregs that drove the evolution of the IDO1 mechanism from one operating in innate and inflammatory responses to pathogens [55, 56] to an effector mechanism of Treg function [57, 58]. Functional plasticity in DCs allows these cells to present antigens in an

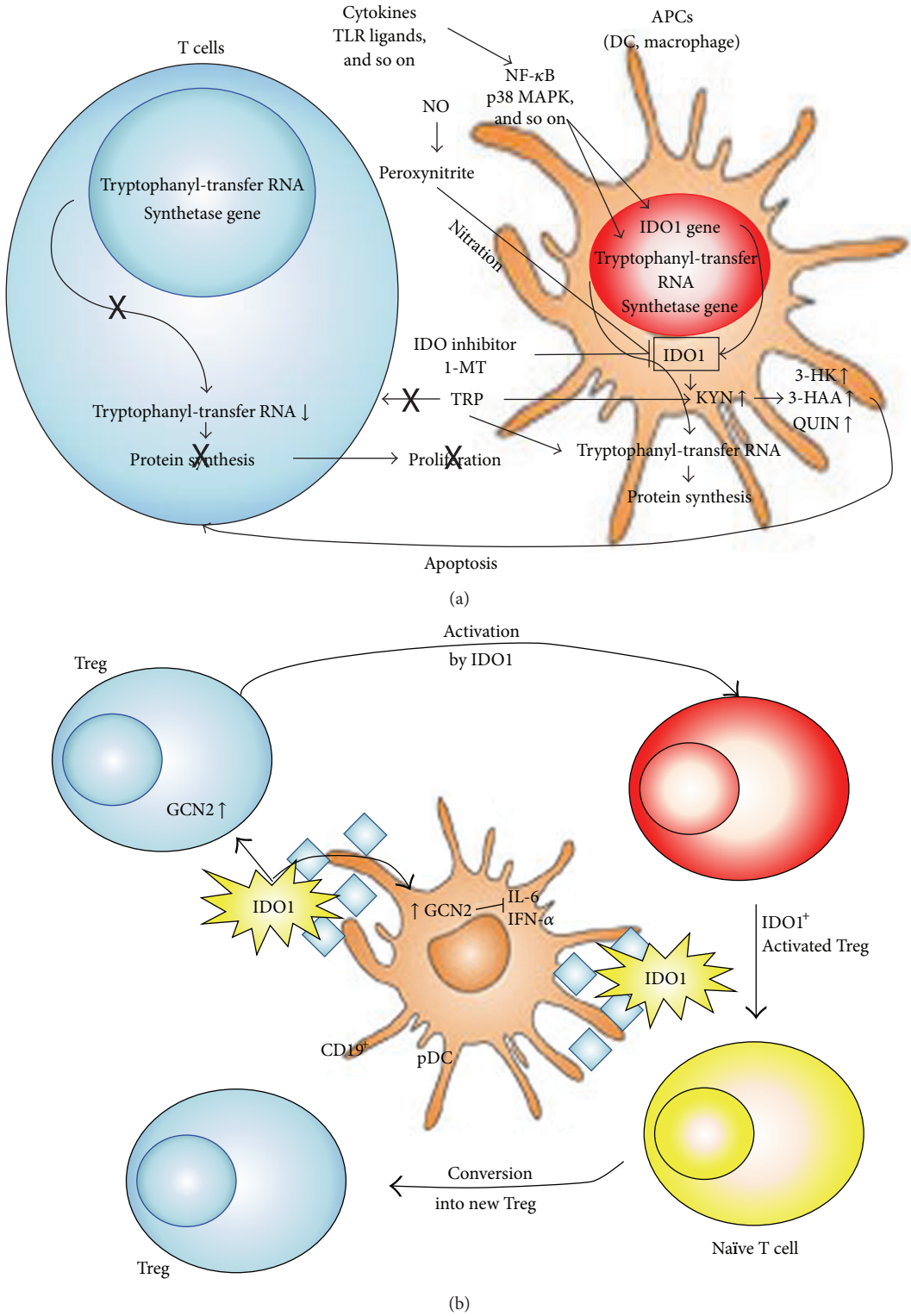

Figure 2: T-cell immune regulation by IDO1. (a) IDO1 is induced by IFN-γ-dependent and/or -independent signal pathways, depending on the variety of immune stimuli by macrophages and dendritic cells (DCs) [28, 83]. IDO1 activity is suppressed by the formation of NO or the competitive enzyme inhibitor, 1MT. Marked increases in IDO1 suppress immune responses by locally depleting L-TRP and preventing T-cell proliferation [44]. Expression of IDO1 has been observed in certain types of activated macrophages and DCs. IDO1-expressing cells deplete TRP from the extracellular milieu and secrete TRP metabolites, including KYN, 3-HK, 3-HAA, and QUIN, which induce T-cell apoptosis and suppress immune responses *in vitro*. (b) $CD19^+$ plasmacytoid DCs (pDCs) express high levels of IDO1, which can activate mature regulatory T (Treg) cells via activation of the protein kinase general control nonderepressing-2 (GCN2) pathway of protein synthesis inhibition [84]. pDC-produced IDO1 and activated Treg can convert naïve T cells into new Treg. IDO1 acts in an autocrine manner to suppress pDC production of IL-6, which prevents the conversion of Treg into IL-17-producing Th17 proinflammatory cells [79]. IDO1 also downregulates type I IFN (IFN-α) production by pDC [80].

immunogenic or tolerogenic fashion, largely contingent on environmental factors [59]. Costimulatory and coinhibitory interactions between DCs and T cells are pivotal in tipping the balance between immunity and tolerance in favor of either outcome. When CD80/CD86 molecules on DCs were engaged to T cells, CTLA-4 (widely expressed by Tregs) was later shown to behave as an activating ligand for CD80/CD86 receptors, resulting in intracellular signaling events. Through an unidentified signal cascade, DCs release type I and type II IFNs that act in an autocrine and paracrine fashion to induce strong IDO1 expression and function [60]. KYN-dependent T-cell differentiation would contribute to expand the pool of Tregs [8]. However, in the long-term control of immune homeostasis and tolerance to self, IDO1 relies on different regulatory stimuli and cytokines, providing a basal function amenable to regulation by abrupt environmental changes (Figure 3) [61].

In a TGF-β-dominated environment and in the absence of IL-6, IDO1 activates a variety of downstream signaling effectors that sustain TGF-β production, production of type I IFNs, and a bias of plasmacytoid DCs (pDCs) toward a regulatory phenotype [62, 63]. IDO1 enhances its own expression and stably tips the balance between proinflammatory and anti-inflammatory NF-κB activation.

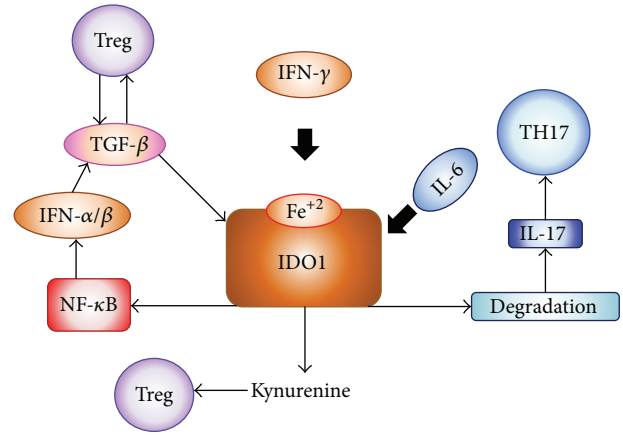

Figure 3: IDO1 induction and proinflammatory cytokines. IFN-γ drives intense enzymatic IDO1 activity, resulting in TRP depletion and high-level production of immunoregulatory TRP metabolites, KYNs, which may foster Tregs expansion. Induced Tregs use TGF-β to maintain an IDO1-dependent regulatory environment, with IDO1 mostly functioning as a signaling molecule. Both mechanisms are interrupted by IL-6, which drives IDO1 degradation as potent inflammatory stimuli enter the local environment [85].

5. Pathophysiologic Significance of Kynurenine Pathway Metabolites

IDO1-expressed DCs are able to lower TRP concentration, increase KYN concentration, and suppress the allogeneic T-cell response [50]. The TRP metabolites KYN, 3-HK, and 3-hydroxyanthranilic acid (3-HAA) inhibit T-cell proliferation by a time-dependent cytotoxic action, an effect which concerns mainly not only the activated T cells, but also B and natural killer (NK) cells. It has also been reported that KYN was able to reduce proliferation of human peripheral blood lymphocytes (PBL) *in vitro* [64]. The cytotoxic action of 3-HK can be attributed to the production of hydrogen peroxides which results in the damaging action of free hydroxyl radical [65]. As with KYN, when 3-HK was administrated exogenously, it effectively reduced symptoms in allergic inflammation [66]. The toxic action of 3-HAA is more complex. Although the final effect of 3-HAA results in the cell death of T cells, thymocytes [51] and monocyte-derived macrophages [67], the mechanisms involved in the cell death, might depend on the cell type. The formation of cytotoxic-free hydroxyl radical may be involved in 3-HAA-induced cell death in monocyte-derived macrophages [67]. L-KYN is considered to be the end product of KYN pathway metabolism in most extrahepatic cells, whereas macrophages produce the largest amount of quinolinic acid (QUIN) in accordance with the highest activities of kynurenine 3-monooxygenase (KMO) and kynureninase [4, 68]. In fact, Heyes et al. showed that macrophages stimulated with IFN-γ may be an important source of accelerated TRP conversion into KYN metabolites in inflammatory diseases [69]. Further, they showed that increased activities of KYN pathway enzymes, including IDO1 and KMO following systemic immune stimulation and HIV infection, in conjunction with macrophage infiltration, resulted in acceleration of the local formation of KYN metabolites, especially QUIN [21, 70]. Therefore, KMO is considered as a secondary regulatory enzyme for the KYN pathway, and macrophages, including monocytes, play a key role in the production of KYN metabolites.

6. Type I IFNs Production and IDO1

IDO1 activities in various tissues are induced by several cytokines after viral infection. However, the role of IDO1 *in vivo* after parasitic or viral infection is not fully understood. Recently, our group demonstrated that inhibition of increased IDO1 activity attenuates *Toxoplasma gondii* replication in the lung, and the inflammatory damage is significantly decreased by the administration of the IDO1 inhibitor after infection [71]. Some *in vitro* studies indicated that IFN-γ-induced antitoxoplasma activities are involved in IDO1-dependent mechanisms. These *in vitro* studies showed that IFN-γ-induced IDO1 degraded TRP in the culture medium, and the depletion of TRP resulted in the suppression of the growth of the parasites [39]. However, our experiments and the most recent study demonstrated that IDO1 ablation reduced local inflammation and parasite burdens, as did pharmacological inhibition of IDO1 *in vivo* [72]. Although IDO1 is certainly not the only regulator that plays a role as an antimicrobial, these studies show that the lack of the IDO1 gene or the inhibition of increased IDO1 activity suppressed the parasites' replication *in vivo* and that TRP degradation and KYNs production are not the only mechanisms of host resistance to early infection with these parasites. On the other hand, Hoshi et al. investigated the role of IDO1 in chronic viral infection diseases in mice infected with LP-BM5 murine leukemia virus (MuLV), including both replication-competent and replication-defective viruses, which resulted

in the development of a fatal immunodeficiency syndrome in mice, known as murine AIDS [73]. Murine AIDS is characterized by activation and proliferation of T and B cells, impaired T- and B-cell function, an aberrant regulation of cytokine production, hypergammaglobulinemia, decreased NK cell function, the development of B-cell lymphoma, and the susceptibility to opportunistic infections [74]. Hoshi et al. used IDO1 gene-deficient (IDO1 K.O.) mice and IDO inhibitor to examine whether IDO1 is an important factor for immune regulation against LP-BM5 infection and especially whether the presence of IDO1 is necessary for the induction of cytokines and IDO1-related molecules, which are important for viral clearance. Remarkably, they demonstrated that absence of IDO1 upregulated type I IFNs and downregulated virus replication in IDO1 K.O. mice with LP-BM5 infection [73]. Their finding is the first piece of evidence that the absence of IDO1 is involved in the clearance of murine retroviral infection via upregulated type I IFNs (Figure 4). Further, they also recently examined the roles of IDO1 in immune regulation in encephalomyocarditis virus (EMCV) infection by using IDO1 K.O. mice or the IDO inhibitor, 1-MT. EMCV, a member of the Picornaviridae family which includes the *Enterovirus* genus, can cause acute myocarditis in various animals. EMCV infection in mice is an established model for viral myocarditis, dilated cardiomyopathy, and congestive heart failure [75]. They demonstrated that type I IFNs are upregulated, resulting in suppressed EMCV replication by IDO1 knockdown or inhibition [76]. They also found that treatment of IDO1 K.O. mice with KYN metabolites eliminated the effects of IDO1 knockdown on the improved survival rates. These results suggested that KYN metabolites regulate the production of type I IFNs by decreasing the number of macrophages. Viruses initially activate the innate immune system, which recognizes viral components through pattern-recognition receptors (PRRs). Currently, three classes of PRRs have been shown to be involved in the recognition of virus-specific components in innate immune cells, which are TLRs, retinoic-acid-inducible-gene-I- (RIG-I-) like receptors (RLRs), and nucleotide-oligomerization-domain- (NOD-) like receptors (NLRs). Of these, TLRs and RLRs are especially important for the production of type I IFNs and various cytokines [77]. Therefore, these reports suggest that the enhancement of TRP breakdown by IDO1 regulates several signal pathways, which is related to IFNs production. TRP metabolites might contribute to the function of IFNs producing cells, like macrophages and DCs. The role of IDO1 may be complex; it may depend on the difference of disease stages (acute/chronic disease) and/or the stimulus pathogens. Kumagai et al. showed that lung infection with Newcastle disease virus (NDV) led to type I IFN, IFN-α production in alveolar macrophages, and conventional DCs (cDCs), but not in pDCs [78]. Specific depletion of macrophages caused a marked defect in initial viral elimination in the lung, and pDCs produced type I IFNs in the absence of macrophage-mediated viral recognition. These results suggest that pDCs work as immune regulators when the first defense line by macrophages is broken. Macrophages are important for the initial response to viral infection in the lung. Besides, a subpopulation of $CD19^+$ pDCs produces high

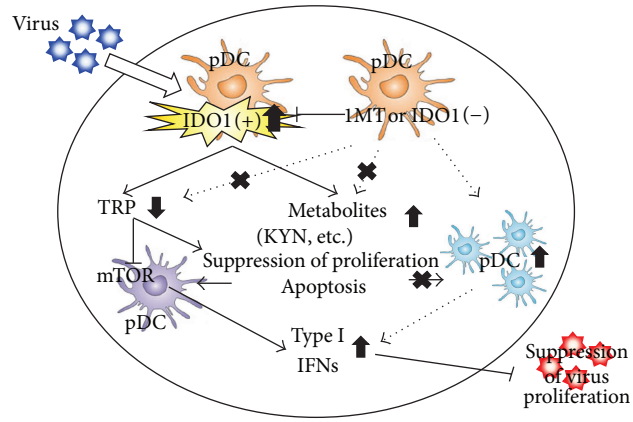

Figure 4: The mechanism of IDO1 regulation on viral infectious diseases. Innate defense occurs when pathogens contact or invade host cells and elicit the production of cytokines and chemokines, which in turn induce an influx of immune cells that affect pathogen clearance. Type I IFNs are critical mediators of innate immunity and limit disease caused by many viruses [73, 86]. The enhancement of TRP breakdown by IDO1 regulates the signal pathway for IFNs production, and TRP metabolites might contribute to the function of IFNs producing cells.

levels of the TRP-catabolizing enzyme IDO1 [44]. Production of IDO1 by pDC has been linked directly to activation of naturally occurring $Foxp3^+$ Treg through modulation of the GCN2 pathway, which leads to inhibition of protein synthesis and Treg activation [63]. IDO1 plays a dual regulatory role by preventing conversion of these Tregs into proinflammatory Th17 cells through autocrine inhibition of IL-6 production via upregulation of GCN2 in pDC [79] and inhibited production of type I IFN and IFN-α, which may limit their ability for activating innate and adaptive antitumor immunity [80]. The mammalian target of rapamycin (mTOR) pathway is also reported in regulating type I IFNs production by pDCs [81]. Additionally, TRP breakdown by IDO1 may regulate mTOR inhibition pathway [82]. Therefore, the degradation of local TRP and increased TRP metabolites by activated IDO1 may stimulate several signal pathways and induce cell death, resulting in the inhibition of IFNs production.

7. Conclusions

IDO1 is not only pivotal in limiting potentially exaggerated inflammatory reactions in response to danger signals and in assisting the effector functions of Treg cells, but also an important component of the regulatory system that presides over long-term control of immune homeostasis. On the other hand, TRP metabolism via KYN pathway is a good example of how metabolism of small molecules can impact the immune system. Therefore, induction of the KYN pathway and/or controlling the systemic TRP concentrations by stimulation of immune cells or by diet might be an effective strategy for treatment of virus infection and immune diseases. In addition, understanding the subsequent steps on the KYN pathway and the physiological mechanisms responsible for regulation of KYN and concentration of its metabolites in

biological fluids may be important for development of drugs in the future. We believe that further findings on the mechanism of immune regulation by IDO1 and TRP metabolites might contribute to the implementation of a novel therapy protocol, which would target several immune disorders.

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Review Article

Macrophage Plasticity and the Role of Inflammation in Skeletal Muscle Repair

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Effective repair of damaged tissues and organs requires the coordinated action of several cell types, including infiltrating inflammatory cells and resident cells. Recent findings have uncovered a central role for macrophages in the repair of skeletal muscle after acute damage. If damage persists, as in skeletal muscle pathologies such as Duchenne muscular dystrophy (DMD), macrophage infiltration perpetuates and leads to progressive fibrosis, thus exacerbating disease severity. Here we discuss how dynamic changes in macrophage populations and activation states in the damaged muscle tissue contribute to its efficient regeneration. We describe how ordered changes in macrophage polarization, from M1 to M2 subtypes, can differently affect muscle stem cell (satellite cell) functions. Finally, we also highlight some of the new mechanisms underlying macrophage plasticity and briefly discuss the emerging implications of lymphocytes and other inflammatory cell types in normal versus pathological muscle repair.

1. Introduction

Tissue regeneration is an evolutionary conserved process in which interactions between infiltrating inflammatory cells and resident cells must be finely coordinated if homeostasis and functionality are to be restored. Perturbation of these interactions leads to unsuccessful regeneration and often compromises survival of the individual [1, 2]. Skeletal muscle, the most abundant tissue of the body, is essential for breathing, posture maintenance, and locomotion, besides serving important homeostatic and metabolic roles, such as heat production and carbohydrate or amino acid storage. Loss of muscle functionality in acute or chronic conditions results in diminished mobility and strength, in addition to metabolic disorders, which can have potentially lethal consequences. Abnormal muscle repair can occur in the context of persistent myofiber degeneration and/or inflammatory infiltration, such as in Duchenne muscular dystrophy (DMD), or when extracellular matrix (ECM) deposition is excessive or inappropriately timed, eventually leading to the substitution of the normal muscle architecture by fibrotic tissue [3]. Therefore, preservation of the capacity of skeletal muscle to regenerate in a coordinated manner in response to direct mechanical trauma (acute injury), or following secondary damage as a consequence of genetic neuromuscular alterations, is of utmost importance.

2. Injury-Induced Skeletal Muscle Regeneration: A Model for Tissue Repair

The capacity of muscle to regenerate relies primarily on a specific population of normally quiescent muscle stem cells, named satellite cells due to their particular position and intimate association with muscle fibers [4]. Many additional cell types also play a role in efficient tissue repair, including resident cells within the skeletal muscle niche such as PICs ($PW1^+$ interstitial cells), mesoangioblasts, FAPs (fibro/adipogenic progenitors), and other ECM-associated cells [5]. However, the inflammatory cells that infiltrate the injured muscle appear to be the most critical, alongside satellite cells, for successful regeneration. Among these

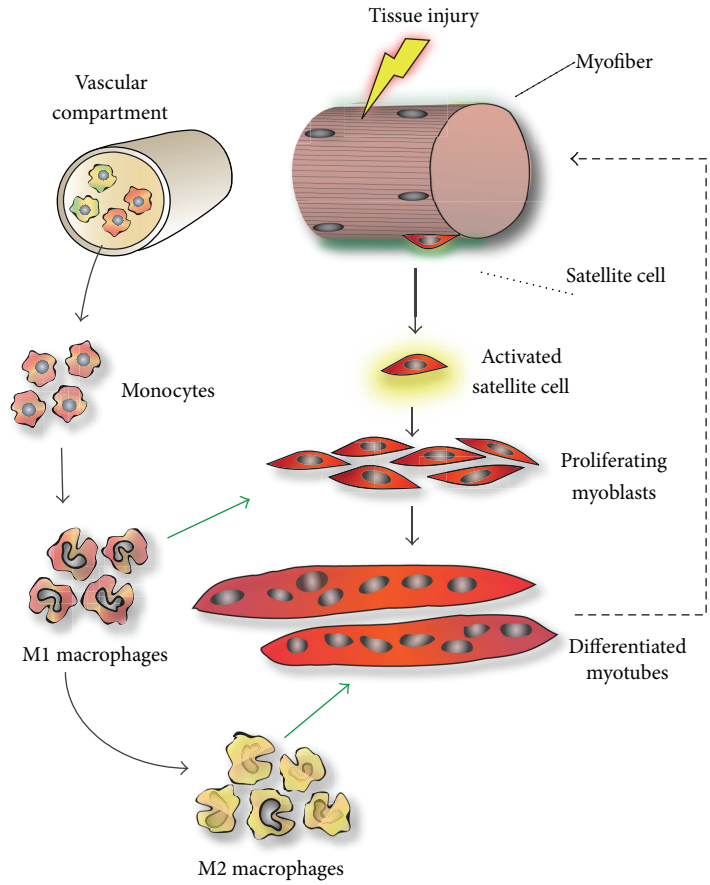

Figure 1: Inflammation and macrophage polarization in skeletal muscle injury and repair. Satellite cells are muscle-resident stem cells which are located underneath the basal lamina of myofibers and are normally quiescent (top right). Upon muscle injury, satellite cells get activated, start to proliferate as myoblasts, and subsequently fuse and differentiate into myotubes that later grow thereby replacing damaged muscle. Several cell types influence the outcome of regeneration, in particular inflammatory cells released from the blood (top left). Proinflammatory monocytes and neutrophils (not shown) extravasate shortly after damage, invading the injured areas where they differentiate into proinflammatory macrophages that phenotypically resemble M1 macrophages. These cells clear the damage and release a number of cytokines that stimulate myoblast proliferation. M2-like macrophages are present locally at later stages of regeneration acting as promoters of myoblast differentiation and fusion. Other cell types such as mast cells and lymphocytes also have less defined roles in muscle repair (not shown).

inflammatory cells, it is the monocytes/macrophages which play the greatest role in this repair process (Figure 1). In response to local vascular damage and signals released by degenerating myofibers, these cells extravasate from the blood and infiltrate the injured areas, to phagocytose myofiber debris. In addition to this critical function, inflammatory cells produce growth factors, cytokines, inflammatory mediators, and damage signals that have a profound impact on satellite cell behavior during the repair process [6]. In concert with monocyte/macrophage recruitment, quiescent satellite cells are activated by damage/inflammation-associated signals and begin to proliferate, thereby providing a sufficient supply of myonuclei for the formation of new myofibers. While most of the proliferating satellite cells will commit to myogenic differentiation, a small population will undergo self-renewal and replenish the pool of quiescent satellite cells, thus maintaining muscle stem cell homeostasis [7].

A further critical step in the repair process is the re-establishment of the ECM around the individual fibers and bundles which helps strengthen the muscle and provides additional support for contraction. Correct remodeling and reorganizing of the muscle ECM after damage is necessary for providing new scaffold structures over which nascent myofibers will be formed, as well as ensuring correct spatial organization of the new myofibers [8]. Excessive and persistent ECM deposition (fibrosis) leads to failure in restoring the previous structure of myofibers, thus provoking a defective regenerative outcome. Although several studies have shown that satellite cell-derived myoblasts may synthesize many components of the ECM, the major matrix-producing cell is the fibroblast [9]. Like satellite cells, resident fibroblasts proliferate and migrate to the injury site immediately after muscle damage, where they function in close proximity to satellite cells and regenerating myofibers. Indeed, recent findings have demonstrated the relevance of the interplay between satellite

cells and fibroblasts and/or FAPs as a determinant factor for the efficiency of the repair process [10–12]. Specific deletion of fibroblasts using genetic approaches resulted in impaired regeneration due to the lack of proliferation of satellite cells and their premature differentiation, strongly suggesting a paracrine action of fibroblasts on muscle cells [10]. An important part of the functional role of ECM in controlling the process of repair is carried out by the basal lamina, a thin layer of nonfibrillar collagen, noncollagenous glycoproteins, and proteoglycans that is in direct contact with the myofiber plasma membrane (see [13] for review). The basal lamina also surrounds satellite cells forming part of the niche that is necessary for maintaining the stem-like properties of quiescent satellite cells. Because of this direct satellite cell contact, the basal lamina composition and integrity also influence the process of repair, by providing guidance cues for satellite cell migration. In the normal repair process, prevention of excessive accumulation of ECM components and restoration of the original basal lamina integrity are controlled by the balanced activities of extracellular proteases and their inhibitors. Dysregulation of these enzymatic activities may cause unrestricted ECM accumulation and altered basal lamina composition, which eventually could lead to fibrosis development and loss of normal muscle architecture [14]. Lastly, proangiogenic factors also need to operate at advanced stages of the repair process to revascularize the newly formed myofibers, thus restoring the vascular network of the damaged tissue [15]. For a recent and comprehensive review focused on macrophage biology in skeletal muscle injury, muscle disease, and fibrosis, see Bosurgi et al. (2011) [16]. In this paper, we focus more specifically on the current knowledge of the inflammatory control of satellite cell-dependent muscle repair in acute injury and highlight several recent findings.

3. Inflammation in Efficient Muscle Repair

Just as satellite cells go through a controlled process of activation from quiescence, proliferation, and self-renewal, and finally differentiation and fusion into new myotubes, the inflammatory response also undergoes a series of carefully regulated stages to ensure an efficient return to tissue homeostasis. That is, the composition of the inflammatory infiltrate is dynamically regulated to facilitate timely initiation of divergent functions, while the duration and intensity of the various inflammatory components must also be coordinated with the degree of muscle damage and the need to change tissue milieu during repair [3, 6, 17]. For example, interfering with the inflammatory response immediately after acute injury disrupts the phagocytosis of necrotic fibers and impedes seeding of new myofibers. Just as detrimental is the prolongation of inflammation which can promote muscle degeneration and fibrosis development, as occurs in severe myopathies such as DMD which are characterized by chronic inflammation [18]. Macrophages have recently been shown to promote survival and proliferation of myogenic precursor cells that were introduced into *mdx* skeletal muscle [19]. Thus, a tightly regulated, transient inflammatory response is required for normal muscle regeneration. Improving our understanding of the different cell subtypes and identifying the factors that regulate their function and the timing of their activity will enable us to improve pharmacological treatment of acute injury and neuromuscular disorders associated with chronic inflammatory responses.

4. Phases of the Inflammatory Response in Acute Muscle Injury

Most studies of skeletal muscle regeneration use acute models of injury and repair, such as sterile destruction of myofibers by either injection of toxins, such as cardiotoxin, notexin, or barium chloride, or by performing freeze crush injuries. These models are useful for synchronizing the repair processes and performing systematic studies, although they do not necessarily reflect the more physiologic repair associated with contraction injuries or replicate the different kinetics of chronic inflammation observed in myopathies. Moreover, there are important contributions of mouse strain to the inflammatory component and kinetics that are briefly discussed below and elsewhere [20, 21]. However, despite these variables, the inflammatory response to experimentally induced muscle repair follows an ordered pattern.

An immediate response to sterile muscle injury is the local activation of the innate immune response via the release of largely unknown factors, but which could include heat shock proteins, high mobility group box 1 (Hmgb1) as well as endogenous myofiber proteins and nucleic acids that become decompartmentalized as the fiber breaks and act as damage-associated molecular patterns (DAMPs) [22]. One of the earliest subsequent events is the invasion of the damaged site by inflammatory cells, particularly monocytes and polymorphonuclear leukocytes, which include neutrophils, that secrete proinflammatory cytokines and phagocytose particles (such as cellular or bacterial debris) [23]. Neutrophils constitute the first wave of inflammatory cells to enter the damaged tissue, reaching elevated numbers as soon as 2 hours after the initial injury. Neutrophils are, however, short-lived cells, whose number declines rapidly, probably through apoptosis, and they are essentially undetectable 3-4 days after injury [6]. The exact role of neutrophils in toxin-induced or freeze-crush injury is not clearly defined. However, several studies on contraction-induced injury show that neutrophils play a key role in repair by causing secondary damage, through the release of reactive oxygen species (ROS) and proteases, as well as facilitating phagocytosis and recruitment of monocytes by the release of cytokines [24, 25]. Neutrophils are known to enter into contraction-damaged muscle via a process called diapedesis that requires CD18 (integrin-β2) [25]. Interestingly, in contraction-injured CD18-deficient mice, neutrophil, but not macrophage, recruitment was impaired, while physiological signs of repair such as fiber size and force were more quickly restored compared to wild-type mice [25].

Recent studies have shown that resident macrophages in the muscle epimysium/perimysium connective tissue orchestrate the innate immune response to injury, which is linked to adaptive immunity through inflammatory dendritic cells (DCs) [26]. In addition to resident macrophages, blood

monocytes also enter the damaged tissue and start differentiating into macrophages shortly after invasion by neutrophils [17]. Other inflammatory cell types, such as mast cells and T cells, have also been implicated in the repair and fibrogenesis of several tissues/organs; however, their role in muscle repair and/or fibrosis is generally limited (see also below) [27]. Monocytes originate in the bone marrow and circulate to the blood and the spleen before entering the muscle after injury [28]. They are equipped with chemokine and adhesion receptors that allow them to migrate from the blood to the injured tissues, where they produce proinflammatory cytokines and phagocytose dying or apoptotic cells. In the blood, circulating monocytes can be classified into at least two populations that are distinguishable by their expression levels of Ly-6C (also known as GR1) and of chemokine receptors CCR2 and CX3CR1 [29]. These two monocyte populations use different mechanisms for extravasation and probably have different functions. The $GR1^+$ monocyte cell pool has been designated as the "inflammatory" population because they efficiently produce proinflammatory cytokines [30]. Through the CCR2/CCl2 axis, they are rapidly recruited to, and accumulate at, the site of inflammation [31, 32]. On the other hand, the $GR1^-$ population of monocytes has an "anti-inflammatory" function, which includes supporting tissue repair and patrolling the vasculature [33]. In contrast to $GR1^+$ cells, $GR1^-$ monocytes enter damaged tissues in a CX3CR1-dependent manner just after the onset of inflammation in models of sterile injury [34–36]. An important consideration beyond the scope of this paper is the known heterogeneity in the use of cell surface markers between mice and humans, with human monocytes broadly defined as expressing different levels of CD14 and CD16 [37]. Thus, as most studies are performed in mice, care will be needed in trying to extrapolate findings to humans and the clinic.

Classically, there are believed to be two waves of tissue-infiltrating monocytes in most experimental wound healing models: a first wave comprising the $GR1^+$ population, endowed with proinflammatory function and a second wave of $GR1^-$ monocytes with an anti-inflammatory function. Interestingly, using an acute muscle injury model, Arnold and colleagues showed that the $GR1^+$ monocyte population is the only one recruited to the injury site, switching subsequently within the damaged tissue into an "anti-inflammatory" macrophage population, thereby dampening the earlier proinflammatory wave and also supporting myogenesis [38]. Distinct macrophage populations have also been associated with the increased fibrosis observed in dystrophic muscle (see also below) [39]. Together these observations suggest that the mechanisms of leukocyte recruitment and maturation could be specific for each type of damage, tissue, and microenvironment.

5. Classification of Macrophage Populations in Tissue Repair: Specific Markers versus Functional Properties

As suggested by the experiments above and additional data from other tissue repair systems [40–42], macrophages exist as different functional populations at different times after injury. Generally, these populations are considered to exhibit opposing activities, being either polarized towards proinflammatory or anti-inflammatory activity [38]. Polarized macrophages are currently classified as either M1 or M2, referring to either classical or alternative activation, respectively [40, 43]. Proinflammatory M1 macrophages arise from exposure to the T-helper (Th)1 cytokines interferon-(IFN)γ and tumor necrosis factor (TNF)α, in addition to lipopolysaccharide (LPS) or endotoxin [43, 44]. However, polarization of M2 macrophages is more complex than M1 polarization, with three possible subtypes currently defined, each one with diverse physiological roles. Alternatively activated or M2a macrophages are commonly associated with advanced stages of tissue repair and wound healing, arising from exposure to Th2 cytokines such as IL-4 and IL-13. As well as promoting the proliferation of nonmyeloid cells, IL-10 is also known to induce M2c macrophages which have an anti-inflammatory function. Similarly, M2b macrophages also have an anti-inflammatory role and can release large amounts of IL-10. M2b share many features with tumor-associated macrophages [45]. Like M1 macrophages, M2b macrophages also release proinflammatory cytokines, such as IL-1β and TNFα, but not IL-12.

Proinflammatory macrophages, observed experimentally in the context of muscle repair, are phenotypically similar to classically activated M1 macrophages, and are usually found at early stages after muscle injury, closely followed by macrophages sharing features with the anti-inflammatory M2c phenotype, so-called because of their role in deactivating M1 macrophages [38]. Early on, M1 macrophages phagocytose necrotic muscle debris and participate in the processing and presentation of antigens. In addition to producing high levels of proinflammatory cytokines, M1 macrophages also express inducible nitric oxide synthase (iNOS), which is required to efficiently metabolize L-arginine, a fundamental reaction for producing an abundance of NO for killing intracellular pathogens during infection. Alternatively activated M2a macrophages are more abundant during the final phase of tissue repair [46]. Importantly, M2a macrophages have also been linked to fibrosis in dystrophic *mdx* mouse muscles [39, 47]. M2b-like macrophages have recently been described in regenerating muscle after acute injury [48], suggesting that a wide range of M2 macrophage subtypes might be functional during the muscle repair process.

Despite the emergence of compelling evidence for the presence of different macrophage subtypes in muscle repair, a clear understanding of their specific functions is still lacking. By analogy with the *in vitro* cytokine proinflammatory profile, monocytes entering the muscle at the onset of inflammation resemble M1 polarized macrophages. Indeed, they produce large amounts of the proinflammatory cytokines TNFα and IL-1β and have an enhanced expression of iNOS. In cell culture models, proinflammatory macrophages have been shown to exert a positive influence on myoblast proliferation while repressing myoblast differentiation [17, 38, 49]. As the process of muscle regeneration advances, they switch their phenotype to resolve inflammation and start to express high levels of IL-10, TGFβ, and other anti-inflammatory cytokines

that dampen the initial cytokine storm. These cytokines have essential roles in promoting proper wound healing, by supporting myogenesis [50], enhancing angiogenesis, and stimulating the transient deposition of the ECM [51]. Similarly, the later wave of anti-inflammatory macrophages stimulates both myoblast differentiation and fusion *in vitro* [17, 38, 49]. The relevance of these inflammatory cells *in vivo* was shown after depletion of blood monocytes exerted negative effects on the regeneration process [38]. Indeed, it is blood monocytes that are the likely source of M1 and M2 macrophages in injured muscle. It is worth noting that although the *in vitro* models of macrophage polarization are useful to establish a theoretical classification, these macrophage populations most likely represent the extremities of a continuum of possible activation states. In addition to problems of classifying polarized macrophages in different tissues and repair models, a recent study has also suggested that there is considerable heterogeneity in the gene expression pattern of different resident macrophage populations in different tissues [52]. Therefore, caution is suggested when classifying wound healing macrophages in general, and muscle infiltrating macrophages in particular, especially when comparing them with *in vitro* polarized macrophages. It is tempting to propose that, rather than belonging to one of these categories, wound healing macrophages could themselves constitute a unique class based on their common characteristics with M1, M2a, M2b, or M2c macrophage subtypes [53]. Thus, to avoid the confusion that can arise from the mixed phenotypes found *in vivo*, some authors propose to classify macrophages regarding their function (i.e., host defense, wound healing, or immune regulation) rather than grouping them on the basis of expression of certain markers [54].

6. Mechanisms of Macrophage Polarization and Deactivation during Muscle Repair

The mechanisms underlying the transition of macrophage phenotypes during muscle repair are poorly understood. However, certain analogies can be established between *in vitro* macrophage responses to endotoxin and the phenotypic transitions that occur during wound healing. For example, the cAMP response element-binding protein (CREB) plays an important role in generating the anti-inflammatory macrophage phenotype in response to LPS. This response is mediated by the mitogen- and stress-activated kinases 1 and 2 (MSK1 and MSK2), which are, in turn, activated by p38 MAP kinase (MAPK) [55, 56]. In a model of toxic contact eczema induced by phorbol-12-myristate-13-acetate, the CREB-induced expression of IL-10, and dual specificity protein phosphatase 1/MAP kinase phosphatase-1 (DUSP1/MKP-1) inhibited the expression of proinflammatory genes associated with M1 macrophage activation, thus supporting a link between p38/MAPK-1 and CREB in macrophage polarization. An important regulatory function for CREB in macrophage polarization has also been revealed during tissue repair. Indeed, M2, but not M1, macrophage gene expression was impaired by deleting two CREB-binding sites from the C/EBPβ gene promoter, resulting in abnormal muscle regeneration [57]. Macrophages from the C/EBPβ promoter mutant mice had a reduced expression of the M2-associated arginase gene after LPS stimulation. It was hypothesized that this may lead to a switch in arginine metabolism from arginase-mediated polyamine synthesis to iNOS-mediated NO production [57]. Importantly, additional studies showed that shifts in macrophage polarization and macrophage competition for arginine metabolism influenced the severity of muscle pathology in *mdx* dystrophic mice [47]. These studies strongly support the idea that CREB might be a pivotal transcription factor in macrophage polarization that functions by promoting M2-associated genes while repressing M1 activation, with CREB transcriptional activity regulated by balance of p38/MSK1/2-MKP-1 activities.

Although macrophages sustain proper healing by secreting growth factors and cytokines that support myogenesis and promote transient ECM deposition, dysregulation of the expression of cytokines such as TGFβ or IL-1β can lead to aberrant repair, including fibrosis development, especially in muscle pathologies and conditions characterized by chronic inflammation [21]. Consequently, efficient muscle repair requires resolution of inflammation, and in particular, deactivation of macrophages, at advanced stages of tissue recovery [58].

The regulatory mechanisms controlling cytokine gene silencing and macrophage deactivation remain largely undeciphered. One recent study investigated the AKT activation status in macrophages of wild-type and MKP-1-deficient mice during the resolution of inflammation after muscle injury. The activity of AKT was higher in MKP-$1^{-/-}$ than in wild-type macrophages in the late stage of muscle repair, correlating with a loss of pro- and anti-inflammatory cytokine gene expression, and this effect could be reverted by pharmacologically inhibiting p38 MAPK activity [49]. Conversely, macrophages from wild-type mice treated with the PI3K/AKT inhibitor wortmannin showed a prolonged activation status presumably by preventing deactivation. Furthermore, levels of the phosphatase PTEN, which functions as a tumor suppressor by negatively regulating the AKT/PKB signaling pathway, were lower in macrophages in the absence of MKP-1 during the later stage of muscle repair. PTEN is also a direct target of miR-21 [59], a miR classically associated with cancer and fibrosis [60, 61], and its expression was previously reported in RAW 264.7 macrophages [62]. Because miR-21 expression was shown to increase in deactivated macrophages in a p38-dependent manner, [49] it is possible that the loss of MKP-1 (through regulation of the miR-21/PTEN/AKT pathway) extends macrophage cell persistence at the site of injury while at the same time provoking their premature deactivation during the tissue repair process. Taken together, these results strongly support a role for MKP-1 in neutralizing p38 MAPK and thereby controlling sequential macrophage activation-deactivation transitions during tissue repair by restraining AKT activation.

7. Additional Immune Cell Types, Such as Lymphocytes, Are Also Implicated in Muscle Repair and Fibrosis

Macrophages are the predominant inflammatory cells in skeletal muscle regeneration, yet other immune cells, in particular T lymphocytes, have also been proposed to influence repair and fibrosis. Like macrophages, T lymphocytes can also differentiate into distinct functional subsets. The two major types are termed Th1 and Th2, which have distinct roles in orchestrating the host response by generating distinct cytokine profiles [44], whereas more newly characterized including Th17 and Treg subtypes have a less defined role in muscle regeneration, although growing evidence suggests they may become important [63]. Cytokines produced in T cells also regulate muscle degeneration and repair. $CD4^+$Th1 cells promote cell-mediated immunity and are able to produce cytokines with antifibrotic properties such as IFNγ, TNFα, IL-12, and IL-2. By contrast, $CD4^+$Th2 cells produce IL-4, IL-5, IL-6, and IL-13, which are cytokines whose primary role is to promote humoral immunity in addition to having profibrotic roles. Importantly, Th1 cytokines inhibit the development of Th2 cells, and conversely, Th2 cytokines inhibit the development of Th1 cells. Clearly, alterations or imbalances in these pathways have the potential to skew repair towards anti- or profibrotic pathways, as witnessed by the importance of Th2 cytokines in the development of liver fibrogenesis [46]. Moreover, T-cell-derived cytokines have a clear role in maintaining the polarized state of macrophages *in vivo*, at least in other models of injury and repair such as after parasite infection [64]. However, the contribution of T cells to macrophage polarization in sterile injury models where T cells are less abundant remains to be explored.

Several studies have suggested roles for T lymphocytes in muscle regeneration. For example, knockout mice lacking the proteolytic activity of the serine protease uPA, and its downstream proteolytic cleavage enzyme plasmin, displayed reduced macrophage and T-lymphocyte infiltration of injured muscle and persistent myofiber degeneration [65–67]. Another study in mice deficient for the *Cbl-b* ubiquitin ligase tumor suppressor gene showed increased infiltration of $CD8^+$ T cells into injured muscles with a subsequent delay in muscle regeneration [68]. Deficiency of *Cbl-b* also significantly increased production of the chemokine CCL5 (RANTES) from macrophages during muscle regeneration, whereas neutralization of CCL5 improved the defective muscle regeneration in *Cbl-b*-deficient mice. All together, these results suggest that *Cbl-b* is an important regulator of $CD8^+$ T-cell infiltration into regenerating muscle, an effect mediated via CCL5 production in macrophages [68]. In another example, athymic BALB/c nude mice, which are T cell deficient, showed significant increase in central nucleation and increased MMP-9 activity in comparison to wild-type BALB/c [21].

Lymphocytes have also been implicated in the deficient regeneration and development of fibrosis observed in some degenerative myopathies. Early studies identified the presence of T cells and several other inflammatory cell subtypes in biopsies of human DMD patients and other myopathies [69, 70]. However, T-cell-mediated cytotoxicity appeared to be limited in DMD patients, despite the appearance of major histocompatibility complex I (MHC I) on regenerating fibers [71]. Dystrophic scid/*mdx* mice, which are deficient in functional T and B lymphocytes, develop much less diaphragm fibrosis with age compared with normal *mdx* mice, concomitant with a decrease in activated TGFβ in skeletal muscle, [72]. In nu/nu/*mdx* mice (immunodeficient nude mice in the *mdx* background), the lack of functional T cells alone was associated with less diaphragm fibrosis at 3 months, supporting the pathogenic role for T cells in *mdx* muscle and revealing this lymphocyte subclass to be an important source of TGFβ [73]. A specific subpopulation of T cells expressing the Vb8.1/8.2 T-cell receptor (TCR) was recently identified and shown to be enriched in *mdx* muscle. These T cells produce high levels of osteopontin, a cytokine that promotes immune-cell migration and survival [74]. Intriguingly, osteopontin levels are increased in patients with DMD and in *mdx* mice after disease onset. Importantly, loss of osteopontin in *mdx* double-mutant mice diminishes the infiltration of natural killer T-cell-(NKT-)like cells, which express both T and NK cell markers and neutrophils. These mice also show reduced levels of TGFβ. These results correlate well with improvements in muscle strength and reduced fibrosis in the diaphragm and heart [74]. Thymectomy at one month of age induces near complete postnatal depletion of circulating T cells in *mdx* mice. When this was followed by anti-CD4 and/or anti-CD8 antibody treatment, it failed to improve diaphragm fibrosis at six months of age [72, 75, 76]. Finally, a recent study investigated the role of lymphocytes in muscle dysferlinopathy using Scid/A/J transgenic mice and showed that the absence of T and B lymphocytes resulted in an improvement of muscle regeneration [77].

Several studies have also shown that mast cells may play a role in normal skeletal muscle repair. Mast cells were shown to accumulate in injured muscles from around 8 hours after saline injection of the gastrocnemius muscle, most of which were recruited from the circulation as very few mast cells are resident in the tissue [78]. Interestingly, mast cells have been linked to development of fibrosis and were shown to be persistently present in *mdx* muscle tissue close to major vessels [78, 79]. Several studies in *mdx* mice and human clinical trials have explored the use of mast cell stabilizers like Oxatomide (Tinset) or Cromolyn (sodium cromoglycate) on the ability to improve muscle repair [80, 81]. Although mast cells are known to release many proinflammatory cytokines such as TNFα and interleukins such as IL-1 and IL-6, consistent with their early appearance in muscle after acute damage, in addition to histamine and proteases such as chymase, their role in macrophage polarization is unknown and their overall contribution to efficient repair requires further investigation.

The above data serves to demonstrate the complexity of the mechanisms that regulate inflammation, muscle repair, and fibrosis development. It is still not clear whether distinct types of Th responses and macrophage subtypes operate in dystrophic muscle, and how they mediate their interactions. Thus, despite our increasing understanding of these immune

cells, the implication of the presence of lymphocytes and their subtypes in muscle repair clearly requires further study.

8. Concluding Remarks and Future Directions

Numerous recent studies have expanded our knowledge of the function of macrophages, which extends far beyond their role in host defense against bacteria or parasites. The progress in this field has led to the discovery of an increasing number of macrophage activation states, rendering their classification more difficult. If the *in vitro* studies on macrophages activation and their subsequent classification in M1 and M2 macrophages have been useful to mirror the Th1 and Th2 polarization of T cells, the M2 designation has expanded to include all of the non-M1 macrophages. Consequently, a growing number of immunologists now classify them in the extended family of M2-like macrophages. However, the plasticity of these cells makes it difficult to assign specific markers to each population, especially since phenotypic changes are temporally dynamic and depend on changes in the microenvironment and on cell intrinsic mechanisms, like in endotoxin tolerance, which represents a switch from a proinflammatory M1 phenotype to an M2-like anti-inflammatory phenotype. The discovery of new markers, together with progress in flow cytometry techniques, will probably increase even more the complexity of classifying macrophages, rendering it essential to rethink the way we create categories of macrophages and forcing us to focus on their function in order to define these different populations more precisely. Finally, in addition to more precisely defining and evaluating macrophage functions in tissue repair, future research should also focus on identifying in greater detail the function of alternative immune cell types, such as lymphocytes, in the correct resolution of tissue injury or, conversely, in facilitating fibrosis development.

Author's Contribution

Y. Kharraz and J. Guerra contributed equally to the paper.

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Research Article

The Role of Mcl-1 in *S. aureus*-Induced Cytoprotection of Infected Macrophages

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As a facultative intracellular pathogen, *Staphylococcus aureus* invades macrophages and then promotes the cytoprotection of infected cells thus stabilizing safe niche for silent persistence. This process occurs through the upregulation of crucial antiapoptotic genes, in particular, *myeloid cell leukemia-1 (MCL-1)*. Here, we investigated the underlying mechanism and signal transduction pathways leading to increased *MCL-1* expression in infected macrophages. Live *S. aureus* not only stimulated *de novo* synthesis of Mcl-1, but also prolonged the stability of this antiapoptotic protein. Consistent with this, we proved a crucial role of Mcl-1 in *S. aureus*-induced cytoprotection, since silencing of *MCL1* by siRNA profoundly reversed the cytoprotection of infected cells leading to apoptosis. Increased *MCL1* expression in infected cells was associated with enhanced NFκB activation and subsequent IL-6 secretion, since the inhibition of both NFκB and IL-6 signalling pathways abrogated Mcl-1 induction and cytoprotection. Finally, we confirmed our observation *in vivo* in murine model of septic arthritis showing the association between the severity of arthritis and Mcl-1 expression. Therefore, we propose that *S. aureus* is hijacking the Mcl-1-dependent inhibition of apoptosis to prevent the elimination of infected host cells, thus allowing the intracellular persistence of the pathogen, its dissemination by infected macrophages, and the progression of staphylococci diseases.

1. Introduction

Staphylococcus aureus is a major cause of community-acquired and nosocomial infections, including localised and systemic life-threatening conditions, such as osteomyelitis, endocarditis, pneumonia, and septicaemia [1]. Despite the increasing morbidity and mortality due to staphylococcal infections, relatively little is known about the molecular mechanisms by which this pathogen disseminates systemically. Recent studies have shown that *S. aureus* not only survives phagocytosis by neutrophils and macrophages but is also able to persist inside these cells [2, 3]. As is the case for *Listeria monocytogenes* and *Mycobacterium tuberculosis* [4–6], long-term survival, especially inside macrophages, may be a mechanism of dissemination of staphylococci. This hypothesis is further supported by the observation that intracellular *S. aureus* manipulates macrophage cell signalling processes and transcription to promote survival of infected phagocytes without bacterial eradication [7]. Therefore, precise elucidation of the mechanism of induction of cytoprotection in macrophages, a potential vehicle for pathogen spreading in the host, is of high importance.

Recently, we showed that live intracellular bacteria induce cytoprotection in human and murine macrophages, allowing the pathogen to silently persist and remain invisible to the immune system [3, 7]. We proposed that the pathogen induces a prosurvival signalling pathway through the induction of expression of antiapoptotic factors, including myeloid cell leukemia-1 (Mcl-1), an antiapoptotic protein of the Bcl-2 family. Mcl-1 possesses BCL-2 homology (BH) domains 1–4,

similar to other antiapoptotic BCL-2 family members (BCL-2, BCL-XL, BCL-W, and A1). It inhibits cell death by sequestering proapoptotic proteins (e.g., BAX, BAK, BIM, PUMA, and BAD), thus stabilising the mitochondrial membrane and preventing release of cytochrome *c* [8, 9]. *MCL1* expression can be induced by survival and differentiation signals such as cytokines and growth factors produced as a result of activation of a number of well-known signal transduction pathways (e.g., MAP kinases, PI3K/Akt, JAK/STAT, and NFκB) [10]. Cellular levels of Mcl-1 are regulated also by alteration of this protein turnover rate, which is dependent on PEST sequences within the N-terminal region of Mcl-1 and other motifs that target the protein for degradation by the proteasome [8].

In the current paper, we documented the elevated level of Mcl-1 in *S. aureus*-infected human primary macrophages and joints in murine model of septic arthritis, associated with infiltration of macrophages into the synovia. We showed for the first time that small inhibitory (si)RNA silencing of *MCL1* in *S. aureus*-infected macrophages abrogates cytoprotection against staurosporine-induced apoptosis, confirming the role of Mcl-1 in sustaining the viability of macrophages during infection. Furthermore, we established that *S. aureus* induced Mcl-1 through signalling pathways that required NFκB and IL-6, since inhibition of these pathways partly suppressed bacterial-mediated enhancement of Mcl-1 expression and cytoprotection. Therefore, we propose a model in which expression of prosurvival genes, such as *MCL1,* enables virulent *S. aureus* strains to circumvent cell death, thus ensuring a safe ecological niche prior to dissemination. Regulation of human macrophage longevity by intracellular *S. aureus* has clinical relevance for understanding the pathogenesis of *Staphylococci*-caused infectious diseases.

2. Materials and Methods

2.1. Cell Culture. PBMCs were isolated from human blood using a Lymphocyte Separation Medium (PAA) density gradient yielding the fraction highly enriched in monocytes (90% CD14-positive) as described previously [7]. Cells were plated at 3×10^6/well in 24-well plates (Sarstedt) in RPMI1640 (PAA) supplemented with 2 mM L-glutamine, 50 μg/mL gentamicin (Sigma), and 10% autological human serum. After 24 h, nonadherent PBMCs were removed by washing with complete medium, and adherent cells were differentiated to hMDMs in this medium for 7 days with fresh medium changed every 2 days. Blood was obtained from the Red Cross, Krakow, Poland. The Red Cross de-identified blood materials as appropriate for human subjects confidentiality assurance. Thus, the current paper adheres to appropriate exclusions from human subjects approval. The murine macrophage cell line RAW 264.7 obtained from American Type Culture Collection was maintained in DMEM (PAA) supplemented with 5% fetal bovine serum.

2.2. Bacterial Strains, Storage, and Growth Conditions. The laboratory *S. aureus* strain Newman was kindly provided by Dr. T. Foster (Trinity College, Dublin, Ireland) and *E. coli* strain was from laboratory stocks. Bacteria were grown to the stationary growth phase at 37°C overnight under constant rotation (180 rpm), then were collected by centrifugation (5,000 ×g, 8 min), washed with phosphate-buffered saline, and resuspended in PBS to the desired $OD_{600\,nm}$. Staphylococci were opsonized by incubation at 37°C for 30 min in heat inactivated FCS, washed, and resuspended in PBS. Numbers of vital bacteria in samples used in phagocytosis assay were routinely verified by plating dilutions on agar plates and counting colonies to determine CFU per mL. Heat treatment (80°C for one hour) was used to kill bacteria.

2.3. Macrophages Infection. Macrophage infection was performed using *S. aureus* and *E. coli* as described previously [7]. Unspecific cell activation by phagocytosis of inert particles was determined using latex beads (1.1 μm; Sigma) as described previously [7]. Phagocytosis assays were carried out for 2 hours at 37°C at a multiplicity of infection (MOI) of 1 : 50 (hMDMs) or 1 : 5 (RAW 264.7), resulting in >85% of macrophages engulfing at least one bacterium. After that time cells were rinsed 4 times with ice-cold phosphate-buffered saline. Any remaining nonphagocytosed bacteria were killed by culturing in medium containing gentamicin (50 μg/mL) for 24 h. The medium was then replaced with fresh media without antibiotics, and cultures were maintained for the desired time. Cells treated identically, but without bacteria, were analyzed in all experiments as a control (mock-infected cells).

For inhibitor experiments, before inoculation with bacteria or applying control stimulus, macrophages were preincubated for 30 min with nontoxic doses (data not shown) of the NFκB inhibitor Bay 11-7082 (2–40 μM). Where indicated, cycloheximide (a final concentration 10 μg/mL) was added 30 min before infection with bacteria and present in media until cells were washed 30 min after infection. This treatment reduced *de novo* translation of proteins in macrophage by 95.5% as determined by ^{35}S-Met incorporation (data not shown).

2.4. Viability Assays. After *S. aureus* phagocytosis and/or treatment with compounds inducing apoptosis, macrophage viability was examined by lactate dehydrogenase (LDH) release. The LDH release assay was performed using a CytoTox96 Non-Radioactive Lactate Dehydrogenase Cytotoxicity Assay kit (Promega). Infected and control hMDMs or RAW 264.7 cells in a 24-well tissue culture plate (3×10^5 cells per well) were treated with 1 μM staurosporine (STS; Sigma) added 2 h or 24 h after-infection as a stimulator of apoptosis. Samples were then incubated for 6 h or 24 h, and 200 μL of culture medium were withdrawn and subjected to analysis as described previously [7].

2.5. Analysis of Caspase-3 Activity. The activity of caspase-3 was determined by release of 7-amino-4-trifluoromethylcoumarin (AFC) from a DEVD-AFC peptide substrate. Cells, both control and exposed to *S. aureus*, with or without apoptotic stimuli, were collected by centrifugation (200 ×g, 5 min, 4°C), washed with ice-cold PBS, and resuspended

in 100 μL of lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, and 0.1% SDS). Samples were then incubated on ice for 20 min and subjected to centrifugation (16,000 ×g, 10 min). The protein content of supernatants was measured using a BCA method, and caspase activity was determined by using a Spectra Max Gemini EM (Molecular Devices) as described previously [7].

2.6. Protein Isolation and Immunoblotting. Whole cellular extracts from control and stimulated cells were prepared using 100 μL of RIPA-lysis buffer (0.25% Na-deoxycholate, 0.5% Nonidet P-40, 0.05% SDS, protease inhibitor cocktail, and 2.5 mM EDTA in PBS) and stored at −20°C. The joints of DBA1 mice were homogenized in 300 μL of RIPA-lysis buffer. Equal amounts of protein (40 μg/well) were separated using SDS-PAGE (12% or 16% gels depending on molecular mass of proteins of interest) and electrotransferred onto nitrocellulose membranes (BioRad) in buffer composed of 25 mM Tris, 0.2 M glycine, and 20% methanol (30 V, overnight). Nonspecific binding sites were blocked with 3% BSA in TTBS buffer (20 mM Tris, 0.5 M NaCl, and pH 7.5 with 0.05% Tween 20) for 1 h, followed by 1-2-hour incubation with the relevant primary antibody: 100-fold diluted anti-Mcl-1 (Santa Cruz), or 3,000-fold diluted anti-β-actin (Sigma). Membranes were washed extensively in TTBS buffer and incubated with secondary horseradish peroxidase-(HRP-) conjugated antibodies, 10,000-fold diluted donkey anti-rabbit IgG, or 20,000-fold diluted sheep anti-mouse IgG, for 1 h in TTBS buffer containing 1% BSA. Membranes were washed (4×15 min) in TTBS buffer, and blots were developed using ECL detection (Western Blotting Detection Reagents; Amersham Biosciences).

2.7. Densitometric Analyses. Densitometric analyses of western blots were performed using Kodak Digital Software. Results are presented as mean values of arbitrary densitometric units corrected for background intensity or as the fold of increase over a level characteristic for nonstimulated cells.

2.8. Quantitative PCR (qRT-PCR). Total cellular RNA was extracted from cultured hMDMs using aRNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. RNA samples were DNase treated, and cDNA was prepared by reverse transcription using RevertAidTM First Strand cDNA Synthesis Kit (Fermentas). Five hundred nanograms of RNA from each sample were used for cDNA synthesis reaction with oligo(dT) primers according to the manufacturer's instructions. Quantitative PCR reaction was performed with an SYBR Green method in a reaction volume of 20 μL, containing 1 μL of cDNA sample, 0.5 μM of each primer, and 1x SYBR Green JumpStart Taq Ready Mix (Sigma). qRT-PCR forward and reverse primers for *IL-6*, *MCL1*, and *MCL1S* genes and for the housekeeping *EF-2* gene (used for normalization) are listed in Table 1. After 5 min of initial denaturation at 95°C, reactions were carried out for 40 cycles at the given conditions: denaturation, 95°C, 20 sec; annealing, 56°C–62°C (as shown in Table 1), 60 sec; extension, 72°C, 60 sec; followed by a final elongation step at 72°C for 10 min. All the reactions were performed in duplicates. Means for threshold cycle (Ct) values were calculated and analyzed using the "delta-delta Ct" quantification method [11]. Routinely, for the evaluation of quality of qRT-PCR reactions, samples were resolved on nondenaturing 1.5% agarose gels and visualized by staining with ethidium bromide.

2.9. Cytokine Assay. Two hundred μL of cell culture supernatants were collected and stored at −80°C until analysis. The level of IL-6 was determined by using commercially available ELISA kits according to the manufacturer's instructions (R&D Systems).

2.10. Transfection with siRNA. Silencing of the *MCL1* gene expression was accomplished by transfection of cells with specific siRNA or with negative control siRNA (Accell Smart pool and Control pool, resp., Thermo Scientific Dharmacon). Briefly, for each of transfected wells, siRNA (final concentration 60 nM) was combined with 3 μL of lipofectamine 2000 (Invitrogen) in Opti-MEM medium (Invitrogen). Transfection was performed for 4 h followed by 24 h of normal cell growth before desired assays were performed.

2.11. Evaluation of NFκB Activity. NFκB activity was measured in nuclear protein extracts by the EMSA method. Nuclear extracts were prepared by a miniextraction procedure as described before [12]. The protein concentration was measured with bicinchoninic acid (BCA method), and the obtained extracts were frozen (−80°C) in 10% glycerol. For the NF-κB-directed EMSA, double-stranded probes were prepared using the pair of primers AGCTTCAGAGGGGACTTTCCGAGAGG and AGTCTCCCCTGAAAGGCTCTCCTCGA. EMSA was performed as described before [12].

2.12. Septic Arthritis Induction and Examination of Infection. Male DBA1 mice (8–12 weeks old) were obtained from the breeding unit of the Department of Human Developmental Biology, Jagiellonian University, School of Medicine. Mice were fed autoclaved food and water. All experiments were conducted according to guidelines of the Animal Use and Care Committee of the Jagiellonian University School of Medicine. Mice were inoculated with *S. aureus* in the tail vein (5×10^7 in 200 μL) at day 0. The overall condition was evaluated by assessment of body weight and general appearance. The hind paws and forepaws were inspected every second day. Animals were observed daily for the presence of arthritis, and the clinical severity of disease was scored for each paw on a scale of 0–4, with the index being the sum of the scores for all four paws. The criteria for the grading were as follows: 0: no evidence of erythema and swelling; 1: mild erythema and swelling of the wrist or the ankle; 2: moderate erythema and swelling from the wrist to the metacarpal joints or from the ankle to the metatarsal joints; 3: severe erythema and swelling of the entire paw including digits; 4: maximal erythema and swelling of the paw. The bacterial load in joints, kidney, and spleen was examined at time of sacrifice (8 day p.i.). The organ homogenates were

Table 1: Oligonucleotide sequences used in qRT-PCR.

Oligonucleotide	Annealing (°C)	Sequence
EF-2 F	62	5′-GACATCACCAAGGGTGTGCAG-3′
EF-2 R	62	5′-TCAGCACACTGGCATAGAGGC-3′
IL-6 F	54	5′-CATCTTTGGAAGGTTCAGGTTTGT-3′
IL-6 R	54	5′-AGCCCTGAGAAAGGAGACATGTA-3′
MCL1 F	62	5′-TAAGGACAAAACGGGACTGG-3′
MCL1 R	62	5′-ACCAGCTCCTACTCCAGCAA-3′
MCL1S F	62	5′-GCAACCACGAGACGGCC-3′
MCL1S R	62	5′-GATGCCACCTTCTAGGTCCTCTAC-3′

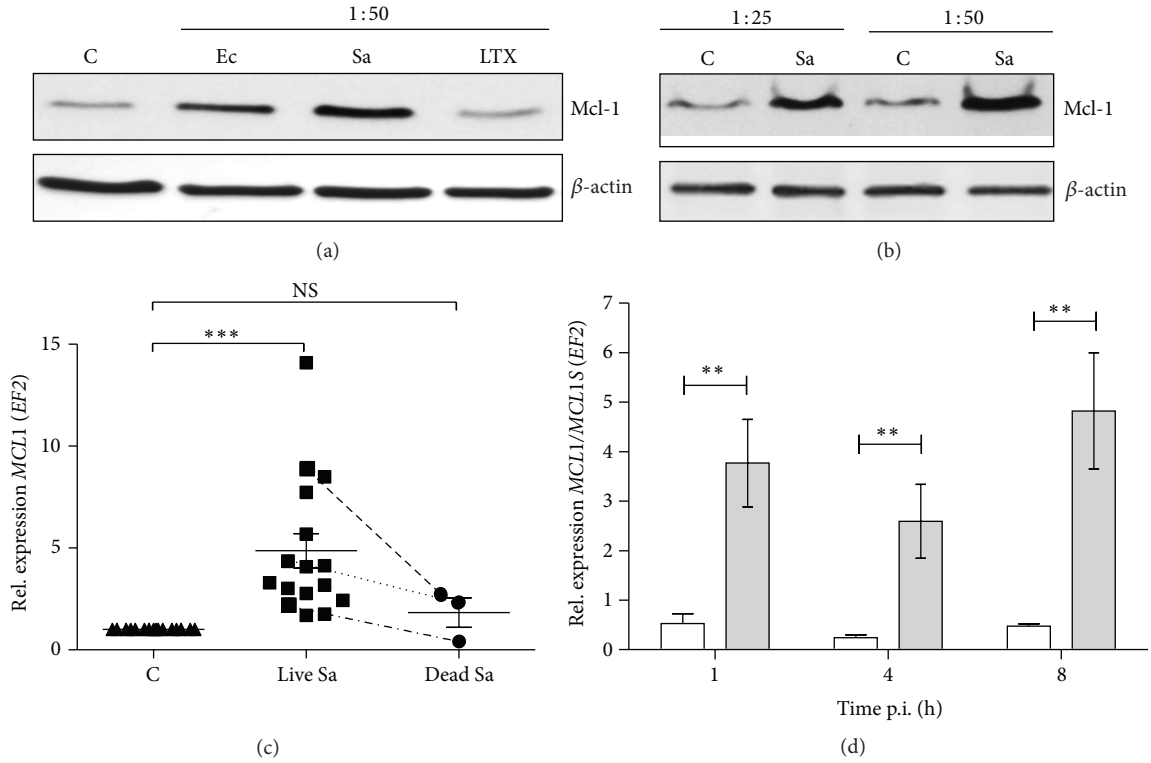

Figure 1: Mcl-1 expression is induced specifically by *S. aureus* phagocytosis and is dependent on bacterial dose and viability. (a) The effect of phagocytosis of *S. aureus* (Sa), *E. coli* (Ec), and latex beads (LTX) on Mcl-1 protein levels was assessed by immunoblot. After phagocytosis, cells were cultured for 8 h and then protein fractions were prepared as described in Section 2. Representative immunoblot from three separate experiments performed on hMDMs derived from different donors is shown. Mcl-1 was visualised by immunoblot using anti-Mcl-1-specific antibodies. (b) The effect of *S. aureus* phagocytosis on Mcl-1 levels is proportional to an infection dose. A representative immunoblot from three separate experiments performed on hMDMs derived from different donors is shown. (c) The effect of phagocytosis of live (Sa) and dead, heat-killed *S. aureus* (Sa HI) on *MCL1* expression was determined by qRT-PCR. Data are from independent reactions using hMDMs derived from different donors. Paired points represent the response of hMDMs obtained from the same donors to both live and dead bacteria. Bars represent relative means ± SD. $^{***}P < 0.001$; NS: not significant. (d) The comparison of expression levels of proapoptotic *MCL1S* (white bars) versus antiapoptotic *MCL1* (filled bars). Results were obtained by qRT-PCR from three separate experiments. $^{**}P < 0.01$.

plated on tryptic soy agar and CFU counted after overnight incubation at 37°C.

2.13. Statistics. Results were analyzed for statistical significance using the nonparametric Student's *t*-test. Differences were considered significant when $P < 0.05$.

3. Results

3.1. S. aureus Specifically Induces Mcl-1 Expression in Human Macrophages. We previously showed that *S. aureus* can protect infected macrophages against apoptosis through upregulation of expression of antiapoptotic genes [7]. Among these genes, *MCL1* plays a key role in macrophage survival

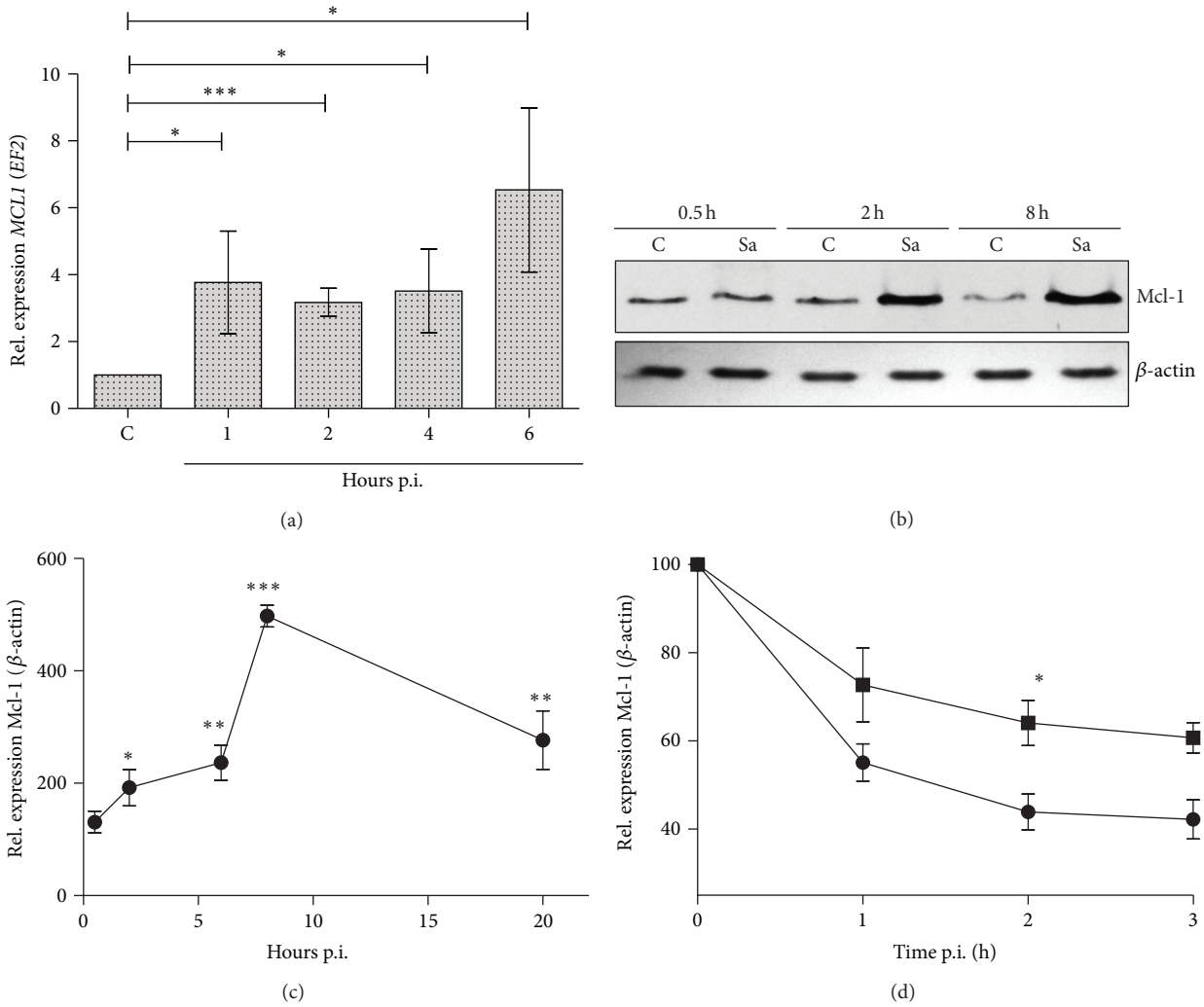

Figure 2: *S. aureus* increases both *de novo* Mcl-1 synthesis and stability. (a) Time course of *MCL1* expression in control and *S. aureus*-infected macrophages (hours after-infection; p.i.) was monitored by qRT-PCR, as described in Section 2. Data represent the mean values calculated from the results of three independent experiments using hMDMs derived from different donors. Bars represent mean relative expression ± SD. $^{*}P < 0.05$; $^{***}P < 0.001$. (b, c) Time course of Mcl-1 protein synthesis following *S. aureus* infection. Mcl-1 levels were measured at different time points between 0.5 and 20 h p.i. by immunoblot. (b) Representative immunoblot from three separate experiments performed on macrophages derived from different donors. (c) Relative Mcl-1 levels obtained by densitometric analyses of western blots. Results from three separate experiments. Data represent means ± SD. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. (d) Mcl-1 stability in macrophages incubated in the presence of cycloheximide (CHX) (10 μg/mL) in the absence (circles) or presence (squares) of *S. aureus* (MOI 1 : 50). At time periods of up to 3 h, Mcl-1 levels were detected by immunoblot in cell lysates. Data represents Mcl-1 levels relative to time 0, which was arbitrarily set as 100%, obtained by densitometric analyses of western blots. Data represent means ± SD of three separate experiments. $^{*}P < 0.05$.

[13, 14]. Here, we investigated potential mechanisms of Mcl-1 regulation, as well as its role in cytoprotection induced by *S. aureus*. The Mcl-1 induction in response to phagocytosis of different bacteria and particles was examined by incubating macrophages with *S. aureus*, *E. coli,* or latex beads for 8 h. *S. aureus* induced the highest level of Mcl-1, about five-times more (4.78 ± 0.96-fold above the control level) than that seen in mock-infected cells (Figure 1(a)). By contrast, no change in Mcl-1 levels was observed after incubation with latex beads (1.06 ± 0.06) and only a slight upshift after *E. coli* (1.78 ± 0.63) (Figure 1(a)). The enhanced Mcl-1 production in response to *S. aureus* was proportional to infection rate (Figure 1(b)) and was exerted only by viable bacterial cells (Figure 1(c)). The latter was clearly apparent from a comparison of relative levels of *MCL1* mRNA induced in response to live or dead bacteria in macrophages derived from different blood donors (8.87 versus 2.75, 4.36 versus 2.35, and 2.24 versus 0.4, resp.). An intriguing feature of *S. aureus*-induced Mcl-1 expression was the synthesis of an alternatively spliced *MCL1* gene product (*MCL1S* proapoptotic isoform), which was observed at the mRNA level early after infection (Figure 1(d)). Significantly, however, the expression of *MCL1S* was at much lower level compared to the full-length, antiapoptotic *MCL1* form (Figure 1(d)).

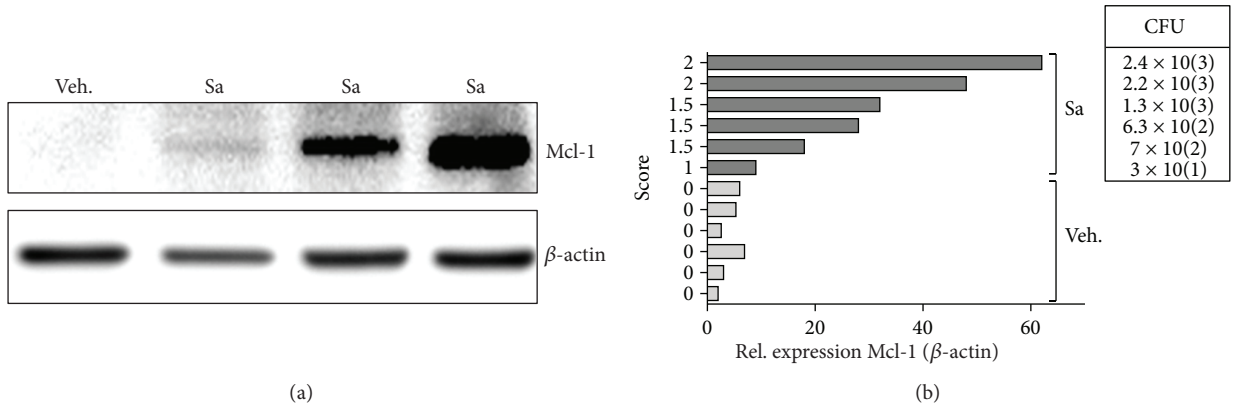

Figure 3: Exposure to *S. aureus* triggers Mcl-1 production *in vivo.* (a) Immunoblot reveals increase in Mcl-1 expression in inflamed joints derived from three individual *S. aureus*-infected mice (Sa) in comparison to noninfected animals (Veh.). A representative immunoblot from three separate experiments is shown. (b) The expression of Mcl-1 in joints correlates with infection score and bacterial load determined as described in Section 2. Data represents Mcl-1 levels in noninfected (Veh.; $n = 6$) and *S. aureus*-infected (Sa; $n = 6$) animals, obtained by densitometric analyses of western blots.

Taken together, these results suggested that stimulation of Mcl-1 expression in macrophages is preferentially induced by viable *S. aureus*.

3.2. S. aureus Influences Mcl-1 Synthesis and Turnover Rate. To correlate *S. aureus*-induced cytoprotection with the expression of *MCL1,* we determined the time dependence of the induction of specific mRNA after macrophage challenge with bacteria. A 4-fold increase of *MCL1* mRNA was observed 1 h after-infection, with sustained upregulation observed for up to 6 h (Figure 2(a)). At the protein level, Mcl-1 levels were significantly increased 2 h after-infection, reached a maximum at 8 h, and remained at 3-fold higher levels compared to mock-infected cells for at least 20 h (Figures 2(b) and 2(c)).

Since the observed high levels of expression of Mcl-1 in *S. aureus*-infected macrophages could be the result of either *de novo* synthesis or the decreased turnover rate, we compared Mcl-1 stability in macrophages treated with cycloheximide in the absence and presence of *S. aureus*. As shown in Figure 2(d), in mock-infected cells, blocking *de novo* biosynthesis resulted in a rapid decrease in the cellular levels of Mcl-1. By contrast, in cells infected with *S. aureus*, the level of Mcl-1 was significantly higher (Figure 2(d)). Cumulatively, these results clearly showed the dual nature of the effects of *S. aureus* on Mcl-1, which involved increased Mcl-1 protein synthesis as well as increased protein stability.

3.3. The Mcl-1 Expression Correlates with Prevalence and Severity of S. aureus-Induced Arthritis. S. aureus is the causative agent in about 60% of nongonococcal bacterial arthritis cases, a disease characterized among others by robust influx of macrophages and their sustain activation in joints [15, 16]. Therefore, we determined Mcl-1 expression in inflamed joints in the previously established murine model of *S. aureus* arthritis [17]. To this end DBA1 mice were injected i.v. with 5×10^7 CFU, a dose causing a low mortality rate (see Supplementary Figure 1(a) in Supplementary Material available online at http://dx.doi.org/10.1155/2013/427021). At day 8 after injection all animals showed clear symptoms of arthritis (Supplementary Figure 1(b)). Bacteriological examination of joints, spleen, and kidneys revealed the abundant load of *S. aureus* in 100% of mice (Supplementary Figure 1(c)). This finding correlates with inflammatory response manifested by IL-6 secretion (Supplementary Figure 1(d)) and splenomegaly (data not shown). Further investigation of the relationship between both clinical and bacteriological signs of arthritis and Mcl-1 expression in joints revealed the significant association (Figures 3(a) and 3(b)). We found Mcl-1 expression being significantly upregulated in *S. aureus*-positive joints (32.83 ± 7.94-fold above the control level) in comparison to noninfected tissues (4.29 ± 0.82-fold above the control level, $P < 0.001$). Furthermore, we also observed positive association between the Mcl-1 expression level and bacterial load in joints. This indicates that *S. aureus* induced Mcl-1 expression also *in vivo* in the inflamed tissue.

3.4. Downregulation of Mcl-1 Interferes with S. aureus-Induced Cytoprotection. As we described previously *S. aureus* protects infected macrophages, both human and murine, against induced cell death [7]. Thus, to further determine whether the survival of infected macrophages in response to proapoptotic stimulants was dependent on Mcl-1 synthesis, RNA interference with siRNA was used to selectively silence *MCL1* gene expression. Treatment of cells with an *MCL1*-specific siRNA, but not a nonspecific control siRNA, resulted in specific and efficient suppression of Mcl-1 protein levels at 24–72 h after-transfection (data not shown) without effect on macrophages viability. The infection of macrophages 24 h after-transfection has not influenced the low level of already silenced protein within 24–48 h after infection (Figure 4(a)). The increasing caspase-3 activity (Figure 4(b)) and a lactate dehydrogenase leaking from macrophages (Figure 4(c)) revealed that the knockdown of *MCL1* expression

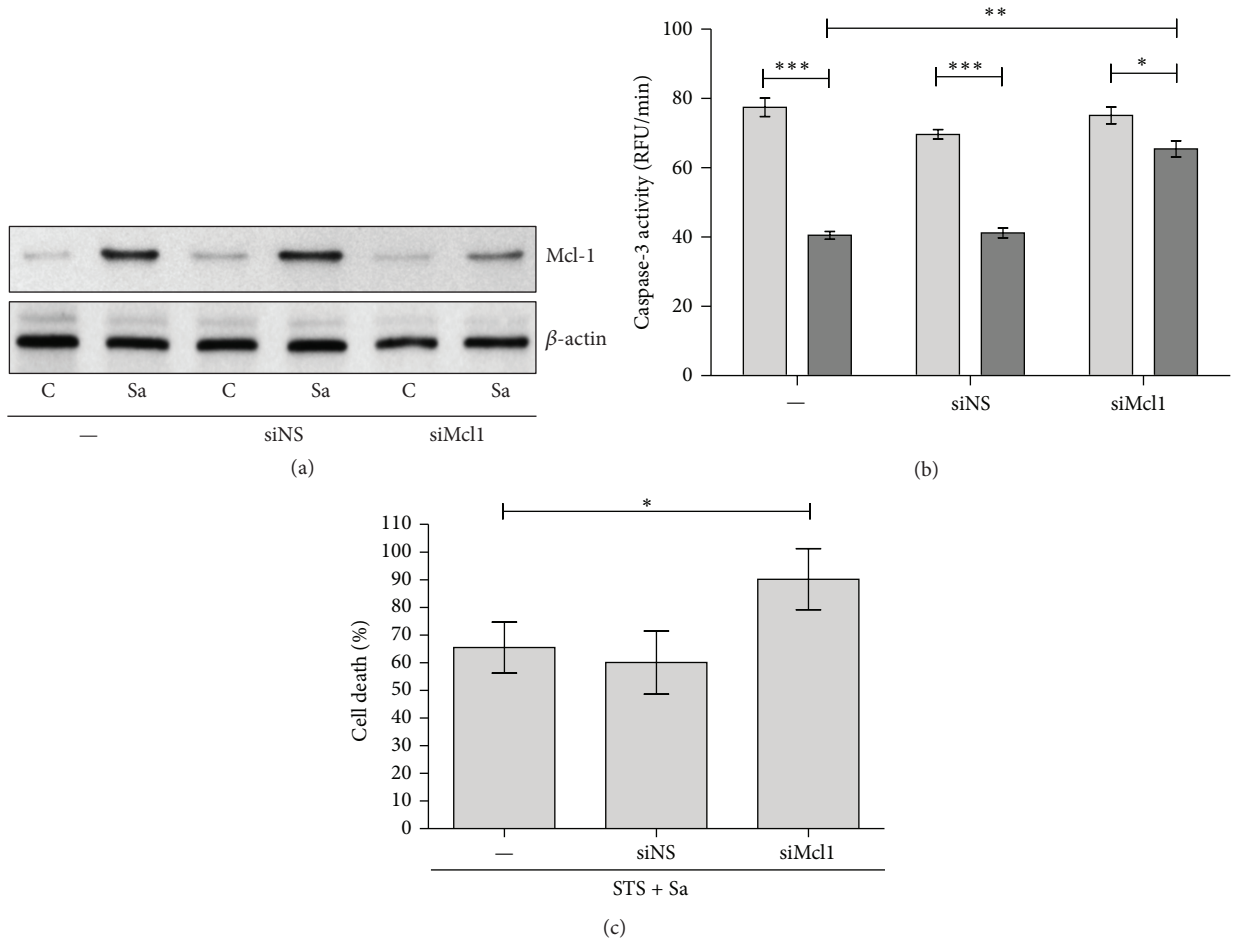

Figure 4: Effect of Mcl-1 expression on cytoprotection induced by *S. aureus* in hMDMs. (a) Human macrophages were treated with an *MCL1* siRNA (siMCL1) or a nonspecific siRNA (siNS). At 24 h following transfection macrophages were infected with *S. aureus* at an MOI 1 : 50. After additional 24 h cells were collected and Mcl-1 expression was detected by immunoblot. Data are representative of three separate experiments using hMDMs derived from different donors. (b) The increase in caspase-3 activity (RFU/min) induced by STS in *MCL1* knockdown macrophages infected with *S. aureus*. Twenty-four hours after treatment with siRNA, hMDMs were infected with *S. aureus* (24 h), followed by treatment with STS at a concentration of 1 μM for 18 h. The measurement of caspase-3 activity (RFU/min) in cell lysates was performed using DEVD-AFC as a substrate. The figure is representative of three experiments, using hMDMs cultures obtained from different donors. Light bars—STS, dark bars—Sa + STS. Data represent means ± SD. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. (c) Increased susceptibility to the cytotoxic effects of staurosporine (STS) in *MCL1* knockdown macrophages infected with *S. aureus*. Twenty-four hours after treatment with siRNA, hMDMs were infected with *S. aureus* (24 h), followed by treatment with STS at a concentration of 1 μM for 24 h. Plasma membrane permeabilisation or cell lysis induced in the hMDMs was assessed by measuring LDH activity in the culture medium. LDH activity in the media of cells treated only with STS was arbitrarily set as 100%. Results were calculated based on data (± SD) from three separate experiments. $^{*}P < 0.05$.

significantly attenuated the *S. aureus*-exerted cytoprotection of cells in a staurosporine-induced cell death model. Our data also indicates (Supplementary Figure 2) that silencing of *Mcl-1* in *S. aureus*-infected macrophages partly ablates the cytoprotection against spontaneous cell death. This observation confirmed that Mcl-1 plays an important role in preventing apoptosis in *S. aureus*-infected human macrophages.

3.5. S. aureus-Dependent Mcl-1 Expression Is Regulated by IL-6. IL-6 upregulates Mcl-1 in human myeloma cells [18]. To determine whether IL-6 was also playing a role in *S. aureus*-induced Mcl-1 expression in hMDMs, macrophages were infected with *S. aureus,* what leads to IL-6 secretion within 24 h after infection (Supplement Figure 3). Both high mRNA and protein Mcl-1 expression observed after bacteria phagocytosis (Figures 5(a) and 5(b), resp.) was markedly reduced upon treatment of cells with IL-6 receptor (R) neutralising antibodies. Together, these results demonstrated that *S. aureus*-induced Mcl-1 expression is partly mediated by IL-6.

3.6. The NFκB Pathway Is Involved in IL-6-Dependent Regulation of Mcl-1 Expression Induced by S. aureus. A variety of intracellular signalling pathways are activated by pathogens.

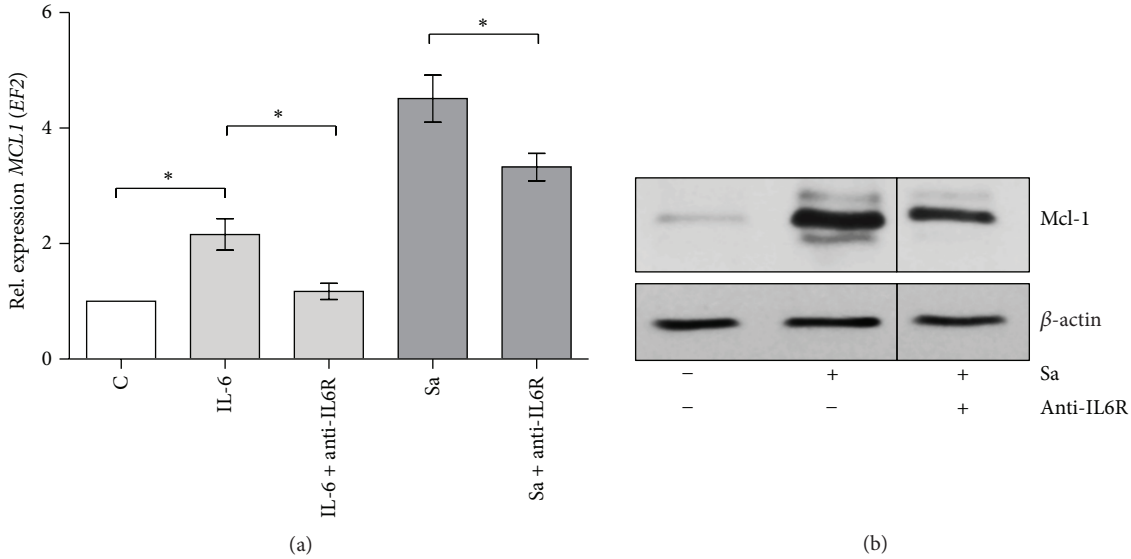

Figure 5: Mcl-1 expression induced by *S. aureus* is mediated by IL-6. (a) hMDMs were preincubated with anti-IL6 receptor antibodies (1 μg/mL) for 30 min and then infected with *S. aureus* at an MOI of 1 : 50 and/or stimulated with IL-6 (200 ng/mL). At 7 h p.i., RNA was extracted and relative *MCL1* expression was measured by qRT-PCR. Diagram shows the mean values calculated from the results of at least three independent real-time reactions using hMDMs derived from different donors. Bars represent mean relative expression ± SD. $^{*}P < 0.05$. (b) hMDMs were preincubated with anti-IL6 receptor (1 μg/mL) antibodies for 30 min and then infected with *S. aureus* Newman at an MOI of 1 : 50. The effect of *S. aureus* on Mcl-1 protein synthesis was measured 20 h after-infection by immunoblot. Shown is a representative immunoblot from three separate experiments performed on hMDMs derived from different donors.

Among them, activation of NFκB has been shown to be critical for cytoprotection of infected cells [19]. Moreover, *S. aureus* is a potent inducer of NFκB activity as was confirmed in infected macrophages by EMSA (Supplement Figure 4). To determine the effect of inhibition of the NFκB pathway on Mcl-1 expression in hMDMs, macrophages were infected with *S. aureus* followed by incubation for 6 h with a specific NFκB-inhibitor, and then the levels of *MCL1* were assessed in comparison to untreated infected cells. As seen in Figure 6(a), inhibition of the NFκB pathway abrogated the *S. aureus*-induced increase in *MCL1* gene transcription. This effect was confirmed at the protein level as well (Figure 6(b)). These results strongly suggested that Mcl-1 expression in *S. aureus*-infected cells is dependent on NFκB. Since IL-6 transcription is upregulated in an NFκB-dependent manner [20], we investigated the possibility that NFκB stimulated *MCL1* expression indirectly, via IL-6. *S. aureus*-induced production of IL-6 in infected macrophages was abrogated by treatment with Bay 11-7095, the NFκB-specific cell-permeable inhibitor, which indicated that IL-6 production was absolutely dependent on NFκB (Figure 6(c)). Taken together, these findings indicate that IL-6-mediated Mcl-1 expression in infected macrophages is regulated through the NFκB pathway.

Since we proved that the *S. aureus* mediated Mcl-1 induction in both human and murine model, therefore, in all further experiments we routinely use the hMDMs and/or RAW 264.7 cell line.

3.7. NFκB and IL-6 Are Required for S. aureus-Induced Inhibition of Macrophage Apoptosis. Based on the fact that *S. aureus* delayed apoptosis through Mcl-1 upregulation in infected macrophages we investigated whether NFκB-dependent pathways and/or IL-6 were necessary for *S. aureus*-induced inhibition of apoptosis. In this experiment we used both hMDMs and murine macrophage RAW 264.7 cell line, the latter to verify the results of Mcl-1 induction in mice joints by *S. aureus* infection. Preincubation of macrophages with an NFκB inhibitor (Bay 11-7095) reversed antiapoptotic effect (measured by caspase-3 activation) induced by *S. aureus* in staurosporine-treated cells (Figure 7(a)). The viability of hMDMs was also assessed after blocking IL-6 signalling. Pretreatment of human macrophages with IL-6R neutralising antibodies partly abolished *S. aureus*-induced protection against cell death exerted by staurosporine, but had no effect on cytoprotection against cycloheximide- (CHX-) mediated cell death (Figure 7(b)). To determine whether IL-6 acted in an autocrine manner to prevent cell death induced by staurosporine, conditioned media from *S. aureus*-infected macrophages containing abundant IL-6 (Supplement Figure 3) was added to noninfected macrophages, followed by treatment of the cells with staurosporine. To confirm the role of secreted upon *S. aureus* infection IL-6 on suppression of macrophages apoptosis we blocked the action of IL-6 using anti-IL6 receptor (1 μg/mL) antibodies. Measurement of caspase-3 activity revealed a significant effect, albeit one that was weaker than that induced by *S. aureus* infection, of

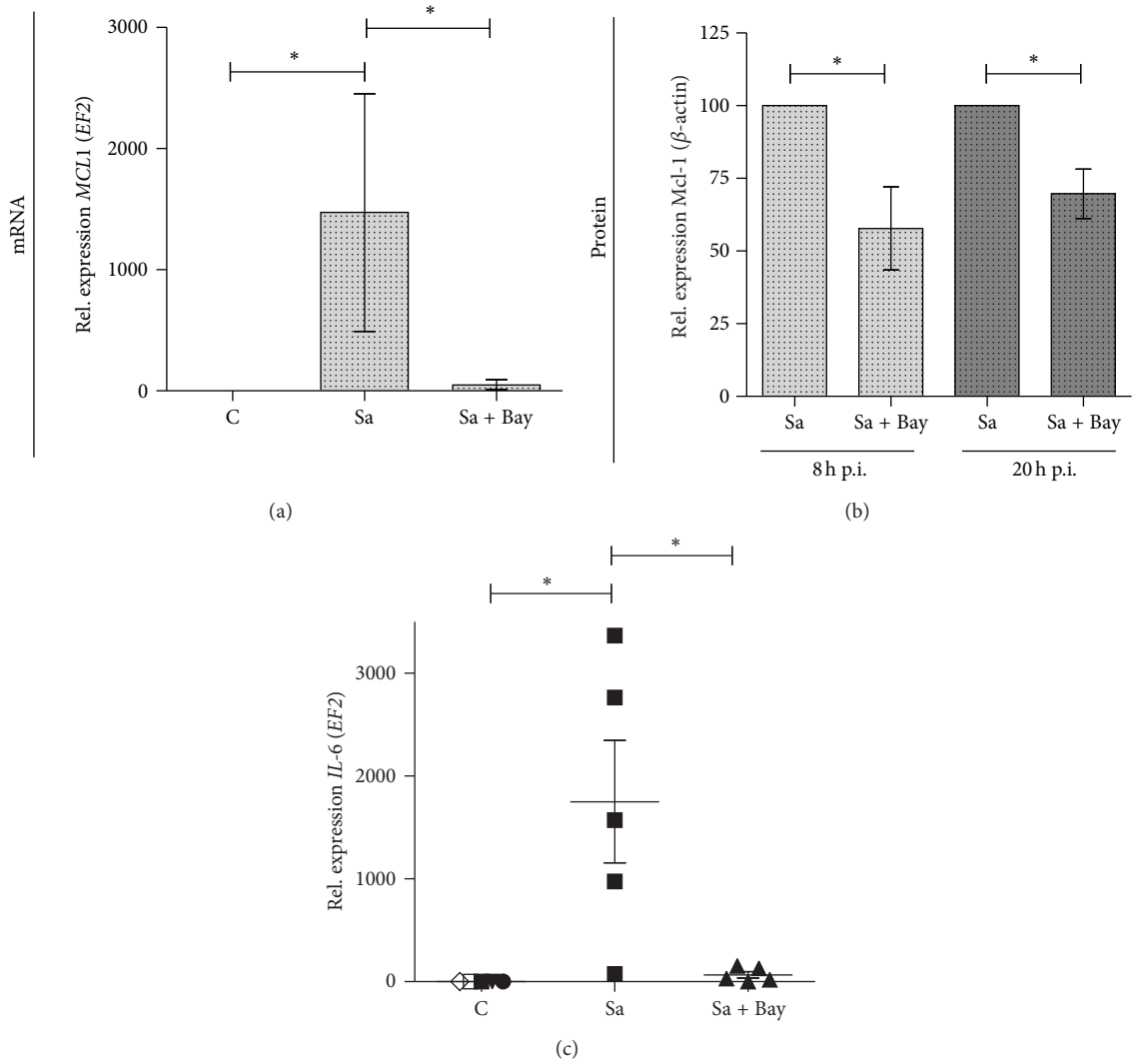

Figure 6: NFκB is required for Mcl-1 expression induced by *S. aureus*. (a, b) hMDMs were pretreated for 30 min with Bay 11-7095 (40 μM) followed by *S. aureus* infection at an MOI of 50. After the indicated times, RNA and protein were extracted and Mcl-1 expression levels were determined by qRT-PCR and immunoblot (a and b, resp.). Data represent the means ± SD from three separate experiments. $^{*}P < 0.05$. (c) The effect of NFκB inhibition on IL-6 expression was measured by qRT-PCR. Shown are the mean values calculated from the results of five independent real-time reactions using hMDMs derived from different donors. Bars represent mean relative expression ± SD; $^{*}P < 0.05$.

the conditioned medium on cytoprotection, which can be partly reversed by IL-6 signalling inhibition (Figure 7(c)). Cumulatively, these data demonstrate that the induction of IL-6 production in macrophages upon *S. aureus* infection is an important factor in the activation of downstream events that lead to suppression of the intrinsic apoptotic pathway through *de novo* synthesis Mcl-1.

4. Discussion

Results from recent *in vitro* studies have demonstrated that macrophages infected with *S. aureus* are resistant to apoptosis (both induced and spontaneous), and this apparent cytoprotective effect is a consequence of the significant upregulation of antiapoptotic genes, especially those involved in the mitochondrial pathway, such as *MCL1* [7]. Here, we investigated this phenomenon in more detail and showed for the first time that *S. aureus*-induced resistance to cell death is highly dependent on Mcl-1 expression since specific silencing of *MCL1* abrogated the effect. In this respect, *S. aureus* clearly resembles *M. tuberculosis* or respiratory syncytial virus (RSV), well-known obligatory intracellular pathogens that employ a similar strategy [21, 22].

Mcl-1 is a short-lived cytoplasmic protein (half-life of approximately 3 h) destined for prompt degradation by the proteasome [23, 24]. This fast proteolytic removal of Mcl-1 was markedly slowed in *S. aureus*-infected macrophages. The level of Mcl-1 rapidly increased within 2 h after bacterial infection, reached a maximum at 6 h, and then remained elevated for up to 24 h. In addition to enhanced *MCL1* gene expression [7], this increase in Mcl-1 levels was due in large part to increased Mcl-1 stability. In contrast to

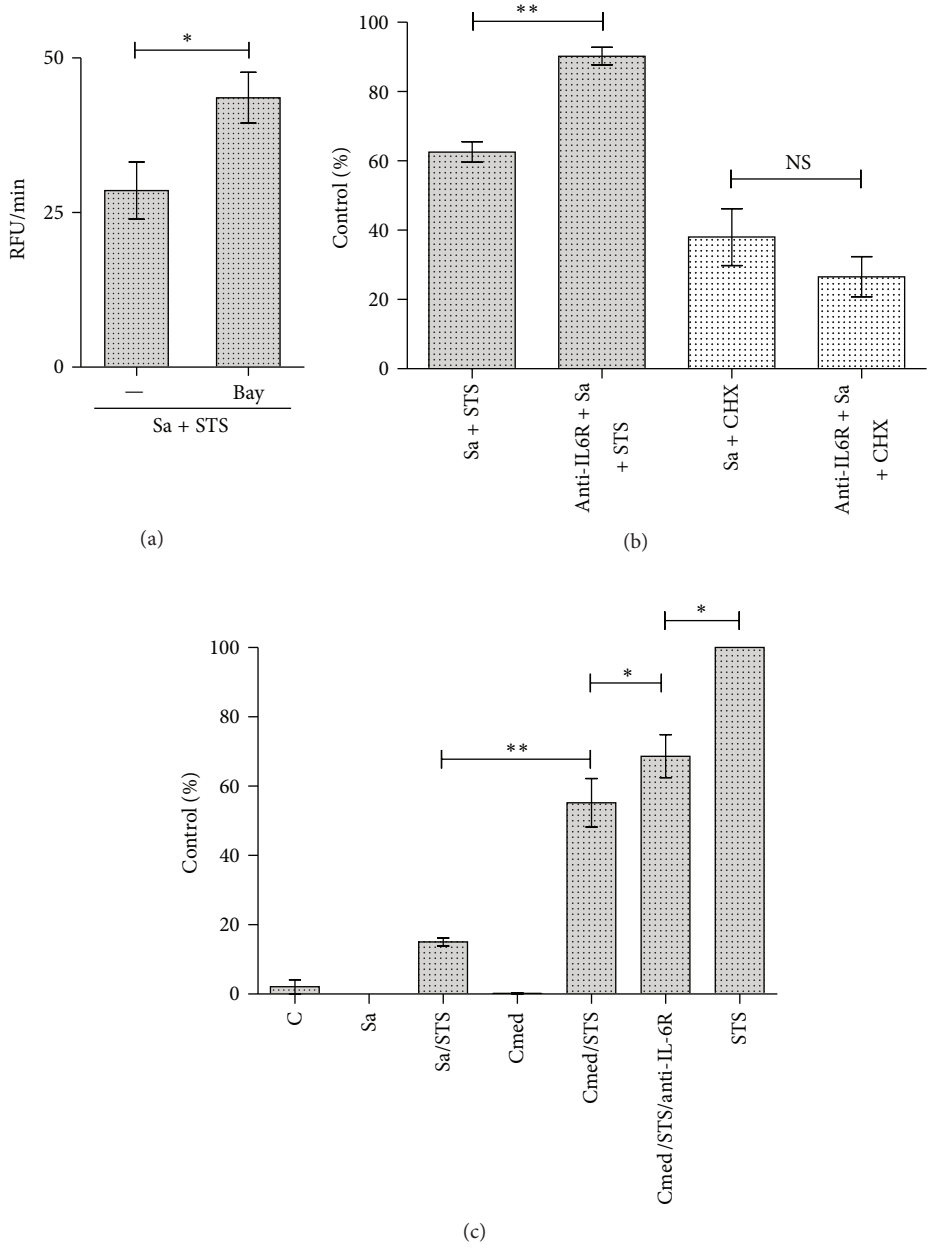

Figure 7: The role of NFκB and IL-6 in cytoprotection induced by *S. aureus*. (a) Inhibition of *S. aureus*-induced cytoprotection upon treatment with an NFκB inhibitor was assessed by measuring caspase-3 activation. RAW 264.7 macrophages were pretreated for 30 min with Bay 11-7095 (4 μM) followed by bacterial infection at an MOI of 5 for 2 h. STS was added for an additional 3 h, and then caspase-3 activity was measured in cell lysates (as described in Section 2). Data represent mean ± SD caspase-3 activity (RFU/min) from three separate experiments. $^{*}P < 0.05$. (b, c) The influence of IL-6 on STS- (1 μM) or CHX- (10 μM) induced cytotoxicity in *S. aureus*-infected macrophages. (b) hMDMs were preincubated with anti-IL6 receptor antibodies (1 μg/mL) for 1 h followed by *S. aureus* infection at an MOI of 1 : 50 for 2. STS or CHX was added for 6 h, and then permeabilisation of the plasma membrane or cell lysis was determined by measuring LDH activity in conditioned media. Cell death after treatment with STS or CHX alone was set as 100%. Diagram shows the mean values calculated from the results of three independent experiments using hMDMs derived from different donors. Bars represent mean relative expression ± SD; $^{**}P < 0.01$; NS: not significant. (c) RAW 264.7 cells were infected with *S. aureus* (MOI 1 : 5) or stimulated with conditioned medium collected from infected RAW 264.7 cells 5 h p.i. alone (cmed) or with anti-IL6 receptor (1 μg/mL) antibodies (cmed + anty-IL6R). At 2 h after stimulation, macrophages were treated with STS for another 4 h and then caspase-3 activity was measured. Caspase-3 activation induced by STS alone was set as 100%. Shown are mean values calculated from the results of three independent experiments. Bars represent mean relative expression ± SD; $^{*}P < 0.05$; $^{**}P < 0.01$.

control cells, the relative levels of Mcl-1 in infected cells were only slightly decreased by inhibition of protein translation. This is consistent with the resistance of *S. aureus* infected macrophages to cell death experimentally induced by cycloheximide. Thus, apoptosis inhibition in macrophages following *S. aureus* infection involves both enhancement of *MCL1* transcription and the prolonged life-time of Mcl-1 in infected cells. This underscores the essential role of antiapoptotic Mcl-1 in *S. aureus*-induced cytoprotection in macrophages and argues that hijacking of Mcl-1 function is essential for survival of infected cells thus facilitating survival of intracellular invaders.

The confirmation of Mcl-1 role in staphylococcal infection was obtained studying *S. aureus*-induced septic arthritis. Our data clearly shows enhanced Mcl-1 expression in infected joints. This suggests that also *in vivo S. aureus* yields cytoprotection to infected cells establishing a safe haven impervious to attack by antibacterial forces of the immune system. Taking into account that the majority of synovial cells in the cartilage-synovium junction that participate in the destructive process are macrophages, the prolonged life-spam of infected phagocytes may be directly linked to the severity of septic arthritis lesions. Our hypothesis is corroborated by both the level of bacterial infection/spreading and the Mcl-1 expression in peritoneal macrophages isolated from *S. aureus*-infected mice, which are higher in comparison to macrophages from infected animals additionally exposed to NFκB or IL-6 signalling inhibitors (data not shown).

IL-6 plays an important role in pathogenesis of septic arthritis promoting synovitis, manifested by stimulation of chemokines and adhesion molecules and infiltration of inflammatory cells, such as macrophages and lymphocytes. IL-6 has been also shown to be essential for Mcl-1 expression thus promoting accumulation of macrophages [25]. Therefore, *S. aureus*-induced IL-6 secretion could be responsible for Mcl-1 upregulation in infected macrophages. In keeping with this, Mcl-1 induction was partly attenuated by specifically blocking the IL-6R, which suggests an autocrine role of IL-6 in the *S. aureus*-induced cytoprotection of infected macrophages through increased expression of Mcl-1. This effect is regulated by the NFκB pathway as it is apparent from the observation that Mcl-1 expression was decreased upon inhibition of NFκB. However, it should be underlined that, since Mcl-1 can be induced by several different survival and differentiation signals, both on transcriptional and posttranscriptional levels, the involvement of NFκB and IL-6 pathways in this process is a part of the very complex regulation network.

Interactions between hosts and pathogens vary depending on the strategies employed by the pathogens to deter host immunity. Therefore, defining the defence mechanisms that are selective for a particular pathogen or, conversely, the precise virulence strategy of the pathogen is a crucial step in predicting the outcome of infection. Described here and in a previous report [7] sustained Mcl-1 expression and cytoprotection of macrophages by *S. aureus* following infection appears to be specific for this bacterium, since engulfment of Gram-negative bacteria or latex beads yielded negligible effects. Such highly specific activation of Mcl-1 expression could be beneficial for the host or advantageous for *S. aureus*. Pathogen persistence or replication in a protected intracellular environment and dissemination before infected cells are removed by Fas-mediated apoptosis argue strongly for the latter option. A similar strategy is employed by *M. tuberculosis*, an obligatory intracellular pathogen, which employs a strategy whereby intracellular replication is prolonged by Mcl-1 upregulation and inhibition of apoptosis, hence promoting chronic persistence [22]. The opposite mode of action has been documented for *Streptococcus pneumoniae* infection, where initial enhancement of macrophage viability allows professional phagocytes to eradicate the infection [26].

Therefore, based on presented data we propose the scenario in which intracellular *S. aureus* makes macrophages resistant to apoptosis thus using these mobile cells as the Trojan horse to disseminate. The follow-up study aimed to unambiguously verify this attractive hypothesis is in progress in our laboratory.

5. Conclusions

To sum up, our work describes the mechanism of Mcl-1 induction and its pivotal role in *S. aureus*-mediated cytoprotection of infected macrophages. Moreover, the upregulation of Mcl-1 *in vivo* indicates that observed phenomenon plays a role during staphylococcal infection.

Acknowledgments

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Research Article

P2X7 Receptor Activation Induces Reactive Oxygen Species Formation and Cell Death in Murine EOC13 Microglia

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The P2X7 purinergic receptor is a ligand-gated cation channel expressed on leukocytes including microglia. This study aimed to determine if P2X7 activation induces the uptake of organic cations, reactive oxygen species (ROS) formation, and death in the murine microglial EOC13 cell line. Using the murine macrophage J774 cell line as a positive control, RT-PCR, immunoblotting, and immunolabelling established the presence of P2X7 in EOC13 cells. A cytofluorometric assay demonstrated that the P2X7 agonists adenosine-5′-triphosphate (ATP) and 2′(3′)-O-(4-benzoylbenzoyl) ATP induced ethidium$^+$ or YO-PRO-1^{2+} uptake into both cell lines. ATP induced ethidium$^+$ uptake into EOC13 cells in a concentration-dependent manner, with an EC_{50} of ~130 μM. The P2X7 antagonists Brilliant Blue G, A438079, AZ10606120, and AZ11645373 inhibited ATP-induced cation uptake into EOC13 cells by 75–100%. A cytofluorometric assay demonstrated that P2X7 activation induced ROS formation in EOC13 cells, via a mechanism independent of Ca^{2+} influx and K^+ efflux. Cytofluorometric measurements of Annexin-V binding and 7AAD uptake demonstrated that P2X7 activation induced EOC13 cell death. The ROS scavenger N-acetyl-L-cysteine impaired both P2X7-induced EOC13 ROS formation and cell death, suggesting that ROS mediate P2X7-induced EOC13 death. In conclusion, P2X7 activation induces the uptake of organic cations, ROS formation, and death in EOC13 microglia.

1. Introduction

Microglia are the resident innate immune cells of the central nervous system (CNS) and play an important role in immune surveillance [1] and in the pathogenesis and progression of a number of CNS disorders [2]. Microglia are constantly mobile, spending time scanning the extracellular space of the CNS [1]. In response to brain injury or immunological stimuli, these cells become activated and undergo dramatic morphological and functional changes, which are highly dependent on the context of their activation [3]. Activated microglia phagocytose debris and peptides, present antigens, and produce a number of soluble factors. These factors may be inflammatory, regulatory, or cytotoxic in nature and include reactive oxygen species (ROS), nitric oxide (NO), proinflammatory and anti-inflammatory cytokines, prostaglandins, and growth factors [4, 5]. Microglia can be both neuroprotective or neurotoxic when activated, depending on the factors they produce and the quantity and context in which they are released, with prolonged or excessive activation of these cells being associated with neuroinflammation and the progression of a number of CNS disorders [6]. The mechanisms behind enhanced microglial activation in these disorders and the features determining the balance between neuroprotection and neurotoxicity are not fully understood.

The P2X7 receptor is a trimeric ligand-gated cation channel belonging to the P2X family of purinergic receptors [7]. P2X7 is predominately expressed on mononuclear leukocytes including macrophages and microglia and plays a role in inflammation and immunity [8]. In particular, P2X7 is currently receiving attention due to its possible roles in neuroinflammation [9]. Activation of P2X7 by extracellular adenosine-5′-triphosphate (ATP), or the most potent P2X7 agonist, 2′(3′)-O-(4-benzoylbenzoyl) ATP (BzATP), causes the passage of small cations including Ca^{2+}, Na^+, and K^+ across the plasma membrane, as well as organic cations, such as the fluorescent dyes ethidium$^+$ and YO-PRO-1^{2+} [7].

Compared to other P2X receptors, P2X7 requires relatively high ATP concentrations for activation, with a half maximal effective concentration (EC_{50}) of 100–300 μM [7]. Activation of P2X7 leads to a number of cell-specific downstream signalling events, including the formation of ROS and reactive nitrogen species [10], and either cell proliferation or death [11, 12].

Using molecular, immunochemical, and pharmacological techniques, we demonstrate in the current study that the murine microglial EOC13 cell line [13] expresses functional P2X7. Activation of P2X7 by ATP in this cell line induces the uptake of organic cations, ROS formation, and cell death.

2. Materials and Methods

2.1. Reagents and Antibodies. RPMI-1640 and DMEM/F12 media, GlutaMAX, normal horse serum (NHS), 0.05% trypsin, YO-PRO-1^{2+}, 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA), and 2′,7′-difluorofluorescein diacetate (DAF-FM DA) were from Invitrogen (Grand Island, NY). Fetal bovine serum (FBS) (heat-inactivated before use) was from Bovogen Biologicals (East Keilor, Australia). ATP, BzATP, ethidium bromide, dimethyl sulfoxide (DMSO), glycerol gelatin, and the P2X7 antagonist Brilliant Blue G (BBG) were from Sigma-Aldrich (St. Louis, MO). The P2X7 antagonists A438079, AZ10606120, and AZ11645373 were from Tocris Bioscience (Ellisville, MO). Primers were from GeneWorks (Hindmarsh, Australia). Protease inhibitor cocktail tablets (complete, Mini, EDTA-free) and Annexin-V-Fluorescein were from Roche Diagnostics (Penzberg, Germany). SuperSignal West Pico Chemiluminescent Substrate was from Pierce (Rockford, IL). The viability dye 7-aminoactinomycin D (7AAD) and the ROS scavenger N-acetyl-L-cysteine (NAC) were from Enzo Life Sciences (Plymouth Meeting, PA). The broad-spectrum ROS inhibitor diphenyleneiodonium (DPI) was from Cayman Chemical (Ann Arbor, MI). Phenyl-methyl-sulfonyl-fluoride (PMSF), n-dodecyl β-D-maltoside, and ethylene glycol tetraacetic acid (EGTA) were from Amresco (Solon, OH).

Cells preincubated with H_2O soluble compounds (BBG, A438079, and AZ10606120) were compared to cells preincubated in the absence of each compound. Cells preincubated with DMSO soluble compounds (AZ11645373 and DPI) were compared to cells preincubated with DMSO alone. Solutions containing 40 mM NAC were prepared in NaCl medium (140 mM NaCl, 5 mM NaOH, 5 mM KCl, 10 mM HEPES, and 5 mM glucose, pH 7.4) and adjusted to pH 7.4; cells were then preincubated in NaCl medium with or without NAC. When studied, antagonists/inhibitors were present during incubations with ATP.

Rabbit anti-mouse P2X7 (extracellular epitope) polyclonal antibody (Ab) and rabbit anti-rat P2X7 (C-termini epitope) Ab (and corresponding blocking peptide) were from Alomone Labs (Jerusalem, Israel). Peroxidase-conjugated goat anti-rabbit IgG Ab was from Rockland Immunochemicals (Gilbertsville, PA). Cy3-conjugated donkey anti-rabbit IgG Ab was from Jackson ImmunoResearch (West Grove, PA). Rat anti-mouse P2X7 monoclonal antibody (mAb) (clone HANO43) was from Enzo Life Sciences. Rat IgG2b isotype control mAb and allophycocyanin- (APC-) conjugated donkey anti-rat IgG Ab were from eBioscience (San Diego, CA).

2.2. Cell Lines. The murine macrophage J774 cell line, the murine microglial EOC13 cell line, and the murine lymphoblast LADMAC cell line, all originally obtained from the American Type Culture Collection (Manassas, VA), were kindly provided by Jasmyn Dunn (University of Queensland, Brisbane, Australia) (J774) and Iain Campbell (University of Sydney, Sydney, Australia) (EOC13 and LADMAC). J774 cells were maintained in RPMI-1640 medium containing 10% FBS and 2 mM GlutaMAX (complete RPMI medium). EOC13 cells were maintained in DMEM/F12 supplemented with 10% FBS, 2 mM GlutaMAX, and 20% LADMAC conditioned medium (complete DMEM medium). Cell lines were maintained at 37°C and 95% air/5% CO_2 and passaged every 3-4 days. Quarterly mycoplasma testing was carried out using the MycoAlert Mycoplasma Detection Kit (Lonza, Rockland, ME), as per the manufacturer's instructions. For experiments, cells were harvested by cell scraping unless otherwise stated.

2.3. Fluorescent Cation Dye Uptake Assay. Cells were washed in NaCl medium (300 × g for 5 min), resuspended in NaCl medium, and equilibrated at 37°C for 5 min (1×10^5 cells/1 mL/tube). Cells were then incubated with 25 μM ethidium$^+$ (or 1 μM YO-PRO-1^{2+} where indicated) in the absence or presence of the P2X7 agonists ATP or BzATP (as indicated) for 5 min. In some experiments, ATP-induced cation uptake was assessed with cells suspended in KCl medium (150 mM KCl, 5 mM glucose, and 10 mM HEPES, pH 7.4) or in NaCl medium containing 1 mM $CaCl_2$ or 100 μM EGTA. In other experiments, cells were preincubated in the absence or presence of P2X7 antagonists or the ROS scavenger NAC (as indicated) for 15 and 30 min, respectively, prior to cation and ATP addition. Incubations with nucleotides were stopped by the addition of an equal volume of ice-cold NaCl medium containing 20 mM $MgCl_2$ ($MgCl_2$ medium) followed by centrifugation (300 × g for 5 min). Cells were washed once with NaCl medium and events collected using a LSR II flow cytometer (BD Biosciences, San Diego, CA) (excitation 488 nm, emission collected with 575/26 and 515/20 band-pass filters for ethidium$^+$ and YO-PRO-1^{2+}, resp.). The mean fluorescence intensity (MFI) of relative cation uptake was determined using FlowJo software (Tree Star, Ashland, OR).

2.4. P2X7 Expression by RT-PCR. Total RNA isolation from cells was performed using the RNeasy Mini Kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions. PCR amplification was performed as described previously [14] using SuperScript III One-Step RT-PCR System Platinum Taq DNA polymerase (Invitrogen) with 500 ng of RNA, and P2X7 forward (5′-ATATCCACTTCCCCGGCCAC-3′) and reverse (5′-TCGGCAGTGATGGGACCAG-3′) primers for 42 cycles (94°C, 1 min; 68°C, 1 min; 72°C, 1 min). PCR products were separated on a 2% agarose gel in Tris-acetate

EDTA buffer and visualised with ethidium bromide staining. Images of gels were collected using a Gel Logic 212 PRO imaging system (Carestream Health, Rochester, NY).

2.5. P2X7 Protein Detection by Immunoblotting. Cells were washed three times with phosphate-buffered saline (PBS) ($300 \times g$ for 5 min) and lysed (1×10^7 cells/mL) over 60 min in ice-cold lysis buffer (50 mM BisTris, 750 mM 6-aminohexanoic acid, 1% n-dodecyl β-D-maltoside, 1 mM PMSF, and protease inhibitor cocktail, pH 7.0). Cells were sheared by passing ten times through a 21 G needle and stored at −20°C until needed. Cells were then thawed and cleared ($16{,}000 \times g$ at 4°C for 10 min). Supernatants (25 μg protein/lane) were separated under reducing conditions (5% β-mercaptoethanol) using a discontinuous SDS-PAGE system with a 4% stacking gel and 10% separating gel. Proteins were then transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA) and blocked at 4°C overnight with Tris-buffered saline (250 mM NaCl and 50 mM Tris, pH 7.5) containing 0.2% Tween-20 and 5% milk powder. The following day, nitrocellulose membranes were incubated at room temperature for 2 h with anti-mouse P2X7 Ab (1 : 500) in Tris-buffered saline containing 0.2% Tween-20 and 5% milk powder. Membranes were washed three times over 30 min with Tris-buffered saline containing 0.2% Tween-20 and then incubated at room temperature for 1 h with peroxidise-conjugated anti-IgG Ab (1 : 1000) in Tris-buffered saline containing 0.2% Tween-20 and 5% milk powder. Membranes were washed as above and visualised using chemiluminescent substrate and Amersham Hyperfilm ECL (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Images of films were collected using a GS-800 Calibrated Densitometer (Bio-Rad).

2.6. Cell Surface P2X7 Protein Detection by Flow Cytometry. Cells in NaCl medium containing 10% NHS and 0.02% NaN_3 (1×10^5 cells/200 μL/tube) were incubated with anti-P2X7 or rat IgG2b isotype control mAb (5 μg/mL) at room temperature for 30 min. Cells were then washed twice with NaCl medium ($300 \times g$ for 5 min) and incubated with APC-conjugated anti-rat IgG Ab (1.3 μg/mL) and 7AAD (to exclude dead cells) for 30 min protected from light. Cells were washed once as above. Events were then collected using a LSR II flow cytometer (excitation 633 nm, emission collected with 660/20 band-pass filter for APC; excitation 488 nm, emission collected with 695/40 band-pass filter for 7AAD). Relative cell-surface P2X7 was determined using FlowJo software and is expressed as the difference in the MFI of specific mAb labelling and isotype control labelling.

2.7. P2X7 Protein Detection by Confocal Microscopy. EOC13 or J774 cells in their respective complete culture medium were plated into 24-well plates with 13 mm glass coverslips (5×10^4 cells/0.5 mL/well) and incubated at 37°C, 95% air/5% CO_2 overnight to allow time to adhere. The following day, cells were fixed with 4% paraformaldehyde in PBS at room temperature for 15 min and then washed three times with PBS over 10 min. Cells were incubated with permeabilisation solution (PBS containing 0.1% DMSO, 2% NHS, and 0.1% Triton X-100) at room temperature for 10 min and washed three times with PBS. Cells were then blocked with 20% NHS in PBS at room temperature for 20 min. Cells were incubated at 4°C overnight with anti-rat P2X7 Ab (5 μg/mL; preincubated for 1 h in the absence or presence of blocking peptide as per the manufacturer's instruction) in PBS containing 1% BSA, 0.2% NHS, and 0.05% NaN_3. Cells were then washed as above and incubated at room temperature for 1 h with Cy3-conjugated anti-rabbit IgG Ab (15 μg/mL) in PBS containing 0.2% NHS. Cells were washed as above and then the coverslips mounted onto slides with 50% (v/v) glycerol gelatin in PBS. Coverslips were sealed with nail varnish. Cells were visualised using a DM IBRE inverted microscope and TCS SP confocal imaging system (Leica, Mannheim, Germany) (excitation 488 nm, emission collected at 560–600 nm). Images were captured using Leica Confocal Software.

2.8. ROS Formation Assay. EOC13 cells in complete DMEM medium were plated into 24-well plates (5×10^4 cells/0.5 mL/well) and incubated at 37°C, 95% air/5% CO_2 overnight. Cells were then incubated with NaCl medium containing 10 μM H_2DCFDA (0.5 mL/well) at 37°C, 95% air/5% CO_2, protected from light for 30 min. The medium was removed, and cells were further incubated in NaCl medium (containing 1 mM $CaCl_2$) in the absence or presence of 2 mM ATP at 37°C, 95% air/5% CO_2 for 15 min. Incubations were stopped by the addition of an equal volume of ice-cold $MgCl_2$ medium. Cells were harvested using 0.05% trypsin (5 min, 37°C) and were washed once with NaCl medium. Events were collected using a LSR II flow cytometer (excitation 488 nm, emission collected at 515/20 nm) and the MFI of relative dichlorofluorescein (DCF) determined using FlowJo software.

In some experiments, ATP-induced ROS formation was assessed in KCl medium, in NaCl medium in the absence of 1 mM $CaCl_2$ or presence of 100 μM EGTA, or in complete DMEM medium in the absence or presence of 10 μM AZ10606120 (15 min preincubation, prior to ATP addition). As free Ca^{2+} lowers the concentration of ATP^{4-} [15], cells incubated in the absence of 1 mM Ca^{2+} were incubated with 1.4 mM ATP to provide equimolar ATP^{4-} concentrations (575 μM), as calculated using the Bound and Determined Program [16]. In other experiments, cells were preincubated in the absence or presence of AZ10606120, NAC, or DPI (as indicated) for 15, 30, and 30 min, respectively, prior to ATP addition. Cells prior to harvesting were also visualised by differential interference contrast (DIC) imaging using an Eclipse TE2000 inverted microscope (Nikon, Tokyo, Japan) to examine cell morphology, and DIC images were captured using Image-Pro AMS (Version 6.1) (Media Cybernetics, Rockville, MD).

2.9. NO Formation Assay. EOC13 cells in complete DMEM medium were plated into 24-well plates (5×10^4 cells/0.5 mL/well) and incubated at 37°C, 95% air/5% CO_2 overnight. Cells were then incubated with NaCl medium containing 10 μM DAF-FM DA (0.5 mL/well) at 37°C, 95% air/5% CO_2,

protected from light for 30 min. The medium was removed, and the cells were washed once. Cells were then preincubated with NaCl medium in the absence or presence of 10 μM AZ10606120 at 37°C, 95% air/5% CO_2 for 15 min. Following this, cells were further incubated in the absence or presence of 1.4 mM ATP for 15 min. Incubations were stopped by the addition of an equal volume of ice-cold $MgCl_2$ medium. Cells were harvested using 0.05% trypsin (5 min, 37°C) and were washed once with NaCl medium. Events were collected using a LSR II flow cytometer (excitation 488 nm, emission collected at 515/20 nm) and the MFI of relative benzotriazole derivative determined using FlowJo software.

2.10. Cell Death Assay. EOC13 cells in complete DMEM medium were plated into 24-well plates (5×10^4 cells/0.5 mL/well) and incubated at 37°C, 95% air/5% CO_2 overnight. Cells were then incubated with filter sterile ATP (as indicated) at 37°C, 5% CO_2 for 24 h. In some experiments, cells were preincubated in the absence or presence of 10 μM AZ10606120 or 40 mM NAC for 15 or 30 min, respectively, prior to ATP addition. In other experiments, cells were preincubated in the absence or presence of 40 mM NAC for 90 min, with 2 mM ATP added in the final 15–60 min, and then the medium replaced and cells incubated at 37°C, 95% air/5% CO_2 for a further 24 h. Following the 24 h incubations, cells were harvested from wells using 0.05% trypsin (5 min, 37°C) and washed once with Annexin-V binding medium (NaCl medium containing 5 mM $CaCl_2$). Cells were then incubated with Annexin-V binding medium containing Annexin-V-Fluorescein and 7AAD at room temperature protected from light for 15 min. Events were collected using a LSR II flow cytometer (excitation 488 nm, emission collected with 515/20 and 695/40 band-pass filters for Annexin-V-Fluorescein and 7AAD, resp.) and the MFI of Annexin-V-Fluorescein and 7AAD determined using FlowJo software. Quadrant markers were used to determine the percentage of Annexin-V^+/$7AAD^-$, Annexin-V^-/$7AAD^+$, and Annexin-V^+/$7AAD^+$ cells. In some experiments, cells prior to harvesting were visualised by DIC imaging to examine cell morphology, and DIC images captured as outlined in Section 2.8.

2.11. Data Presentation and Statistical Analyses. Data is presented as the mean ± SD. Differences between multiple treatments were compared by ANOVA paired with Tukey's HSD posttest using Prism 5 for Windows (Version 5.04) (GraphPad Software, San Diego, CA), with differences considered significant for $P < 0.05$. Concentration response and inhibition curves were fitted using Prism 5 and assuming a variable slope, with normalised and nonnormalised response curves, respectively, selected to obtain the best fit. Estimates of EC_{50} values and half maximal inhibitory concentrations (IC_{50}) were obtained from individual fits of these plots.

3. Results

3.1. P2X7 Antagonists Inhibit ATP-Induced Ethidium$^+$ Uptake into J774 Macrophage Cells in a Concentration-Dependent Manner. The murine macrophage J774 cell line is well known to express functional P2X7 [17]. Moreover, our group has demonstrated the presence of functional P2X7 in various cell types using a fixed-time fluorescent cation uptake assay (e.g., [14, 18]). Therefore, this technique was used to confirm the presence of P2X7 in J774 cells and to validate the use of this cell line as a positive control. Incubation of J774 cells with the P2X7 agonist ATP and the most potent P2X7 agonist BzATP induced significant ethidium$^+$ uptake into these cells compared to cells incubated in the absence of nucleotide (Figure 1(a)). In addition, incubation of J774 cells with ATP induced significant YO-PRO-1^{2+} uptake compared to cells incubated in the absence of ATP (Figure 1(b)). However, ATP-induced YO-PRO-1^{2+} uptake was significantly lower than ATP-induced ethidium$^+$ uptake (Figure 1(b)).

A number of highly specific P2X7 antagonists, including A438079 [19], AZ10606120 [20], and AZ11645373 [21], have recently become available. In addition, BBG is commonly used as a P2X7 antagonist *in vitro* and *in vivo* (e.g., [22, 23]). Therefore, to determine the optimum concentrations of these antagonists required to inhibit murine P2X7, J774 cells were preincubated in the absence or presence of varying concentrations of BBG, A438079, AZ10606120, and AZ11645373 and the ATP-induced ethidium$^+$ uptake assessed. Each antagonist impaired 1 mM ATP-induced ethidium$^+$ uptake in a concentration-dependent manner, with IC_{50} values of 1.8 ± 0.2, 7.9 ± 0.4, 1.0 ± 0.1, and 1.5 ± 0.1 μM, respectively (Figure 1(c)). AZ10606120 and A438079 completely inhibited ethidium$^+$ uptake at respective concentrations of 10 and 100 μM. In contrast, BBG and AZ11645373 were partial antagonists at the ATP concentration used (1 mM).

3.2. EOC13 Microglial Cells Express P2X7. To determine whether EOC13 microglial cells express P2X7, a series of experiments using J774 cells as a positive control were performed. Firstly, RNA was isolated from EOC13 and J774 cells and amplified by RT-PCR using primers for P2X7. Similar to J774 cells, EOC13 cells were found to express P2X7 mRNA, as evident from the 230 base pair band corresponding to the size of the predicted product (Figure 2(a)). The presence of total P2X7 protein was determined by probing separated whole lysates of both cell lines with an anti-P2X7 Ab. Immunoblotting revealed one major protein band of 75 kDa for both EOC13 and J774 cells (Figure 2(b)), corresponding to the predicted size of glycosylated P2X7. Moreover, both cell lines were incubated with an anti-P2X7 mAb and the presence of cell-surface P2X7 determined by flow cytometry. Immunolabelling demonstrated cell-surface P2X7 on both EOC13 and J774 cells, with MFIs of 13 ± 2 and 14 ± 4, respectively ($n = 3$) (Figure 2(c)). Finally, both cell lines were stained with an anti-P2X7 Ab and analysed by confocal microscopy. This similarly demonstrated the presence of cell-surface P2X7, as well as intracellular P2X7, with bright staining observed on all cells (Figure 2(d)). Preincubation of the anti-P2X7 Ab with blocking peptide completely abrogated the detection of P2X7 in both cell lines (data not shown). Together, these results indicate that P2X7 is expressed in EOC13 cells.

3.3. EOC13 Microglial Cells Express Functional P2X7. To determine whether P2X7 was functional in EOC13 cells,

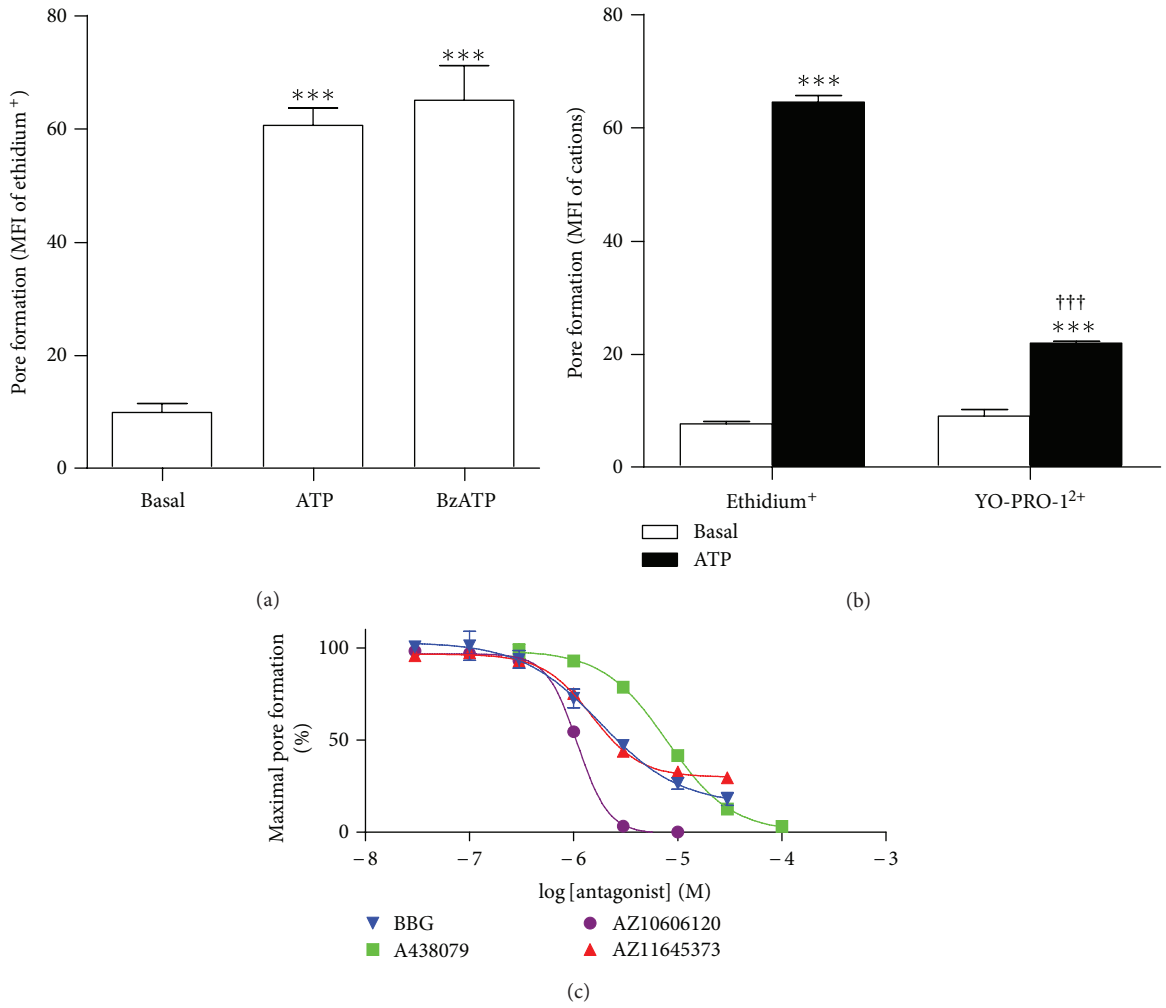


FIGURE 1: P2X7 antagonists inhibit ATP-induced ethidium$^+$ uptake into J774 macrophage cells in a concentration-dependent manner. (a and b) J774 cells in NaCl medium were incubated with (a and b) 25 μM ethidium$^+$ or (b) 1 μM YO-PRO-1^{2+} in the absence (basal) or presence of (a and b) 1 mM ATP or (a) 0.1 mM BzATP at 37°C for 5 min. (c) Cells in NaCl medium were preincubated with Brilliant Blue G (BBG), A438079, AZ10606120, and AZ11645373 (as indicated) at 37°C for 15 min. Ethidium$^+$ (25 μM) was then added, and cells were incubated in the absence or presence of 1 mM ATP at 37°C for 5 min. (a–c) Incubations were stopped by the addition of $MgCl_2$ medium and centrifugation. Mean fluorescence intensity (MFI) of fluorescent cation uptake (pore formation) was determined by flow cytometry. (a and b) Results shown as means ± SD, $n = 3$; $^{***}P < 0.001$ compared to corresponding basal; $^{†††}P < 0.001$ compared to corresponding ATP. (c) Curves presented as a percentage of the maximal ATP-induced ethidium$^+$ uptake and expressed as the mean ± SD, $n = 3$-4.

the fixed-time ethidium$^+$ uptake assay was performed. Both ATP and BzATP were found to induce significant ethidium$^+$ uptake into EOC13 cells compared to cells incubated in the absence of nucleotide (Figure 3(a)). Next, EOC13 cells were incubated with increasing concentrations of ATP. ATP induced ethidium$^+$ uptake in a concentration-dependent manner, with maximal uptake obtained at an ATP concentration of 1 mM and with an EC_{50} of 130 ± 30 μM (Figure 3(b)). Subsequent characterisations of P2X7 in EOC13 microglia were performed using this maximal concentration of ATP (1 mM).

To determine if the observed ATP-induced ethidium$^+$ uptake into EOC13 cells was mediated by P2X7, cells were preincubated in the absence or presence of P2X7 antagonists at inhibitory concentrations optimal for 1 mM ATP-induced ethidium$^+$ uptake in J774 cells, as demonstrated above (Figure 1(c)). Preincubation of EOC13 cells with 30 μM BBG, 100 μM A438079, 10 μM AZ10606120, and 30 μM AZ11645373 resulted in significant impairment of ATP-induced ethidium$^+$ uptake by 75 ± 2, 90 ± 1, 100 ± 0, and 99 ± 1%, respectively (Figure 3(c)). None of the P2X7 antagonists except AZ11645373 significantly altered the basal ethidium$^+$ uptake into EOC13 cells. Again with the exception of AZ11645373, which reduced the amount of gated viable cells by ~30%, cell viability (as assessed by forward and side scatter) was similar between treatments (data not shown).

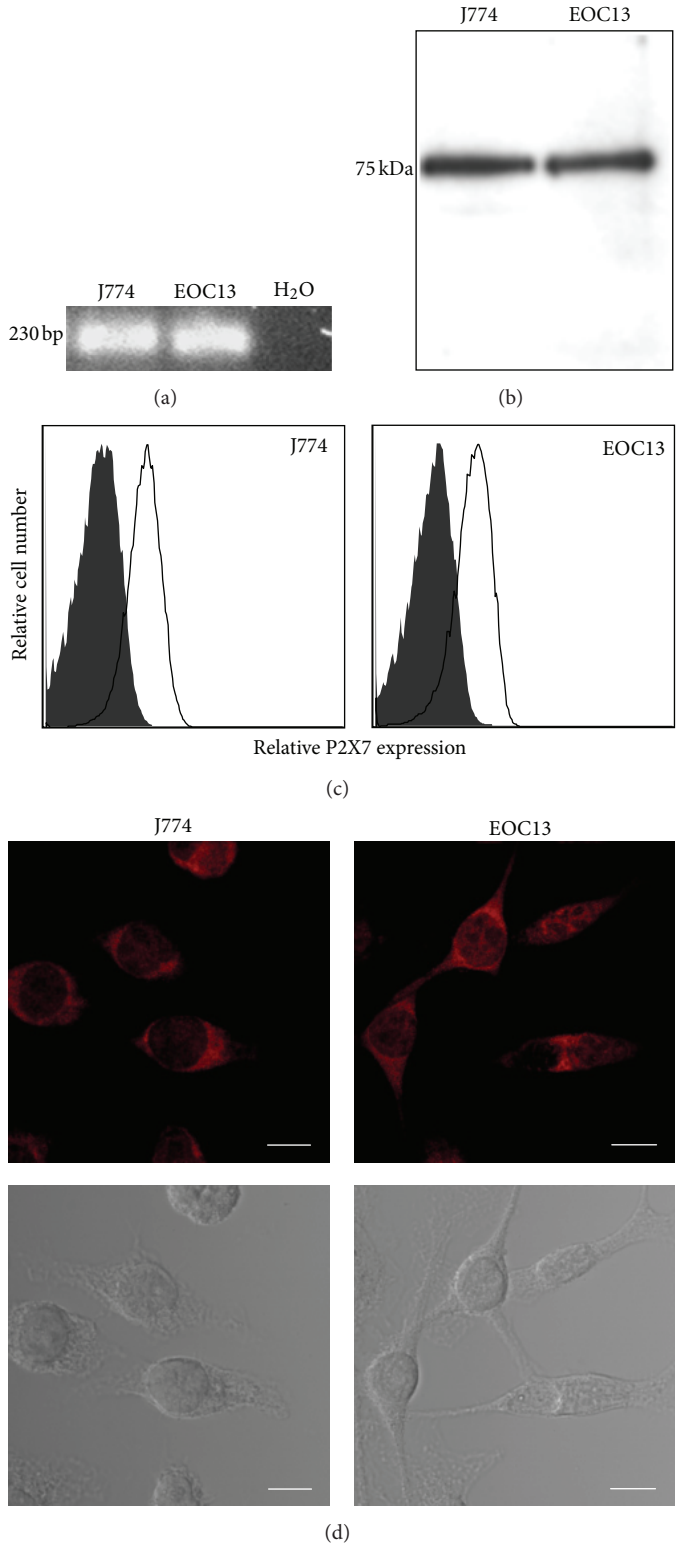


Figure 2: EOC13 microglial cells express P2X7. (a) RNA from EOC13 and J774 cells was amplified by RT-PCR using primers for P2X7. Water in place of RNA was included as a negative control in the PCR reaction. PCR products were separated and visualised with ethidium bromide staining. (b) EOC13 and J774 cell lysates were separated by SDS-PAGE, transferred to nitrocellulose, and probed with an anti-P2X7 Ab. (c) EOC13 and J774 cells were labelled with an anti-P2X7 (solid line) or isotype control (shaded) mAb and then with APC-conjugated anti-IgG Ab and 7AAD (to exclude dead cells). Relative P2X7 expression (mean fluorescence intensity) was determined by flow cytometry. (d) Fixed and permeabilised EOC13 and J774 cells were labelled with an anti-P2X7 Ab and then with Cy3-conjugated anti-IgG Ab. P2X7 (top panels) and phase contrast (bottom panels) images were assessed by confocal microscopy. Bars represent 10 μm. (a–d) Results are representative of 2-3 experiments.

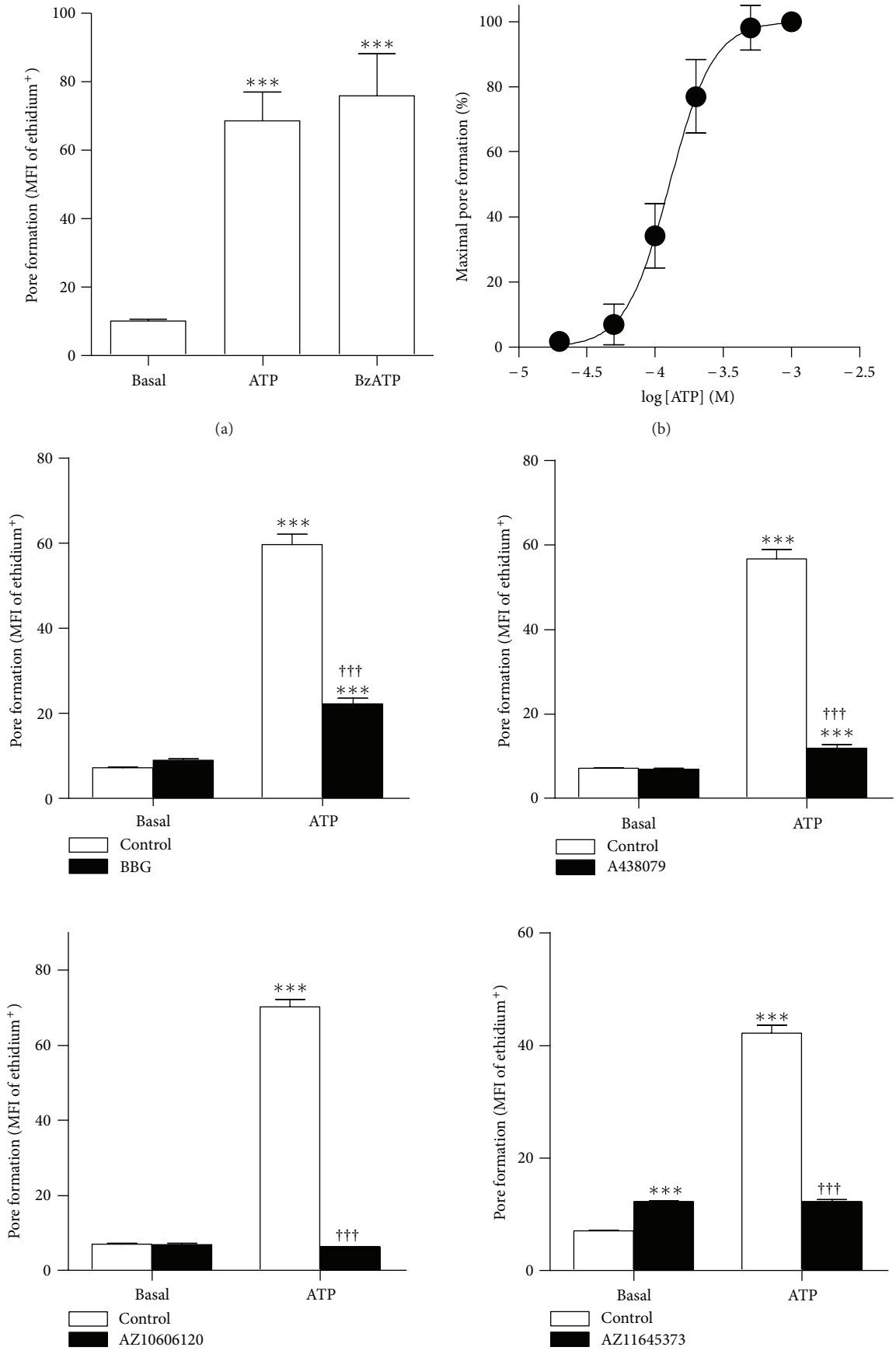


Figure 3: Continued.

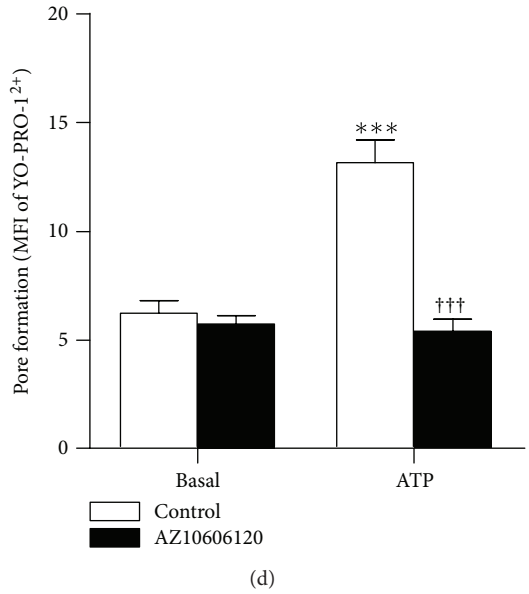
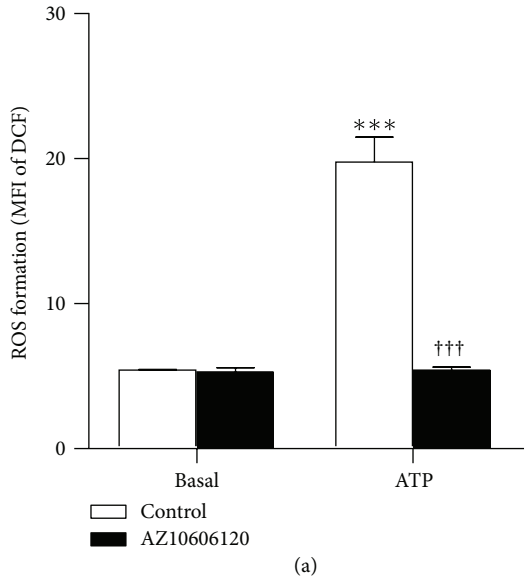


Figure 3: EOC13 microglial cells express functional P2X7. (a and b) EOC13 cells in NaCl medium were incubated with 25 μM ethidium$^+$ in the absence (basal) or presence of (a) 1 mM ATP, 0.1 mM BzATP, or (b) varying concentrations of ATP (as indicated) at 37°C for 5 min. (c and d) Cells in NaCl medium were preincubated in the absence (control) or presence of (c) 30 μM Brilliant Blue G (BBG), 100 μM A438079, 30 μM AZ11645373, or (c and d) 10 μM AZ10606120 at 37°C for 15 min. (c) Ethidium$^+$ (25 μM) or (d) YO-PRO-1^{2+} (1 μM) was then added, and (c and d) cells were incubated in the absence (basal) or presence of 1 mM ATP at 37°C for 5 min. (a–d) Incubations were stopped by the addition of $MgCl_2$ medium and centrifugation. Mean fluorescence intensity (MFI) of fluorescent cation uptake (pore formation) was determined by flow cytometry. (a, c, and d) Results shown as means ± SD, $n = 3$; $^{***}P < 0.001$ compared to corresponding basal; $^{\dagger\dagger\dagger}P < 0.001$ compared to corresponding ATP in the absence of antagonist. (b) Curve presented as a percentage of the maximal ATP-induced ethidium$^+$ uptake and expressed as the mean ± SD, $n = 3$.

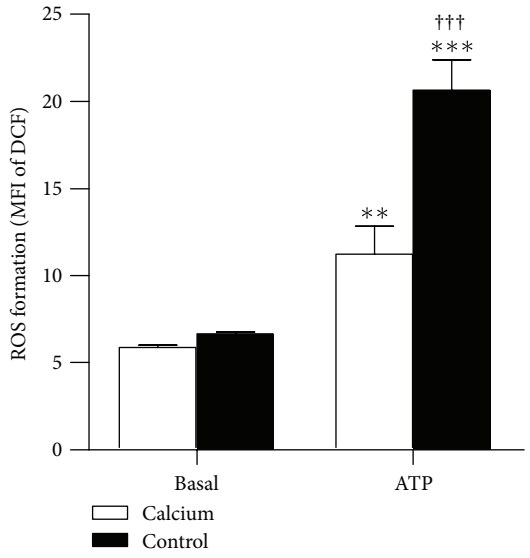
To determine if P2X7 activation could induce the uptake of a second organic cation into EOC13 cells, cells were preincubated in the absence or presence of AZ10606120, and ATP-induced YO-PRO-1^{2+} uptake examined. Similar to ethidium$^+$ uptake, 1 mM ATP induced significant YO-PRO-1^{2+} uptake into EOC13 cells compared to cells incubated in the absence of ATP (Figure 3(d)). Moreover, preincubation of cells with 10 μM AZ10606120 resulted in complete inhibition of ATP-induced YO-PRO-1^{2+} uptake (Figure 3(d)). Incubation with AZ10606120 did not significantly alter the basal YO-PRO-1^{2+} uptake (Figure 3(d)). Furthermore, cell viability (as assessed by forward and side scatter) was similar between treatments (data not shown). Collectively, these results indicate that P2X7 is functional in EOC13 cells.

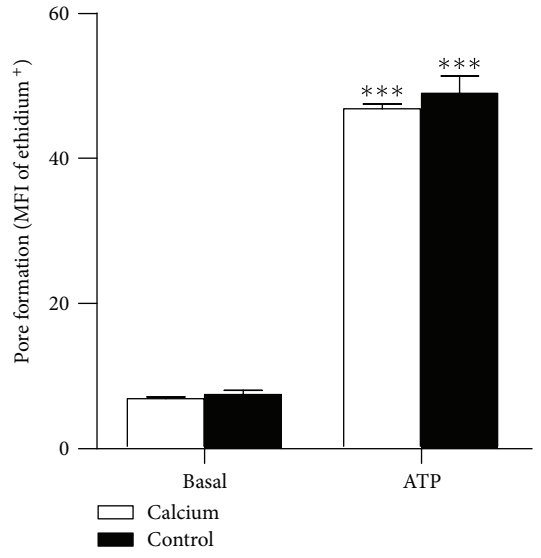
3.4. P2X7 Activation Induces ROS Formation in EOC13 Microglial Cells. P2X7 activation has been reported to induce ROS formation in a number of cell types, including primary microglia [24, 25]. Thus, ATP-induced ROS formation in the EOC13 cell line was investigated using the ROS sensitive probe DCF. Cells loaded with H_2DCFDA (which is converted to DCF inside cells) were incubated in the absence or presence of ATP, and the subsequent ROS formation analysed by flow cytometry. As extracellular Ca^{2+} has been reported to be important for P2X7-induced ROS formation in a number of cell types [24, 26, 27], assays were initially conducted in the presence of 1 mM Ca^{2+}. However, due to the inhibitory action of Ca^{2+} on P2X7 [15], assays were initially conducted with 2 mM ATP. Incubation with 2 mM ATP induced significant ROS formation in EOC13 cells compared to cells incubated in the absence of ATP (MFI of ROS formation 16.3 ± 0.6 and 5.18 ± 0.06, resp., $P < 0.001$, $n = 3$). Furthermore, preincubation of cells with 10 μM AZ10606120 resulted in complete inhibition of ATP-induced ROS formation (Figure 4(a)), indicating that this process is mediated by P2X7 activation. As for cation uptake (Figure 3), AZ10606120 did not significantly alter the basal ROS formation (Figure 4(a)) or cell viability (as assessed by forward and side scatter) (data not shown).

P2X7 is a ligand-gated cation channel [7]; therefore the possible roles for cation fluxes in P2X7-induced ROS formation were next investigated. P2X7-induced ROS formation has been reported to be partially dependent on Ca^{2+} influx in human promyelocytes [26] and rat submandibular gland cells [27]. Thus, to determine if Ca^{2+} influx is required for P2X7-mediated ROS formation in EOC13 cells, ATP-induced ROS formation in the absence and presence of Ca^{2+} was investigated. For this comparison, equivalent amounts of ATP^{4-} (575 μM) were used by adding 1.4 or 2 mM ATP to NaCl medium nominally free of Ca^{2+} or containing 1 mM Ca^{2+}, respectively. ATP induced significant ROS formation in both the absence and presence of 1 mM Ca^{2+} compared



(a)





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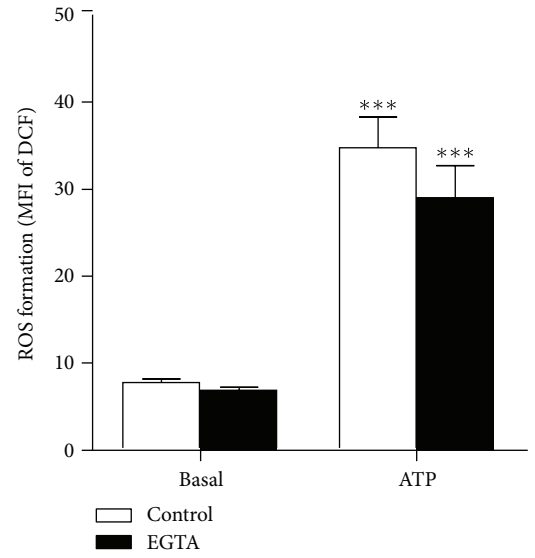

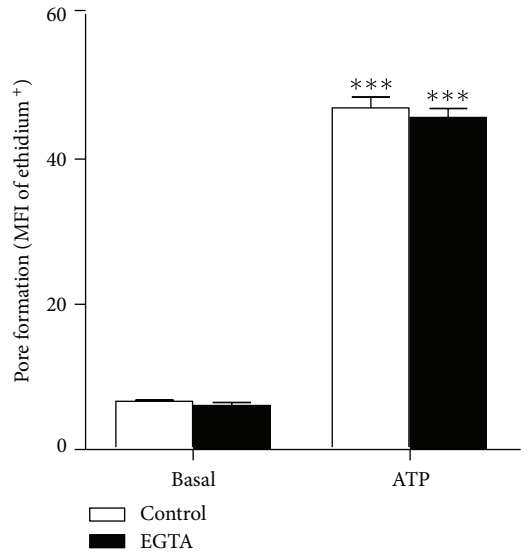

(c)

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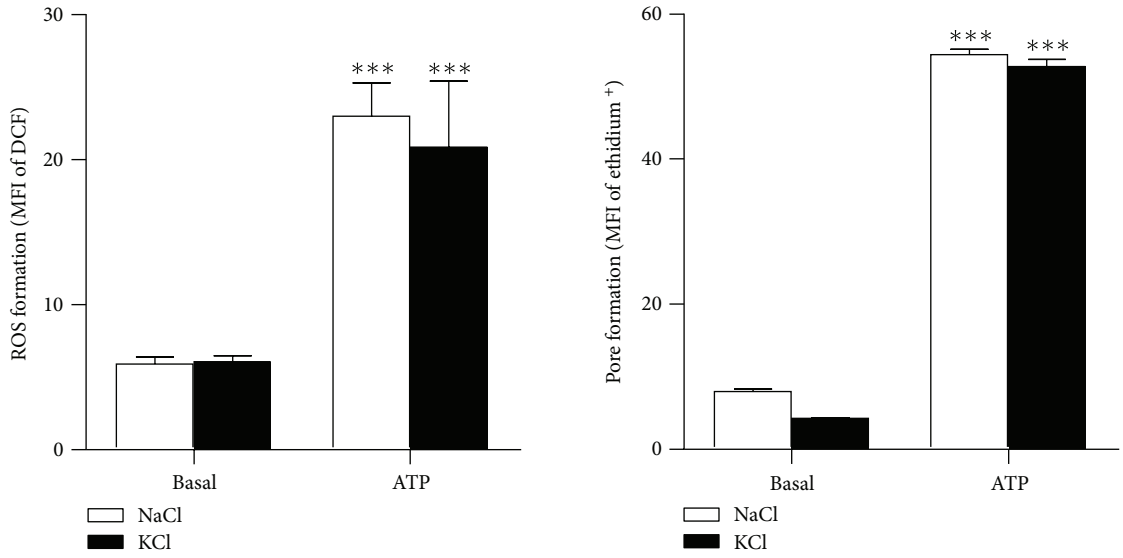


(d)

Figure 4: P2X7 activation induces ROS formation in EOC13 microglial cells. (Left panels) Adherent DCF-loaded EOC13 cells or (right panels) suspended EOC13 cells in (a) NaCl medium containing 1 mM Ca^{2+} (preincubated in the absence (control) or presence of 10 μM AZ10606120 at 37°C for 15 min), (b) NaCl medium in the absence (control) or presence of 1 mM Ca^{2+}, (c) NaCl medium in the absence (control) or presence of 100 μM EGTA, or (d) NaCl or KCl medium were (a–d) incubated in the absence (basal) or presence of 575 μM ATP^{4-} (2 mM or 1.4 mM ATP as explained in Section 2.8) at 37°C for (left panels) 15 min or (right panels) 5 min in the presence of 25 μM ethidium$^+$. (a–d) Incubations were stopped by the addition of $MgCl_2$ medium and centrifugation. Mean fluorescence intensities (MFI) of (left panels) DCF (ROS formation) or (right panels) ethidium$^+$ uptake (pore formation) were determined by flow cytometry and results shown as means ± SD, $n = 3$; $^{***}P < 0.001$ or $^{**}P < 0.01$ compared to corresponding basal; $^{\dagger\dagger\dagger}P < 0.001$ compared to corresponding ATP.

to similarly treated cells in the absence of ATP (Figure 4(b)). Cells incubated in the absence of Ca^{2+} had significantly higher ATP-induced ROS formation compared to those incubated in the presence of Ca^{2+}. In contrast, ATP-induced ethidium$^+$ uptake (P2X7 function) was similar in cells incubated in the absence or presence of Ca^{2+} (Figure 4(b)), indicating that the differences in ATP-induced ROS formation were not due to altered P2X7 function.

NaCl medium may contain nominal amounts of Ca^{2+}. Thus, to further exclude a role for Ca^{2+} in P2X7-mediated ROS formation in EOC13 cells, ATP-induced ROS formation was investigated in the absence and presence of the Ca^{2+} chelator EGTA. Incubation with 1.4 mM ATP induced significant but similar amounts of ROS formation in both the absence and presence of 100 μM EGTA compared to similarly treated cells in the absence of ATP (Figure 4(c)). Again, ATP-induced ethidium$^+$ uptake was similar in cells incubated in the absence or presence of EGTA (Figure 4(c)).

Finally, the role of K^+ in P2X7-induced ROS formation in EOC13 cells was investigated. Both ROS and K^+ efflux have been reported to be involved in interleukin-1β (IL-1β) release from monocytes, although whether these downstream processes are linked is yet to be established [28]. Thus, to determine if K^+ efflux is involved in P2X7-mediated ROS formation in EOC13 cells, ATP-induced ROS formation was compared with cells in NaCl medium and KCl medium, which prevents the loss of intracellular K^+. Incubation with 1.4 mM ATP induced significant ROS formation in both NaCl and KCl media, with similar levels of ROS formation in both media (Figure 4(d)). Likewise, ATP-induced ethidium$^+$ uptake was similar in NaCl and KCl media (Figure 4(d)), indicating that the inability of high extracellular K^+ to impair ATP-induced ROS formation was not due to altered P2X7 function.

To confirm that P2X7 activation induced ROS formation in EOC13 microglia, DCF-loaded cells in NaCl medium were preincubated in the absence or presence of the ROS scavenger NAC, before incubation in the absence or presence of ATP. As above (Figure 4), 1.4 mM ATP induced significant ROS formation (Figure 5(a)). Preincubation with 40 mM NAC inhibited ATP-induced ROS formation by 73.7 ± 0.3% (Figure 5(a)). Basal ROS formation (Figure 5(a)) and cell viability (as assessed by forward and side scatter) (data not shown) were similar between treatments. Preincubation of cells with 40 mM NAC inhibited ATP-induced ethidium$^+$ uptake by 30 ± 2% (Figure 5(b)). Thus, the inhibitory effect of NAC on P2X7-induced ROS formation may be partially attributed to inhibition of P2X7 itself. However, incubation with NAC and ATP, but not either compound alone, reduced the amount of gated viable cells by ~40% in the ethidium$^+$ uptake assay (as assessed by forward and side scatter) (data not shown). This suggests that the inhibitory action of NAC on ATP-induced ethidium$^+$ uptake may be a result of cytotoxicity in this assay.

To confirm that NAC did not induce morphological changes or cause cytotoxicity under the conditions used for

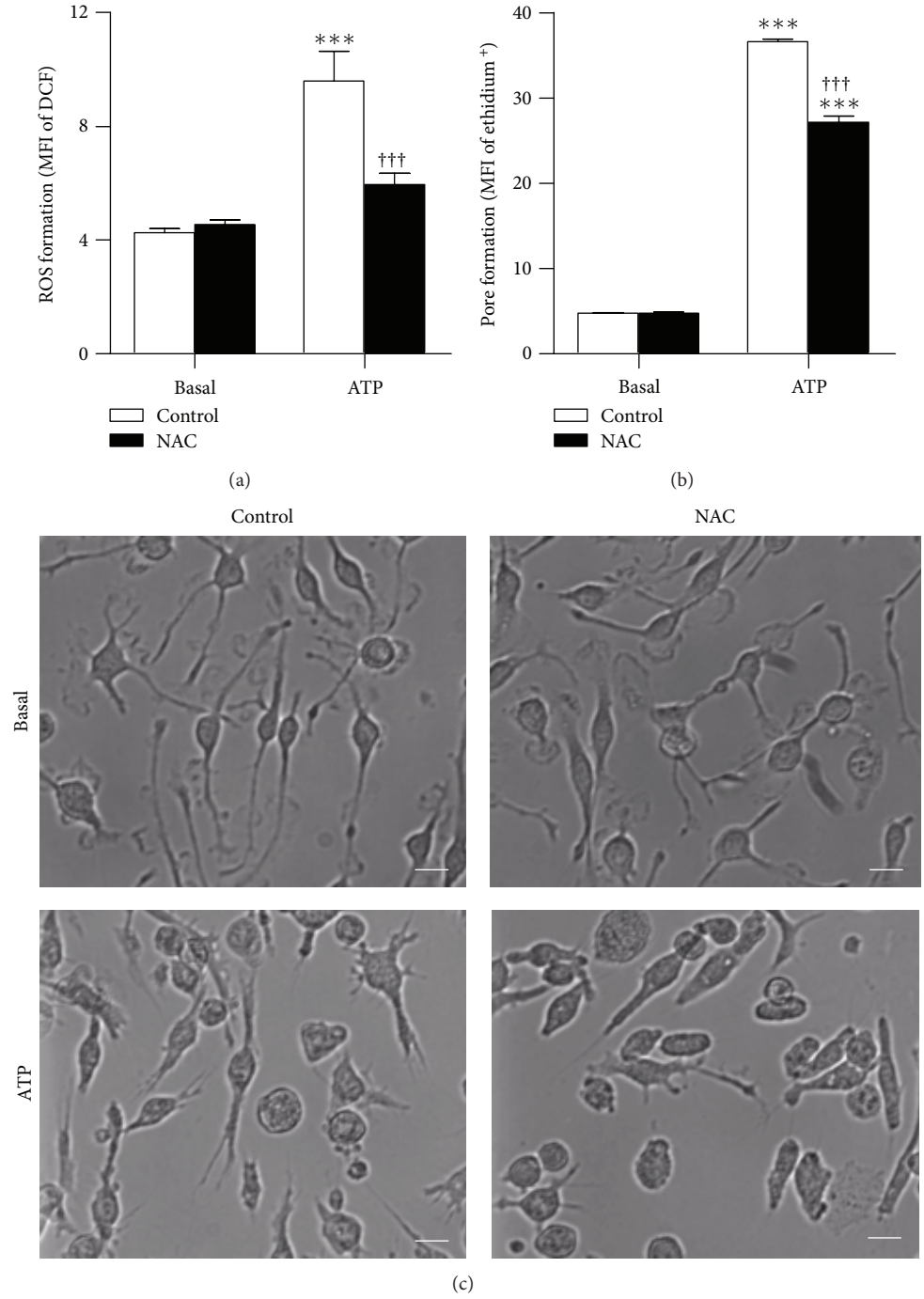


Figure 5: The ROS scavenger NAC inhibits P2X7-induced ROS and pore formation in EOC13 microglial cells. (a and c) Adherent DCF-loaded EOC13 cells or (b) suspended EOC13 cells in NaCl medium were preincubated in the absence (control) or presence of 40 mM NAC at 37°C for 30 min and then in the absence (basal) or presence of 1.4 mM ATP for (a and c) 15 min or (b) 5 min in the presence of 25 μM ethidium$^+$. (a–c) Incubations were stopped by the addition of $MgCl_2$ medium and (a and b) centrifugation. (a and b) Mean fluorescence intensities (MFI) of (a) DCF (ROS formation) or (b) ethidium$^+$ uptake (pore formation) were determined by flow cytometry and results shown as means ± SD, $n = 3$; $^{***}P < 0.001$ compared to corresponding basal; $^{†††}P < 0.001$ compared to corresponding ATP in the absence of NAC. (c) DIC images of cell morphology were acquired by microscopy. Bars represent 20 μm. Results are representative of 2 experiments.

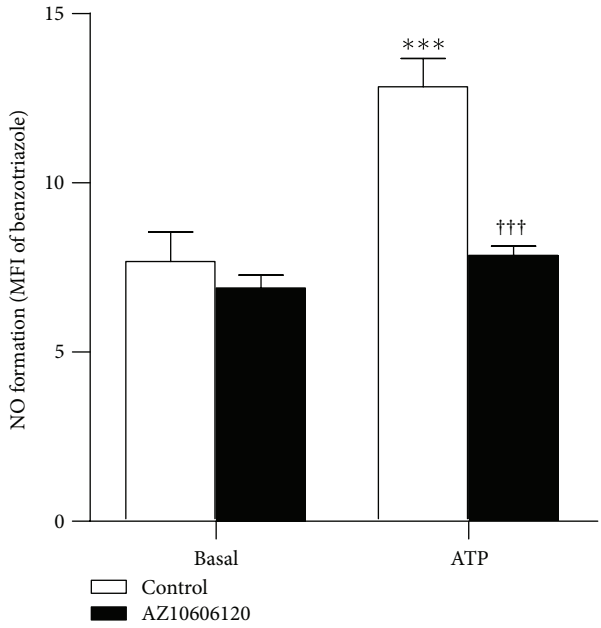


Figure 6: P2X7 activation induces NO formation in EOC13 microglial cells. Adherent DAF-FM DA-loaded EOC13 cells in NaCl medium were preincubated in the absence (control) or presence of 10 μM AZ10606120 at 37°C for 15 min and then in the absence (basal) or presence of 1.4 mM ATP for 15 min. Incubations were stopped by the addition of $MgCl_2$ medium and centrifugation. Mean fluorescence intensities (MFI) of benzotriazole (NO formation) were determined by flow cytometry and results shown as means ± SD, $n = 3$; $^{***}P < 0.001$ compared to corresponding basal; $^{\dagger\dagger\dagger}P < 0.001$ compared to corresponding ATP.

the ROS assay, DIC images of adherent cells were acquired following incubation in the absence or presence of ATP. Cells incubated in the absence or presence of NAC (without ATP) displayed discrete cell bodies with long, spindled shaped processes (Figure 5(c)), as previously observed [13]. Cells incubated in the absence or presence of NAC (with ATP) also displayed discrete cell bodies, but with retracted and branched processes (Figure 5(c)), typical of ATP causing membrane changes [29]. Therefore, in the ROS assay, EOC13 cell morphology was not altered by NAC when compared to the corresponding treatment.

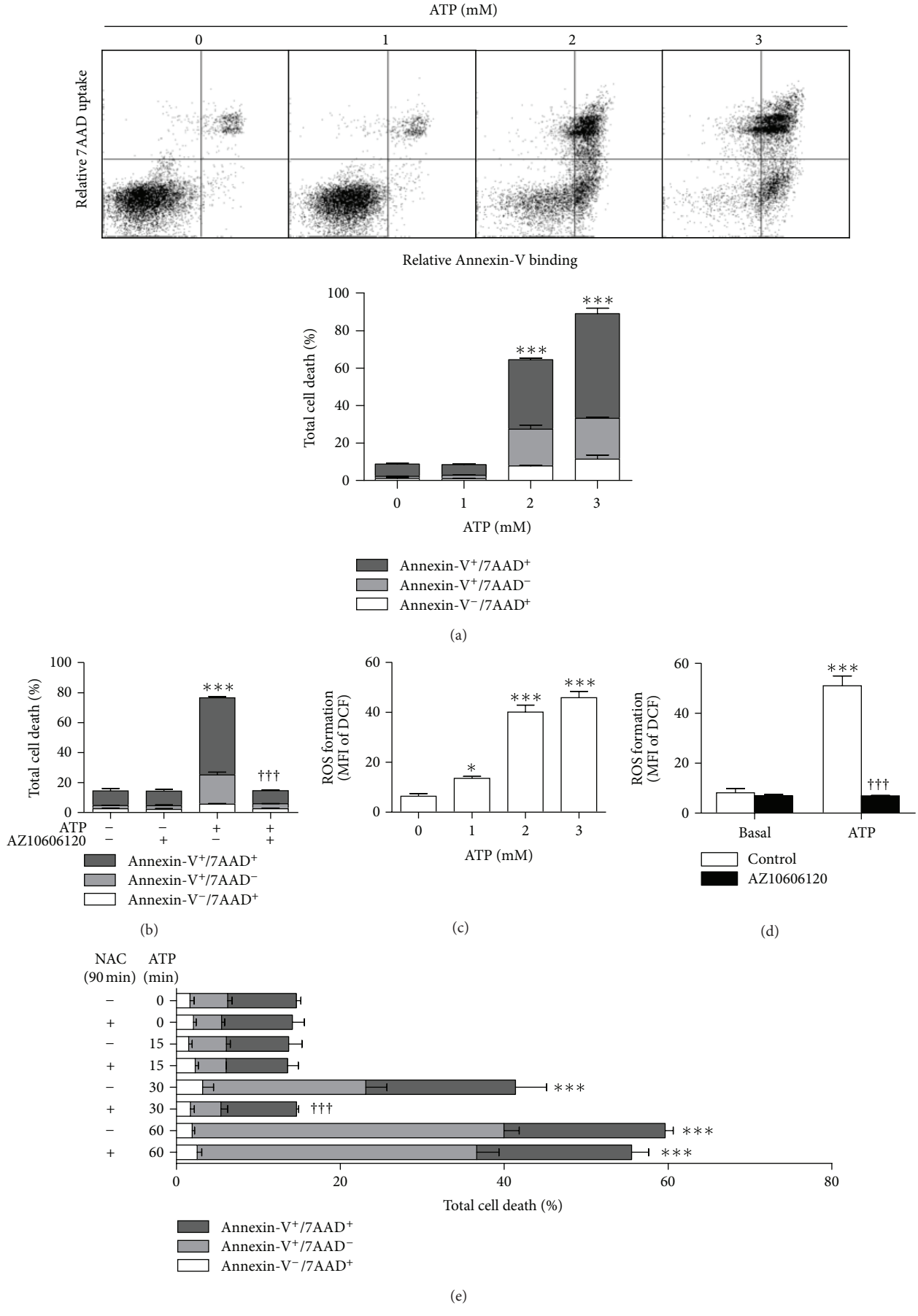
DCF-loaded cells were also preincubated in the absence or presence of the broad-spectrum ROS inhibitor DPI and the ATP-induced ROS formation investigated. However, a 30 min preincubation with DPI at various concentrations (5–40 μM) led to high amounts of cell death (data not shown), and thus this compound was not examined further.

To further verify that P2X7 activation induces the formation of reactive species in EOC13 cells, ATP-induced NO formation was investigated using the NO sensitive probe DAF-FM DA. Cells loaded with DAF-FM DA (which reacts with NO to form a fluorescent benzotriazole) were preincubated in the absence or presence of AZ10606120, followed by incubation in the absence or presence of ATP, and the subsequent NO formation analysed by flow cytometry. Incubation with 1.4 mM ATP induced significant NO formation in EOC13 cells compared to cells incubated in the absence of ATP (Figure 6). Furthermore, preincubation of cells with 10 μM AZ10606120 inhibited ATP-induced NO formation by 82 ± 11% (Figure 6), indicating that this process is mediated by P2X7 activation. Again, AZ10606120 did not significantly alter the basal NO formation (Figure 6) or cell viability (as assessed by forward and side scatter) (data not shown).

3.5. P2X7 Activation Induces Cell Death in EOC13 Microglial Cells. P2X7 activation results in the death of various cell types [11, 12]. To determine whether ATP induces the death of EOC13 microglia, cells in complete DMEM medium were incubated in the absence or presence of ATP for 24 h, and then the percentage of Annexin-V^+/7AAD$^-$, Annexin-V^-/7AAD$^+$, and Annexin-V^+/7AAD$^+$ cells examined by flow cytometry (Figure 7(a)). Cell death is expressed as the total of dying (Annexin-V^+/7AAD$^-$) and dead (Annexin-V^-/7AAD$^+$ and Annexin-V^+/7AAD$^+$) cells. Incubation with either 2 or 3 mM ATP but not 1 mM ATP resulted in significantly higher percentages of total cell death compared to cells incubated in the absence of ATP (Figure 7(a)). Next, to determine if the ATP-induced EOC13 death was mediated by P2X7 activation, cells were preincubated in the absence or presence of AZ10606120 and then incubated in the absence or presence of ATP for 24 h. As above (Figure 7(a)), 2 mM ATP induced significant cell death, with higher percentages of total cell death compared to cells incubated in the absence of ATP (Figure 7(b)). Preincubation with 10 μM AZ10606120 completely inhibited ATP-induced cell death (Figure 7(b)), indicating that this process is mediated by P2X7 activation.

P2X7-induced death of murine RAW264.7 macrophages is mediated by ROS formation [30]. Therefore, a role for ROS in P2X7-induced death of EOC13 microglia was investigated. To confirm that P2X7 induced ROS formation under conditions used to induce cell death, DCF-loaded EOC13 cells in complete DMEM medium were incubated in the absence or presence of ATP, and then subsequent ROS formation determined by flow cytometry. Similar to ATP-induced cell death (Figure 7(a)), incubation with 2 or 3 but not 1 mM ATP induced significant ROS formation in EOC13 cells compared to cells incubated in the absence of ATP (Figure 7(c)). The requirement for higher ATP concentrations to induce cell death (Figure 7(a)) or ROS formation (Figure 7(c)) compared to pore formation (Figure 3(b)) is in line with the inhibitory action of divalent cations [15], which are present in the culture medium but not in the NaCl medium used. To confirm that this ROS formation was mediated by P2X7, cells were preincubated with AZ10606120 and the amounts of ATP-induced ROS formation determined. To parallel the cell death experiments, 2 mM ATP was utilised. Again, ATP induced significant ROS formation compared to cells incubated in the absence of ATP (Figure 7(d)). Preincubation with 10 μM AZ10606120 completely inhibited this ATP-induced ROS formation (Figure 7(d)).

Finally, the effect of NAC on P2X7-induced cell death was investigated. EOC13 cells were preincubated in the absence or presence of NAC followed by ATP for 24 h. However, 24 h incubation with 40 mM NAC in the absence of ATP led to



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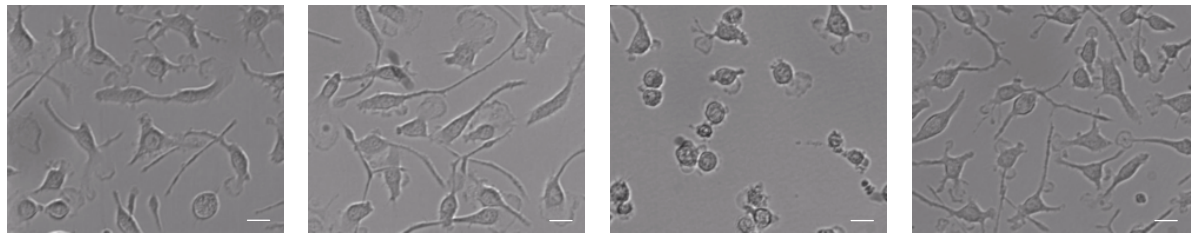

(f)

Figure 7: P2X7 activation induces cell death in EOC13 microglial cells. (a) Adherent EOC13 cells in complete DMEM medium were incubated in the absence or presence of varying concentrations of ATP (as indicated) at 37°C for 24 h. (b) Adherent cells in complete DMEM medium were preincubated in the absence or presence of 10 μM AZ10606120 at 37°C for 15 min and then in the absence or presence of 2 mM ATP for 24 h. (e and f) Adherent cells in complete DMEM medium were incubated in the absence or presence of 40 mM NAC at 37°C for 90 min and incubated in the absence or presence of 2 mM ATP for the final (e) 15–60 min or (f) 45 min (of the 90 min incubation), and then the medium replaced with fresh complete DMEM medium for 24 h. (a, b, and e) Cells were harvested, labelled with Annexin-V-Fluorescein and 7AAD, and the percentage of Annexin-V$^-$/7AAD$^+$, Annexin-V$^+$/7AAD$^-$, and Annexin-V$^+$/7AAD$^+$ cells (together representing total cell death) determined by flow cytometry. (f) DIC images of cell morphology were acquired by microscopy. Bars represent 20 μm. (c) Adherent DCF-loaded cells in complete DMEM medium were incubated in the absence (basal) or presence of varying concentrations of ATP (as indicated) at 37°C for 15 min. (d) Adherent DCF-loaded cells in complete DMEM medium were preincubated in the absence (control) or presence of 10 μM AZ10606120 at 37°C for 15 min and then in the absence (basal) or presence of 2 mM ATP for 15 min. (c and d) Incubations were stopped by the addition of $MgCl_2$ medium and centrifugation. Mean fluorescence intensity (MFI) of DCF (ROS formation) was determined by flow cytometry. Results shown as (a) dot plots of one representative set of data demonstrating the quadrant markers and (a–e) means ± SD, $n = 3$; $^{***}P < 0.001$ or $^{*}P < 0.05$ compared to (a and c) 0 mM ATP, (b and d) corresponding basal, or (e) corresponding 0 min ATP; $^{\dagger\dagger\dagger}P < 0.001$ compared to corresponding ATP in the absence of (b and d) AZ10606120 or (e) NAC.

significant amounts of EOC13 cell death (data not shown). Therefore, to reduce the total exposure to 40 mM NAC, cells were incubated in the absence or presence of NAC for 90 min, with ATP added in the final 15–60 min. The medium was then replaced with fresh complete DMEM medium and the cells incubated for 24 h. Incubation with 2 mM ATP for 30 or 60 min but not 15 min resulted in significant cell death compared to cells incubated in the absence of ATP (Figure 7(e)). In contrast to the 24 h incubation with NAC, 90 min incubation with 40 mM NAC (without ATP) did not induce significant cell death compared to cells incubated for the same length of time in the absence of both NAC and ATP (Figure 7(e)). A 60 min preincubation with NAC inhibited cell death induced by 30 min incubation with ATP by 99 ± 6% (Figure 7(e)). In contrast, a 75 and 30 min preincubation with NAC, followed by 15 and 60 min ATP treatment, respectively, had no effect on the percentage of cell death compared to cells incubated for the same time length with ATP in the absence of NAC (Figure 7(e)).

To further confirm that NAC did not induce morphological changes or cause cytotoxicity under the conditions used for the cell death assay, DIC images of adherent cells were acquired following the 24 h incubation. As above (Figure 5(c)), cells incubated in the absence or presence of NAC (without ATP) displayed discrete cell bodies with long, spindled shaped processes (Figure 7(f)). In addition, cells incubated in the presence of NAC and ATP displayed a similar morphology to that of cells incubated in the absence of ATP (Figure 7(f)). In contrast, cells preincubated with ATP alone displayed rounded and granular cell bodies with no or few processes (Figure 7(f)), characteristic of cell death. Furthermore, wells preincubated with ATP alone had a high amount of nonadherent cells compared to the other treatments (data not shown). Thus, preincubation with NAC prevented the morphological changes associated with ATP incubation, but NAC alone had no effect on cell morphology.

4. Discussion

The current study demonstrates for the first time that the murine microglial EOC13 cell line expresses functional P2X7. Firstly, the presence of P2X7 mRNA and protein was established using RT-PCR and immunoblotting techniques. In addition, the presence of cell-surface P2X7 was demonstrated using immunofluorescence staining. Moreover, P2X7 on EOC13 microglia was functional, as the P2X7 agonists ATP and BzATP induced significant ethidium$^+$ or YO-PRO-1^{2+} uptake into these cells. In these experiments, ATP induced ethidium$^+$ uptake with an EC_{50} value which falls within the typical range for ATP-induced cation fluxes mediated by recombinant murine P2X7 [31]. Furthermore, pretreatment of cells with P2X7 antagonists inhibited ATP-induced organic cation uptake. Lastly, ATP could induce ROS formation in and the death of EOC13 cells, and both of these events, which are often associated with P2X7 activation [10–12], could be inhibited by the P2X7 antagonist AZ10606120. The presence of functional P2X7 on EOC13 microglia is consistent with the presence of this receptor on primary microglia and microglial cell lines (including N9, N13, BV-2, and NTW8 cells) [32–35].

P2X7 activation induced ROS formation in EOC13 microglia. P2X7-induced ROS formation has been reported in primary microglia [24, 25], and a role for this process in microglia has been highlighted by several studies. P2X7 activation induces the production of the ROS, superoxide, in primary rat microglia, while this receptor is upregulated in a transgenic mouse model of Alzheimer's disease [25]. Although a direct link between P2X7, superoxide, and Alzheimer's disease was not established, the authors proposed a link between these molecules and this disease. This link is supported by subsequent observations by others, where fibrillar β-amyloid peptide, which is associated with Alzheimer's disease, caused ATP release and autocrine activation of P2X7 leading to ROS formation in primary rat microglia [24]. In addition, another group demonstrated that P2X7-induced superoxide release from primary rat microglia induced injury of rat cortical neurons [36]. Collectively, this data indicates that P2X7-induced ROS formation from microglia may be involved in various neuroinflammatory and neurodegenerative disorders. This may be of particular importance in diseases where microglial P2X7 is reported to be upregulated such as in Alzheimer's disease, multiple sclerosis, and amyotrophic lateral sclerosis [25, 37]. It should be noted, however, that DCF, as employed in the current study and as widely used by others to detect ROS, can also propagate ROS formation [38]. Nevertheless, our observation that P2X7 activation also induces the formation of NO in EOC13 cells supports a role for this receptor in the formation of reactive species.

The current study excluded an essential role for Ca^{2+} influx in P2X7-induced ROS formation in EOC13 microglia. This finding is similar to other observations with other murine cell types, including submandibular glands [39] and erythroid cells [40]. In contrast, P2X7-induced ROS formation in primary rat microglia [24] and rat submandibular glands [27] is dependent on an influx of Ca^{2+}. The reason for this difference between these two species remains unknown but may reflect differences in experimental protocols or differences in signalling molecules between mice and rats. The current study also excluded an essential role for K^+ efflux in P2X7-induced ROS formation in EOC13 microglia. Both ROS formation and K^+ efflux are involved in IL-1β release from monocytes, although whether these downstream processes are linked has not been established [28]. Thus, our results indicate that P2X7-induced ROS formation does not require K^+ efflux and that ROS formation and K^+ efflux may be independent events in IL-1β release from myeloid cells.

P2X7 activation also induced cell death in EOC13 microglia. Use of an Annexin-V/7AAD assay suggested that this process was mediated by apoptosis. However, in the absence of other markers of apoptosis and necrosis, this remains to be established, especially since P2X7 activation induces both apoptosis and necrosis in the microglial N13 cell line [41]. Nevertheless, our observations support previous studies in which P2X7 activation induced death in primary microglia and other microglial cell lines [41, 42]. The physiological role of P2X7-induced microglia death is unclear. Further obscuring this is the known role of P2X7 activation in inducing the proliferation of microglia [43]. This paradoxical role of P2X7 is thought to be related to the relative ATP concentration, with high concentrations promoting cell death and low concentrations promoting cell proliferation [44]. In support of this, our study observed that ATP only induced EOC13 cell death at 2 or 3 but not 1 mM ATP. Moreover, our data also showed that a transient incubation with ATP of 30–60 but not 15 min induced cell death in EOC13 microglia. This suggests that transient ATP release and subsequent P2X7 activation may be sufficient to kill microglia *in vivo*.

The current study examined a potential link between P2X7-induced ROS formation and death in EOC13 microglia. A previous study demonstrated that the ATP-induced death of murine RAW264.7 macrophages was mediated by ROS derived from NADPH oxidase downstream of P2X7 activation [30]. This contrasts with another study, which found that P2X7-induced ROS formation, but not death, was attenuated in primary macrophages from NADPH oxidase deficient mice [45]. Our data using the ROS scavenger NAC supports a role for ROS formation in the P2X7-induced death of EOC13 microglia. The capacity of NAC to prevent P2X7-induced EOC13 microglia death was dependent on the preincubation time with NAC, as well as the total incubation time with ATP, with only 45–60 min preincubations with NAC preventing cell death induced by transient 30–45 min exposures to ATP. In contrast, 24 h incubation with NAC induced significant amounts of EOC13 microglia death, equivalent to that induced by ATP alone. This cytotoxicity of NAC may have occurred due to increased toxic metabolic byproducts such as reduced glutathione [46]. Alternatively, scavenging of ROS by NAC may indicate that low amounts of ROS are important for EOC13 cell homeostasis. Of note, the ROS inhibitor DPI also induced the death of EOC13 microglia, albeit over a much faster time course. Finally, it should be noted that NAC inhibition of P2X7-induced death and ROS formation in EOC13 microglia may have been partly due to direct inhibition of P2X7. NAC inhibited ATP-induced pore formation by 30% compared to a 74 and 99% inhibition of ATP-induced ROS formation and cell death, respectively. This direct inhibition of P2X7 by NAC was not due to an acidic pH, which is known to impair P2X7 function [47], as the NAC-containing solutions were adjusted to pH 7.4 before each assay. Thus, our results indicate that either cellular signalling involving ROS may modulate P2X7 activation in EOC13 microglia or that NAC may directly impair P2X7 at 40 mM. The concentration of NAC used in these experiments (40 mM) is 4–8-fold higher than that used in a number of similar studies (e.g., [48]). The requirement for this high concentration of NAC remains unknown but may reflect a reduced ability of NAC to cross the plasma membrane or to be converted to glutathione in EOC13 cells.

The presence of functional P2X7 on J774 macrophage cells was confirmed in the current study. P2X7 is present in this cell line [17], and activation of P2X7 leads to the release of mature IL-1β [49], the formation of macrophage-derived multinucleated giant cells [50–52], and cell death [53]. In this

study, the potency of four P2X7 antagonists against 1 mM ATP, the ATP concentration most commonly used to study P2X7, was determined. The IC_{50} values for BBG, A438079, and AZ11645373 (1.8, 7.9, and 1.5 μM, resp.) were within one log range of those published for recombinant murine P2X7 [54, 55]. In contrast, the IC_{50} value for AZ10606120 has not been reported for murine P2X7, although this compound has been shown to specifically bind to and inhibit rat and human P2X7 [20]. In the current study, this highly specific P2X7 antagonist also completely impaired ATP-induced ethidium$^+$ uptake, ROS formation, and death of murine EOC13 cells. Thus, AZ10606120 will be useful for future studies of murine P2X7.

In the CNS, extracellular ATP acting through P2X7 on microglia is an important mediator of neuroinflammation [9]. ATP acts as a neurotransmitter and is released from neurons during synaptic transmission and from dying cells [56]. Under normal physiological conditions, extracellular ATP concentrations in the CNS are estimated to be in the nanomolar to micromolar range, depending on the balance between ATP release and degradation, while intracellular microglial ATP concentrations are in the millimolar range [57]. After CNS injury, however, extracellular ATP concentrations increase and can reach as high as the millimolar range [57]. Furthermore, it is hypothesised that ATP may act on microglial P2X7 at very close range where the concentration of ATP may be quite high. Activation of P2X7 on primary microglia and microglial cell lines leads to the release of proinflammatory IL-1β and tumour necrosis factor-α [33, 58] and ROS formation [24]. Although proinflammatory factors are important for immunity [1], prolonged or inappropriate release of such factors from chronically activated microglial can be highly toxic to neurons and can promote neuroinflammation and neurodegeneration [5]. There are a number of diseases in the CNS characterised by the presence of activated microglia, including Alzheimer's disease, prion infection, cerebral ischemia, multiple sclerosis, and amyotrophic lateral sclerosis. In such diseases, P2X7 has also been reported to be upregulated [25, 37, 59, 60]. This raises questions of possible roles for P2X7 in mediating inappropriate microglial responses in CNS disorders.

5. Conclusions

This study demonstrates that EOC13 microglial cells express functional P2X7. Activation of this receptor by ATP resulted in organic cation uptake, ROS formation, and death in these cells. Moreover, the EOC13 cell line may be useful for investigating P2X7-mediated events in microglia and the role of this receptor in microglia-mediated inflammatory disorders.

Abbreviations

7AAD: 7-Aminoactinomycin D
Ab: Polyclonal antibody
APC: Allophycocyanin
ATP: Adenosine-5′-triphosphate
BBG: Brilliant Blue G
BzATP: 2′(3′)-O-(4-Benzoylbenzoyl) ATP
CNS: Central nervous system
DAF-FM DA: 2′,7′-Difluorofluorescein diacetate
DCF: Dichlorofluorescein
DIC: Differential interference contrast
DMSO: Dimethyl sulfoxide
DPI: Diphenyleneiodonium
EC_{50}: Half maximal effective concentration
EGTA: Ethylene glycol tetraacetic acid
FBS: Fetal bovine serum
H_2DCFDA: 2′,7′-Dichlorodihydrofluorescein diacetate
IC_{50}: Half maximal inhibitory concentration
IL-1β: Interleukin-1β
mAb: Monoclonal antibody
MFI: Mean fluorescence intensity
NAC: N-Acetyl-L-cysteine
NHS: Normal horse serum
NO: Nitric oxide
PBS: Phosphate-buffered saline
PMSF: Phenyl-methyl-sulfonyl-fluoride
ROS: Reactive oxygen species.

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Review Article

Pivotal Roles of Monocytes/Macrophages in Stroke

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Stroke is an important issue in public health due to its high rates both of morbidity and mortality, and high rate of disability. Hypertension, cardiovascular disease, arterial fibrillation, diabetes mellitus, smoking, and alcohol abuse are all risk factors for stroke. Clinical observations suggest that inflammation is also a direct risk factor for stroke. Patients with stroke have high levels of inflammatory cytokines in plasma, and immune cells, such as macrophages and T-lymphocytes, are noted within stroke lesions. These inflammatory events are considered as a result of stroke. However, recent studies show that plasma levels of inflammatory cytokines or soluble adhesion molecules are high in patients without stroke, and anti-inflammatory therapy is effective at reducing stroke incidence in not only animal models, but in humans as well. Statins have been shown to decrease the stroke incidence via anti-inflammatory effects that are both dependent and independent of their cholesterol-lowering effects. These reports suggest that inflammation might directly affect the onset of stroke. Microglial cells and blood-derived monocytes/macrophages play important roles in inflammation in both onset and aggravation of stroke lesions. We review the recent findings regarding the role of monocytes/macrophages in stroke.

1. Introduction

Stroke is the third leading cause of death and a major cause of disability in industrialized countries. Ischemic stroke is the most common type of stroke, occurring in approximately 80% of all strokes [1]. A less common type of stroke is hemorrhagic stroke, which occurs due to a subarachnoid hemorrhage and/or an intracerebral hemorrhage. Hypertension, cardiovascular disease, arterial fibrillation, diabetes mellitus, obesity, smoking, and alcohol abuse are risk factors for stroke [2], even if there are slight differences in the influence of these factors between ischemic stroke and hemorrhagic stroke. However, some stroke patients do not have any of these risk factors, suggesting that other risk factors exist. For many years, clinical observations showed that plasma levels of inflammatory cytokines were increased after stroke onset, and immune cells, especially monocytes/macrophages and T-lymphocytes, existed in stroke lesions and related to exaggerate brain damage. In the clinical setting, elevated plasma levels of inflammatory cytokines, C-reactive protein (CRP), and chemokines are associated with future cardiovascular risk [3]. Plasma levels of soluble intercellular adhesion molecule-1 (sICAM-1) and sE-selectin were observed to be increased both in large intracranial artery disease and small-artery disease [4], and plasma levels of ICAM-1 and monocyte chemoattractant protein-1 (MCP-1) were noted to be high in patients with ischemic stroke and myocardial infarction [5, 6]. Epidemiological studies have shown that elevated leukocyte count was associated with the risk for first-time myocardial infarction and ischemic stroke [7–9] and the risk of recurrent myocardial infarction and ischemic stroke in a high-risk population [10]. These observations indicate that inflammatory events occur in stroke patients and increase the risk of stroke recurrence. Recently, both clinical and animal studies revealed that these inflammatory events occurred prior to stroke onset. Plasma levels of soluble vascular cell adhesion molecule-1 (sVCAM-1), sICAM-1, sE-selectin, and MCP-1 were elevated in patients with essential hypertension in the absence of other diseases [11–13]. Anti-inflammatory strategies were shown to suppress the incidence of stroke in both human and animal models. These reports suggest that inflammation might be a risk factor for stroke. We review the recent findings regarding the

role of inflammation, especially monocytes/macrophages, in ischemic stroke which is predominant type of strokes.

2. Monocytes/Macrophages and Stroke

2.1. Atherosclerosis. Atherosclerosis is one of the major risk factors for stroke, and monocytes/macrophages affect the brain indirectly by inducing unstable plaques and plaque rupture in atherosclerotic lesions [14]. It is well recognized that atherosclerosis is an inflammatory disease and macrophages play important roles in the initiation and the progression of atherosclerotic lesion [15]. Accumulation of monocytes/macrophages in the vascular wall occurs early during atherosclerosis [15]. In addition to phagocytosis of oxidized low-density lipoproteins, macrophages secrete interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and transforming growth factor-β1 (TGF-β1). These inflammatory cytokines and growth factors induce endothelial dysfunction, smooth muscle cell migration and proliferation, and extracellular matrix production as fibrous plaques. During later disease stages, activated macrophages secrete several classes of neutral extracellular proteases, including serine proteases, cathepsins, and matrix metalloproteinases (MMPs) [16]. Blood monocytes already express low levels of a few MMPs [17]; however, contact with matrix leads to rapid upregulation of a broad spectrum of MMPs. Cell biology experiments identify mechanisms by which excessive MMP production can cause plaque rupture, either directly by destruction of extracellular matrix [18] or indirectly through actions that promote death of macrophages [19] and vascular smooth muscle cells [20]. Rupture of unstable plaques may lead to thrombotic stroke onset.

2.2. At the Brain. Monocytes/macrophages directly play important roles in stroke at the brain. Microglial cells, the resident macrophages of the brain, and blood-derived monocytes/macrophages have morphologically and functionally similar roles in stroke [21, 22]. Microglial cells are activated rapidly in response to brain injury [23]. This activation occurs within minutes of ischemia onset and induces production of inflammatory cytokines, including IL-1β and TNF-α, which exacerbate tissue damage [24–26]. Following the rapid activation of resident microglial cells, blood-derived immune cells infiltrate into the brain tissue within hours to a few days [21, 22]. Most current data from mice models and humans show that blood-derived macrophages are recruited into the ischemic brain tissue, most abundantly at days 3 to 7 after stroke [27–29]. In contrast, resident microglial cells are already activated rapidly on day 1 after focal cerebral ischemia. Resident microglial cells exist in lesions even at days 4 through 7. These reports suggest that the resident microglial cell activation is induced immediately after brain injury and then blood-derived macrophage infiltration follows. On the other hand, it is reported that macrophages exist in the brain before onset of stroke in stroke-prone spontaneously hypertensive rats (SHRSP) [30, 31]. These findings suggest that the alteration of the blood-brain barrier and macrophage activation occurs before the onset of stroke, and these changes might induce stroke onset.

2.3. Activation of Immune Cells. Neutrophils and lymphocytes are also observed in stroke lesions. In ischemic stroke mice model, macrophages started to appear already at 12 hours after ischemia. On the other hand, lymphocytes (B- and T-lymphocytes) and neutrophils were significantly increased at 3 days after ischemia [32]. According to this observation, it was reported that macrophages produce inflammatory cytokines and upregulate adhesion molecules in endothelial cells, thereby promoting neutrophil accumulation and migration into the brain [33]. These data suggest that macrophage infiltration occurs prior to other immune cells and macrophage activation attracts other immune cells into stroke lesions. Different subtypes of T-lymphocytes play differential roles in the stroke. $CD4^+$ TH1 cells may progress stroke through releasing proinflammatory cytokines, including IL-2, IL-12, IFN-γ, and TNF-α, whereas $CD4^+$ TH2 cells may play a protective role through releasing anti-inflammatory cytokines such as IL-4, IL-5, IL-10, and IL-13 [34]. However, exact role of neutrophils in the stroke is still unclear.

3. Relationship between Monocytes/Macrophages and Hypertension

Hypertension is the principal risk factor for stroke and is a leading cause of cognitive decline and dementia [35]. There is a linear relationship between blood pressure and stroke mortality [36]. Hypertension might induce endothelial cell dysfunction along with macrophage activation and infiltration into the brain. There is emerging evidence that monocyte/macrophage infiltration contributes to hypertension [37].

3.1. Endothelial Cell Dysfunction. Endothelial cell dysfunction is the first step of monocytes/macrophages infiltration into brain. Hypertension might induce endothelial cell dysfunction [38], vascular inflammation on the vascular lumen [39], and monocyte adhesion [40]. It was reported that hypertension promoted or aggravated endothelial dysfunction, which induced the expression of ICAM-1, P-selectin, and monocyte adhesion in a rat model [40]. High intraluminal pressure activated NFκB in an organ culture model of mouse carotid arteries [41]. In humans, the association of chronically or acutely elevated blood pressure with markers of inflammation has also been documented. Circulating levels of sICAM-1, sVCAM-1, sE-selectin, and MCP-1 are increased in patients with essential hypertension [13, 42]. Increasing levels of adhesion molecules and chemoattractant molecules could induce monocyte adhesion on the vascular surface and migration into subendothelial lesions in both aortae and the brain.

3.2. Monocyte/Macrophage Activation. Hypertension might affect blood monocytes directly. The total number of blood

monocytes and activated monocytes is greater in spontaneously hypertensive rats compared with Wistar Kyoto rats, which represent the normotensive control [43, 44]. On the other hand, reducing blood pressure with angiotensin converting enzyme inhibitors suppresses endothelial dysfunction and the number of subendothelial macrophages in the aorta [45]. In humans, circulating monocytes from patients with essential hypertension are preactivated compared with those in normotensive healthy individuals. IL-1β secretion of peripheral blood monocytes stimulated by angiotensin II was shown to be significantly higher in patients with essential hypertension compared with normotensive healthy individuals [46].

3.3. Renal Dysfunction. Inflammatory cells accumulate in perivascular regions in the kidney, and in and around glomeruli in hypertensive rats [47, 48] and hypertensive subjects [49]. There is extensive perivascular infiltration of leukocytes in the kidney of double transgenic rats harboring human renin and angiotensinogen genes. In a study that emphasized the role of inflammation in blood pressure elevation, pyrrolidine dithiocarbamate, an inhibitor of NFκB, prevented monocyte/macrophage infiltration in animals, reduced expression of ICAM-1 and inducible nitric oxide synthase, and reduced blood pressure [48]. There is also evidence of macrophage infiltration in the glomeruli of hypertensive animals [50] and humans [49]. Monocytes/macrophages in the kidney modulate blood pressure via the production of inflammatory cytokines and modulation of renin-angiotensin-aldosterone system [51, 52]. On the other hand, drugs acting on the renin-angiotensin-aldosterone system prevent or modulate inflammation [53]. Monocytes/macrophages might play some important roles in the reciprocal influence between inflammation and hypertension.

4. Animal Models

4.1. Stroke-Prone Spontaneously Hypertensive Rats. SHRSPs are unique genetic model that mimic both microvessel and parenchymal changes in spontaneous stroke [54, 55]. The microvascular changes and brain parenchymal damage may not simply be the result of hypertension, and endothelial cell dysfunction [56] and inflammation may play a role in brain damage [55]. This animal model has been used to examine the contributions of inflammation (macrophages) to stroke. In SHRSP, fed a high-salt diet, rosuvastatin treatment significantly delayed the onset of stroke and attenuated the transcription of inflammatory biomarkers (MCP-1, TGF-β1, IL-1β, and TNF-α) [57]. Pioglitazone, peroxisome proliferator-activated receptor-γ agonist, reduced the risk of recurrent stroke in patients with type 2 diabetes [58]. In SHRSP, pioglitazone delayed the onset of stroke by improving vascular endothelial dysfunction, inhibiting brain inflammation, and reducing oxidative stress [59]. A low dose of acetylsalicylic acid (aspirin) delayed the onset of stroke in SHRSP by suppressing inflammation [60]. In addition to drug treatments, dietary restriction has been shown to delay the onset of stroke in SHRSP via suppression of systemic and local inflammation including macrophage infiltration into the brain [31].

4.2. Middle Cerebral Artery Occlusion. Permanent or transient middle cerebral artery occlusion is an established method for inducing focal ischemic stroke in mice or rats. Middle cerebral artery occlusion produces highly reproducible lesions, and macrophages primarily infiltrate into the core of the ischemic lesion [61]. The focal ischemic stroke model is a closer approximation to human stroke and produces a heterogeneous pathology that includes a necrotic core and salvageable penumbra [62]. However, small differences in surgical technique may account for different effects on the infarct [63, 64]. Furthermore, due to variances in cerebral vascular anatomy, different mouse strains show a different outcome [65, 66]. In addition, conditions of animals during surgery, such as blood pressure, blood gases, body temperature, and anesthesia influence outcome. Thus, standardization and quality control are very important when using this animal model.

4.3. Hypertensive Mice with Salt Loading. There are a lot of hypertensive animal models [67]; however, surgical intervention is needed to induce stroke in these models. Recently, these hypertensive mice have been used to research spontaneous stroke. Excessive salt intake induced frequent thoracic or abdominal cavity hemorrhage in Tsukuba hypertensive mice, which are human renin and angiotensinogen transgenic mice [68]. Hemorrhaging occurred due to the development of aortic aneurysms and rupture at the aortic arch and aorta near the renal arteries. Vascular lesions progressed with structural degeneration of the aortic media. Unfortunately, cerebral pathology was not assessed in this model [68]. Subsequently, a spontaneous stroke model using human renin and angiotensinogen transgenic hypertensive mice, but not Tsukuba hypertensive mice, was reported [69]. In this report, high-salt diet and L-NAME diet induced hemorrhage in the brain stem, cerebellum, and basal ganglia, which were reasonably similar to those observed in patients with hypertension. It is not clarified whether these mice models show ischemic stroke; however, these hypertensive mice, especially renin and angiotensinogen transgenic mice, are useful for experimental stroke research.

5. Inflammatory Cytokines

Inflammatory cytokines, such as IL-1β, IL-6, and TNF-α, are secreted by activated microglial cells and macrophages in stroke lesions and induce the expression of chemokines, which recruit more circulating monocytes/macrophages into lesions and lead to further brain damage. However, the role of each cytokine in stroke is complicated.

5.1. Interleukin-1β. Recently, IL-1β has been considered a therapeutic target for stroke. Chronic increases in IL-1β expression in the brain led to leukocyte infiltration and increased MCP-1 and ICAM-1 expressions in a mouse model

[70], which is a phenotype also seen in stroke lesions. In addition, a number of studies have demonstrated that inhibiting the release or action of IL-1 markedly reduced ischemic cerebral damage in animal models. IL-1α and IL-1β double knockout mice exhibited dramatically reduced ischemic infarct volume compared with wild-type mice [71]. In a meta-analysis of animal model studies, IL-1 receptor antagonist (IL-1Ra), which represents the most advanced approach to modify IL-1 action, reduced infarct volume in models of focal cerebral ischemia [72]. In humans, a phase II clinical trial of intravenous IL-1Ra compared with placebo in patients with acute stroke is currently underway [73]. Further, IL-1Ra gene polymorphism represents a risk factor for ischemic stroke [74, 75]. These reports suggest that inhibition of IL-1β signals can prevent the onset of stroke.

5.2. Interleukin-6. A prospective cohort study and systemic review revealed that plasma levels of IL-6 were associated with poor outcome after both ischemic and hemorrhagic strokes [76]; however, it was not clear whether IL-6 increased before or after stroke onset. Animal models showed less association between IL-6 and stroke. IL-6 could not induce adhesion molecules and MCP-1 mRNA expressions in cerebrovascular endothelial cells derived from SHRSP [31]. Mice deficient in IL-6 showed similar stroke lesion volume and neurological function as control mice in an acute ischemic injury model [77]. Furthermore, IL-6 mediates anti-inflammatory effects in addition to its proinflammatory role [78]. Therefore, its manipulation can have either detrimental or beneficial effects. Further studies are required to clarify the role of IL-6 in stroke.

5.3. Tumor Necrosis Factor-α. Increased serum and cerebrospinal fluid levels of TNF-α have been found in patients 24 hours, 1 week, and 2 weeks after stroke, and these increases correlate with infarct volume and severity of neurological impairment [79]. However, previous reports suggest that TNF-α has a dual role in brain injury [80, 81]. Blockade of TNF-α actions reduced infarct volume after permanent middle cerebral artery occlusion in BALB/C mice with the dimeric type I soluble TNF receptor, which binds to TNF-α and antagonizes its action [82]. In contrast, TNF-α pretreatment was neuroprotective against permanent middle cerebral artery occlusion in BALB/C mice with reduction of infarct size, macrophages, and CD11b-positive neutrophils [83]. In addition to these observations, pentoxifylline, an anti-inflammatory agent, attenuated damage of stroke via the dual role of TNF-α. Pentoxifylline treatment increased serum levels of TNF-α, but not IL-1β and IL-6, and dose dependently prevented the occurrence of spontaneous brain damage by reducing macrophage infiltration into lesion in SHRSP [84], suggesting a protective role of TNF-α. On the other hand, pentoxifylline reduced brain edema in a rat model of transient focal cerebral ischemia through a decline in TNF-α production [85], suggesting an deleterious role of TNF-α. Although anti-TNF-α strategies have proved beneficial in other clinical settings such as inflammatory bowel disease, there are no clinical trials of anti-TNF-α agents in stroke. Further studies are required to clarify the role of TNF-α in stroke.

5.4. MCP-1. CC chemokine ligand (CCL2) is known as MCP-1 and is a potent mononuclear cell attractant. MCP-1 is synthesized by several cell types, such as monocytes/macrophages, T-lymphocytes, smooth muscle cells, endothelial cells, and even cerebrovascular endothelial cells. Expression of MCP-1 is upregulated by inflammatory cytokines. Serum levels of MCP-1 are high in patients with ischemic stroke and myocardial infarction [5, 6], which might be interpreted as a stroke-induced increases in inflammatory events. On the other hand, there is one report that serum CCL2 levels in acute ischemic stroke patients did not differ from that in controls at 1 to 3 days after stroke onset [86]. In this paper, details of controls were not shown, but one of the possibilities is that control subjects were hypertensive. It is reported that plasma levels of MCP-1 were elevated in patients with essential hypertension in the absence of other diseases [13]. The MCP-1-deficient mice model is a unique model to elucidate the role of macrophages in stroke [87]. Compared with control mice, infarct volume was smaller in MCP-1-deficient mice 24 hours after middle cerebral artery occlusion, and a reduction of phagocytic macrophage accumulation within infarcts and the infarct border in MCP-1 deficient mice 2 weeks after middle cerebral artery occlusion. In addition, MCP-1 deficient mice produced less IL-1β in ischemic tissue. This means that MCP-1 and IL-1β are key factors of macrophages in stroke lesions.

5.5. Adipokines. Obesity is also recognized as the risk factor for stroke, because obesity is associated with hypertension and inflammation via secretion of adipokines, such as adiponectin, leptin, resistin, adipsin, plasminogen activator inhibitor-1, and inflammatory cytokines [88–90]. It is well known that macrophage infiltration into adipose tissue induces inflammation in adipose tissue and influences these adipokine secretions [91, 92]. The most commonly studied adipocytokines are leptin and adiponectin. There are a lot of reports about the association of leptin and adiponectin with stroke, and leptin and adiponectin show differential association patterns with ischemic stroke [93]. It is reported that higher leptin levels and lower adiponectin levels were found in stroke patients [94]. On the other hand, there are controversial reports that adiponectin, but not leptin, levels are recognized as a predictor of the risk for stroke [95], or that leptin, but not adiponectin, levels are recognized as a predictor of the risk for stroke in men, but not women [96]. It is not clear whether adiponectin and leptin are useful predictors of stroke in obese subjects; however, adiponectin and leptin might directly influence stroke incidence. It is reported that leptin stimulates macrophages and that adiponectin suppresses it. Leptin increases the mRNA and protein levels of IL-1β, IL-6, IL-12, TNF-α, cyclooxygenase-2, and MCP-1 in macrophages and endothelial cells [97, 98]. Adiponectin inhibits pro-inflammatory signaling in human macrophages [99] and promotes macrophage polarization toward an anti-inflammatory phenotype [100]. Adiponectin also increases

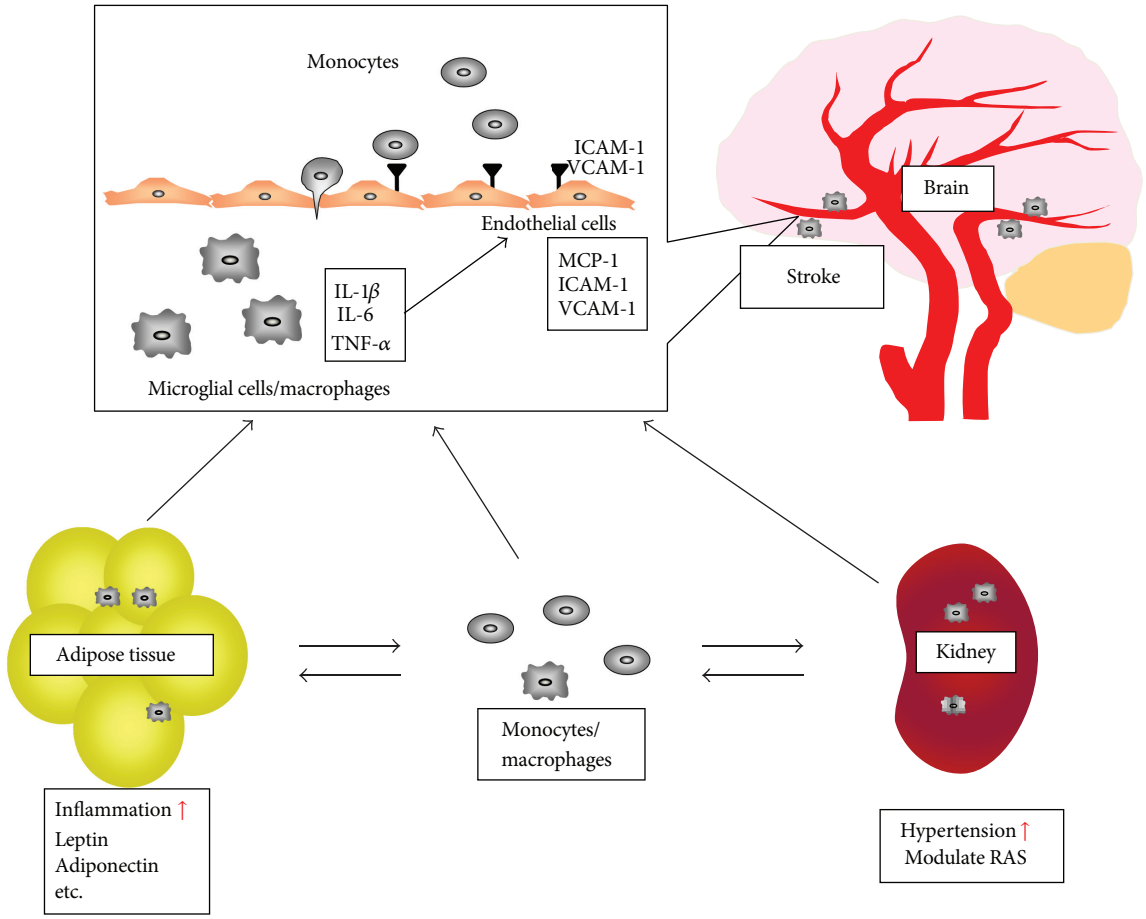

Figure 1: Monocytes/macrophages modulate adipose tissue and kidney functions and accelerate stroke. Monocytes/macrophages infiltration into adipose tissue stimulates secretion of leptin and inflammatory cytokines and suppresses secretion of adiponectin, which induce systemic inflammation, endothelial cell dysfunction, and monocytes/macrophages activation. Monocytes/macrophages infiltration into kidney modulates renin-angiotensin system and increase blood pressure, which also induces endothelial cell dysfunction and monocytes/macrophages activation. Endothelial cells express MCP-1 and adhesion molecules, which induce monocytes chemotaxis, adhesion, and migration into subendothelial lesions. Microglial cells and infiltrated monocytes/macrophages in brain induce cerebrovascular damages and cause stroke onset.

IL-10, an anti-inflammatory cytokine, as well as mRNA expression in human monocyte-derived macrophages [101]. In addition, both adiponectin and leptin receptors are expressed in the brain, suggesting that these adipokines might be directly associated with stroke [102, 103].

6. Anti-Inflammatory Strategies

There are several reports that treatment with drugs that have anti-inflammatory properties can prevent stroke not only in animal models, but also in humans.

6.1. Statins. Rosuvastatin treatment significantly delayed the onset of stroke and attenuated the transcription of inflammatory biomarkers [57]. Clinical studies using statins already use inflammatory events as endpoints for stroke prevention. In healthy persons without hyperlipidemia but with elevated high-sensitivity CRP levels, rosuvastatin, which lowered high-sensitivity CRP as well as cholesterol levels, reduced the incidence of stroke and myocardial infarction by 50% relative to placebo [104]. A meta-analysis of statin trials showed that statins might reduce the incidence of all strokes by decreasing LDL-cholesterol without increasing the incidence of hemorrhagic stroke [105]. In addition to cholesterol-dependent effects, cholesterol-independent effects of statins on stroke have also been recognized [106, 107]. However, statin treatment increases the risk of hemorrhagic stroke in patients with a history of cerebrovascular disease, even though it also clearly decreased the risk of ischemic stroke [108]. Therefore, patients undergoing statin treatment should be carefully monitored to avoid achieving very low level of cholesterols, which are known risk for hemorrhagic stroke [109].

6.2. Thiazolizinediones. Thiazolidinediones, including rosiglitazone and pioglitazone, are peroxisome proliferator-activated receptor-γ (PPAR-γ) agonists used in the treatment of type 2 diabetes. A systemic review showed that

rosiglitazone and pioglitazone were similarly effective in reducing infarct volume and protecting neurologic function in a rodent model of focal or global cerebral ischemia [110]. Pioglitazone delayed the onset of stroke by improving vascular endothelial dysfunction and brain inflammation in SHRSP. Pioglitazone suppressed macrophage infiltration, MCP-1 and TNF-α gene expressions in the brain [59]. Rosiglitazone induced upregulation of CD36 in macrophages and enhanced the ability of microglia to phagocytose red blood cells, which helped to improve hematoma resolution, and improved functional deficits in an intracerebral hemorrhage mouse model [111]. In humans, the PROspective pioglitAzone Clinical Trial In macroVascular Events (PROACTIVE) [112] showed that pioglitazone significantly reduced the risk of recurrent stroke in high-risk patients with type 2 diabetes [58]. On the other hand, one report showed that compared with pioglitazone, rosiglitazone was associated with an increased risk of stroke, heart failure, and all-cause mortality and an increased risk of the composite of acute myocardial infarction, stroke, heart failure, or all-cause mortality in patients of 65 years or older [113].

6.3. Other Anti-Inflammatory Drugs. Low-dose acetylsalicylic acid (aspirin) also delayed the onset of stroke in SHRSP via suppression of inflammation [60]. Aspirin reduced salt-induced macrophage accumulation and MMP-9 activity at the stroke-negative area in the cerebral cortex of SHRSP [60]. Frequent aspirin use might also confer a protective effect for risk of stroke in humans [114, 115]. Terutroban, a specific thromboxane/prostaglandin endoperoxide receptor antagonist, decreased cerebral mRNA expressions of IL-1β, transforming growth factor-β, and MCP-1 and increased survival in SHRSP [116]. These effects were similar to rosuvastatin and aspirin [116]. The Prevention of cerebrovascular and cardiovascular Events of ischemic origin with terutroban in patients with a history of ischemic stroke or transient ischemic attack (PERFORM) study was started in February 2006 [117]. Recently, it was reported that PERFORM study did not meet the predefined criteria for noninferiority, but showed similar rates to terutroban and aspirin for the primary endpoint, such as a composite of fatal or nonfatal ischemic stroke, fatal or nonfatal myocardial infarction, or other vascular death [118]. These reports indicate that antiplatelets agents, which also have anti-inflammatory properties, could suppress inflammation and prevent stroke onset.

7. Beneficial Roles after Stroke

It is generally believed that the activated microglial cells in ischemic injury are neurotoxic, and results of several recent studies revealed that microglial cells might exert neuroprotective effects in certain conditions [119, 120]. In addition to the primary role of macrophages, which is the phagocytosis of cellular and fibrillar debris resulting from stroke, activated microglial cells and macrophages are involved in regulation of the regenerative state and remodeling of the brain by producing brain-derived neurotrophic factor [121, 122], insulin growth factor 1 [123, 124], several other growth factors [125], neuroprotective gene Ym1 [126], and nitric oxide which are known to regulate synaptic functions [127]. As described previously, some cytokines secreted from microglial cells and macrophages, such as IL-6 and TNF-α, and attenuate brain damage. In addition to these mediators, intracranial transplantation of monocyte-derived multipotential cells enhances recovery after ischemic stroke [128]. Whether activated microglial cells and macrophages act as toxic or neuroprotective factors might depend on the time and severity of stroke lesions.

8. Summary

Microglial cells and monocytes/macrophages play important roles in the onset and aggravation of stroke via expression of several inflammatory cytokines at the brain, adipose tissue, and kidney (Figure 1). However, it is also reported that these inflammatory events are important in the reduction of and recovery from brain damage. However, it is clear that suppression of inflammation is effective in the prevention of primary stroke, and macrophages might be therapeutic targets to prevent stroke.

Conflict of Interests

The authors have no conflict of interests to disclosure.

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Review Article

Macrophage Autophagy in Atherosclerosis

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Macrophages play crucial roles in atherosclerotic immune responses. Recent investigation into macrophage autophagy (AP) in atherosclerosis has demonstrated a novel pathway through which these cells contribute to vascular inflammation. AP is a cellular catabolic process involving the delivery of cytoplasmic contents to the lysosomal machinery for ultimate degradation and recycling. Basal levels of macrophage AP play an essential role in atheroprotection during early atherosclerosis. However, AP becomes dysfunctional in the more advanced stages of the pathology and its deficiency promotes vascular inflammation, oxidative stress, and plaque necrosis. In this paper, we will discuss the role of macrophages and AP in atherosclerosis and the emerging evidence demonstrating the contribution of macrophage AP to vascular pathology. Finally, we will discuss how AP could be targeted for therapeutic utility.

1. Introduction

Atherosclerosis-related cardiovascular diseases are the leading cause of mortality worldwide. In addition to lipid dysfunction and arterial lipid accumulation, immune-inflammatory responses are major factors in directing the initiation and development of atherosclerosis [1, 2]. Macrophages play a central role in each stage of disease pathogenesis [3]. Interestingly, recent investigation into macrophage autophagy (AP) has demonstrated a novel pathway through which these cells contribute to vascular disease [4–7]. In this paper, we will discuss the role of macrophages and AP in atherosclerosis and the contribution of macrophage AP to vascular pathology. Finally, we will discuss how AP could be targeted for therapeutic utility in atherosclerosis.

2. The Origin of Vascular Macrophages

Macrophages are defined as diverse, scavenging, and bactericidal tissue-resident cells with critical immune functions. They are present in every endothelial and epithelial surface of the body, exhibit stellate morphology, and express markers including F4/80, CD11b, CD115, macrosialin (CD68), and CD83. They also express an array of Fc receptors, receptors for complement components, scavenging receptors, and pathogen recognition receptors such as Toll-like receptors (TLRs) and Nod-like receptors (NLRs). When activated, tissue macrophages phagocytose and kill microorganisms and secrete proinflammatory cytokines. In addition, the proinflammatory cytokines and chemokines they release upon activation contribute to the recruitment and activation of lymphocytes. However, it is these very functions that drive their well-established role in inflammatory conditions such as atherosclerosis.

The origin of tissue macrophages has been receiving much attention recently, with many long-held concepts proving incorrect. Indeed, many tissue macrophage populations do not arise from blood monocytes but maintain themselves locally in tissues after they are seeded by yolk sac macrophages [8, 9]. However, to our knowledge, the origin of vascular macrophages in the steady state is unclear and during inflammation, it is clear that input from circulating

monocytes is critical [10]. Monocytes originate from common CSF-1R$^+$CX3CR1$^+$Flt3$^+$ macrophage/dendritic cell precursors (MDPs) [11] and expand in response to macrophage colony-stimulating factor (M-CSF) [12]. Monocytes in the mouse can be divided into 2 subsets, classical (Ly6Chi CCR2+) and nonclassical monocytes (Ly6Clo CCR2lo) [13], with analogous subsets present in humans [14]. Classical monocytes exit the bone marrow in a CCR2-dependent manner to seed sites of inflammation [15], whereas it is as yet unclear how and if nonclassical monocytes arise from the bone marrow [16].

A central feature of atherosclerosis is the accumulation in the lesion of monocyte-derived, lipid-laden macrophages termed foam cells and, indeed, monocyte recruitment into plaques is critical for, and increases with, disease progression [10, 17, 18]. Consistent with this, mice deficient in M-CSF-derived macrophages (op/op) have reduced development of atherosclerosis [19]. However, perhaps the most compelling evidence of the role of monocyte-derived cells in atherosclerosis is borne out of successful therapeutic studies in mice targeting chemokine/chemokine receptors critical for monocyte chemoattraction to the plaque [20, 21]. Activation of blood vessel endothelium results in the arrest and extravasation of circulating monocytes into the plaque [22], and the extent of recruitment is regulated at least in part by blood monocyte levels [23]. Hypercholesterolemia correlates with an increase in the frequency of classical monocytes, and it is primarily this subset of monocytes that seeds the plaque [24]. Nevertheless, the capacity of nonclassical monocytes to patrol blood vessel walls [11] could be pertinent to the inflammatory process during atherosclerosis, and indeed this subset has been demonstrated to enter plaques [25, 26].

3. The Role of Macrophages in Atherosclerosis

Upon entry into the vascular wall, monocytes undergo maturation into macrophages that are critical for the inflammatory response. Although there is undoubtedly heterogeneity in plaque macrophages, the majority of macrophages in the plaque are classically rather than alternatively activated, and this is discussed in recent reviews [27]. Concomitant with this maturation process, macrophages engulf vast amounts of lipid in the form of apoB-containing lipoproteins into membrane-bound droplets to form foam cells [3]. Macrophages utilize scavenger receptors like CD36 and scavenger receptor type A *inter alia* to recognize modified low-density lipoproteins (LDL) [28, 29], and uptake of oxLDL alone can drive inflammatory gene expression in macrophages through a novel recognition pathway involving a CD36-dependent TLR4-TLR6 heterodimer [30]. In addition, signals through scavenging and c-type lectin-like receptors, TLRs, and numerous intracellular sensors can drive macrophage activation. Intriguingly, several endogenous ligands for TLRs such as heat shock proteins (HSPs) can be found in high concentration in atherosclerotic plaques [31] and have been proposed as a potential pathway to activate macrophages and perpetuate atherosclerosis [32]. Interestingly, recent work focusing on the effects of cholesterol crystals on macrophages has uncovered a novel pathway of macrophage activation in atherosclerosis [33, 34]. The intracellular apparatus consisting of NLRP3, ASC, and caspase-1 all cooperate to drive the generation and subsequent release of active IL-1β and IL-18 by macrophages in response to cholesterol crystals and play an important role in the development of atherosclerosis [33]. Thus, there are a plethora of described pathways, and additional putative mechanisms, that drive macrophage activation during atherosclerosis.

Once activated, macrophages produce an array of proinflammatory cytokines such as TNFα, IL-12, IL-6, IL-1β [35], and leukotrienes [36] that drive inflammation during atherosclerosis. This, together with their production of inflammatory chemokines such as MCP-1, IL-8, and MIP-3α, results in further recruitment of monocytes, neutrophils and other inflammatory cells. Macrophage-derived TNFα and IL-1β also activate the vascular endothelium to upregulate adhesion molecules and chemokines [22, 37] and thus promote monocyte migration as part of a positive feedback loop. Activation of macrophages is also enhanced by IFNγ released by NK cells and T cells, and macrophage-derived cytokines, such as IL-12 and IL-15, can in turn drive proatherogenic T cells [38]. In addition to the production of inflammatory mediators, macrophage activation results in the induction of several bactericidal systems such as the NADPH oxidase enzyme. This converts oxygen into the superoxide anion and other free radicals, and these reactive oxygen intermediates (ROIs) are toxic to microbes but can damage host tissue due to their capacity to cause DNA degradation and inactivation of metabolic enzymes, and indeed perpetuate atherosclerosis [39]. Activated macrophages also release nitric oxide (NO) which combined with superoxide, generate peroxynitrite which causes cell injury [39, 40]. Further, myeloperoxidase-(MPO-) generated reactive nitrogen species from monocytes contributes to the conversion of LDL to an atherogenic form [41]. In addition, macrophages express nonspecific esterase, lysosomal hydrolases, and ectoenzymes [42], and secrete an array of cathepsins [43] and matrix metalloproteinases (MMPs) [44] that degrade collagens and the extracellular matrix, likely contributing to thinning of the fibrous cap and plaque instability.

Combined with overzealous macrophage activation in atherosclerotic plaques is the impairment of macrophage functions critical for the control and resolution of inflammation. Indeed, an important function of macrophages under both resting and inflammatory conditions is the rapid uptake of apoptotic cells from tissues, termed efferocytosis. Efferocytosis is mediated by a range of receptors such as CD36 [45] and MerTK [46], and chronic apoptosis of lipid-filled foam cells is combined with defective efferocytosis during atherosclerosis [47], likely contributing to the formation of the necrotic core. Interestingly, the receptors involved in the recognition of apoptotic cells, such as CD36 and $\alpha v\beta 3$, may also be involved in the recognition of necrotic cells [48]. This may be pertinent when one considers that the vast majority of cell death in advanced plaques is the result of necrosis, a process that drives inflammation and formation of the necrotic core [49].

Finally, an increasing body of evidence indicates that macrophages have developed several strategies to survive

and proliferate in the atherosclerotic plaque, such as the unfolded protein response and AP [50]. In particular, recent investigation into macrophage AP in atherosclerosis has demonstrated another pathway through which these cells contribute to vascular pathology [4–7].

4. Autophagy Machinery and Regulation

AP (derived from Greek words, "auto" meaning "self" and "phagy" meaning "to eat") is an evolutionarily conserved controlled cellular catabolic process involving the delivery of cytoplasmic contents to the lysosomal machinery for ultimate degradation and recycling. In mammalian cells, several types of AP have been identified; they are differentiated on the basis of their physiological functions and the mode of cargo delivery to the lysosomal compartment, such as chaperone-mediated AP, microAP, macroAP, and others [51]. MacroAP has been studied most extensively as compared with other types of AP and this paper will focus on macroAP (herein referred to as "AP"). The AP mechanism involves the formation of characteristic double-membrane vesicles, called autophagosomes or autophagic vacuoles, in which cytoplasmic material is sequestered. The origins of this structure remain incompletely understood; it may be generated from multiple sources including the endoplasmic reticulum (ER) [52, 53], the outer mitochondrial membrane [52, 54], and the plasma membrane [55, 56]. The autophagosomes are targeted to lysosomes to form single-membraned autolysosomes with degradative capacity. During the degradative phase, a series of lysosomal enzymes (e.g., cathepsins and other acid hydrolases) digest the contents of autolysosomes, that are then released to the cytosol for recycling or reuse for anabolic pathways and to get rid of toxic harmful cellular substances [57, 58].

In mammalian systems, basal AP is a continuous process serving as a quality control system to clear and recycle damaged and/or unwanted components of the cell including organelles and protein aggregates. This pathway is stimulated by numerous cellular or subcellular stresses, together with nutrient or growth factor deprivation, reactive oxygen species (ROS), hypoxia, DNA damage, protein aggregates, dysfunctional organelles, or intracellular pathogens to counter the stress for cell survival [58]. AP is mostly considered as a cell survival and cytoprotective process but under chronic stress situations, it is also associated with cell death (hence called "autophagic cell death" rather than "cell death with autophagic features"), though the meaning of AP in these situations remains controversial [59]. It is now well acknowledged that AP can exert a critical and decisive influence on a great variety of human physiological and pathophysiological processes, such as cancer, neurodegenerative disorders and cardiovascular diseases [60]. Moreover, the AP machinery also orchestrates various responses to exogenous stimuli such as microorganisms [61]. For instance, AP plays a key role in the defense against bacterial infection [62, 63]. AP is also required for antigen presentation via major histocompatibility complex (MHC) class II, which plays a key role in immune driven diseases [64], including atherosclerosis [65].

Recently, progress has been made in characterizing the AP protein machinery and signaling cascades. It has been demonstrated that, in mammalian cells, the proteins encoded by AP-related genes (Atg) generally form multiprotein complexes that are crucial for autophagosome formation. The core machinery of mammalian AP incorporates five functional subgroups: (i) the unc-51-like kinase (ULK) complex, including ULK1/2, Atg13, and FIP200, involved in AP induction; (ii) the class III phosphatidylinositol 3-kinase (PI3K) complex, consisting of Vps34, Beclin 1, p150, and Barkor (Atg14-like protein). The lipid kinase activity of Vps34 is indispensable for generating phosphatidylinositol (3)-phosphate (PI3P) at the PAS (phagophore assembly site) for the recruitment of other Atg proteins and the formation of the autophagosome; (iii) two ubiquitin-like protein (Atg12 and LC3) conjugation systems involved in the expansion of autophagosome membranes; (iv) Atg9 and its recycling system that contributes to the delivery of membranes forming the autophagosome; (v) the proteins needed for the fusion between autophagosomes and lysosomes for ultimate degradation. The detailed literature concerning these topics can be found elsewhere [51, 58]. However, several non-Atg proteins and different macromolecular signaling complexes are shown to contribute in the regulation of this process [51, 66, 67].

Mammalian target of rapamycin (mTOR), and particularly its complex 1 (mTORC1), acts as a major checkpoint. In normal conditions (presence of growth factors and nutrients), mTORC1 induces phosphorylation of ULK1/2 and Atg13, which inhibits ULK complex activity and, in turn, AP. Many diverse signals, such as growth factors, amino acids, glucose, energy status, and different forms of stress, regulate the mTOR pathway [68]. In conditions that trigger AP, such as nutrient starvation or stimulation with the antibiotic rapamycin, the mTORC1 serine/threonine kinase activity is inhibited; mTORC1 dissociates from the ULK complex, which becomes active. mTORC1 also incorporates upstream activating signals that inhibit AP via the class I PI3K (and protein kinase B, PKB, also known as Akt) pathway [69]. AMPK (adenosine $5'$-monophosphate-activated protein kinase) and Sirtuin1 (Sirt1) also control starvation-induced AP through a coordinated fashion depending upon the energy status (ATP/AMP levels) of the system [70], but at the same time AMPK negatively regulates mTORC1 [71, 72]. A second regulatory step required for the autophagosome formation involves the "Beclin 1 core complex." There are several Beclin 1 complexes: the UVRAG (ultraviolet irradiation resistant-associated gene) [73] or the Rubicon (RUN domain and cysteine-rich domain containing, Beclin 1-interacting) [74, 75] complex, with UVRAG or Rubicon, in place of Barkor, respectively. These 3 complexes act differently: the Barkor complex has a role in the formation of autophagosomes, the UVRAG complex acts in autophagosome maturation, whereas the Rubicon complex inhibits autophagosome maturation [76, 77]. Moreover, other Beclin 1 binding partners have been shown to modulate AP, including ambra-1 (activating molecule in Beclin 1-regulated AP) [78] or Bif-1 (Bax-interacting factor 1) [79]. Under resting conditions, antiapoptotic Bcl-2 protein family members, such as Bcl-2 and Bcl-X_L,

constitutively bind Beclin 1 and act as negative regulators of AP, showing intricate interlinked complex control between AP and apoptosis processes [80–82]. The tumor suppressor gene, *p53*, has been reported to play a dual role in AP [83]. p53 can induce AP through activation of AMPK [84] and upregulation of DRAM (damage-regulated modulator of AP) [85], where cytoplasmic p53 can inhibit AP (transcription independent activity) [86]. The NF-κB transcription factor, and its certain upstream regulators, connects and integrates diverse stress response signals including immune signals with the AP pathway [87–89]. Others regulators that induce AP include tumor suppressors, such as PTEN, TSC1 and TSC2 complexes, and the death-associated kinase (DAPK); stress-activated signaling molecules, such as c-Jun N-terminal kinase 1 (JNK1), and those that respond to endoplasmic reticulum (ER) stress (PERK, eIF2α-kinase, and IRE1), and molecules involved in innate immune signaling, such as TLRs and immunity-related GTPases [90].

5. AP and Macrophages

In the last decade, it has been established that AP exerts important functions in many aspects of immune and inflammatory responses [91, 92]. AP is under the control of immune receptors and cytokine signaling [91, 92] and is stimulated upon microbial recognition by pattern recognition receptors (PRRs) [93–96] or activation with T helper 1 cytokines [97].

AP plays a critical role in host defense by promoting the elimination of pathogens via autolysosomes (referred to as xenophagy) as well as the delivery of microbial nucleic acids and antigens to endo/lysosomal compartments for activation of innate and adaptive immune responses [98]. *In vitro*, it has been demonstrated that AP has a crucial role in macrophage phagocytosis of different pathogens, such as *Listeria monocytogenes*, *Mycobacterium tuberculosis*, *Herpes simplex virus type I*, *Toxoplasma gondii* and many others [99, 100]. Immunity-related GTPase family M, an IFN-inducible GTPase, promotes AP that is involved in the elimination of mycobacteria in macrophages [101, 102]. Consistent with this, macrophages lacking Atg7 fail to eliminate live yeasts in phagolysosomes [103]. In mice, knockout of Atg5 in macrophages and neutrophils increases susceptibility to infection with *L. monocytogenes* and the protozoan *T. gondii* [104].

It has been shown that AP machinery and pathways interact with several PRRs. Firstly, TLRs were connected with AP [61, 103, 105]. Activated TLRs recruit adaptor proteins such as myeloid differentiation marker 88 (MyD88) and tumor necrosis factor receptor- (TNFR-) associated factor 6 (TRAF6), an E3 ubiquitin ligase and scaffold protein. In macrophages, both MyD88 and another adaptor protein, TRIF (TIR domain-containing adaptor inducing interferon-beta), interact with Beclin 1. Exposure of murine macrophages to a TLR4 ligand (lipopolysaccharide, LPS) also reduces the association between Beclin 1 and Bcl-2. TLR4 triggers AP via TRAF6 that ubiquitinates Beclin 1 and determines the Beclin 1/Bcl-2 dissociation [106]. Furthermore, it has been reported that TLR7-induced AP in murine macrophage cell lines depends on MyD88 and Beclin 1 [96]. The stimulation of TLR2 with zymosan (a cell component of fungi) triggers the recruitment of LC3 to the phagosomes although the signaling pathways seem MyD88-independent [94]. The soluble TLR2 ligand, Pam3CSK4, only when fused with latex beads, is able to induce the maturation of phagosomes in primary macrophages, suggesting that TLR2 signaling is necessary but not sufficient for the induction of AP [94].

Others receptors involved in the detection of invasive pathogens, the cytosolic Nod- (nucleotide-binding oligomerization-domain-) like (NLRs) and RIG-I-like (RLRs) receptors, have been demonstrated to be players in the autophagic response to bacteria [107]. Among the members of the NLR family, Nod1 and Nod2 detect intracellular bacteria through their ability to sense bacterial peptidoglycan [108]. Activation of Nod1 and Nod2 initiates a proinflammatory response dependent mainly on the activation of the transcription factor NF-κB and on the recruitment of the adaptor protein RIP2 [109, 110]. Similar to TLRs, mutations in genes encoding Nod proteins have been associated with chronic inflammatory disorders [111].

Travassos and colleagues demonstrated that, in mouse macrophages and other cell lines, Nod1 and Nod2 recruited the AP protein ATG16L1 to the plasma membrane at the bacterial entry site by a mechanism independent of the adaptor RIP2 and transcription factor NF-κB [107]. In contrast to Nod2, NLRC4 (Ipaf) and NLRP4 exert inhibitory effects on AP. NLRC4 acts negatively at the initiation stage, whereas NLRP4 acts at both the initiation and maturation stages. NLRs, including those essential for inflammasome assembly and activation such as NRLP3, are found in complexes with Beclin 1 in several human cell lines including acute monocytic leukemia cells [112].

Recently, the autophagic adaptors, sequestosome 1/p62-like receptors (SLRSs), have been proposed as a new category of PRRs in order to link AP and innate immunity signaling [113]. The autophagic adaptors, NBR1 and p62, present at the earliest stages of autophagosome formation, have been studied independently of AP as complex inflammatory signaling platforms [114, 115]. A recent study performed with both *Drosophila* blood cells and mouse macrophages show that constitutive p62-mediated selective AP is required for cell spreading and Rho1-induced cell protrusions. It is suggested that p62 may mediate selective autophagic degradation of a regulator of the Rho pathway. Moreover, it is becoming apparent that proteins, organelles, and pathogens can be targeted for autophagic clearance by selective mechanisms, although the extent and roles of such degradation are unclear. These results illuminate a specific and conserved role for AP as a regulatory mechanism for cortical remodeling, with implications for immune cell function [116]. Moreover, AP can drive the rapid cellular changes necessary for proper differentiation. In fact, it has been shown that this process plays a crucial role in monocyte differentiation into macrophages when this differentiation is induced by colony stimulating factor-1 (CSF-1) as well as by M-CSF [117, 118]. SLRs can participate in the promotion of autophagic killing of intracellular microbes. The p62 adaptor protein can deliver specific ribosomal and bulk ubiquitinated cytosolic proteins to

autolysosomes where they are proteolytically converted into products capable of killing *M. tuberculosis*. Thus, p62 brings cytosolic proteins to autolysosomes where they are processed from innocuous precursors into neoantimicrobial peptides, explaining in part the unique bactericidal properties of autophagic organelles [119]. However, there are also potential links between AP and conventional antimicrobial peptides, such as cathelicidin, a peptide obtained by conventional proteolysis from larger precursors. Cathelicidin expression and its antimycobacterial action are induced by vitamin D3, an activator of AP, in human monocytes/macrophages [120]. Moreover, vitamin D3 triggers AP in human macrophages that inhibit HIV-1 infection [121].

AP and the inflammasome undergo complex functional interactions. Indeed, damage-associated molecular patterns (DAMPs), toxins, and several particulates and nanomaterials can induce AP directly or via the activation of the inflammasome pathway [113, 122, 123]. Cytokines can induce AP in some cases or, conversely, suppress it in other cases (reviewed in [103, 124, 125]). Some of the immune signals that induce AP include IFN-γ, TNF, and CD40-CD40L interactions. In contrast, AP is negatively regulated by the T helper type 2 cytokines, IL-4 and IL-13 [61]. Several observations have revealed a link between autophagic protein deficiency and proinflammatory cytokine secretion in macrophages. Macrophages lacking Atg16L1 and Atg7 produce high amounts of IL-1β and IL-18, but not TNF or IFN-β, in response to LPS. In these macrophages, the enhanced IL-1β production is induced by Toll/IL-1 receptor domain-containing adaptor inducing IFN-β (TRIF)-dependent generation of ROS. Further, deletion of Atg5 in macrophages enhances retinoic acid-inducible gene-I-like receptor- (RLR-) mediated type I IFN production in response to single-stranded RNA viruses. These data indicate the importance of Atg in the inflammatory response [103, 126, 127]. Increased activation of IL-1β and IL-18 has been observed in macrophages and monocytes genetically deficient in Beclin 1 and LC3B; this occurs through the increased activation of the NLRP3 (NALP3) inflammasome pathway [128, 129]. IFN-γ has also been reported to induce AP in macrophage cell lines, but less in primary mouse and human macrophages [97]. Taken together, this evidence suggests that AP in macrophages can be triggered directly via at least some PRRs and indirectly via certain cytokines induced upon PRR activation [130].

In addition, it has been reported that cells undergoing autophagic cell death can induce a proinflammatory response in human macrophages. Indeed, upon engulfing MCF-7 cells undergoing autophagic cell death, human macrophages generate a proinflammatory response involving the secretion of IL-6, TNFα, IL-8, and the anti-inflammatory cytokine IL-10 [131]. Interestingly, AP also regulates phagocytosis of dead cells [132, 133]. Importantly, AP can influence adaptive immunity by regulating antigen presentation and the maintenance of lymphocyte function and homeostasis [134].

In conclusion, AP can influence inflammatory responses through several pathways in a cell-intrinsic manner, affecting both pro- and anti-inflammatory signaling and subsequent immune-driven diseases such as atherosclerosis.

6. Role of AP in Atherosclerosis

AP in atherosclerosis has been extensively investigated with particular focus on vascular smooth muscle cells (SMCs) and endothelial cells (ECs). Transmission electron microscopy (TEM) of SMCs in the fibrous cap of experimental or human plaques reveals features of AP such as formation of myelin figures [135]. This data is supported by western blot analysis of advanced human plaques showing elevated levels of LC3-II [136].

Several AP triggers are present in the atherosclerotic plaque, such as inflammatory mediators [137], ROS production [138] and accumulation of oxidized LDL [139, 140]. TNFα stimulation increases the number of vacuolated cells and the expression of LC3-II and Beclin 1 in SMCs isolated from atherosclerotic plaques [137]. Osteopontin, a protein involved in vascular inflammation, and advanced glycation end products (AGEs) have been shown to induce AP in human and rat SMCs, respectively [141, 142]. Similarly, 7-ketocholesterol, one of the major oxysterols present in atherosclerotic plaques, triggers extensive vacuolization and intense protein ubiquitination and increases the LC3-II expression in cultured SMCs [143]. SMCs in the fibrous cap are surrounded by a thick layer of basal lamina and therefore are subjected to hypoxia, caused by inadequate vascularization [144], and experience nutrient and growth factor deprivation, well-known conditions leading to the induction of AP. Dying SMCs in the fibrous cap of advanced human plaques show ubiquitinated inclusions in their cytoplasm and may undergo autophagic death [143, 145].

AP in vascular endothelial cells can be induced by several compounds in the circulation or in the subendothelial layer of the plaque. For example, oxLDL intensifies AP in human umbilical vein EA.hy926 cells [139], and mitochondrial-derived ROS activate AMPK that in turn increases AP leading to EC survival [146]. AP plays an important role in preserving vascular endothelial function by reducing oxidative stress, increasing nitric oxide bioavailability and reducing vascular inflammation [147]. Importantly, AP is reduced with ageing in vascular tissues [147].

The general consensus is that basal AP can protect plaque cells against oxidative stress by degrading damaged intracellular material [148] and promoting cell survival. The protective role of AP in stabilizing the plaque was confirmed *in vitro* showing that SMC death induced by low concentrations of statins was attenuated by the AP inducer 7-ketocholesterol [149]. Similarly the exposure of ECs in culture to oxLDL or AGEs [139, 150] induced AP which protected against EC injury [151]. Moreover, Salabei et al. [152] showed that the antiproliferative effect of verapamil in SMCs, beneficial in controlling vascular-injury-induced neointimal formation, was associated with the onset of AP. Although verapamil strongly upregulated AP, it did not promote SMC cell death yet appeared to suspend cell division resulting in an anti-proliferative state.

In contrast to basal AP, excessive stimulation of AP in SMCs or ECs may cause autophagic cell death [151], leading to reduced synthesis of collagen, thinning of the fibrous cap, plaque destabilization, lesional thrombosis, and acute clinical events [136].

7. Macrophage AP in Atherosclerosis

The investigation of macrophage AP in atherosclerosis has been complicated by the strong phagocytic activity of these cells. It is difficult to determine via conventional electron microscopy whether the vacuoles in their cytoplasm result from autophagocytosis or heterophagocytosis [136, 151]. In addition, the autophagosomal marker protein LC3 is poorly expressed in macrophages and overexpression of other lysosomal marker proteins may give rise to false-positive signals in immunoelectron microscopy [136, 151].

Importantly, pharmacological modulation of macrophage AP has been shown to affect vascular inflammation. Stent-based delivery of everolimus (mTOR inhibitor; a well-known AP inducer; see Section 8) in atherosclerotic plaques of cholesterol-fed rabbits leads to a marked reduction of macrophages via autophagic cell death without altering the SMC plaque content [4]. The observed macrophage cell death was characterized by bulk degradation of long-lived proteins, processing of LC3, and cytoplasmic vacuolization, which are all markers of AP [4]. Further, local administration of imiquimod (TLR7 ligand) to rabbit atherosclerotic carotid arteries induced macrophage AP, without affecting SMCs and ECs. However, in this case the induction of macrophage AP triggered cytokine production and the upregulation of VCAM-1 and enhanced leukocyte infiltration in the artery [5]. The authors speculate that the moderate AP induced in the macrophages of imiquimod-treated lesions does not lead to cell death but to plaque inflammation. Of note, TLR7 stimulation could lead to the activation of other immune cells homing to atherosclerotic vessels such as plasmacytoid dendritic cells, recently shown to play a key role in promoting experimental atherosclerosis in mice [153–155].

Two recent elegant papers add new dimensions to the understanding of the role of macrophage AP in regulating atherosclerotic plaque development [6, 7]. Razani et al. [6] demonstrated that during lesion formation, autophagic markers (p62 and LC3/Atg8) were expressed in the atherosclerotic plaques of apolipoprotein-E (apoE)$^{-/-}$ mice, colocalizing mainly with monocyte-macrophages (MOMA-2, CD11b +ve) and plaque leukocytes (CD45 +ve). The AP protein, p62/SQSTM1, is known to accumulate when autophagy flux through lysosomes is defective [156]. AP induction by prolonged fasting resulted in decreased levels of p62 protein in the aorta of apoE$^{-/-}$ mice, suggesting that changes in aortic p62 protein reflect *in vivo* AP status. p62 levels are raised with increasing age/plaque burden in atherosclerotic aortas, suggesting that initially AP is functional and becomes severely compromised with disease progression.

Beclin 1/Atg6 heterozygous-deficient (Beclin-Het) mice on the apoE$^{-/-}$ background showed similar extent of atherosclerosis compared to apoE$^{-/-}$ mice that were wild-type at the Beclin locus, demonstrating that autophagy haploinsufficiency had no effect on pathology. On the contrary, complete deficiency of macrophage AP increased vascular inflammation and plaque formation. To pursue this notion, the authors used macrophage-specific Atg5-null (Atg5-mϕKO) mice with complete absence of an AP gene in macrophages. Plaque formation, serum IL-1β levels, and aortic IL-1β expression were all increased in Atg5-mϕKO/apoE$^{-/-}$ mice fed a high-fat diet (HFD) as compared to apoE$^{-/-}$ controls. In addition, AP deficiency was associated with elevated plaque macrophage content.

Given the increased levels of IL-1β observed in Atg5-null mice, the authors suggest a link between AP deficiency and inflammosome hyperactivation. Indeed, deficient AP, through mechanisms that might include lysosomal leakage, generation of ROS, and impaired mitophagy, could result in the activation of the inflammasome. However, the effects of AP on IL-1β production are complex and context dependent and the assumption that AP suppresses the inflammosome in vascular inflammation merits further investigation.

The protective role of macrophage AP in atherosclerosis was confirmed by Liao et al. [7]. The authors provide evidence that AP prevents macrophage apoptosis and defective efferocytosis, both promoting plaque necrosis in advanced atherosclerosis. Firstly, authors demonstrated, in primary macrophages from mice transgenic for a GFP-tagged version of the AP effector LC3-II, that several proatherosclerotic stimuli induced AP and promoted autophagic flux through lysosomes. Inhibition of AP by silencing Atg5 or in Atg5-deficient macrophages enhanced apoptosis and NADPH oxidase-mediated oxidative stress, rendering the apoptotic cells less recognizable to efferocytosis. Importantly, the same findings were confirmed *in vivo*. Aortic root lesions of HFD-fed GFP-LC3/low-density lipoprotein receptor (LDLr)$^{-/-}$ mice contained macrophages expressing Atg5 and displaying the punctate pattern of GFP-LC3 fluorescence typical of AP. The number of macrophages expressing p62 increased as lesions progressed. These data confirm the presence of AP in atherosclerotic vessels and suggest that autophagic flux through lysosomes decreases as disease progresses. Lesion size and necrotic area were higher in Atg5$^{fl/fl}$Lysmcre$^{+/-}$/LDLr$^{-/-}$ versus control mice. The number of lesional macrophages was not increased, however, macrophage-rich regions of the Atg5$^{fl/fl}$Lysmcre$^{+/-}$/LDLr$^{-/-}$ plaques had more apoptotic cells positive for TUNEL, activated caspase-3, DHE, and p47. In summary, macrophage AP deficiency increased apoptosis and oxidative stress in plaque macrophages, promoted plaque necrosis, and impaired lesional efferocytosis in LDLr$^{-/-}$ mice. These data complement results obtained by Razani et al. [6], confirming a protective role played by macrophage AP in the two most widely used mouse models of atherosclerosis.

Another important aspect to consider is the contribution of lipophagy to vascular pathology. As recently reviewed [157–159], lipophagy, a special kind of AP, contributes in cholesterol egress from lipid-laden cells to high-density lipoprotein (HDL) via lysosomal lipases. AP can play a role in

the hydrolysis of stored cholesterol droplets in macrophages, thus facilitating cholesterol efflux [160]. Interestingly, Wip1 phosphatase, a known negative regulator of Atm-dependent signaling, has been recently shown to play a major role in controlling AP and cholesterol efflux in apoE$^{-/-}$ mice. Deletion of Wip1 resulted in suppression of macrophage conversion into foam cells, thus preventing the formation of atherosclerotic plaques [161].

In conclusion, macrophage AP becomes dysfunctional in atherosclerosis and its deficiency promotes vascular inflammation, oxidative stress, and plaque necrosis, suggesting a mechanism-based strategy to therapeutically suppress atherosclerosis progression.

8. Pharmacological Manipulation of Autophagic Pathways

Several pharmacological agents that are able to modulate AP have already been identified, such as mTOR inhibitors, AMPK modulators, IP_3 and calcium lowering agents, and lysosome inhibitors [162, 163].

mTOR inhibitors are the most studied AP inducers, and among them, rapamycin (also known as sirolimus) was the first drug to be identified. Rapamycin, a lipophilic macrolide antibiotic already in use to prevent the rejection of transplanted organs and to block restenosis after angioplasty, binds to the immunophilin FK506-binding protein of 12 kDa (FKBP12) inhibiting the kinase activity of mTOR, particularly TORC1 [164, 165]. AP induction with rapamycin enhances the clearance of toxic substrates, such as intracellular aggregate-prone proteins associated with neurodegenerative diseases, and protects against toxicity of these substrates in cell and animal models [166–168]. However, rapamycin also regulates numerous physiological processes that are independent of AP [68]. Indeed, rapamycin inhibits the translation of numerous proteins, causes immunosuppression and cell cycle arrest and alters cell size [169]. These side effects may be unwelcome consequences if rapamycin is used as an AP enhancer, and consequently there is a need for nonimmunosuppressive AP-inducing drugs. Of note, rapamycin and its analogs (temsirolimus, everolimus, and deforolimus) have had limited success as anticancer drugs, may be because they inhibit mTORC1, but not mTORC2 [170]. Consequently, a more complete blockade of the mTOR pathway has led to the development of ATP-competitive mTOR inhibitors of both mTORC1 and mTORC2 (e.g., PP242, AZD8055, WYE132, and Torin 1). Although these compounds clearly show preclinical evidence of antitumor activity, their effectiveness in the clinical setting has yet to be demonstrated [171–174].

Recently, further mTORC1 inhibitors, such as perhexiline, niclosamide, and rottlerin, have been identified as compounds that increase autophagosome number but further work is required to completely understand their activity [175].

Interestingly, several other drugs with well-known pharmacological actions can induce AP by mTOR-independent pathways. For example, mood-stabilizing drugs, such as carbamazepine, valproic acid, and lithium have been identified as AP inducers by reducing IP_3 levels [176, 177]. Furthermore, L-type calcium channel antagonists (e.g., verapamil), and antiarrhythmic drugs (e.g., amiodarone) induce AP by inhibiting levels of calcium [162]. The anticancer drug tamoxifen appears to function in part by upregulating the level of Beclin 1 and inducing AP [178]. Finally, the antidiabetic drug metformin has been shown to induce AP of several cancer cell types by activating AMPK [179–181].

A useful strategy for pharmacological manipulation of AP based on additive effects of drugs could be obtained using mTOR inhibitors in combination with mTOR-independent AP enhancers. It has been demonstrated that trehalose and small molecule enhancer rapamycin (SMERs) exerted an additive effect on the clearance of aggregate-prone proteins associated with Huntington's disease and Parkinson's disease when associated to rapamycin [168, 182].

AP inhibitors can be classified in: (i) early stage inhibitors including 3-methyadenine (3-MA), wortmannin and LY294002 that target the class III PI3K; (ii) late stage inhibitors, including chloroquine (CQ) or hydroxychloroquine (HCQ), bafilomycin A1, and monensin that prevent fusion of autophagosomes with the lysosomes [183]. Currently, several ongoing clinical trials registered with the National Cancer Institute (http://www.cancer.gov/clinicaltrials) are evaluating the efficacy of the combination of HCQ with cytotoxic drugs in a variety of cancers.

Importantly, many of the aforementioned pharmacological agents have been shown to be effective in the treatment of cardiovascular disorders, including cardiomyopathy, and heart failure in which AP is involved [184]. For example, reduction of infarct size has been demonstrated in mice treated with rapamycin [185]. Rapamycin, AICAR, and metformin improve cardiac function, reduce cardiac hypertrophy, and delay the onset of heart failure during overload pressure [186, 187]. On the other hand, a strong activation of AP, due to Beclin 1 upregulation, is observed in response to severe pressure overload, and this could be responsible for the transition from compensatory ventricular hypertrophy to pathological remodeling [188]. Finally, several indications support AP as a therapeutic target in experimental atherosclerosis [189]. Sirolimus and everolimus are antiatherogenic in mice [190–193]; on the other hand, AP induction by calorie-deprivation reduced atherosclerosis [194]. However, no clinical data are available regarding the efficacy of AP modulators in cardiovascular diseases.

In conclusion, several drugs that regulate AP have been identified, suggesting that the autophagic signaling may be manipulated to treat human disease. However, considering the dual role of AP in cytoprotection and cell death, there is a need for more specific molecules in order to target the pathways that control AP.

9. Conclusions

This paper summarizes recent evidence showing a protective role played by macrophage AP in atherosclerosis. AP becomes dysfunctional in atherosclerosis and its deficiency promotes vascular inflammation, oxidative stress, and plaque

necrosis. However, further work is needed to obtain a better understanding of this phenomenon in all stages of the pathology. We need to understand how AP is induced in atherosclerotic lesions, how lipophagy contributes to the pathology, the mechanisms governing the crosstalk between AP and apoptosis within the arterial wall, how they could influence plaque stability, and if they may prove to be effective therapeutic targets.

What degree of AP deficiency is proatherogenic? Is AP induction anti-atherogenic? The answer to these questions requires further investigation. Importantly, to date no studies have addressed the potential effect of AP on the multiple leukocyte subsets which have been shown to infiltrate the naïve and inflamed vessels playing a significant role in plaque formation and development [1, 195–197]. For example, AP regulates antigen presentation [198], T-cell development and homeostasis [113, 199], T-cell and dendritic cell activation [200, 201], and degranulation of mast cells [202]. However, none of these AP functions have been investigated in the context of vascular inflammation. As these studies progress we can expect to learn more about whether AP is indeed a good target for therapeutic intervention in atherosclerosis.

Conflict of Interests

The authors declare that there is no conflict of interests.

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Research Article

The Role of Glucocorticoid Receptors in Dexamethasone-Induced Apoptosis of Neuroprogenitor Cells in the Hippocampus of Rat Pups

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Background. Dexamethasone (Dex) has been used to reduce inflammation in preterm infants with assistive ventilation and to prevent chronic lung diseases. However, Dex treatment results in adverse effects on the brain. Since the hippocampus contains a high density of glucocorticoid receptors (GCRs), we hypothesized that Dex affects neurogenesis in the hippocampus through inflammatory mediators. *Methods*. Albino Wistar rat pups first received a single dose of Dex (0.5 mg/kg) on postnatal day 1 (P1) and were sacrificed on P2, P3, P5, and P7. One group of Dex-treated pups (Dex-treated D1D2) was given mifepristone (RU486, a GCR antagonist) on P1 and sacrificed on P2. Hippocampi were isolated for western blot analysis, TUNEL, cleaved-caspase 3 staining for cell counts, and morphological assessment. Control pups received normal saline (NS). *Results*. Dex reduced the developmental gain in body weight, but had no effect on brain weight. In the Dex-treated D1D2 group, apoptotic cells increased in number based on TUNEL and cleaved-caspase 3 staining. Most of the apoptotic cells expressed the neural progenitor cell marker nestin. Dex-induced apoptosis in P1 pups was markedly reduced (60%) by pretreatment with RU486, indicating the involvement of GCRs. *Conclusion*. Early administration of Dex results in apoptosis of neural progenitor cells in the hippocampus and this is mediated through GCRs.

1. Introduction

Corticosteroids are used in preterm infants to suppress inflammation, to facilitate extubation, and/or to prevent chronic lung diseases [1–3]. However, such early dexamethasone (Dex) therapy can result in an adverse neurodevelopmental outcome [4, 5]. For example, Dex treatment reduces cerebral gray matter volume without affecting white matter and the basal ganglia, suggesting that Dex affects only certain cells in the brain [6–8].

Neurons in the dentate gyrus (DG) of the hippocampus continue to divide after term and therefore remain vulnerable to the adverse effects of steroids during the early postnatal period [9–11]. Cells in the hippocampus have a high density of glucocorticoid receptors (GCRs) [12] suggesting that they could be affected by Dex [8–13]. Dex is known to change synaptic plasticity in the hippocampus [14]. As rat and human develop over different embryonic time scales [15], rat pups on P1 correspond to the human fetus at about week 22 to 24 of gestation. The equivalence in development is reflected in brain weight, neurochemistry, electroencephalography, and synaptogenesis [16]. Therefore, rat pups can be used as an animal model for human preterm infants undergoing drug exposure [17].

Here, we studied the effects of single-dose Dex therapy and the role of GCRs on hippocampal development.

2. Materials and Methods

2.1. Animals. The study was approved by the Animal Care and Use Committee of National Cheng Kung University. Time-dated pregnant Albino Wistar rats (body weight 250–300 g) were used. Food and water were provided *ad libitum*. The dams were allowed to deliver naturally on

gestational day 21 ± 1. Animals were kept in a ventilated room at 22 ± 2°C under a 12/12 h light/dark cycle. The day of birth was designated P0.

2.2. Study Design. In a preliminary study, each litter was divided into two groups: the Dex group received a dose of Dex phosphate (0.1, 0.2, or 0.5 mg/kg, i.p.) (Oradexon, 4 mg/mL, Organon, the Netherlands) and controls received an equal volume of normal saline (NS). Compared with the controls, changes in apoptosis were only observed with Dex 0.5 mg/kg, so this dose was used in the subsequent experiments. On day 1 (P1) pups received a single dose of 0.5 mg/kg Dex or NS. On P2, P3, P5, and P7, pups were anesthetized and euthanized with 10% chloral hydrate (W/V, 300 mg/kg, Riedel-de Haen, Germany) and then infused transcardially with NS. The body and isolated brain weights were measured to the nearest milligram. Brain tissues were processed histologically following standard protocols and cut serially at 5 μm in the coronal plane.

2.3. Immunohistochemical and Immunofluorescence Staining. Brain sections were matched to the E22 coronal plates 12 to 15 of the prenatal rat brain development atlas [18]. Sections were stained by immunohistochemical (IHC) and/or immunofluorescence (IF), TUNEL, and double-IF methods, using the protocols recommended by the manufacturers or described previously [19]. The following TUNEL detection kits and primary antibodies were used: TdT-Frag EL DNA fragmentation detection kit, ApopTag Red *in situ* apoptosis detection kit (Calbiochem, San Diego, CA), antibrain-derived neurotrophic factor (diluted 1 : 2000), and rabbit antiglucocorticoid (diluted 1 : 50) (Santa Cruz Biotechnology, Santa Cruz, CA), mouse antinestin (diluted 1 : 200; Santa Cruz Biotechnology), anti-OX-6 (diluted 1 : 50; AbD Serotec, Oxford, UK), and anti-NeuN (diluted 1 : 200; Chemi-Con, Temecula, CA), mouse antiactin and anti-pCNA (diluted 1 : 10000 and 1 : 1000, resp.; Millipore, Billerica, MA), and cleaved caspase 3 (diluted 1 : 100; Cell Signaling, Boston, MA). All sections were matched to the same anatomical sites for comparing cell counts between the Dex-treated and control groups. For IF staining, biotinylated anti-rabbit and anti-mouse IgG (1 : 400; Victor, Burlingame, CA) were used as the secondary antibodies; for IHC staining goat anti-rabbit IgG, H&L Chain specific Texas Red conjugate and rat anti-mouse IgG H&L Chain specific fluorescein conjugate (diluted 1 : 400; Calbiochem, San Diego, CA) were used as secondary antibodies. Sections were also double stained using the above primary antibodies and dilutions.

2.4. TUNEL Assay. The sections were subjected to TUNEL (Oncogene Research, Cambridge, MA) and IF staining. The signal was detected by the streptavidin-horseradish peroxidase conjugate and diaminobenzidine reaction.

2.5. Counts of Cells Stained for TUNEL, Cleaved-Caspase 3, and NeuN. Sections were examined by light microscopy and the images captured by a video camera coupled to a desktop computer (Eclipse 80i, Nikon, Japan). TUNEL-positive cells were identified and counted at 400x magnification. The numbers of TUNEL-positive cells in the DG, cornu ammonias 1 (CA1), CA2, and CA3 in the hippocampus were counted. For comparison with the control, sections at the same level were analyzed. Four sections were counted for each pup and the results were averaged. Cleaved-caspase 3- and NeuN-positive cells were counted at 400x magnification in four, 1 mm^2 areas in the DG. Data were validated by TissueGnostics FACS-like Tissue Cytometry software (Vienna, Austria). This method was also applied to IHC, IF, and double-stained cells in subsequent experiments.

2.6. Tissue Dissection and Western Blot Analysis. The hippocampi were dissected from the pups on P2, one day after Dex or NS treatment (D1D2). Western blots were performed on cytosolic and nuclear fractions of the hippocampus homogenates as described previously [19]. Briefly, 20 μg protein homogenate, determined by the Bradford protein assay (Bio-Rad, Hercules, CA), was separated by SDS-PAGE, blotted onto nitrocellulose (Hy-Bond, Amersham, Arlington Heights, IL) and blocked with nonfat dry milk. Blots were incubated with specific primary antibody, followed by incubation with horseradish-peroxidase-conjugated secondary antibodies (Abcam, Cambridge, UK) and detected by enhanced chemiluminescence (Bio-Rad). Samples were normalized to actin and proliferating cell nuclear antigen (pCNA) proteins (EMD Millipore). Each experiment was repeated at least three times.

2.7. Mifepristone (RU486) Treatment. The GCR antagonist, RU486 (Tocris Bioscience, Ellisville, MO), was dissolved in 100 mM DMSO. Thirty minutes prior to Dex injection, P1 pups received 25 mg/kg RU486 or vehicle intraperitoneally [20]. The RU486- and DMSO-treated pups (D1D2) with or without Dex treatment were euthanized on P2, and morphological and biochemical analyses were carried out as described above.

2.8. Statistical Analysis. Quantitative results are expressed as mean ± standard error (SE). Statistical analyses were performed using one- or two-way ANOVA with a multiple comparisons posttest or the Wilcoxon signed-rank test as appropriate. P values < 0.05 were considered statistically significant. The body and brain weight results were analyzed by mixed-model ANOVA with age as the within-subject factor and Dex as the between-subject factor.

3. Results

3.1. Body and Brain Weights. Pups that had received Dex (0.5 mg/kg) showed reduced body weights. In contrast, no difference in the developmental growth of brain weight was found between the Dex and control groups (Table 1).

3.2. Apoptotic Cell Death. More TUNEL-positive and cleaved-caspase 3-positive cells were found in the DG of P1 D1D2 Dex-treated group (Figures 1(b) and 1(e)), with

Table 1: Effects of dexamethasone on rat pup body weight and brain weight.

Age (D)	Body weight, Mean ± SE, g ($N = 6$)		Brain weight, Mean ± SE, g ($N = 6$)	
	NS	Dex	NS	Dex
P1 group				
1[a]	7.17 ± 0.07	6.58 ± 0.06		
2	8.13 ± 0.04	6.63 ± 0.03	0.373 ± 0.013	0.376 ± 0.008
3	9.33 ± 0.14	7.50 ± 0.07[*]	0.387 ± 0.008	0.373 ± 0.006
5	12.45 ± 0.11	9.83 ± 0.16[†]	0.530 ± 0.013	0.549 ± 0.011
7	16.90 ± 0.33	12.70 ± 0.23[†]	0.699 ± 0.017	0.721 ± 0.007

N: number of pups; [a]days of normal saline (NS) or dexamethasone (Dex) administration to the rat pups.
In P1 group, after Dex or NS injection on day 1, the pups were sacrificed on days 2, 3, 5, and 7.
Compared pups injected with dexamethasone (Dex) and normal saline (NS) by using two-way ANOVA.
[*] $P < 0.05$; [†] $P < 0.001$.

an increase to 2.7- to 3-fold (Figures 1(c) and 1(f)) with respect to the control (Figures 1(a) and 1(d)).

3.3. Cell Counts and Coexpression of Apoptosis and Neuronal Maturation Markers. Representative results of TUNEL, nestin, and NeuN staining are shown in Figure 2. We found about twice as many TUNEL-positive cells in the Dex-treated D1D2 group (38.1 ± 1.1) compared with the control (21.8 ± 1.2; $P < 0.05$) (Figure 3(a)). The Dex-treated group had a greater proportion of TUNEL-positive cells (0.75 ± 0.01) that coexpressed nestin than control (0.61 ± 0.01) (Figure 3(b)). The proportion of TUNEL-positive cells coexpressing NeuN was also greater in the Dex-treated group (0.68 ± 0.10) than in control (0.64 ± 0.01; $P < 0.05$) (Figure 3(c)).

3.4. Glucocorticoid Receptors (GCRs) and Mifepristone (RU486) in Dex-Induced Apoptosis. Western blot analysis showed that the nuclear fractions of GCRs in the hippocampus were upregulated in the Dex-treated D1D2 group (Figure 4). The Dex-retarded developmental gain in body weight was blocked by RU486 while neither Dex nor RU486 affected the brain weight in this group (Table 2). Furthermore, Dex-induced apoptosis in the P1 D1D2 group was reduced by the preadministration of RU486 (Figure 5). Dex treatment alone increased the apoptotic cell count (41.3 ± 0.60) compared to the control (23.1 ± 0.18); $P < 0.05$); the number of apoptotic cells in pups treated with DMSO (22.4 ± 0.3) or DMSO plus Dex (41.9 ± 0.56) was similar to that in pups treated with NS or Dex alone ([§] $P < 0.05$). RU486 treatment had no additional effect on apoptosis when compared with the NS or DMSO group. Pretreatment with RU486 followed by Dex reduced the apoptotic cell count (29.2 ± 0.45) ($P < 0.05$) (Figure 5).

3.5. Identification of Inflammatory Cells. Eosin/hematoxylin or IHC staining did not reveal any inflammatory cells in the NS and Dex-treated D1D2. Positive controls after implantation of rat brain tumor cells revealed OX-6 positive cells (stained brown) which helped identification of microglia in the brain (Figure 6).

4. Discussion

Steroids have long been used in the treatment of respiratory problems in preterm infants [21]. The complications of adverse neurological effects (such as increased risk of cerebral palsy and neurodevelopment impairment [4, 22, 23] demand the reevaluation of steroid-based therapeutic strategies in postnatal practice [23–26]. The safe timing and dosage of Dex remain undecided for preterm patients [4]. Our results from the P1 rat pups, equivalent to 24-week preterm infants, showed that Dex retarded the developmental gain in body weight, consistent with earlier reports [27–29]. Furthermore, neonatal Dex exposure leads to delayed neurodevelopment and physical maturation, suggesting that Dex permanently alters neuronal functions during this period, particularly those associated with the hypothalamic-pituitary-adrenal axis [27–29]. Other studies showed that neonatal Dex exposure reduces brain weight [10, 28–32]. On the contrary, our data showed no such change. We attribute this discrepancy to the differences in timing, dosage regimes, and/or the preparation of Dex [28, 31, 32]. Among clinicians, the general consensus is that a lower dose of Dex (0.1–0.2 mg/kg/day) facilitates tracheal extubation and reduces the risk of chronic-lung disease. This study revealed no deleterious effects on the brain with single low doses of Dex (0.1–0.2 mg/kg), although it became harmful at a higher dose (0.5 mg/kg). The subgranular zone of the DG contains a reserve of neuroglial progenitor cells [33]. Apoptosis is crucial during neuronal development by eliminating excess cells and ensuring proper synaptic connectivity [34, 35]. GCRs are known to be involved in Dex-induced apoptosis [36] and are present at high levels in the hippocampus where progenitor cells capable of dividing reside in the DG. Our results showed increased apoptosis throughout the DG, suggesting that perinatal development in the hippocampus is vulnerable even to a single dose of Dex when given at a critical time. How this effect is related to the GCR density, receptor types, and the proliferation of progenitor cells remains to be studied.

It has been reported that administration of 3.0 mg/kg Dex to P7 mice increases the apoptosis of cerebellar progenitor cells and reduces the number of cerebellar neurons [37, 38]. It is likely that the dosage and timing of Dex treatment and

Table 2: Effects of dexamethasone and RU486 on D1D2 rat pup body weight and brain weight.

Treatment	Body weight, Mean ± SE, g ($N = 6$)	Brain weight, Mean ± SE, g, ($N = 6$)
NS	7.75 ± 0.04	0.314 ± 0.004
Dex	6.75 ± 0.08*	0.293 ± 0.004
DMSO	7.50 ± 0.09	0.309 ± 0.004
RU486	7.16 ± 0.08	0.312 ± 0.004
DMSO/Dex	6.83 ± 0.04	0.301 ± 0.004
RU486/Dex	7.08 ± 0.03	0.319 ± 0.004

N: number of pups.
Compared pups injected with dexamethasone (Dex) and normal saline (NS) by using Wilcoxon rank sum test, $^*P < 0.05$.
D1D2: administration on postnatal day 1 and sacrificed on day 2; RU486: mifepristone;
DMSO: dimethyl sulfoxide; DMSO/Dex: dimethyl sulfoxide plus dexamethasone;
RU486/Dex: mifepristone plus dexamethasone.

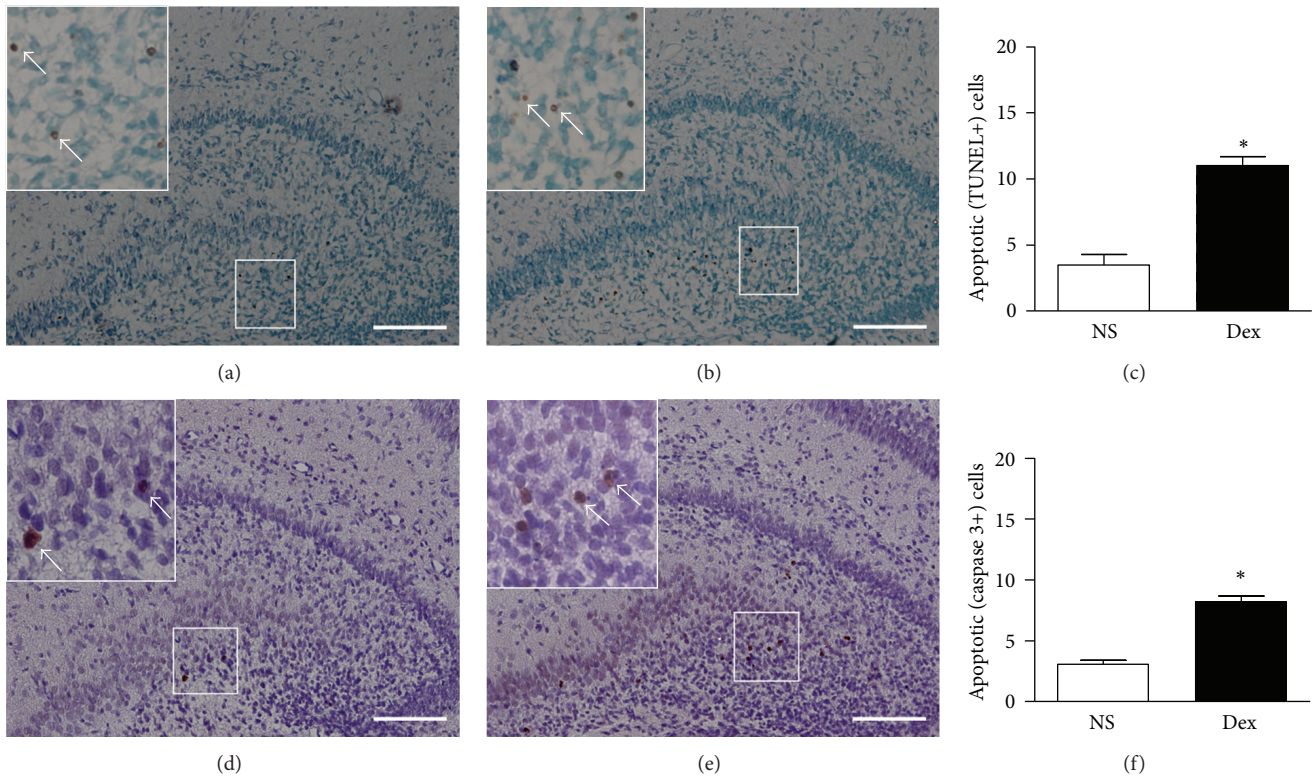


Figure 1: TUNEL staining of dentate gyrus (DG) P1 D1D2 pups treated with dexamethasone (Dex) or normal saline (NS) shows apoptotic cells stained brown (arrows). The Dex-treated D1D2 DG had more apoptotic cells (b) than NS control (a). High magnification photomicrographs of TUNEL-positive cells are shown in the upper left corner. TUNEL-positive cell counts revealed that the number of apoptotic cells in the Dex-treated D1D2 DG was increased when compared to that of NS control pups ($n = 6$, $^*P < 0.05$) (c). Cleaved-caspase 3 staining of the DG from P1 D1D2 pups with or without Dex treatment showed apoptotic cells (arrows) ((d) and (e)). High magnification photomicrographs of cleaved-caspase 3-positive cells are shown in the upper left corner. The numbers of apoptotic cells in Dex-treated pups were increased compared to those of NS control (f) ($n = 6$, $^*P < 0.05$). Magnification, 200x; scale bars, 100 μm; inset magnification, 400x. P1 D1D2: P1 pups receiving treatment on postnatal day 1 and sacrificed on day 2.

vulnerability of neural progenitor cells to glucocorticoids together determine the effects on neonatal brain development.

Exposure to Dex in the neonatal period results in marked apoptosis among the nestin-expressing cells in the DG [39]. The importance of Dex-induced apoptosis in the hippocampus and the type of cells affected remain unknown. Our results from double staining in the Dex-treated pups showed higher ratios of cells coexpressing TUNEL and nestin to TUNEL-positive cells, indicating that Dex-induced apoptosis affects the neuroprogenitor cells. This result is consistent with previous reports that neuroprogenitor cells are sensitive to Dex during the early neonatal period [39, 40]. Since the ratio of TUNEL and NeuN coexpressing cells to TUNEL-positive cells also increased, Dex treatment might also cause apoptosis in mature neurons and be associated with a transient

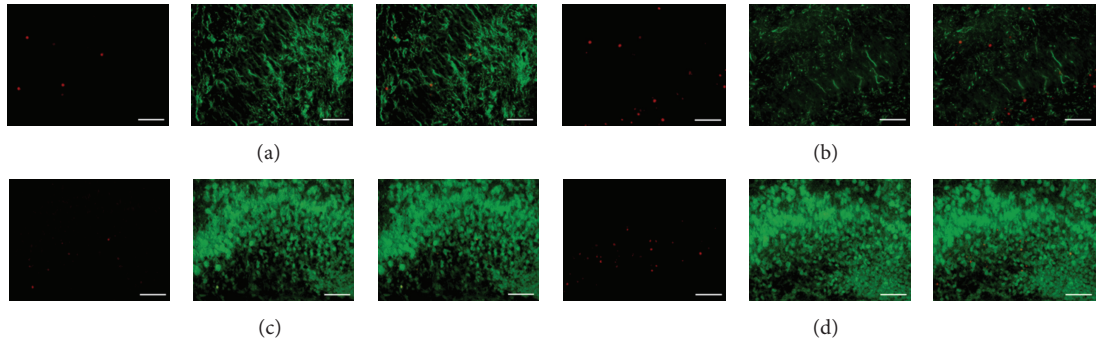


Figure 2: TUNEL, nestin, and NeuN expression in the hippocampus of D1D2 pups treated with normal saline (NS) or Dex. Representative double-IF micrographs demonstrate TUNEL (red) and nestin (green) staining ((a) and (b)), and TUNEL (red) and NeuN (green) staining ((c) and (d)). Cells coexpressing two proteins are merged and show as yellow or orange. Magnification, 400x, scale bars, 50 μm. Dex-treated D1D2: P1 pups receiving dexamethasone on postnatal day 1 and sacrificed on day 2.

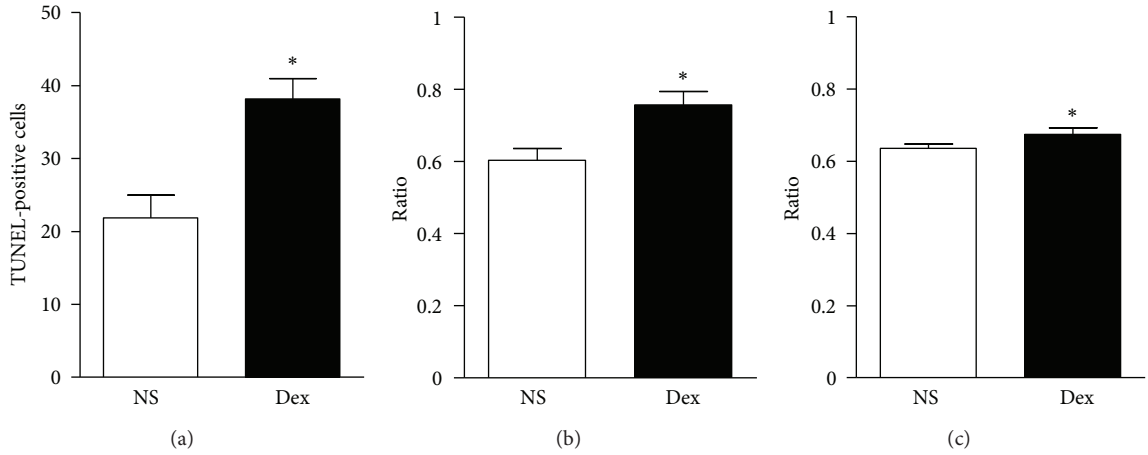


Figure 3: TUNEL-positive cells were more numerous in the hippocampus of Dex-treated D1D2 pups than in control ($n = 6$, $^*P < 0.05$) (a) and the ratio of TUNEL-positive cells which coexpressed nestin to total TUNEL-positive cells was higher in the Dex treated than in the control groups ($n = 6$, $^*P < 0.05$) (b). The ratio of TUNEL-positive cells coexpressing NeuN to total TUNEL-positive cells was higher than that of control ($n = 6$, $^*P < 0.05$) (c). Dex-treated D1D2: P1 pups receiving dexamethasone on postnatal day 1 and sacrificed on day 2.

and acute slowing of cell proliferation during hippocampal development [41, 42]. Whether these neurons were derived from the proliferating progenitors cells or represent existing mature neurons in the DG remains to be determined.

Mifepristone (or RU-486) is a synthetic steroid with both antiprogesterone and antiglucocorticoid properties. In a recent study, mifepristone was the only GCR antagonist found to increase both mineralocorticoid receptor (MCR) and GCR binding in the rat frontal cortex [43]. The effects of mifepristone on corticosteroid receptor expression could explain the neurocognitive improvement it is reported to induce [43, 44]. RU486 has no effect on Dex regarding thymocyte composition and maturation [45]. MCR-mediated responses to glucocorticoids, rather than GCRs, are important in steroid-responsive hearing disorders [46]. GCR activity levels are high in the hippocampus during the first postnatal week [40]. The anti-inflammatory effects of glucocorticoids require the presence of GCRs [47]. Interestingly, results of these studies and those of our own (60% reduction in Dex-induced apoptosis by RU486) are consistent, suggesting that the type of receptor and the timing of Dex treatment determine the effects of glucocorticoids on the hippocampus. Examining pup brain tissues stained with eosin/hematoxylin or IHC revealed no inflammatory cells in the NS and Dex-treated D1D2 groups. These results suggest that while GCRs are likely to be key players in Dex-induced neuroprogenitor cell death, inflammatory cells are not.

In summary, brain development is a dynamic process in which the growth spurt, differentiation, and cell responses to endogenous (and exogenous) steroids occur at critical times. Species differences add further complexity to the process. These species-specific developmental schedules allowed the design of animal models to study the effects of drug treatment in preterm babies, as in this study. Here, we have demonstrated that timing is a major factor in determining Dex-induced apoptosis in the hippocampus, the vulnerable cells are neuronal precursors, and the process is partly regulated by GCRs. We also provided cytological evidence that the administration of a single dose of Dex can result in deleterious effects in the brain.

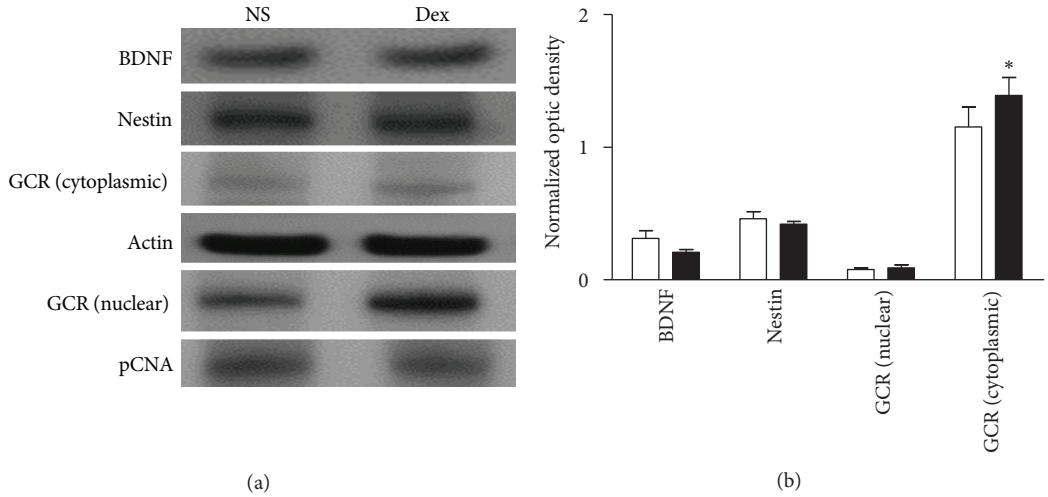


Figure 4: Dexamethasone (Dex) treatment increased levels of nuclear glucocorticoid receptors (GCRs), but not cytosolic GCRs, total brain-derived neurotrophic factor (BDNF) or total nestin in the hippocampus of P1 pups. (a) Representative western blots of hippocampal protein extracts obtained from P1 D1D2 pups, normal saline (NS, control) (left lane), and Dex (right lane). (b) Signals from BDNF, nestin, and cytosolic GCRs were normalized to actin and signals from nuclear GCRs were normalized to pCNA. Quantitative results showed no difference in BDNF, nestin, and cytosolic GCR expression between Dex-treated and control pups. The nuclear fraction of GCR expression in Dex-treated pups differed from control ($n = 6$, $^{*}P < 0.05$). P1 D1D2: P1 pups receiving treatment on postnatal day 1 and sacrificed on day 2; white and black bars represent control and Dex-treated pups, respectively.

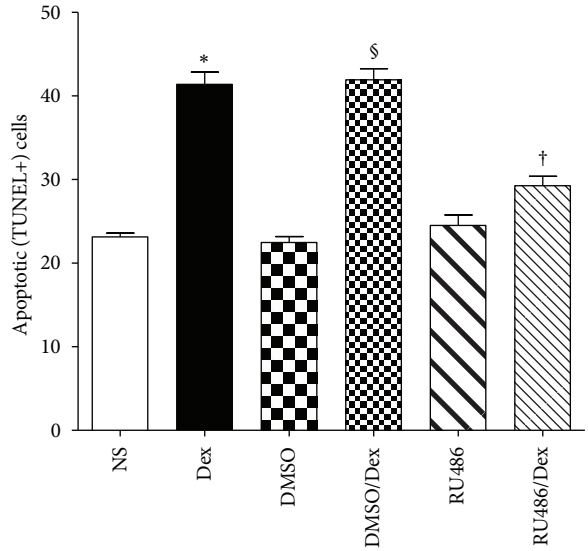
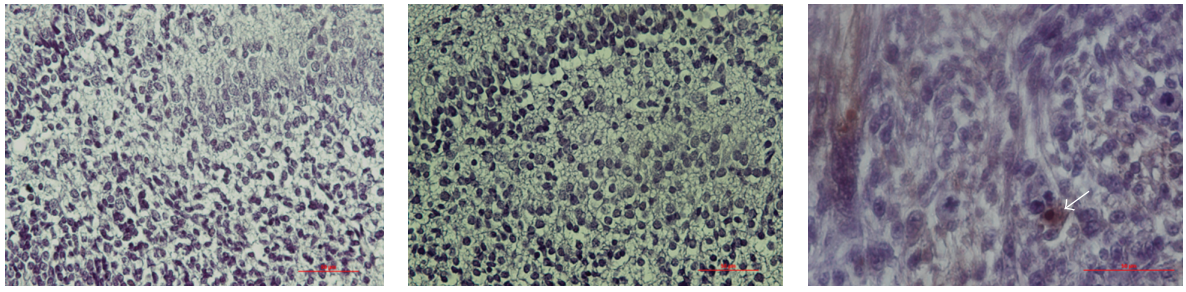


Figure 5: Dexamethasone-(Dex-) induced apoptosis in P1 D1D2 pups was reduced by preadministration of RU486. Dex treatment increased apoptotic cell counts in the hippocampus compared to control ($n = 6$, $^{*}P < 0.05$); P1 D1D2 pups treated with DMSO or DMSO plus Dex showed results similar to those from pups treated with either normal saline (NS) or Dex alone ($n = 6$, $^{\S}P < 0.05$). RU486 treatment showed apoptotic cell counts similar to those from pups treated with NS or DMSO. Pups treated with RU486 plus Dex had lower apoptotic cell counts than those with Dex or DMSO plus Dex ($n = 6$, $^{\dagger}P < 0.05$). P1 D1D2: P1 pups receiving treatment on postnatal day 1 and sacrificed on day 2.

List of Abbreviations

Dex:	Dexamethasone
GCR:	Glucocorticoid receptor
DG:	Dentate gyrus
CA:	Cornu ammonias
RU486:	Mifepristone
BDNF:	Brain-derived neurotrophic factor
NS:	Normal saline
P1/P0:	Day1/day 0 newborn pups
D1D2:	Pups receiving dexamethasone injection on day 1 and sacrificed on day 2
IHC:	Immunohistochemical
IF:	Immunofluorescence
TUNEL:	Terminal deoxynucleotidyl transferase dUTP nick end labeling.



(a) (b) (c)

Figure 6: OX-6 IHC staining in the DG. No OX-6 positive cells were identified in P1 D1D2 pups treated with either NS (a) or Dex (b). Brain tumor implant staining as a positive control showed OX-6 positive cells stained brown (c), arrow). Magnification, 400x; scale bar, 50 μm.

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Review Article

Alternatively Activated Macrophages in Types 1 and 2 Diabetes

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Macrophages are innate immune cells derived from monocytes, which, in turn, arise from myeloid precursor cells in the bone marrow. Macrophages have many important roles in the innate and adaptive immune response, as well as in tissue homeostasis. Two major populations have been defined: The classically activated macrophages that respond to intracellular pathogens by secreting proinflammatory cytokines and reactive oxygen species and alternatively activated macrophages which are induced during Th2 responses displaying anti-inflammatory activities. Both macrophage populations are central players in diabetes, the first one triggering inflammatory responses which initiates insulitis and pancreatic β cell death during type 1 diabetes, whereas the second population decreases hyperglycemia, insulitis, and inflammation in the pancreas, thereby negatively regulate type 1 diabetes. Obesity is an important factor in the development of type 2 diabetes; classically activated macrophages are a dominant cell population involved in the establishment of the inflammatory profile, insulin resistance, and activation of inflammatory signals during the development and progression of this disease. In contrast, alternatively activated macrophages regulate the release of proinflammatory cytokines, attenuating adipose tissue inflammation. Here, we review the advantages and disadvantages of these two macrophage populations with regard to their roles in types 1 and 2 diabetes.

1. Macrophages

Mφs have important roles in the immune response and tissue homeostasis. The huge capacity of Mφs for phagocytosis renders them effective at microbial killing and the clearance of apoptotic and necrotic cells, and through their expression of MHC-II molecules and secretion of pro- and anti-inflammatory cytokines, they can also trigger CD4+ T-cell activation and differentiation into Th1, Th2, Th17, and Treg subsets [1–3]. Importantly, Mφs have diverse roles in the regulation of glucose and lipid metabolism, as well as in the inflammation of adipose tissue [4].

In recent years, it has been clearly demonstrated that macrophages display high plasticity depending on the microenvironment in which they are found. Two major macrophage phenotypes have been described, specifically, classically activated macrophages (CAMφs) and alternatively activated macrophages (AAMφs) [2]. CAMφs are induced by stimulation with Th1-cell-derived IFN-γ and microbial products, such as bacterial lipopolysaccharide (LPS) [5], and respond to microbial infection with an enhanced phagocytic microbicidal capability through the expression of the CAMs marker, inducible nitric oxide synthase (iNOS), which catalyzes the conversion of L-arginine into ROS, such as NO. These macrophages produce several proinflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α), interleukin-12 (IL-12), IL-1β, and IL-23, as well as toxic mediators, such as reactive oxygen species (ROS) and nitric oxide (NO), through the expression of inducible nitric oxide synthase (iNOS). These macrophages also have an enhanced antigen presenting ability [6].

In contrast, AAMφs are induced during Th2-type responses, such as those elicited by helminthic infection and during allergic responses. The activation of these macrophages is dependent upon stimulation with IL-4/IL-13 [16] through the IL-4Rα receptor [17] and signal transducer and activator of transcription factor 6 (STAT6) [18], as well as with several helminth antigens [19–22]. AAMφs produce

moderate levels of IL-10 and TGF-β and low or null levels of the proinflammatory cytokines secreted by CAMφs. Additionally, AAMφs produce urea, polyamines, and L-ornithine, due to the high expression of the enzyme arginase-1 (Arg-1), which competes for its common substrate, L-arginine, with iNOS, thereby lowering the levels of NO secretion [6, 23]. AAMφs have enhanced expression of Ym-1, which induces eosinophil recruitment [24]; these cells, in turn, can potentiate the Th2 response and the alternative activation of macrophages by the secretion of the anti-inflammatory cytokines IL-4/IL-13. Further, AAMφs can express high levels of PD-1 ligands (Program-Death 1), PDL-1 and PDL-2, thereby inhibiting the proliferative response of activated T-cells [25].

AAMφ populations have been identified as an essential part of the immune response against almost any helminth parasite, such as *Taenia crassiceps* [25, 26], *Brugia malayi* [27, 28], *Schistosoma mansoni* [29, 30], *Litomosoides sigmodontis* [31], *Nippostrongylus brasiliensis* [32], *Heligmosomoides polygyrus* [33], *Fasciola hepatica* [19], *Hymenolepis diminuta* [34], and *Echinococcus granulosus* [35].

Of importance for this paper, helminth-induced AAMφs have been linked with decreased T1D-triggering inflammation, as well as glucose tolerance induction during obesity [4], by which these macrophages may participate in inhibiting the initiation and development of both TD1 [7] and TD2 [15].

This paper focuses on the different roles that CAMφs and AAMφs display in both types of diabetes, emphasizing the role of AAMφs as essential players in diabetes regulation.

2. Diabetes Mellitus

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia as a result of the impairment of insulin secretion, its action, or both. The chronic hyperglycemia of diabetes is associated with dysfunction and failure of various organs, such as the eyes, kidneys, heart, and blood vessels [36]. It has been estimated that the number of deaths caused by diabetes worldwide is 4.6 million per year. Thus, diabetes remains a major cause of death and is considered to be an epidemic. Diabetes mellitus is divided into two categories: type 1 diabetes (T1D) and type 2 diabetes (T2D), and at least 90% of all cases belong to the latter [37].

2.1. Type 1 Diabetes. T1D is an autoimmune disease that has increased in prevalence over the last 30 years in developed countries. It is known that more than 5.3 million people in the world have T1D, and more than 218,000 may develop the disease each year [40]. T1D is caused by the selective destruction of the insulin-producing β cells located in pancreatic Langerhans' islets by autoantigen-specific inflammatory T cells. Insulin, glutamic acid decarboxylase (GADA/GAA), and protein tyrosine phosphatase (IA-2AA) are the most common autoantigens involved in this process. When the majority of β cells are destroyed, the pancreas's ability to secrete insulin in response to blood glucose levels is impaired, resulting in the disruption of glucose homeostasis [36].

CAMφs and CD4+ and CD8+ autoreactive lymphocytes are the first cells that infiltrate the Langerhans islets, and the levels of cytokines, such as TNF-α, IL-1β, and IL-6, as well as NO, are increased in the pancreas during inflammation (Figure 1), where they activate different signaling pathways [38]. IL-1β and TNF-α induce the NF-κB (nuclear factor κB) signaling pathway, which promotes apoptosis of β cells by increasing the expression of FAS. TNF-α and IFN-γ act synergistically to activate the transcription factor signal transducer and activator of transcription-1 (STAT-1) signaling, thus inducing iNOS overexpression and secretion of NO and thereby promoting apoptosis of β cells by the p53 pathway [38, 39]. Free radicals, in turn, can induce apoptosis and necrosis of β cells by activating the caspase pathway and inducing excessive cell stress, respectively [39]. During this process, chemokines, such as MCP-1 (or CCL2), are also secreted; this chemokine is important in the recruitment of CAMφs, inflammatory monocytes, dendritic cells, and T cells into the pancreatic islets [40, 41]. Another cytokine that has been involved in T1D is the macrophage migration inhibitory factor (MIF). MIF is associated with MCP-1, which facilitates monocyte transmigration [42]. In a mouse model with MLD-STZ, the levels of MIF were elevated in diabetic mice, and the use of MIF inhibitors reduced the inflammatory response and insulitis [43].

A study performed in diabetic patients showed increased numbers of monocytes, as well as higher levels of IL-1β, IL-6, and TNF-α, in the pancreas of sick patients compared with healthy people. The enhanced expression of CD80 and PDL1 in the infiltrating monocytes suggests a proinflammatory profile for these cells [44]. Several studies have attempted to verify the role of CAMφs as important cells in the initiation and development of T1D. In experimental models, Martin et al. [41] demonstrated that the increased expression of CCL2 (using RIPCCL2 transgenic mice) promotes the recruitment of inflammatory monocytes to the pancreatic islets, thereby initiating inflammation and destruction of β cells. These data suggest that monocytes are needed for the development of diabetes. Also, the experimental depletion of CAMφs in NOD mice by the intraperitoneal injection of clodronate liposomes resulted in a decrease in insulitis and inflammation [45, 46].

Recently, a new subpopulation of CD4+ T lymphocytes, known as Th17 cells, have been described, which are characterized by their ability to secrete high levels of IL-17, thereby promoting an inflammatory profile. The differentiation of Th17 cells is dependent upon IL-6 and transforming-growth factor-β (TGF-β) stimulation, and the presence of this subpopulation of CD4+ cells has been correlated with the onset and progression of autoimmune diseases, such as T1D [47]. IL-23 is an inflammatory cytokine involved in the expansion and commitment of Th17 cell populations, and one of its main sources is CAMφs. In diabetic mice induced by streptozotocin (STZ), it has been shown that the administration of IL-23 increases IL-17, TNF-α, and IFN-γ secretion, which is associated with the onset of extremely severe T1D, implicating CAMφs in the recruitment, differentiation, and expansion of pathogenic Th17 lymphocytes, contributing to β cell death and T1D induction [48]. Therefore, CAMφs

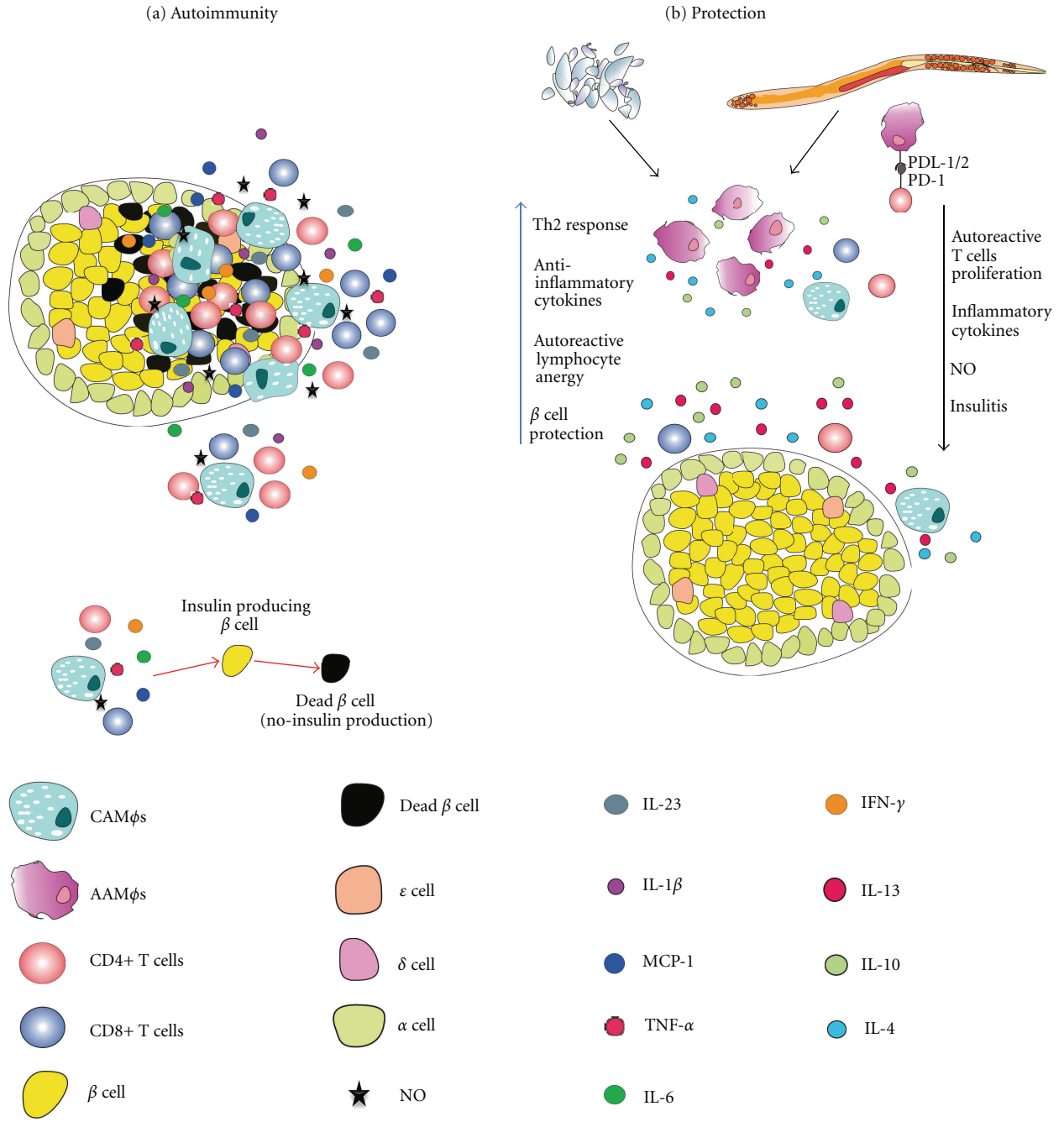

Figure 1: (a) In type 1 diabetes, CAMφs and autoreactive T cells are the first cells that infiltrate the islets of Langerhans and release proinflammatory cytokines and NO, which induce β cell apoptosis or necrosis; (b) the release of anti-inflammatory cytokines, AAMφ induction and PD-1/PD-ligand-dependent lymphocyte anergy induction by helminths, the antigens of which have the ability to decrease NO, as well as proinflammatory cytokine, secretion, thereby reducing insulitis and β cell death.

and Th17 cells, together with CD8+ cytotoxic T cells, are considered to be the main cell populations favoring the development of T1D.

However, certain pathogens (mainly viruses) can induce the development of T1D, including Rubella, enterovirus, rotavirus, cytomegalovirus, and mumps, by diverse mechanisms [49]. Several viruses may break self-tolerance by the expression of viral antigens; additionally, certain viral proteins show homology with autoantigens of β cells (known as molecular mimicry). Furthermore, several viruses can express superantigens, which results in an increase in the autoreactive T-cell populations, or induce the cytolysis of β cells, including Coxsackievirus [50] and Encephalomyocarditis (EMC) virus [51]. In the case of humans, rubella virus

Table 1: Helminths that reduce types 1 and 2 diabetes.

Helminths	Disease/model	Infection/antigen	Effect	Reference
Taenia crassiceps	T1D/MLD-STZ	Inf	Increased Th2 response, AAMφ induction, and decreased TNF-α, therefore less hyperglycemia and no insulitis	[7]
Schistosoma mansoni	T1D/NOD/MLD-STZ	Inf/Ag	Increased anti-inflammatory cytokines, such as IL-4, IL-10, IL-5, and IL-13, as a result, loop of Th2 response; Treg, eosinophil, and AAMφ generation	[8–11]
Heligmosomoides polygyrus	T1D/NOD	Inf	Th2 response induction; IL-4, IL-13, and IL-10 augmentation; AAMφs in pancreatic and peripheral lymph nodes; inflammation and insulitis reduced; no Treg generation	[12]
Litomosoides sigmodontis	T1D/NOD	Inf/Ag	High IL-4 and IL-5; AAMφ and Treg induction; reduced inflammation and glycemia	[13]
Trichinella spiralis	T1D/NOD	Inf/Ag	Amplification of Th2 response; less injury in pancreas and glycemia	[14]
Nippostrongylus brasiliensis	T2D/obese	Inf	Th2 response; recruitment of eosinophils and AAMφs; decreased obesity and insulin resistance	[15]

infection correlates with an increased incidence of T1D, and one possible mechanism of induction is molecular mimicry. Other examples are rotavirus and reovirus, which have been shown to induce lysis of β cells and release of autoantigens, suggesting the first mechanism of induction of T1D [49, 51]. Conversely, other pathogens may have protective roles and T1D. Epidemiological observations have pointed out an increase in the incidence and prevalence of T1D and other autoimmune diseases, mainly in developed countries, which have been correlated with a decrease in the incidence of bacterial and parasitic infections, particularly helminth infections. These observations prompted the proposal of the *hygiene hypothesis*, which states that the lack of intense infections that actively modulate the balance of the immune response toward Th2 or anti-inflammatory profiles (such as those that can be found in helminth infections) favors the induction of strong Th1 immune responses against autoantigens, thereby favoring the development of autoimmune responses [40, 52].

Helminths share a unique ability to exert profound regulatory effects on the immune system of their hosts by inducing strong Th2-type responses and increasing the numbers of regulatory cell populations, such as Tregs and AAMφs. The results of several experiments in murine models of autoimmunity and its regulation by helminth infections support the protective role of helminth-induced Th2 responses proposed by the hygiene hypothesis [3, 53, 54]. For example, it has been shown that the infection of nonobese diabetic (NOD) mice with *Heligmosomoides polygyrus* has a protective effect in T1D, resulting in the regulation of hyperglycemia and reduced incidence of diabetes; these effects were accompanied by reduced numbers of macrophages, dendritic cells, and CD4+ and CD8+ T cells in the inflammatory infiltrate in the pancreas, as well as a reduction on β cell damage. Importantly, higher numbers of AAMφs were found in the pancreatic and peripheral lymph nodes of NOD mice compared to noninfected mice [12]. Interestingly, in other studies, the experimental infection of mice with *Schistosoma mansoni* or their treatment with either helminth or soluble worm extracts (SWA) or soluble egg antigen (SEA) could prevent diabetes in NOD mice, with a direct relationship being observed between the lower incidence of T1D and reduced insulitis and higher numbers of AAMφs [8–10]. Other regulatory cell populations, such as Treg cells, which can inhibit inflammation and suppress several autoimmune diseases, including T1D, also increased in number during *Schistosoma mansoni* infection and antigen administration [10]. Other parasites, such as *Litomosoides sigmodontis,* have also been shown to reduce T1D [13]. We have shown that previous *Taenia crassiceps* infection of diabetic mice, which were induced by multiple low doses of streptozotocin (MLD-STZ), significantly decreased the incidence of T1D, hyperglycemia, and the inflammatory infiltration of islets of Langerhans. These effects were accompanied by a significant increase in the secretion of IL-4 and the expansion of the AAMφs population compared with noninfected, diabetic mice, suggesting that AAMφs induced by *T. crassiceps* infection can be important in the protection against T1D [7]. In a recent study, the adoptive transfer of AAMφs, which were induced *in vitro* by IL-4 and IL-13, into diabetic mice reduced kidney injury, hyperglycemia, and insulitis in the pancreas, clearly suggesting that AAMφs may have a protective role against T1D [55]. In another recent study, the adoptive transfer of AAMφs, which expressed PDL-2, FcγRIIb, IL-10, and TGF-β prevented 80% of NOD mice from developing this disease [56]. Collectively, these data suggest that AAMφs may have important roles in the inhibition and prevention of T1D (Table 1 and Figure 1).

2.2. Type 2 Diabetes. T2D is a metabolic disease, and its incidence has increased significantly in recent years. It is estimated that in 2000, there were approximately 171 million

people with this disease, and it has been predicted that by 2030, the prevalence of T2D will increase to 366 million people [57]. T2D is characterized by a peripheral resistance to the action of insulin and a rise in insulin production by β cells in a process called "compensatory hyperinsulinemia" to force glucose uptake in peripheral tissues. Regardless, during T2D, there is a chronic deficiency of glucose uptake and insulin action, mainly in the liver, skeletal muscle, and adipose tissue (AT), causing hyperglycemia, hypercholesterolemia, and hyperlipidemia [58, 59].

AT is composed of adipocytes, preadipocytes (which are immature adipocytes that have not yet loaded any lipids), endothelial cells, leukocytes, fibroblasts, and macrophages [60]. During obesity, lipid accumulation causes a high degree of stress on adipocytes, activating them and promoting the production and subsequent release of free fatty acids (FFA), proinflammatory adipocytokines (such as leptin and resistin), and cytokines, such as IL-1β, IL-6, TNF-α, MCP-1, and MIF, as well as ROS [61–63], ensuring that in addition to its well-known capacity to store energy, AT has the capability to function as an endocrine organ. In fact, this endocrine ability of AT triggers inflammation, leading to insulin resistance and the development of T2D.

Several data show that macrophages are recruited into AT and classically activated due to adipocytokine secretion, contributing to the establishment of an inflammatory profile and insulin resistance in this tissue. A deficiency of MCP-1 (CCL2) or CCR2 (CCL2 receptor) in mice during obesity results in the impairment of CAMφ recruitment to adipose tissue, thus impeding the induction of insulin resistance by a high-fat diet (HFD) [64, 65] and suggesting an important role for CAMφs in T2D initiation and development (see Figure 2). Additionally, the stressed AT secreted the adipocytokines leptin and resistin, which have been implicated in the recruitment and activation of monocytes and CAMφs in adipose tissue, inducing these cells to produce higher levels of TNF-α, IL-12, and IL-6 [61]. Besides the production of resistin by stressed AT, stressed AT also induces the expression of MCP-1 and cellular adhesion molecules, such as V-CAM and ICAM, in adipose tissue and its vascularization [66]. Furthermore, FFA can be recognized by Toll-like receptors (TLRs) with low affinity, leading to the activation of macrophages, which release more TNF-α [67, 68]. TNF-α (one of the cytokines most abundantly secreted by CAMφs) has the ability to reduce the expression of important genes in the glucose regulation process, such as the glucose transporter GLUT-4 [4]; in fact, TNF-α receptor knock out mice are resistant to diabetes induction [69], suggesting that the endocrine function of AT is important in the recruitment and activation of CAMφs and the induction of insulin resistance. Consistent with these observations, a recent report on a model of T2D (induced with a single high dose of streptozotocin) in MIF KO mice showed that these mice had a reduced inflammatory response, such as reduced TNF-α production, and failed to develop T2D, demonstrating that MIF is also important in promoting the disease [70].

Secretion of IL-1β, TNF-α, and ROS by AT CAMφs induces the activation of JNK and NF-κB signaling in various leukocytes. NF-κB is a transcription factor with an important role in the induction of inflammatory responses and the activation of CAMφs, whereas JNK (c-Jun amino-terminal kinase), also known as the protein kinase activated by stress (SAPK), is activated by oxidative stress. Therefore, the activation of these signaling pathways induces the production of more IL-1β, TNF-α, and MCP-1 and high levels of iNOS expression, contributing to insulin resistance in different tissues [71–73].

When insulin binds to its receptor, IRS-1 and IRS-2 (insulin-receptor substrates 1 and 2) are recruited to its cytoplasmic region, which permits the binding and activation of two important kinases, the first of which is PI3K (phosphatidylinositol 3-kinase), and the second of which is AKT (a protein kinase B) [74]. Once activated, these kinases can regulate glucose and lipid metabolism. However, activated JNK can induce the phosphorylation of serine residues on IRS-1/2, inhibiting their ability to couple to PI3K and thereby promoting insulin resistance. In fact, the expression of JNK and NF-κB is increased in diabetic patients [73], suggesting an important role for these molecules in diabetes. In myeloid-specific I$\kappa\kappa$-β (an activator of NF-κB)-deficient mice, a decrease in proinflammatory cytokine production (IL-1β, IL-6, TNF-α, and MCP-1) and the inhibition of NF-κB activation has been reported, avoiding, in this way, the development of insulin resistance [75].

CAMφs have been confirmed to be directly involved in diabetes because it has been found that 30% of the transcripts expressed in the adipose tissue of HFD-treated mice encode characteristic macrophage proteins associated with this subpopulation [76]. Also, the expression of transcripts for MIP-1α, MCP-1, MAC-1, F4/80, and CD68 was associated with insulin subproduction and TNF-α release [77]. In addition, macrophage polarization to CAMφs had a direct relationship with the development of lipid droplets [78]. These characteristics relate the activation of CAMφs to the promotion of AT accumulation and insulin resistance.

Interestingly, a macrophage phenotypic switch has been reported in the AT of HFD-treated mice compared with normal diet-treated mice. Lumeng et al., 2007 [4], reported the presence of a natural AAMφ population within the AT of lean mice, and interestingly, the phenotype of these cells shift to CAMφs when the mice were HFD-treated. The authors also showed that the IL-10 produced by AAMφs had the ability to block the pathological effects of TNF-α in adipose tissue during insulin sensitivity [4, 78, 79], suggesting that while CAMφs have insulin resistance-inducing effects, AAMφs have a protector role within AT. Recently, another inflammatory chemokine has been shown to be involved in the resistance to insulin and T2D. A-ZIP transgenic mice (these animals are insulin-resistant and hyperlipidemic), which have a deficiency in MCP-1, displayed decreased hyperglycemia, hyperinsulinemia, and hepatomegaly; moreover, these mice had increased levels of markers for AAMφs, such as *Arg1* and *Chi313* [80].

Also of note, PPARs are ligand-dependent transcription factors that have important functions in FA transport, synthesis, storage, mobilization, activation, and oxidation. Three distinct types of PPARs have been characterized: PPARα, PPARδ, and PPARγ. PPARα and PPARδ are involved in the

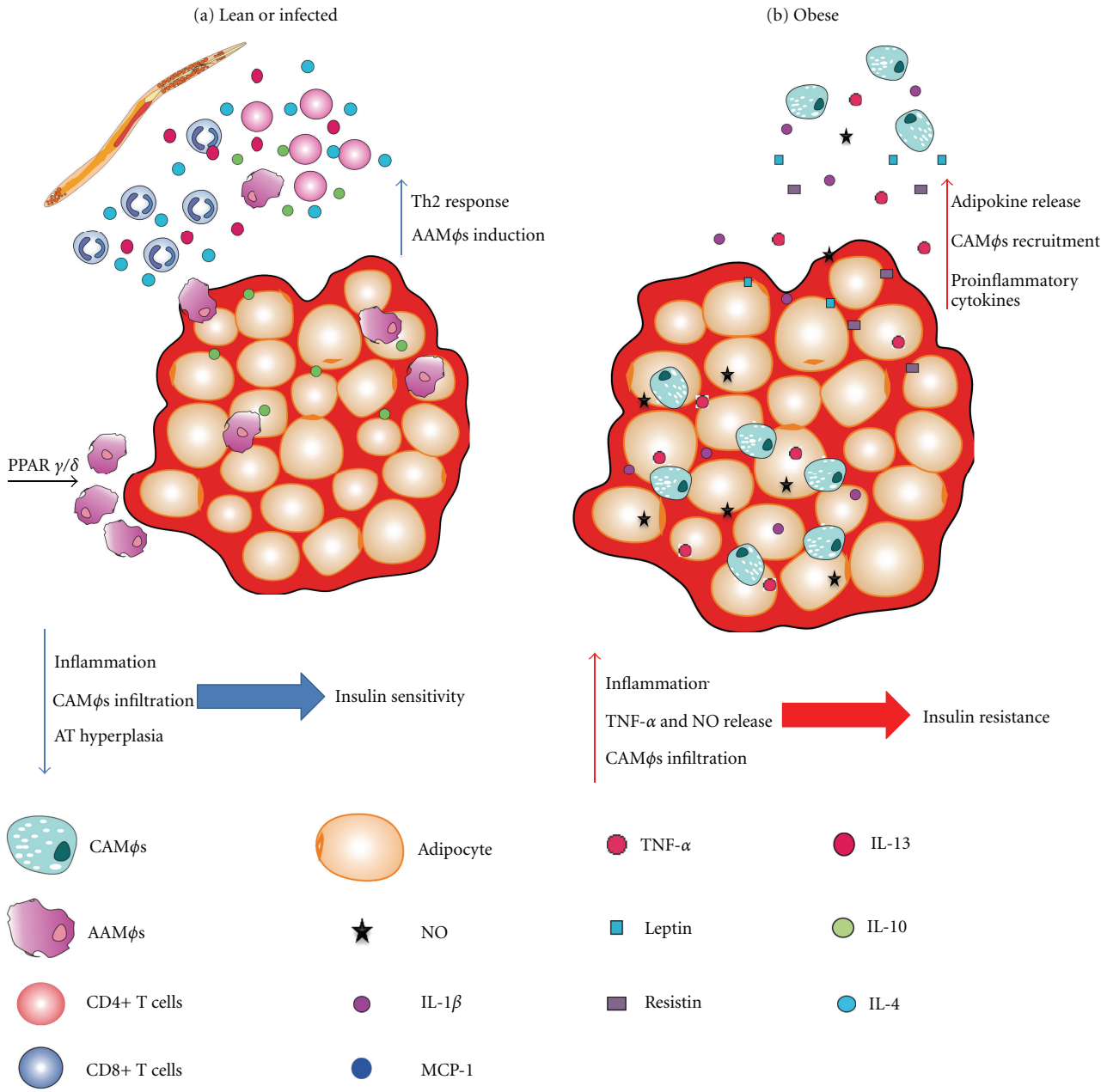

Figure 2: (a) Lean individuals have AAMφs in their AT, which protect them from insulin resistance by secreting IL-10. An helminth infection can recruit Th2 lymphocytes, IL-4/13-secreting eosinophils and AAMφs, thereby increasing protection. The natural AAMφ population in the lean AT is sustained by PPARγ/δ; (b) obesity induces resistin and leptin secretion, as well as proinflammatory adipocytokines, thereby promoting CAMφ recruitment into the AT. CAMφs in turn induce insulin resistance by secreting NO and TNF-α.

oxidation of FFA, while PPARγ contributes to adipogenesis and the storage of FA. PPARγ expression is induced in Mφs by IL-4/IL-13 [81–83]. Recent reports have shown that PPARγ is required for AAMφs induction and maturation, and the absence of this molecule enhances obesity and insulin resistance in HFD mice [81]. Moreover, PPARδ-deficient Kupffer cells cannot be alternatively activated, predisposing mice to develop hepatic steatosis and insulin resistance [84]. As mentioned above, AAMφ development is dependent on IL-4/IL-13 stimulation, which activates the transcription factor STAT-6. STAT-6-deficient mice are more prone to obesity, and oxidative stress in their AT makes them more susceptible to T2D development, which, in turn, is associated with the absence of AAMφs [85].

The role of other cells in the regulation of insulin sensitivity is recognized principally because of evidence in experimental models. Eosinophil-deficient mice have a smaller AT-AAMφ population and gain more weight, which indicates that eosinophils are an important source of IL-4 in adipose tissue [15]. Likewise, *Nippostrongylus brasiliensis* infection

induced the recruitment of eosinophils and AAMφs, which promoted a strong Th2 response and decreased obesity and insulin resistance [15], suggesting that eosinophils contribute to AAMφ induction and prevent T2D.

Collectively, these findings suggest that adipose tissue is an important source of inflammatory molecules during obesity and can induce insulin resistance due to the increased recruitment of CAMφs, which, in turn, can amplify the inflammatory response, promoting development of T2D, while high numbers of AAMφs in the adipose tissue have been involved in glucose tolerance and diabetes prevention (Figure 2).

3. Conclusions

There is no doubt that the incidence of diabetes has increased in recent years, perhaps reflecting changes in lifestyle with regard to diet and/or hygiene. One explanation for the increased incidence of T1D is the hygiene hypothesis, which suggests that low or null exposure to parasites, especially helminths or their antigens, promotes the development of autoreactive leukocytes that attack β cells, initiating the disease. Helminth infections in mice with T1D have proved to prevent the inflammatory cascade through a mechanism associated with AAMφ induction. AAMφs have been implicated in the regulation of other autoimmune diseases, such as experimental autoimmune encephalomyelitis [86] and autoimmune colitis, suggesting that AAMφs have a strong immunoregulatory role in the induction of autoantigen tolerance [87]. Therefore, it is likely that these cells are the main players in the regulation of T1D.

The importance of AAMφs extends beyond the regulation of autoimmunity, which we reviewed in this paper. AAMφs can also inhibit the development of T2D, mainly by reducing obesity and insulin resistance, two major etiological factors in the induction of this disease, while CAMφs are associated with increasing inflammation, obesity, and insulin resistance. Interestingly, the use of helminth parasites to induce AAMφs has proved to be effective in disease treatment by reducing hyperglycemia, obesity, and the incidence of T2D.

Finally, while CAMφs have a major role in the injury and inflammatory response in diabetes, AAMφs appear to reduce inflammation during type 1 and type 2 diabetes, suggesting that these macrophage populations may be therapeutic targets. Thus, based on the results of the various reports reviewed in this paper, we can highlight the possible therapeutic use of diverse immune-modulatory molecules to counteract or negatively influence specific inflammatory and cytotoxic T-cell-activating properties of macrophages.

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Research Article

Urinary Macrophage Migration Inhibitory Factor Serves as a Potential Biomarker for Acute Kidney Injury in Patients with Acute Pyelonephritis

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Conventional markers of kidney function that are familiar to clinicians, including the serum creatinine and blood urea nitrogen levels, are unable to reveal genuine injury to the kidney, and their use may delay treatment. Macrophage migration inhibitory factor (MIF) is a proinflammatory cytokine, and the predictive role and pathogenic mechanism of MIF deregulation during kidney infections involving acute kidney injury (AKI) are not currently known. In this study, we showed that elevated urinary MIF levels accompanied the development of AKI during kidney infection in patients with acute pyelonephritis (APN). In addition to the MIF level, the urinary levels of interleukin (IL)-1β and kidney injury molecule (KIM)-1 were also upregulated and were positively correlated with the levels of urinary MIF. An elevated urinary MIF level, along with elevated IL-1β and KIM-1 levels, is speculated to be a potential biomarker for the presence of AKI in APN patients.

1. Introduction

Even minor increases in the serum creatinine level are associated with an increased risk of inhospital morbidity and mortality [1]. A modest decline in the glomerular filtration rate and kidney injury should be used to diagnose kidney damage to facilitate early detection and intervention [2, 3]. Therefore, the RIFLE (risk, injury, failure, loss, and end-stage kidney disease) criteria replace the term "acute renal failure" with "acute kidney injury" (AKI) [4]. However, traditional tools, including the serum creatinine and blood urea nitrogen (BUN) levels and urinary markers (urinary output and urine sodium excretion) are not sufficiently sensitive to provide an early diagnosis of AKI, and their use may delay treatment [5, 6]. It is expected that injury biomarkers, in addition to the functional markers, will facilitate the early detection of renal injury.

Macrophage migration inhibitory factor (MIF) is a potent proinflammatory cytokine that activates macrophages and promotes the synthesis of cytokines, including tumor necrosis factor-α, interleukin (IL)-1β, and IL-8 [7, 8]. MIF is released from an intracellular pool in response to pathological stimuli including infection and inflammatory activation. MIF has been shown to bind to CD74 and recruits CD44 to form a receptor complex, resulting in the phosphorylation of extracellular signal-regulated kinase through Src tyrosine kinase [8–10]. MIF also activates transcription factors of the E-twenty-six family and upregulates Toll-like

receptor (TLR) 4 expression and signaling to enhance the inflammatory response [11–14]. Renal MIF is a constitutive expression in normal kidneys and is upregulated in patients with glomerulonephritis and renal allograft rejection. The upregulation of MIF is associated with leukocyte infiltration, histopathological damage, and renal dysfunction in patients with inflammatory kidney disease [15–20]. The concentration of urinary MIF is significantly correlated with the upregulation of renal MIF expression, instead of the serum MIF level, suggesting that the elevated level of urinary MIF is due to MIF production in and secretion by the injured kidney [17, 18]. The association between the urinary MIF level and renal damage makes MIF a candidate marker for renal injury in humans.

Acute pyelonephritis (APN) is a common infection in which bacteria invade the renal epithelial cells and is an important cause of renal insufficiency [21]. Although elevated levels of urinary MIF are found in individuals with urinary tract infections (UTIs), the upregulation of MIF in the context of a UTI has only been found in patents with APN [22–24]. The correlation between the urinary MIF level and significant renal dysfunction has not yet been defined. Therefore, the aim of this study was to investigate the ability of aberrant urinary MIF levels to detect AKI in patients with kidney infections.

2. Materials and Methods

2.1. Subjects and Study Design. Patient serum and urine samples were prospectively collected between January 2010 and December 2010 in the emergency department of the National Cheng Kung University Hospital, Tainan, Taiwan. The diagnostic criteria for APN included fever (body temperature above 38.3°C), flank pain and/or costovertebral angle tenderness with or without painful micturition, and pyuria. Thirty-nine patients who were diagnosed with symptomatic and culture-proven APN were enrolled in the study. Patients who presented with shock, urinary tract malignancy, or glomerulonephritis were excluded from the study. Patients with serum creatinine levels more than 50% above baseline were defined as having AKI according to the criteria of RIFLE [25]. The estimated glomerular filtration rate (eGFR) was calculated using the modification of diet in renal disease (MDRD) formula relative to the serum creatinine level based on age, race, and sex [26]. The blood samples for the laboratory analysis, which included a hemogram and analyses of the current renal function, C-reactive protein level, and serum MIF level, were collected within 2 hours of hospital arrival. Data including demographic information, data on comorbidities, clinical features (including blood pressure, oxygen saturation, respiratory rate, and consciousness levels, which were necessary for calculating severity scores), and baseline renal function were collected from the patients' medical records. Receiver operating curve (ROC) analysis was used to determine the ability of the urinary MIF level to predict AKI. To investigate the ability of MIF to distinguish AKI from chronic kidney disease, we also conducted a subgroup analysis including APN patients with renal dysfunction (eGFR < 60 mL/min/1.73 m^2). The severity scores, including the Rapid Emergency Medicine Score (REMS) and the Rapid Acute Physiology Score (RAPS), were used as a measure of initial patient care [27, 28]. The REMS and RAPS scoring systems are truncated versions of the Acute Physiology and Chronic Health Evaluation (APACHEII), and they were calculated at the time of the patient's arrival at the hospital. We also included patients without UTIs as control subjects. The protocols and procedures were approved by the institutional review board of the National Cheng Kung University Hospital, Tainan, Taiwan.

2.2. Measurement of the Urinary Levels of MIF, IL-1β, and Kidney Injury Molecule (KIM)-1. Serum and urine samples were collected from APN patients and normal controls. The levels of MIF, IL-1β, and KIM-1 were measured using standard enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's recommendations. All measurements were performed in triplicate. After the reaction, the plates were washed, and 100 μL of *o*-phenylenediamine substrate was added to each well. The plates were incubated for 30 min at room temperature, after which 50 μL of 4 N sulfuric acid was added to each well. The plates were read at 450 nm using a microplate reader (SpectraMax 340PC; Molecular Devices, Inc., Sunnyvale, CA), and the data were analyzed (using Softmax Pro software). The levels of urinary cytokines were calculated as ratios relative to the urinary creatinine level.

2.3. Statistical Analysis. Values are expressed as the means ± SD. Groups were compared using Student's two-tailed unpaired *t*-test or one-way analysis of variance using SPSS 17.0 (SPSS, IBM, West Grove, USA). Pearson correlation coefficients were used to analyze the correlations between the urinary MIF level, IL-1β level, KIM-1 level, and white blood cell (WBC) count. A value of $P < 0.05$ was considered to be statistically significant. A receiver operating characteristic curve was used to analyze the ability to diagnose AKI based on several parameters, and the area under the curve (AUC) for each parameter was determined.

3. Results

3.1. There Is an Increase in Urinary MIF Levels in APN Patients with AKI. To determine the clinical implications of urinary MIF in patients with kidney infections, cytokine levels and renal biochemical parameters were analyzed in patients with APN. Thirty-nine APN patients were enrolled in our study. Based on the RIFLE criteria [25], the patients were divided into two groups according to the presence of AKI. The two groups, which included 13 patients with AKI and 26 without AKI, did not differ significantly with respect to age, gender, comorbidities, laboratory data, disease severity scores, or serum MIF levels except urinary MIF levels and renal function (present BUN, creatinine, and eGFR), as shown in Table 1. The patients with AKI had an increase in urinary MIF compared to patients without AKI (17.0 ± 13.2 ng/mg versus 4.2 ± 3.5 ng/mg, $P = 0.004$).

Table 1: Demographic data and clinical characteristics of patients with APN.

Clinical variables	Number of cases (%)		P value
	Without AKI ($n = 26$)	With AKI ($n = 13$)	
Age (yrs)	66 ± 18	73 ± 10	0.204
Male	9 (35)	4 (31)	1.000
Comorbidity			
Diabetes mellitus	13 (50)	6 (46)	1.000
Hypertension	15 (58)	9 (69)	0.728
CKD (eGFR < 60 mL/min/1.73 m^2)	7 (27)	6 (46)	0.290
Baseline eGFR[a]	79 ± 33	67 ± 46	0.322
Laboratory data			
White blood cell count (k/μL)	12.7 ± 4.8	13.9 ± 8.4	0.565
Absolute neutrophil count (k/μL)	10.8 ± 4.8	11.1 ± 7.5	0.858
C-reactive protein (mg/dL)	99 ± 107	115 ± 67	0.669
Creatinine (mg/dL)	1.4 ± 1.3	3.4 ± 2.0	0.003
Blood urea nitrogen (mg/dL)	28.9 ± 29.6	68.7 ± 36.2	0.001
Current eGFR[b]	78 ± 40	22 ± 15	<0.001
Urine MIF (ng/mg)[c]	4.2 ± 3.5	17.0 ± 13.2	0.004
Serum MIF (ng/mL)	259.6 ± 240.9	279.8 ± 248.1	0.810
Gram-negative bacteria[d]	24 (92)	12 (92)	1.000
Severity score			
REMS	7.5 ± 3.6	7.7 ± 2.8	0.893
RAPS	2.1 ± 2.0	3.2 ± 2.3	0.174

Categorical variables are expressed as a number (percentage), and continuous variables are expressed as the mean ± SD. AKI: acute kidney injury; CKD: chronic kidney disease; eGFR: estimated glomerular filtration rate; MIF: macrophage migration inhibitory factor; REMS: rapid emergency medicine score; RAPS: rapid acute physiology score.
[a]The baseline eGFR was estimated using the MDRD equation, and the units are mL/min/1.73 m^2.
[b]The current eGFR was estimated by the MDRD equation using the serum creatinine level while the patient arrived at the emergency department.
[c]The concentrations of MIF in the urine were measured by ELISA and were normalized based on the urinary creatinine levels. We divided the urine levels of MIF by urine creatinine to measure the adjusted urine MIF (ng/mg).
[d]The bacterial pathogens of APN were identified and proven as Gram-negative bacteria.

Table 2: Microbiological analysis of 39 patients with APN.

Invaded pathogens[a]	Number of cases (%)	
	Without AKI ($n = 26$)	With AKI ($n = 13$)
Gram-negative bacteria	24 (92)	12 (92)
E. coli	17 (65)	10 (77)
Proteus mirabilis	2 (8)	0 (0)
Klebsiella pneumoniae	1 (4)	2 (15)
Pseudomonas aeruginosa	3 (12)	0 (0)
Providencia stuartii	1 (4)	0 (0)
Gram-positive bacteria	2 (8)	1 (8)
Enterococcus species	2 (8)	0 (0)
Coagulase-negative staphylococcus	0 (0)	1 (8)

Variables are expressed as a number (percentage). AKI: acute kidney injury.
[a]The bacterial pathogens of APN were identified and proven as shown later.

According to the power analysis for a two-group independent sample t-test, a sample size of 39 subjects had a reasonable power (0.97) to distinguish the two groups based on urinary MIF expression. The Gram-negative bacteria are common pathogens of UTI, including strains of *Escherichia coli, Klebsiella* spp., and *Proteus* spp. Among them, *E. coli* accounts for the 70–95% of community-acquired UTI. The microbiological analysis of the invaded pathogens was shown in Table 2. The majority of invaded pathogens in the APN patients were Gram-negative bacteria (92%), and the percentage of Gram-negative bacteria was consistent between the two groups (92%, $P = 1.000$, Table 1). To adjust the bacterial factor in altering urinary MIF expression, subgroup analysis of patients whose pathogens were identified as Gram-negative bacteria or *E. coli* was conducted in Table 3. In APN patients, invaded pathogens were identified as Gram-negative bacteria, and the urinary MIF was higher in patients with AKI compared to patients without AKI ($n = 36$, 16.5 ng/mg ± 13.8 ng/mg versus 4.4 ng/mg ± 3.5 ng/mg, $P = 0.011$). In patients whose invaded pathogens were proven as *E. coli*, there was consistently an increase in urinary MIF in APN patients with AKI compared to those without AKI ($n = 27$, 15.2 ng/mg ± 11.6 ng/mg versus 4.0 ng/mg ± 3.4 ng/mg, $P = 0.013$). MIF has been reported to increase and participate in the pathogenesis of diabetic nephropathy

Table 3: Subgroup analysis of urinary MIF levels between APN patients with diabetes, renal dysfunction, or microbiological analysis.

Clinical characteristics of APN patients	Number of cases	Without AKI	With AKI	*P* value
Gram-negative bacteria[a]	36	4.4 ± 3.5	16.5 ± 13.8	0.011
E. coli	27	4.0 ± 3.4	15.2 ± 11.6	0.013
Diabetes mellitus	19	4.1 ± 4.1	15.2 ± 8.7	0.024
Renal dysfunction on arrival[b]	21	2.9 ± 2.4	17.0 ± 13.2	0.002

Urinary MIF levels (ng/mg) are expressed as the mean ± SD. AKI: acute kidney injury; eGFR: estimated glomerular filtration rate.
[a]The bacterial pathogens of APN were identified and proven as Gram-negative bacteria.
[b]The patients who presented with renal dysfunction (defined as eGFR < 60 mL/min/1.73 cm^2) upon arrival at our emergency department were included.

[29]. Because it remains unclear whether diabetes confounds the function of urinary MIF in detecting AKI, we analyzed the level of urinary MIF in diabetic patients (Table 3). There was an increase in urinary MIF levels in diabetic patients with AKI compared to patients without AKI ($n = 19$, 15.2 ± 8.7 ng/mg *versus* 4.1 ± 4.1 ng/mg, $P = 0.024$).

3.2. The Urinary Levels of MIF, IL-1β, and KIM-1 Are Elevated in APN Patients with AKI. The urinary IL-1β level has been reported to be elevated in patients with APN [30], and urinary KIM-1 is a sensitive biomarker for AKI and is not influenced by UTIs or chronic kidney disease [31, 32]. We therefore evaluated the diagnostic utility of the urinary MIF, IL-1β, and KIM-1 levels as biomarkers for AKI during kidney infection. The levels of urinary MIF (Figure 1(a)), IL1β (Figure 1(b)), and KIM-1 (Figure 1(c)) were significantly higher in APN patients with AKI than in patients without AKI or normal controls.

3.3. The Urinary Levels of MIF Were Positively Correlated with the Urinary Levels of IL-1β and KIM-1. The Pearson correlation coefficient was used to analyze the urinary levels of MIF, IL-1β, and KIM-1 and the urinary WBC count. The level of urinary MIF was positively correlated with urinary levels of IL-1β ($R^2 = 0.512$, $P < 0.001$, Figure 2(a)) and KIM-1 ($R^2 = 0.319$, $P < 0.001$, Figure 2(b)). However, no correlation was found between the urinary level of MIF and the urinary WBC count ($R^2 < 0.001$, $P = 0.926$, Figure 2(c)).

3.4. The AUC of the Urinary Levels of MIF for Detecting AKI among APN Patients. The ROC curve for detecting the presence of AKI in APN patients included the urinary levels of MIF, IL-1β, and KIM-1. The AUC for the urinary MIF level reached 0.871 in all APN patients (Figure 3(a)). In patient with normal renal function on arrival (defined as eGFR ≥60 mI/min/1.73 cm^2, $n = 18$), our result revealed an elevated urinary MIF indicating the presence of kidney infection compared to normal controls (4.7 ± 3.8 ng/mg versus 0.7 ± 0.5 ng/mg, $P < 0.001$). Encountering patients with abnormal renal function test, it is doubtable to determine whether patients have AKI or preexisting chronic kidney disease (CKD). We, therefore, analyzed the enrolled patients who presented with renal dysfunction (defined as eGFR< 60 mI/min/1.73 cm^2 on arriving at our emergency department, $n = 21$). These patients with AKI had an increase in urinary MIF compared to patients without AKI (17.0 ± 13.2 ng/mg *versus* 2.9 ± 2.4 ng/mg, $P = 0.002$,) in Table 3. The AUC for the urinary MIF level in detecting the presence of AKI reached 0.923 in patients with renal dysfunction (Figure 3(b)). Urinary MIF helps to distinguish the presence of AKI from pre-existing CKD.

4. Discussion

The current criteria for the diagnosis of AKI based on elevated levels of serum creatinine or BUN are often inadequate for the early detection of renal injury. Injury to the renal tubules may not be sufficiently severe to cause changes in the serum creatinine or BUN levels. Therefore, injury biomarkers for the detection of tubular damage, used in addition to the functional markers, may facilitate the early recognition of renal injury. Our study revealed the elevation of the urinary MIF level in APN patients with kidney infection, and urinary MIF serves as an injury biomarker of AKI in these patients.

In mice models of endotoxic shock or *E. coli* peritonitis, elevated serum MIF was detected, and MIF neutralizing antibodies protected the mice from lethal shock and sepsis [33, 34]. Calandra et al. have reported that streptococcal and staphylococcal exotoxin induced MIF secretion in macrophage, and anti-MIF antibody increases survival in mice model of exotoxin-induced shock [35]. Taken together, these findings indicate that MIF has an important role in bacterial infections. Several clinical studies have indicated that septic patients with high serum MIF levels appear to have a higher risk of mortality than patients with lower serum MIF levels [36–38]. In our investigation, however, the serum MIF level was not sufficient to detect the presence of AKI. We found that the urinary MIF level is a more sensitive indicator of kidney injury than the systemic MIF level, as suggested by Brown et al. [17, 18]. Previous studies have focused on the use of elevated urinary MIF levels to detect the presence of UTIs and to distinguish kidney infections from acute cystitis [23, 24]. In patient with normal renal function, consistent with previous study, our study revealed that elevated urinary MIF indicated the presence of APN. Our investigation further demonstrated that high urinary levels of MIF suggest the presence of AKI. In patients with elevated serum creatinine or BUN levels, it is a clinical puzzle to determine whether patients have AKI or pre-existing CKD. In subgroup analysis of patients with renal dysfunction on arrival, we revealed that the patients with AKI had an

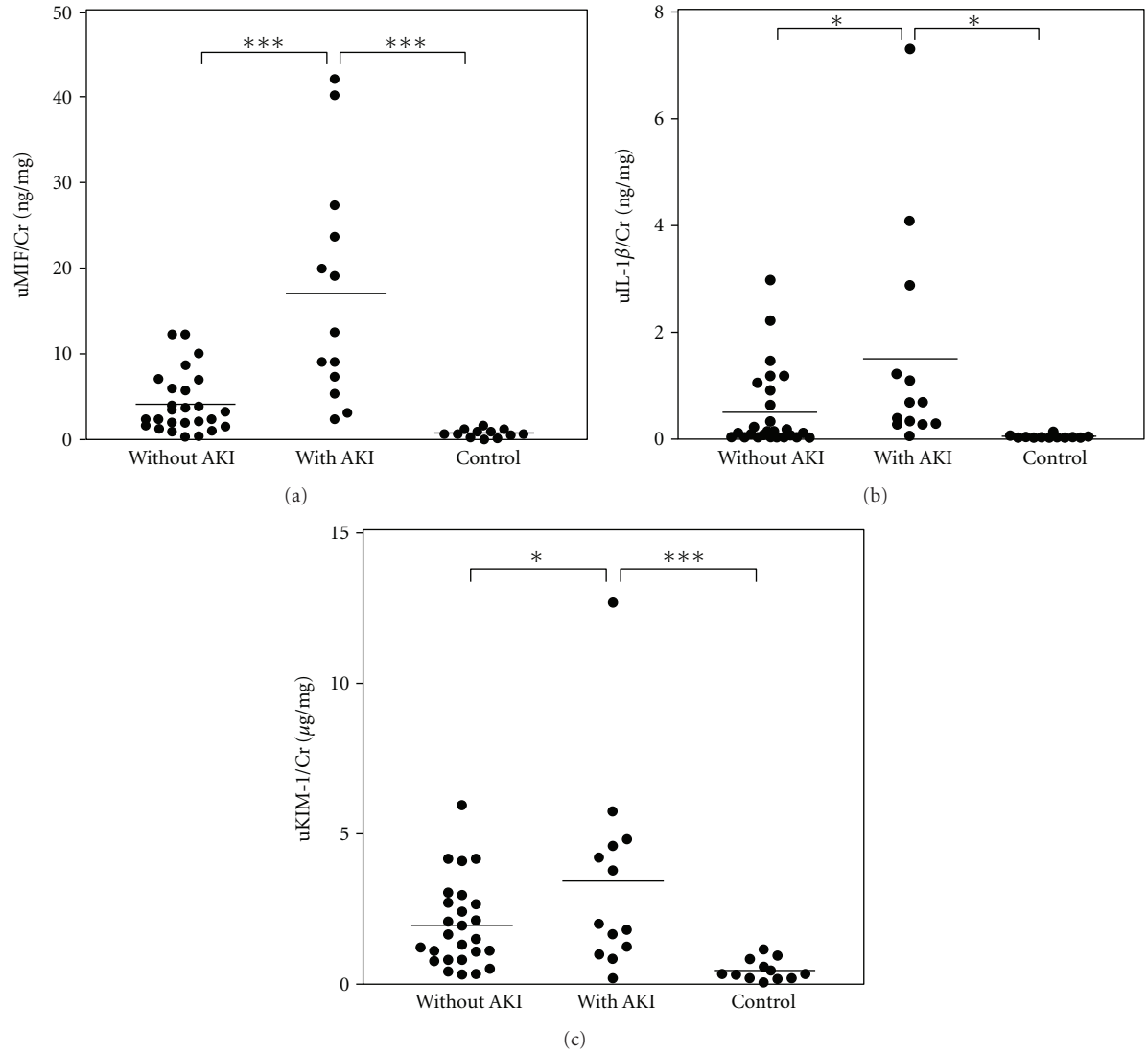

Figure 1: The urinary MIF level is markedly elevated in the presence of AKI in patients with kidney infections. The concentrations of MIF, IL-1β, and KIM-1 in the urine were measured by ELISA and were normalized based on the urinary creatinine (Cr) levels. The urinary levels of MIF (uMIF/Cr) (a), IL-1β (uIL-1β/Cr) (b), and KIM-1(uKIM-1/Cr) (c) were measured in APN patients with ($n = 13$) or without ($n = 26$) AKI and in normal controls ($n = 12$). Data are the means SD. $^{***}P < 0.001$, and $^{*}P < 0.05$.

increase in urinary MIF compared to patients with pre-existing CKD. MIF has been reported to be an injury marker in kidney inflammatory disease, and consistently, our results revealed the utility of urinary MIF in determined AKI under infection. We, therefore, provide evidence supporting the ability of the urinary MIF level to identify patients with AKI and to discriminate AKI from CKD in patients with renal dysfunction.

Our study of APN patients also revealed increased urinary IL-1β levels in AKI patients. The urinary IL-1β level has been previously reported as a marker for APN [30]. IL-1β is secreted in biological fluids and thought to be a primary initiator of the inflammatory cascade during bacterial infection [39]. The levels of urinary IL-1β were correlated with the levels of urinary MIF in the present study. The regulatory role of MIF in IL-1β production has been demonstrated in previous studies, which demonstrated that MIF promotes inflammation through autocrine and paracrine effects to induce the production of IL-1β by nearby tissues or immune cells [8, 40]. Furthermore, elevated levels of MIF in the urine have been found in individuals with the progressive form of glomerulonephritis and those experiencing renal allograft rejections. All of these reports reinforce the role of MIF in renal damage [17, 18].

The urothelium contains a rich store of preformed MIF. During cystitis, MIF is upregulated in the bladder, released from the bladder, and detected in the urine as a potential marker for cystitis [24, 41]. Otukesh et al. have revealed an increase in urinary MIF in patient with APN compared to that in patient with cystitis, suggesting renal origin, in addition to cystic origin, for excretion of urine MIF [23]. In human glomerulonephritis, elevated concentrations

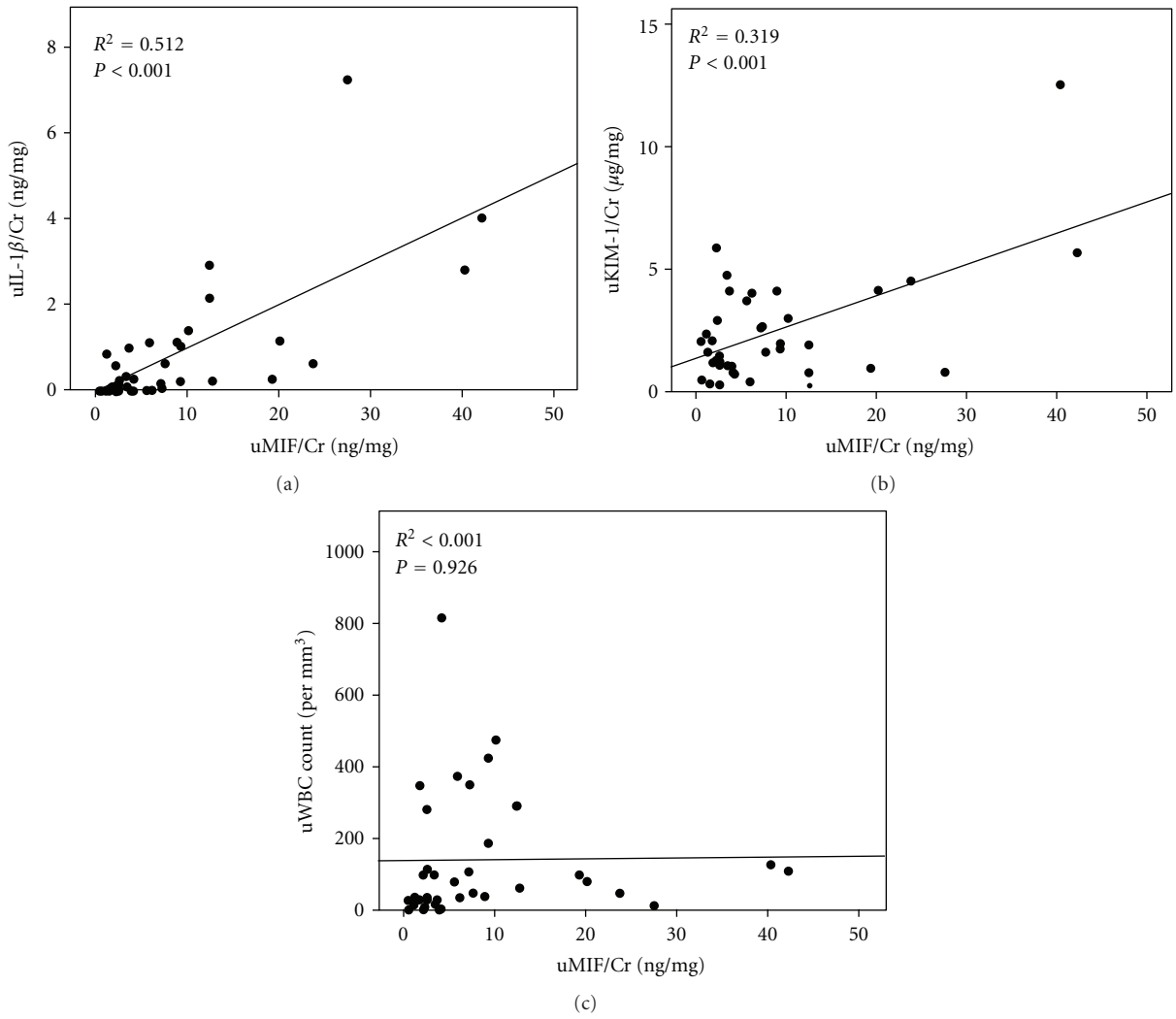

Figure 2: The pairwise correlations between the urinary levels of MIF (uMIF/Cr) and the urinary level of IL-1β (uIL-1β/Cr), the urinary level of KIM-1 (uKIM-1/Cr) and the urinary WBC (uWBC) count. Pearson correlation coefficients were used to analyze the correlation between two variables. Each circle represents a single individual, and lines represent linear approximations.

of urinary MIF reflect the severity of renal injury and AKI. A significant correlation of urinary MIF and renal MIF implicates a renal origin for the excreted urine MIF during AKI [17]. Based on the study mentioned earlier, our investigation confirmed the applicability of urinary MIF in detecting APN-related AKI. The stepwise increase in urinary MIF may originate from both bladder and kidney, reflecting the extent of bacterial invasion.

The urinary levels of MIF were correlated with the levels of KIM-1 but not with the urinary WBC count. KIM-1 is highly and specifically overexpressed by the proximal tubular cells under conditions of nephrotoxic AKI and is, therefore, a sensitive urinary biomarker to detect renal tubular injury [42, 43]. MIF is constitutively expressed in renal tubules in normal kidneys and released and then performs its biological function related to renal inflammatory disease [8]. The positive correlation between the urinary levels of MIF and KIM-1 suggests that renal tubules are one of the origins of urinary MIF. Renal tubular cells expressing TLRs contribute to the activation of the inflammatory response during ischemia-reperfusion injury in rat kidneys [44, 45]. TLR4 on renal epithelial cells activates the immune response and participates in the renal clearance of uropathogenic *E. coli* [46]. Additionally, our previous studies and those of others have shown that inhibition of MIF suppresses TLR4-induced inflammatory cytokine production via alternations in ERK, p38 mitogen-activated protein kinase, and NK-κB activation [13, 14]. The relationship between increased MIF levels in patients with UTI-related renal inflammation and TLR4 is, therefore, speculative.

In summary, we found that the urinary MIF level is significantly elevated in AKI patients during kidney infection. The elevated level of MIF was significantly correlated with the urinary IL-1β and KIM-1 levels, which are indicative of injury to the renal tubules. These findings suggest that urinary MIF is a potential biomarker and that the

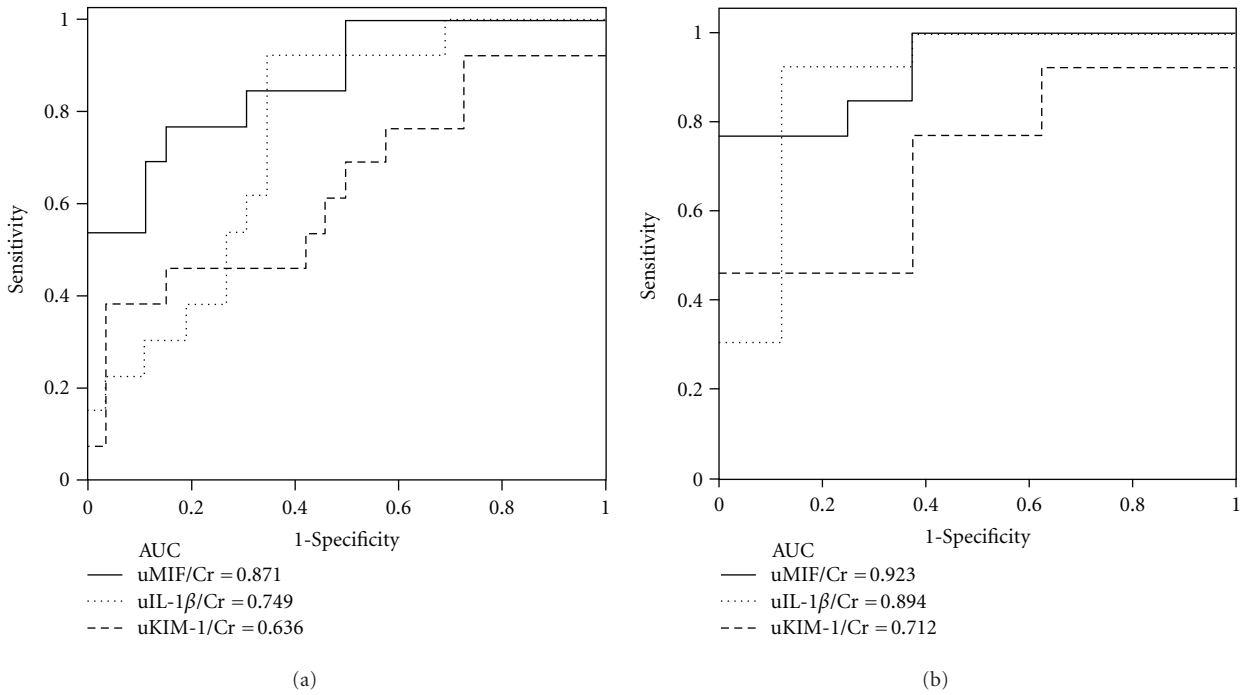

Figure 3: Urinary levels of MIF detected the presence of AKI in APN patients. The receiver operating characteristic curves for the laboratory parameters for the detection of AKI in all APN patients ($n = 39$) (a) or in the subgroup with renal dysfunction (eGFR < 60 ml/min/1.73 cm^2, $n = 21$) (b). The AUCs of the urinary MIF, IL-1β, and KIM-1 levels, which were normalized based on the urinary creatinine (Cr) levels, are shown.

measurement of the urinary MIF level may serve as a useful tool for recognizing nephrotoxicity in APN patients.

Conflict of Interests

The authors declare that there is no conflict of interests.

Authors' Contribution

M.-Y. Hong, C.-L. Chen, and C.-F. Lin were involved in the conception and design of the study. M.-Y. Hong, C.-C. Tseng and S.-H. Lin analyzed the data. M.-Y. Hong, C.-C. Tseng, C.-C. Chuang, C.-L. Chen, and C.-F. Lin interpreted the results of the experiments. M.-Y. Hong and C.-F. Lin drafted the paper.

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Research Article

Activation of Peroxisome Proliferator-Activated Receptor γ by Rosiglitazone Inhibits Lipopolysaccharide-Induced Release of High Mobility Group Box 1

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Peroxisome proliferator-activated receptors (PPARs) are shown to modulate the pathological status of sepsis by regulating the release of high mobility group box 1 (HMGB1), a well-known late proinflammatory mediator of sepsis. Ligand-activated PPARs markedly inhibited lipopolysaccharide- (LPS) induced release of HMGB1 in RAW 264.7 cells. Among the ligands of PPAR, the effect of rosiglitazone, a specific ligand for PPARγ, was superior in the inhibition of HMGB1 release induced by LPS. This effect was observed in cells that received rosiglitazone before LPS or after LPS treatment, indicating that rosiglitazone is effective in both treatment and prevention. Ablation of PPARγ with small interfering RNA or GW9662-mediated inhibition of PPARγ abolished the effect of rosiglitazone on HMGB1 release. Furthermore, the overexpression of PPARγ markedly potentiated the inhibitory effect of rosiglitazone on HMGB1 release. In addition, rosiglitazone inhibited LPS-induced expression of Toll-like receptor 4 signal molecules, suggesting a possible mechanism by which rosiglitazone modulates HMGB1 release. Notably, the administration of rosiglitazone to mice improved survival rates in an LPS-induced animal model of endotoxemia, where reduced levels of circulating HMGB1 were demonstrated. Taken together, these results suggest that PPARs play an important role in the cellular response to inflammation by inhibiting HMGB1 release.

1. Introduction

High mobility group box 1 (HMGB1) is a highly conserved nonhistone nuclear protein that exhibits diverse functions according to its cellular location. In the intracellular compartment, it participates in a number of fundamental cellular processes such as transcription, replication, and DNA repair [1]. In addition to its intracellular functions, extracellular HMGB1 plays an important role in inflammatory responses when actively secreted from stressed cells [2]. Proinflammatory properties of HMGB1 as a crucial cytokine were first documented in a report demonstrating that HMGB1 is actively secreted by activated macrophages, serving as a late mediator of lethality in a mouse model of sepsis [3]. Furthermore, circulating HMGB1 levels were elevated with delayed fashion in the mouse model and in patients with sepsis characterized by overwhelming inflammatory and immune responses, leading to tissue damage, multiple-organ failure and death [3–5]. Recent reports indicated that HMGB1 is a late mediator of sepsis, acting as a key regulator in acute and chronic inflammation [2, 3]. In fact, the administration of anti-HMGB1 antibodies or inhibitors, such as ethyl pyruvate and nicotine, significantly protected mice from LPS-induced acute tissue injury and lethal endotoxemia [3, 4, 6–8].

Notably, these reagents against HMBG1 conferred cellular protection to delayed endotoxin lethality, even when applied at a time after the acute-phase cytokine responses had peaked and resolved [3, 6, 8, 9].

Peroxisome proliferator-activated receptors (PPARs), members of the nuclear hormone receptor family, are ligand-activated transcription factors with multiple biological functions [10, 11]. Three different PPAR isoforms have been identified, PPARα (NR1C1), PPARβ/δ (NR1C2), and PPARγ (NR1C3), and are encoded by different genes that show substantial amino acid similarity, especially within the DNA and ligand-binding domains [11]. All PPARs act as heterodimers with the retinoid X receptor (RXR) and exhibit pleiotropic effects in the regulation of lipid and glucose metabolism, as well as cellular differentiation and proliferation [10–12]. Recently, there has been a great deal of interest in the involvement of PPARs in inflammatory processes [13]. PPAR ligands inhibit the expression of inflammatory genes and can negatively interfere with proinflammatory transcription factor-signaling pathways in vascular and inflammatory cells [14–16]. Furthermore, PPAR levels are differentially regulated in a variety of inflammatory disorders in human, indicating that ligands for PPAR represent new promising therapies for the treatment of diseases associated with inflammation [14].

Although PPARs have shown anti-inflammatory effects in monocyte/macrophages and vascular cells [14–16], little is known about their involvement in the endotoxin-mediated release of HMGB1. Here, we demonstrate that PPARs are involved in the regulation of LPS-induced HMGB1 release in RAW 264.7 cells, and the administration of rosiglitazone, a specific ligand for PPARγ, attenuated endotoxin lethality by inhibiting HMGB1 release in a mouse model of sepsis.

2. Materials and Methods

2.1. Materials. GW501516, WY-14643, and GW9662 were obtained from Calbiochem (La Jolla, CA, USA). 5-[[4-(2-[methyl-2-pyridinylamino]ethoxy)phenyl]methyl]-2,4-thiazolidinedione (rosiglitazone) was obtained from Cayman Chemical Company (Ann Arbor, MI, USA). Polyclonal antibodies specific for PPARα, PPARβ/δ, PPARγ, monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor-α (TNF-α), macrophage inflammatory protein-1β (MIP-1β), and horseradish peroxidase (HRP)-conjugated IgG were supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal antibody specific for β-actin, lipopolysaccharide (LPS, *Escherichia coli* 0111:B4), Polyinosinic-polycytidylic acid (Poly (I:C)), and Ponceau S solution were purchased from Sigma-Aldrich Co. (St. Louis., MO, USA). Monoclonal antibodies specific for HMGB1, phospho-IκBα, inducible nitric oxide synthase (iNOS), and myeloid differentiation primary responses gene 88 (MyD88) were purchased from Epitomics (Burlingame, CA, USA) and BD Bioscience (San Jose, CA, USA), respectively. TIR-domain-containing adaptor-inducing interferon-β (TRIF) was purchased from abcam (Cambridge, UK). Other reagents were of the highest grade available.

2.2. Cell Culture and Stimulation. RAW 264.7 cells, a murine macrophage-like cell line, were obtained from American Type Culture Collection (Manassas, VA, USA). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 100 U/mL penicillin and 100 μg/mL streptomycin, supplemented with 10% heat-inactivated fetal bovine serum at 37°C, under an atmosphere of 95% air and 5% CO_2. RAW 264.7 cells (2×10^6 cells) were plated in 60 mm culture dishes. At 60% confluency, the cells were incubated with serum-free DMEM medium for 24 h and then stimulated with LPS (100 ng/mL) in the presence or absence of indicated reagents.

2.3. Western Blot Analysis. Cells treated with the indicated reagents were washed with ice-cold PBS and lysed in PRO-PREP Protein Extraction Solution (iNtRON Biotechnology, Seoul, Korea). An aliquot of the cell lysate was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a Hybond-P^+ polyvinylidene difluoride membrane (Amersham Biosciences UK Ltd., UK). Membranes were blocked overnight at 4°C, with 5% nonfat milk in Tris-buffered saline (TBS) containing 0.1% Tween 20. Membranes were then incubated overnight at 4°C, with the indicated specific antibodies in TBS containing 1% BSA and 0.05% Tween 20. Finally, membranes were incubated for 2 h at room temperature with peroxidase-conjugated goat antibody diluted 1 : 3000. After extensive washing in TBS containing 0.1% BSA and 0.1% Tween 20, immunoreactive bands were detected using West-ZOL Plus (iNtRON Biotechnology, Seoul, Republic of Korea).

2.4. Determination of HMGB1. An equal aliquot of conditioned culture media from an equal number of RAW 264.7 cells was used to determine the amount of HMGB1 released into culture media. Equal volumes of conditioned culture media were mixed with 80% ice-cold acetone and incubated at −20°C for 1 h. The protein pellet was precipitated following centrifugation at 16,000 g for 10 min at 4°C. After washing with 80% ice-cold acetone, the pellets were resuspended in SDS-PAGE sample buffer and subjected to Western blot analysis.

2.5. Construction of Short Hairpin (sh)RNA against PPARγ and Gene Silencing. Two complementary 55-mer siRNA template oligonucleotides, encoding mouse PPARγ short hairpin (sh)RNA with *Bam*HI-*Hind*III overhangs, were designed to knock down PPARγ. The oligonucleotides used were (sense) 5′-GATCCGGATGCAAGGGTTTCTTCC-TTCAAGAGAGGAAGAAACCCTTGCATCCTTA-3′ and (anti-sense) 5′-AGCTTAAGGATGCAAGGGTTTCTT-CCTCTCTTGAAGGAAGAAACCCTTGCATCCG-3′. The oligonucleotides were then annealed by incubating the mixed oligonucleotides in a PCR thermocycler, using the following profile: 90°C for 3 min, followed by 37°C for 60 min. Annealed oligonucleotides were cloned into the *Bam*HI-*Hind*III-digested expression vector pSilencer 4.1-CMV hygro plasmid (Ambion, Austin, TX, USA). The same vector encoding a single hairpin siRNA sequence not

found in the mouse database was constructed and used as a scrambled shRNA control. All DNA oligonucleotides were synthesized by Cosmo Co., Ltd. (Seoul, Republic of Korea). The sequence of the oligonucleotide (5′-AAGGATGCAAGGGTTTCTTCC-3′) was targeted to the PPARγ sequence corresponding to positions 547–564 within the PPARγ mRNA. Transfected RAW 264.7 cells were selected with 100 μg/mL hygromycin, and the efficiency of knockdown was confirmed by Western blot. Small interfering (si)RNA study was performed as described previously [15].

2.6. Plasmid Construction. The mammalian expression vector pcDNA3.1-PPARγ was constructed as described previously [17].

2.7. Real-Time PCR Analysis. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and reverse transcribed into cDNA by TOPscript RT DryMIX kit (Enzynomics, Seoul, Republic of Korea). Equal amounts of cDNA were diluted, amplified by real-time PCR using Rotor Gene RG-3000 (Corbett life Science, Sydney, Australia) in a 10 μL reaction volume containing 1 x SYBR PCR master mix (QIAGEN, Valencia, CA, USA) and 10 μM primers. After an initial denaturation step for 5 min at 95°C, conditions for cycling were 40 cycles of 10 s at 95°C, 10 s at 58.5°C, and 10 s at 72°C. For normalization of each sample, GAPDH primers were used to measure the amount of GAPDH cDNA. The primers used as follows: MyD88, forward 5′-GGAGATGATCCGGCAACTAGAA-3′; reverse 5′-ATTAGCTCGCTGGCAATGGA-3′; TRIF, forward 5′-TTCCAGCCACTCCATTCTCATC-3′; reverse 5′-GTAACGTATGTCCCCAACTCCA-3′; GAPDH, forward 5′-CATGGCCTTCCGTGTTCCTA-3′; reverse 5′-CCTGCTTCACCACCTTCTTGAT-3′. The fold change in target gene cDNA relative to the GAPDH control was determined by delta delta CT method [18].

2.8. Animal Model of Endotoxemia and Serum Analysis. All animal studies were approved by the Institutional Animal Care Committee of Konkuk University. Endotoxemia was induced in BALB/c mice (male, 6-7 weeks, 20–25 g) by intraperitoneal injection of bacterial endotoxin (10 mg/kg, *Escherichia coli* LPS 0111:B4), as described previously [3, 6]. Briefly, BALB/c mice were obtained from Koatech (Pyeongtaek, Korea) and housed in a pathogen-free environment. Standard sterilized laboratory diet and water were available *ad libitum* under controlled environmental conditions, with a 12 h light/dark cycle (light on 06:00). BALB/c mice were randomly assigned to one of four groups: injection of LPS (10 mg/kg), injection of LPS (10 mg/kg) plus rosiglitazone (10 mg/kg), injection of LPS (10 mg/kg) plus rosiglitazone (10 mg/kg) plus GW9662 (1 mg/kg), or injection of GW9662 (1 mg/kg) alone. Another group of BALB/c mice were treated with rosiglitazone (10 mg/mL) after LPS (10 mg/kg) infusion. Mortality was recorded for up to 2 weeks after LPS injection to ensure that no additional late deaths occurred. For measurement of plasma HMGB1 levels, BALB/c mice were subjected to sepsis by LPS injection in the presence or absence of rosiglitazone as described above. After 20 h, blood was collected, allowed to clot for 2 h at room temperature, and then centrifuged for 20 min at 1,500 g. The levels of circulating HMGB1 in serum were determined by Western blot analysis.

2.9. Statistical Analysis. Data are expressed as means ± SE. Statistical significance was determined by Student's t-test or ANOVA with a post hoc Bonferroni test. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Activation of PPARs by Ligand Inhibits LPS-Induced Release of HMGB1 in RAW 264.7 Cells. To investigate whether PPARs exhibit biological functions in RAW 264.7 cells, constitutive expression of PPARs was examined. Expression of three PPAR isoforms was observed (Figure 1(a)), suggesting that all of the PPAR isoforms may be biologically active in RAW 264.7 cells. To determine whether the activation of PPARs by ligand affects endogenous HMGB1 endogenous expression or release, RAW 264.7 cells were treated with LPS for 24 h, and the release of HMGB1 was measured. Levels of secreted HMGB1 were significantly increased upon LPS treatment, and this increase was markedly suppressed in the presence of PPAR ligands, suggesting the involvement of PPARs in the inhibition of LPS-induced HMGB1 release (Figure 1(b)). Among the ligands for PPAR, rosiglitazone, a specific ligand for PPARγ, was superior to others in the inhibition of LPS-induced HMGB1 release. In contrast to that of secreted levels, neither LPS nor ligands of PPARs affected the expression levels of endogenous HMGB1 (Figure 1(c)). These results indicate that PPARs are involved in the regulation of LPS-induced HMGB1 release, but not in the regulation of HMGB1 expression. Under the concentrations of ligands used in these experiments, cells retained good viability within the experimental time frames used, as determined by trypan blue exclusion method (see Supplementary Figure 1 available online at doi:10.1155/2012/352807).

3.2. Rosiglitazone Inhibits Poly (I:C)-Induced HMGB1 Release in RAW 264.7 Cells. To examine whether rosiglitazone has a specific effect against LPS stimulation in the inhibition of HMGB1 release, RAW 264.7 cells were stimulated with Poly (I:C), a well-known ligand of Toll-like receptor (TLR) 3, in the presence or absence of rosiglitazone for 24 h. Rosiglitazone significantly inhibited Poly (I:C)-induced HMGB1 release, but not affect expression levels of HMGB1 (Figure 2), indicating that effect of rosiglitazone on the inhibition of HMGB1 release is not limited to the LPS.

3.3. Rosiglitazone Also Attenuates LPS-Induced Release of HMGB1 in RAW 264.7 Cells When Administered Following LPS Treatment. Because administering rosiglitazone to cells prior to treatment with LPS was effective in the inhibition of LPS-induced release of HMGB1, the effect of rosiglitazone when supplied at time points following LPS treatment was

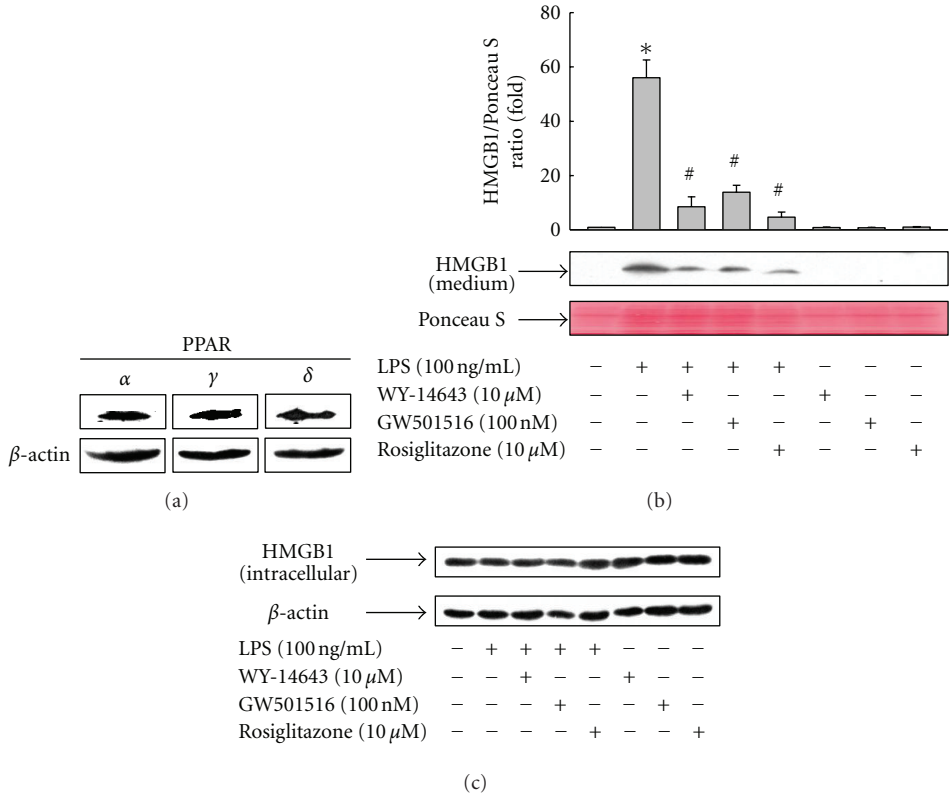

Figure 1: Activation of PPARs by ligand inhibits LPS-induced release of HMGB1 in RAW 264.7 cells. (a) Cells were harvested and the expression levels of PPARs were detected by using Western blot with indicated antibodies, as described in Section 2. (b) Cells grown to 60% confluency were incubated with serum-free medium for 24 h and then stimulated with LPS in the presence or absence of ligands for 24 h. Equal volumes of conditioned media were subjected to Western blot analysis. Ponceau S staining was used as a loading control. (c) At the same time, total protein was extracted, fractionated by electrophoresis, and immunoblotted with the indicated antibodies. Representative blots and densitometric measurements from three independent experiments are shown. The results are expressed as the means ± S.E. ($n = 3$). $^{*}P < 0.01$ compared to untreated group; $^{\#}P < 0.01$ compared to LPS-treated group.

examined. When cells were treated with LPS, an increase in the level of released HMGB1 was detected at 24 h, and this increase was markedly reduced by supplying rosiglitazone to cells following LPS treatment. This effect was observed in cells when rosiglitazone was administered up to 6 h after LPS treatment and also, to a lesser extent, in cells receiving rosiglitazone up to 18 h after LPS treatment (Figure 3), suggesting that rosiglitazone could be useful in treatment, as well as in the prevention of HMGB1 release.

Since PPARγ was reported to mediate inflammatory responses by inhibiting proinflammatory cytokines [19, 20], the effects of rosiglitazone on the secretion of inflammatory cytokines such as TNF-α, MCP-1, and MIP-1β were examined. A marked increase in the levels of MCP-1, MIP-1β, and TNF-α was observed in RAW 264.7 cells treated with LPS for 24 h, whereas simultaneous administration of rosiglitazone clearly reduced the effect of LPS on the level of MCP-1 and MIP-1β, but not on the level of TNF-α (see Supplementary Figure 2). Finally, the effect of rosiglitazone on the expression of inducible nitric oxide synthase (iNOS) was examined. The LPS-induced upregulation of iNOS expression was markedly attenuated in the presence of rosiglitazone, corroborating the effects of rosiglitazone observed in the regulation of inflammation induced by LPS (see Supplementary Figure 3).

3.4. Rosiglitazone-Mediated Inhibition of HMGB1 Release Is Dependent on PPARγ in RAW 264.7 Cells Treated with LPS. To examine the role of PPARγ in rosiglitazone-mediated inhibition of HMGB1 release induced by LPS, RAW 264.7 cells were treated with siRNA against PPARγ or GW9662, an irreversible inhibitor of PPARγ [21]. In LPS-treated RAW 264.7 cells, the addition of PPARγ siRNA or GW9662 almost completely abolished the rosiglitazone-mediated inhibition of HMGB1 release (Figures 4(a) and 4(b)). To further ascertain the effect of endogenous PPARγ on LPS-induced HMGB1 release, knockdown or overexpression of PPARγ using a specific shRNA or vector-host systems, respectively, was carried out. RAW 264.7 cells stably expressing PPARγ shRNA were established and shown to exhibit a reduced level of PPARγ expression, whereas PPARγ expression in cells transfected with a vector expressing scrambled shRNA was unaffected (Figure 4(c)). This PPARγ-shRNA-mediated downregulation of PPARγ counteracted the inhibitory effects of rosiglitazone on the HMGB1 release induced by LPS (Figure 4(d)). Furthermore, overexpression of PPARγ had

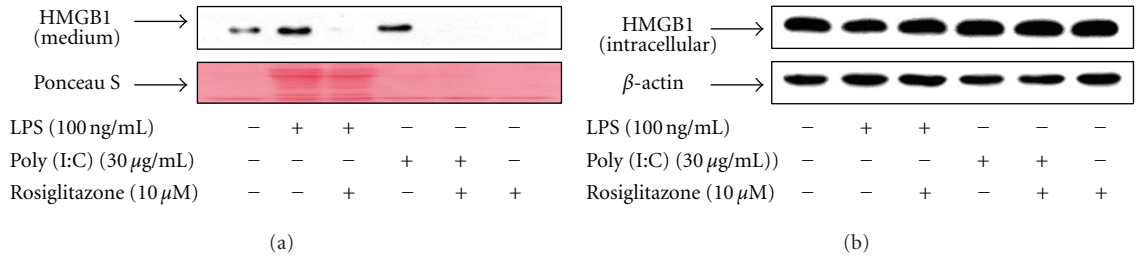

Figure 2: Rosiglitazone inhibits Poly (I:C)-induced HMGB1 release in RAW 264.7 cells. (a) Cells grown to 60% confluency were incubated with serum-free medium for 24 h and then stimulated with LPS and/or Poly (I:C) in the presence or absence of rosiglitazone for 24 h. Equal volumes of conditioned media were subjected to Western blot analysis for the detection of HMGB1. Ponceau S staining was used as a loading control. (b) At the same time, total protein was extracted, fractioned by electrophoresis, and immunoblotted with the indicated antibodies. The results shown are representative of three independent experiments.

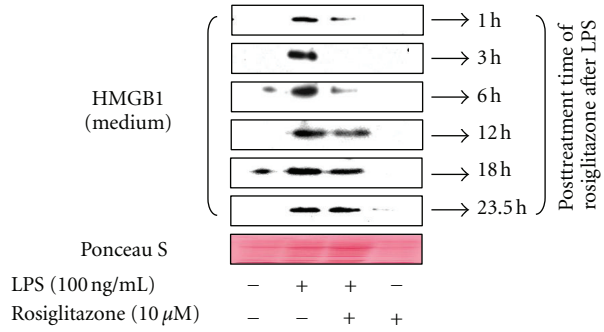

Figure 3: Administration of rosiglitazone post-LPS treatment also attenuates LPS-induced release of HMGB1 in RAW 264.7 cells. Cells were grown to 60% confluency, incubated with serum-free medium for 24 h, and then treated with LPS. Rosiglitazone was administered at the indicated time points post-LPS treatment for 24 h. Conditioned medium was subjected to Western blot analysis for the determination of HMGB1 levels. Ponceau S staining was used as a loading control. The results shown are representative of three independent experiments.

more pronounced effects in HMGB1 release (Figures 4(e) and 4(f)). These data clearly indicate that PPARγ directly regulates LPS-induced HMGB1 release in RAW 264.7 cells.

3.5. Rosiglitazone Inhibits TLR4 Signal Pathway Stimulated by LPS. Since TLR4 is involved in the regulation of LPS-induced HMGB1 release [22, 23], we examined whether rosiglitazone affects TLR4 signal pathway in LPS-treated RAW 264.7 cells. Expression of MyD88 and TRIF, key adaptor molecules of TLR4, was increased in RAW264.7 cells treated with LPS for 6 h, whereas simultaneous administration of rosiglitazone significantly reduced the levels of MyD88 and TRIF (Figures 5(a) and 5(b)). In addition, rosiglitazone also significantly inhibited the LPS-induced phosphorylation of IκBα (Figure 5(c)), indicating that the activation of PPARγ by rosiglitazone modulates TLR4 signal pathway by inhibiting the LPS-induced expression of MyD88/TRIF, and consequent blocking its effector NF-κB.

3.6. Rosiglitazone Attenuates Endotoxin-Induced Lethality through PPARγ-Mediated Inhibition of HMGB1 Release. To further investigate the *in vivo* relevance of these *in vitro* results, an initial evaluation of rosiglitazone as a therapeutic agent was performed using a standard model of murine endotoxemia. Injection of LPS dramatically increased the mortality of mice, whereas the administration of rosiglitazone prior to LPS treatment significantly improved the survival rates (Figure 6(a)). This effect of rosiglitazone was significantly reduced in the presence of GW9662, indicating the involvement of PPARγ in the rosiglitazone-mediated improvement of survival rates. Late deaths in rosiglitazone and/or GW9662-administered animals were not observed during the 2 weeks following endotoxin injection (data not shown), indicating that administration of rosiglitazone conferred protection to mice against lethal endotoxemia. Furthermore, the posttreatment of rosiglitazone after LPS also improved the survival rates until after 3 h (Figure 6(b)), demonstrating that rosiglitazone has an extended therapeutic window. Because endotoxin lethality is corelated to HMGB1 release [3, 4, 6–8], the effects of rosiglitazone on the level of circulating HMGB1 in blood were determined. HMGB1 blood levels significantly increased by LPS injection, whereas the administration of rosiglitazone almost completely abolished the release of HMGB1 into the blood, which was reversed by the presence of GW9662 (Figure 6(c)). These results indicated that rosiglitazone prevents endotoxin lethality *in vivo* by blocking HMGB1 release.

4. Discussion

In the present study, ligand-activated PPARs were shown to inhibit LPS-induced release of HMGB1, indicating a role for

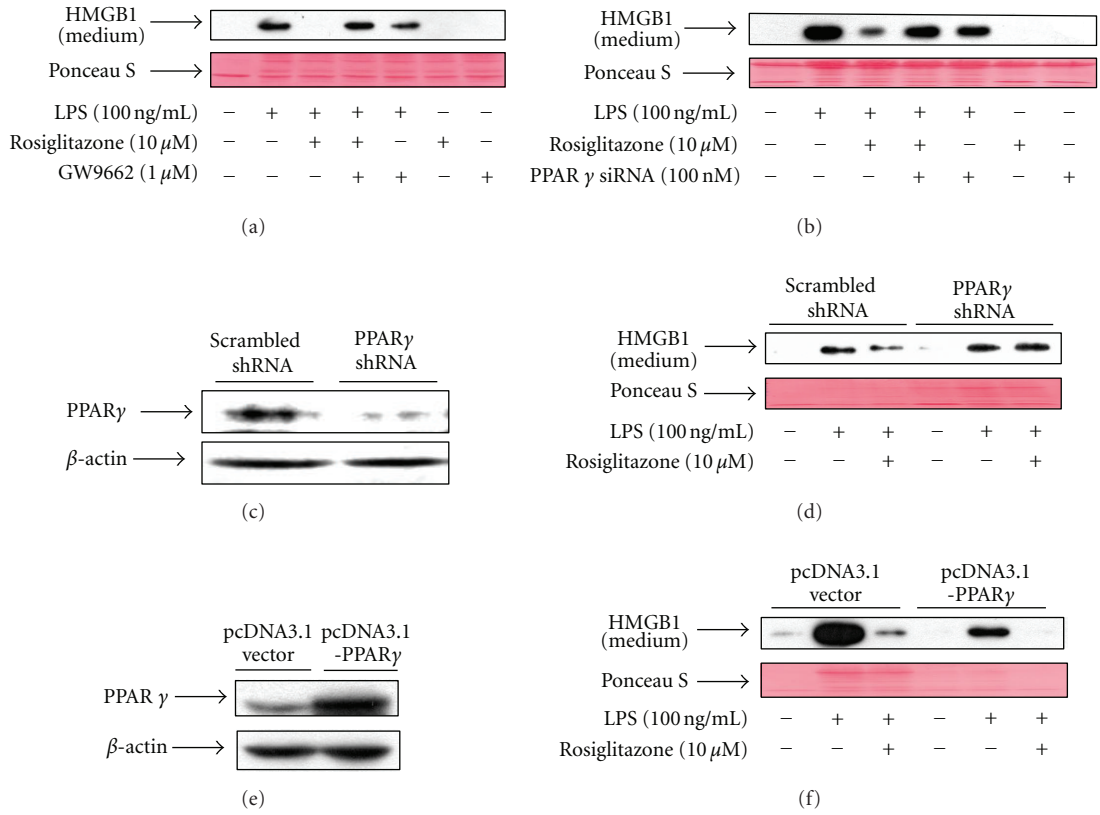

Figure 4: PPARγ regulates LPS-induced release of HMGB1 in RAW 264.7 cells. (a) Cells pretreated for 1 h with GW9662 were stimulated with LPS in the presence or absence of rosiglitazone for 24 h. Conditioned medium was collected and subjected to Western blot analysis for determination of HMGB1 levels. (b) Cells transfected with PPARγ siRNA for 38 h were incubated with serum-free medium for 24 h, and then treated with LPS in the presence or absence of rosiglitazone for 24 h. Equal volumes of conditioned media were subjected to Western blot analysis. (c) Cells were transfected with a vector encoding one hairpin siRNA against PPARγ or encoding a scrambled shRNA control. Stable transfectants were selected with 100 μg/mL hygromycin, and the expression levels of PPARγ were determined by Western blot analysis. (d) Cells expressing PPARγ shRNA or scrambled control shRNA were treated with LPS in the presence or absence of rosiglitazone for 24 h. Conditioned medium was subjected to Western blot analysis for the determination of HMGB1 levels. (e) Cells transfected with pcDNA3.1-PPARγ, or pcDNA3.1 vector for 48 h were harvested and subjected to Western blot analysis with indicated antibodies. (f) Cells transfected with pcDNA 3.1 or pcDNA3.1-PPARγ for 48 h were incubated with serum-free medium for 24 h and then stimulated with LPS in the presence or absence of rosiglitazone for 24 h. Equal volumes of conditioned media were subjected to Western blot analysis for the detection of HMGB1. Ponceau S staining was used as a loading control. The results shown are representative of three independent experiments.

PPARs as regulatory molecules of HMGB1 release. Rosiglitazone, a specific ligand for PPARγ, is superior to other PPAR ligands in the inhibition of HMGB1 release induced by the presence of LPS. This is the first report demonstrating that ligand-activated PPARs inhibit LPS-stimulated HMGB1 release in RAW 264.7 cells. A recent report demonstrated that telmisartan, a non-selective ligand for PPARγ, protected against postischemic injury by partially inhibiting the inflammatory reaction via a PPARγ-dependent HMGB1-inhibiting mechanism [24]. On the other hand, a different line of investigation showed that PPARγ agonist troglitazone inhibited HMGB1 expression in endothelial cells [25]. This is in contrast to the present findings that endogenous HMGB1 expression was unaffected by ligand activation of PPARs, whereas ligand activation of PPARs caused HMGB1 release in RAW 264.7 cells treated with LPS. No conclusive data are available at present although the induction of HMGB1 expression by PPAR ligands was not observed even at ligand concentrations as high as 100 μM (data not shown). However, the possibility remains that this discrepancy could be due to the different ligands or different cell types investigated in these reports. Further studies are, therefore, necessary to clarify the role of PPARγ in the regulation of HMGB1 induced by LPS.

Of particular interest is the possibility that ligand activation of PPARγ may participate in the pathophysiology of sepsis. The ligand-activated PPARγ caused a marked attenuation in the LPS or Poly (I:C)-induced release of HMGB1. Moreover, the administration of rosiglitazone either pre- or post-LPS treatment inhibited LPS-induced release of HMGB1, indicating that HMGB1 regulation by rosiglitazone is effective in both the treatment and prevention of HMGB1 release. Furthermore, the administration of PPARγ ligand decreased the endotoxin-induced lethality of LPS in mice and reduced levels of circulating HMGB1, indicating that the effects of PPARγ on septic shock are HMGB1 dependent.

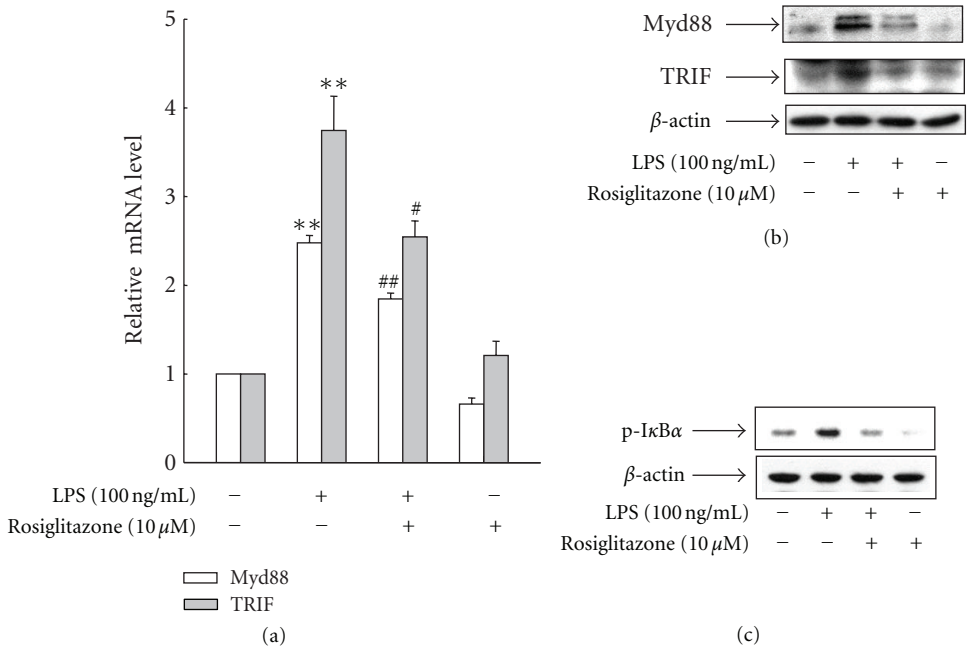

Figure 5: Rosiglitazone inhibits LPS-induced TLR4 signal pathway in RAW 264.7 cells. (a) Cells incubated with serum-free medium for 24 h were treated with LPS in the presence or absence of rosiglitazone for 6 h. The mRNA levels of indicated genes were determined by real-time PCR using SYBR Green. GAPDH was used as an internal standard. (b) Cells were incubated in serum-free medium for 24 h and then stimulated with LPS in the presence or absence of rosiglitazone for 9 h. Total protein was extracted, fractionated by electrophoresis, and immunoblotted with the indicated antibodies. (c) Cells incubated with serum-free medium for 24 h were treated with LPS in the presence or absence of rosiglitazone for 1 h. An aliquot of protein was immunoblotted with phospho-IκBα (p-IκBα) and β-actin antibodies. The results are expressed as the means ± S.E. ($n = 3$). $^{**}P < 0.01$ compared to untreated group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared to LPS-treated group.

This finding is in line with previous studies in which high levels of HMGB1 were demonstrated in patients with severe sepsis and in animals models of endotoxemia [3, 4, 26, 27], suggesting that HMGB1 may play a crucial role in the process of sepsis. PPARγ has also been reported to mediate inflammatory responses by inhibiting proinflammatory cytokines, such as TNF-α, interleukin (IL)-6, and iNOS [19, 20, 28]. However, little is known about the regulatory role of PPARγ in HMGB1 release, a late phase mediator of inflammatory responses *in vitro* and *in vivo*. Accordingly, these results suggested that, under pathological conditions, PPARγ may play a major role as an anti-inflammatory molecule through the inhibition of early and late phase mediators.

The mechanism by which PPARγ controls LPS-induced HMGB1 release remains unclear. As a transcription factor, PPARγ primarily regulates gene expression through its binding with its heterodimeric partner RXR to a specific recognition site, termed the peroxisome proliferator response element (PPRE), in the promoter region of a target gene [29]. However, the consensus PPRE motif was not identified in either the rat or human promoter regions of HMGB1 [25]. Thus, the regulatory mechanism of PPARγ for HMGB1 release may be a result of a secondary effect causing modifications of HMGB1 related to translocation, such as acetylation or phosphorylation [30, 31]. Posttranslational modification of HMGB1 appears to be critical for HMGB1 release [30, 31]. In fact, it has been reported that HMGB1 is extensively modified, hyperacetylated in LPS-activated monocytes, and released to participate in the inflammatory response [31, 32]. Although our present data showed that TLR4 signal pathway was affected by PPARγ activation, extrapolation of our present data awaits further study to reveal the PPARγ-mediated regulatory mechanisms underlying the release of HMGB1 induced by LPS.

In summary, data presented here demonstrate that PPARγ changes the cellular responses of cells to bacterial endotoxin *in vitro* and *in vivo*, hence supporting the hypothesis that PPARγ is involved in inflammatory processes by attenuating mediators released during exposure to endotoxin. Accordingly, this study provides new insights into the pleiotropic roles of PPARγ via the regulation of HMGB1 release and may lead to a better understanding of the clinical efficacy of rosiglitazone in the treatment of inflammation-related disorders.

Abbreviations

HMGB1: High mobility group box 1
iNOS: Inducible nitric oxide synthase
LPS: Lipopolysaccharides
MIP-1β: Macrophage inflammatory protein-1β
MCP-1: Monocyte chemoattractant protein-1
MyD88: Myeloid differentiation primary responses gene 88
Poly (I:C): Polyinosinic-polycytidylic acid
PPAR: Peroxisome proliferator-activated receptor

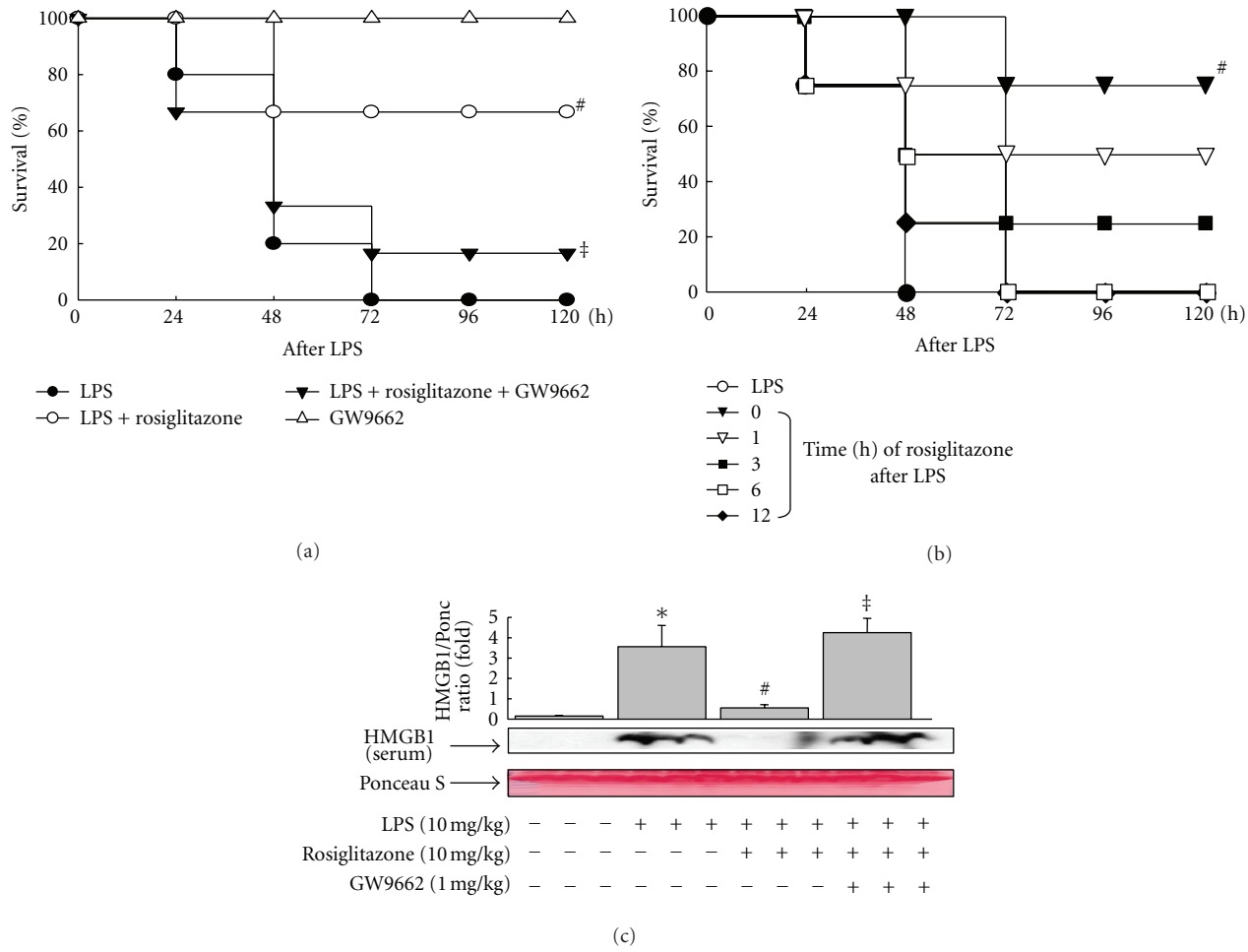

Figure 6: Rosiglitazone prevents endotoxin lethality by attenuating HMGB1 release *in vivo*. (a) BALB/c mice (n = 10-11 per group) were injected with a single dose of rosiglitazone (10 mg/kg, i.p.) with or without GW9662 (1 mg/kg, i.p.), followed 30 min later by a lethal infusion of endotoxin (LPS, 10 mg/kg, i.p.). (b) BALB/c mice (n = 4 per group) infused with endotoxin (LPS, 10 mg/kg, i.p.) were treated with rosiglitazone (10 mg/kg, i.p.) 0, 1, 3, 6, and 12 h later. Survival was monitored daily for up to 2 weeks. (c) In a parallel group of rosiglitazone-administered mice, circulating levels of HMGB1 were detected by Western blot analysis using sera prepared from samples collected at 20 h post-LPS injection. Representative blots from four independent experiments and densitometric measurements are shown. Ponceau S staining was used as a loading control. The results are expressed as the means ± S.E. (n = 3). $^{*}P < 0.01$ compared to the untreated group; $^{\#}P < 0.01$ compared to the LPS-treated group; $^{\ddagger}P < 0.05$ compared to the LPS plus rosiglitazone-treated group.

PPRE: Peroxisome proliferator response element
RXR: Retinoid X receptor
shRNA: Small hairpin RNA
siRNA: Small interfering RNA
TLR: Toll-like receptor
TNF-α: Tumor necrosis factor-α
TRIF: TIR-domain-containing adaptor-inducing interferon-β.

Authors' Contribution

J. S. Hwang and E. S. Kang contributed equally to this work.

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Review Article

Macrophages, Inflammation, and Tumor Suppressors: ARF, a New Player in the Game

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The interaction between tumor progression and innate immune system has been well established in the last years. Indeed, several lines of clinical evidence indicate that immune cells such as tumor-associated macrophages (TAMs) interact with tumor cells, favoring growth, angiogenesis, and metastasis of a variety of cancers. In most tumors, TAMs show properties of an alternative polarization phenotype (M2) characterized by the expression of a series of chemokines, cytokines, and proteases that promote immunosuppression, tumor proliferation, and spreading of the cancer cells. Tumor suppressor genes have been traditionally linked to the regulation of cancer progression; however, a growing body of evidence indicates that these genes also play essential roles in the regulation of innate immunity pathways through molecular mechanisms that are still poorly understood. In this paper, we provide an overview of the immunobiology of TAMs as well as what is known about tumor suppressors in the context of immune responses. Recent advances regarding the role of the tumor suppressor ARF as a regulator of inflammation and macrophage polarization are also reviewed.

1. Introduction

Immune system constitutes one of the first-line defenses to prevent tumor development due to its ability to identify and destroy tumor cells. This process defined as cancer immunosurveillance was initially described by Ehrlich and subsequently revisited by Thomas and Burnet [1–3], gaining considerable attention in last years. Compelling evidence that immune system modulates cancer has emerged over the last decade from gene-targeted mice studies. Mice deficient in several immune effector cells and molecules including interferon (IFN)-γ receptor or signal transducer and activator of transcription 1 (STAT1) [4], natural killer (NK) cells, NK-T cells [5, 6], $\gamma\delta$ T cells [7, 8], IL-12 [9], perforin [10], and granulocyte-macrophage colony stimulating factor (GM-CSF) [11] have been demonstrated to be more susceptible to tumor development. Collectively, these studies strongly support the concept that the immune response is essential in the development of tumors.

2. Macrophages: Key Immune Cells

Macrophages are one of the major widely distributed innate immune cells and present essential roles in the primary response to pathogens, maintenance of tissue homeostasis, inflammation, and immunity.

Macrophages are derived from bone marrow progenitors as immature monocytes. After circulating in the blood stream, monocytes migrate into tissues where they differentiate into resident macrophages [12].

Macrophages are dynamic cells that might modify their functional profiles in response to a variety of stimuli polarizing to functionally different phenotypes. Two distinct subsets

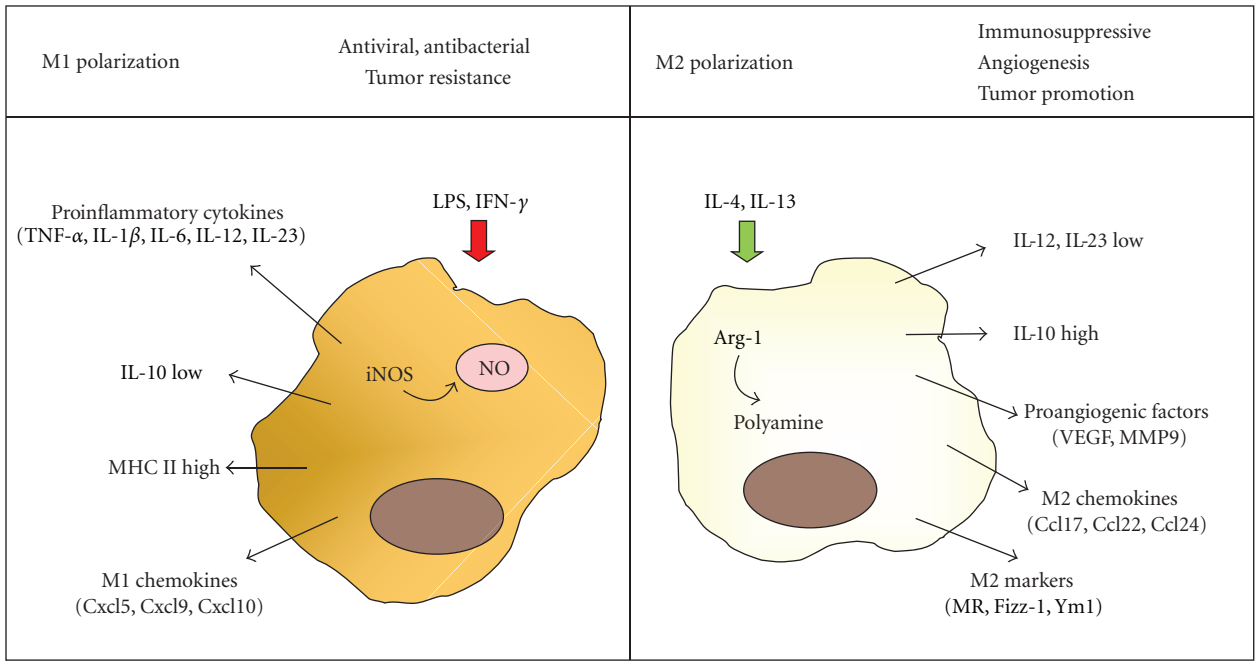

Figure 1: Simplified scheme for M1 and M2 activation of macrophages. M1 macrophages are induced by LPS or IFN-γ secreting high levels of classical proinflammatory cytokines such as TNF-α, IL-1, IL-6, IL-12, or IL-23, chemokines (e.g., Cxcl9, Cxcl10, and Cxcl5) and increasing their concentrations of NO. In addition, they express high levels of MHC I. IL-4/IL-13 stimulation induces M2 macrophages that downregulate IL-12 and IL-23 expression, release Ccl17, Ccl22 and Ccl24 chemokines and proangiogenic factors, and show increased expression of IL-10. Additionally, they are characterized by expression of MR, Fizz-1, and Ym1.

of macrophages have been proposed, including classically activated (M1) and alternatively activated (M2) macrophages [13] (Figure 1). M1 macrophages are induced by IFN-γ either alone or cooperating with microbial stimuli such as lipopolysaccharide (LPS) or cytokines (e.g., tumor necrosis factor (TNF)-α and GM-CSF). These cells secrete high levels of classical proinflammatory cytokines such as TNF-α, interleukin (IL)-1, IL-6, IL-12, or IL-23 and increase their concentrations of nitric oxide (NO), superoxide anions, and oxygen radicals [14, 15]. Furthermore, M1 macrophages can express high levels of major histocompatibility complex (MHC) I and class II antigens and secrete complement factors that facilitate complement-mediated phagocytosis [16].

In contrast, IL-4/IL-13 stimulation induces M2 macrophages that reduce IL-12 and IL-23 expression while upregulate the anti-inflammatory cytokine IL-10 [17, 18]. Additionally, they are characterized by the expression of the scavenger receptors mannose receptor (MR) [13], stabilin-1 [19], CD163 [20], and some genes involved in tissue remodelling such as Found in Inflammatory Zone 1 (Fizz-1) and chitinase 3-like 3 (Ym1) [21].

An alternative metabolic pathway of L-arginine, catalyzed by arginase-1 (Arg-1) provides another feature of distinction among the two macrophage activation states. M1 macrophages upregulate iNOS to catabolize L-arginine to NO and citrulline, but M2 macrophages are characterized by high expression of Arg-1, a cytosolic enzyme which metabolizes L-arginine to ornithine and polyamines, which are precursors for collagen synthesis and cellular proliferation [22].

M1 and M2 macrophages also express a different chemokine repertoire. M1 macrophages produce proinflammatory chemokines such as (C-X-C motif) ligand 9 (Cxcl9), Cxcl10, and Cxcl5 [16], whereas M2 polarization is accompanied by production of Ccl17, Ccl22, and Ccl24 [16].

This plethora of molecules and genes leads macrophages to display distinct functions in virtue to their polarization state. Thus, classically activated macrophages are vital components in the initiation and maintenance of inflammation, as well as in host defense and priming antitumor immune response [13]. For instance, the IL-12 produced by M1 macrophages promotes the differentiation of naive T cells into Th1 cells, stimulates growth of both T and NK cells, and increases bactericidal activity of phagocytes. Moreover, IL-12 exerts an antiangiogenic activity through the increment of the chemokine inducible protein-10 (IP-10 or Cxcl10) [23]. Additionally, M1 chemokines such as Cxcl9, Cxcl10, and Cxcl5 induce the recruitment of Th1 cells, Tc1 cells, and NK cells [16].

Opposite, M2 macrophages have poor antigen-presenting capability, produce factors that suppress T-cell proliferation and activity, and mainly participate in parasite clearance, tissue remodeling, immune modulation, and tumor progression [15]. Thus, IL-10 expressed by M2 macrophages promotes the production of IL-4 and IL-13 by Th2 cells [24], inhibits the synthesis of proinflammatory cytokines such as IFN-γ, IL-2, IL-3, TNF-α and GM-CSF, and also suppresses the antigen-presentation capacity of antigen presenting cells. Furthermore, Ccl17, Ccl22, and Ccl24 production favors

the attraction of immune-inhibitory cells such as regulatory T-cells (Treg) [25].

3. Macrophages and Tumor Microenvironment

Compelling evidence has emerged in recent years for macrophages playing an important function in tumor development. Although the role of macrophages in tumors is still controversial, in most human cancers such as breast, prostate, ovarian, cervical, lung carcinoma, and cutaneous melanoma, a macrophage-rich microenvironment has been correlated with a poor prognosis [26, 27]. These tumor-associated macrophages (TAMs) share many common features with the alternatively activated macrophages, showing a typical M2 marker profile with high expression of C-type lectin receptors, stabilin-1, and Arg-1 [25].

Among the cell surface molecules expressed by TAMs, several members of structurally related C-type lectin receptors such as MR and Macrophage galactose-type C-type lectin 1/2 (Mgl-1/2) are included [28]. The MR is an endocytic and phagocytic receptor that was initially described as a bridge between innate immunity and homeostasis [29] due to its ability to bind carbohydrate moieties on several pathogens such as bacteria, fungi, parasites, and viruses. Mgl-1/2 are induced on macrophages by parasitic infections or allergic asthma [28]. In the tumor microenvironment, MR and Mgl-1/2 have been documented to act as recognition molecules for glycosylated antigens on cancer cells. MR and Mgl-1/2 recognize specifically highly glycosylated molecules such as mucins present in the tumor microenvironment [30], leading to the expression of the immunoregulatory cytokine IL-10 that favors the attraction of Treg cells [25]. Indeed, Mgl-1/2 is predominantly expressed by TAMs from human ovarian carcinoma [31], on lung metastasis produced by mouse metastatic ovarian tumor cells [32], and also they have been detected after challenging tumor conditioned medium [33].

Chitinase 3-like 3 (Ym1) is a member of the mammalian chitinase family that also includes Ym2 and BRP-39 in mice, and YKL-40 in humans [34]. Increased expression of these proteins has been associated with inflammatory diseases, in particular with allergic asthma, with the induction of alternative activation of macrophages, and with progression of cancer [35–39].

Fizz-1, also known as resistin-like molecule α (RELM-α), plays a key role in the regulation of cell growth/proliferation and differentiation. Initially described in lung allergic inflammation [40], it is highly expressed in bleomycin-induced lung fibrosis and after IL-4 or IL-13 activation [41, 42]. Fizz-1 exhibits multiple functions including cell proliferation, angiogenesis, and inflammation [43], and its expression is an indicator of M2 polarization [21, 39].

Stabilin-1, a type-1 transmembrane receptor that mediates clearance of "unwanted self" components, is another marker for M2 macrophages that has been found to be expressed by TAM in several murine tumor models (e.g., B16 melanoma, pancreatic insulinoma, breast carcinoma) [19, 44]. Stabilin-1 mediates internalization of extracellular secreted protein acidic and rich in cysteine (SPARC), regulating its concentration and thereby promoting extracellular matrix remodeling, angiogenesis, and tumor progression [45].

4. Role of TAMs in Tumors

TAMs derive from circulating monocytic precursors previously recruited to the tumor region in response to the chemokines and cytokines secreted by cancer cells. In the tumor mass, TAMs exert immunosuppressive functions through the release of anti-inflammatory cytokines, modulate the tumor microenvironment producing survival/growth factors (e.g., vascular endothelial growth factor, VEGF), and facilitate the progression of tumors via proangiogenic factors release [25, 26].

4.1. TAMs and Immunosuppression. A relevant function of TAMs is to diminish the effective antitumor immune response. Several cytokines and proteases derived from TAMs, such as transforming growth factor (TGF)-β, IL-10 and Arg-1, make a significant contribution to the immunosuppressive condition [46–48].

TGF-β inhibits the antitumor response through different mechanisms including (i) inhibition of the cytolytic activity of NK cells [48, 49], (ii) differentiation of $CD4^+$ T cells into Th2 cells [50], (iii) inhibition of the $CD8^+$ T cells antitumoral activity [48], and (iv) maintenance of Treg cell differentiation [48].

IL-10 promotes the immune evasion impeding the production of IL-12, a cytokine known to stimulate both the proliferation and cytotoxicity of T and NK cells [51], as well as the release of the cytokine IFN-γ, which is the main factor that stimulates naive T-cell differentiation [52]. Additionally, it has been reported that IL-10 decreases the ability of epidermal antigen presenting cells (APCs) to present tumor-associated antigens, therefore interfering the induction of antitumor immune responses [51].

High Arg-1 activity has been described in TAMs from 3LL murine lung carcinoma [22], human papillomavirus E6/E7-expressing murine tumors [53], and CD11b+/CD14− myeloid cells from renal carcinoma patients [54]. Elevated Arg-1 expression might promote tumor growth via several mechanisms including downregulation of NO-mediated tumor cytotoxicity [55], increasing cellular proliferation through its participation in polyamine and proline synthesis, dysregulating the T cell receptor (TCR) signaling and subsequently inducing $CD8^+$ T cell unresponsiveness [47] and enhancing the capacity of myeloid suppressor cells to inhibit T cell proliferation [22].

Finally, TAMs release chemokines that play fundamental roles in immunosuppression. Ccl13, Ccl18 (human only), Ccl22, and to a lesser extent Ccl17 are important chemoattractant for immune-inhibitory cells, such as Treg, which might inhibit antitumor immunity resulting in tumor growth and decreased patient survival [16]. Moreover, Ccl2 and Ccl5 suppress the T-cell responses [16].

4.2. TAMs and Angiogenesis. Accumulating evidence indicates that TAMs exert a critical function in regulating angiogenesis, the process by which new blood vessels sprout from the existing vasculature. TAMs depletion studies in mice showed reduction of blood vessel density in the tumor tissue [56] and numerous correlations between increased TAM numbers and high vascular grades have been reported for many tumor types [57–62].

TAMs support tumor cell invasion by secreting a broad repertoire of molecules, including growth factors, cytokines, proteases, and chemokines. For instance, TAMs release potent proangiogenic cytokines such as IL-8 (or Cxcl8) and growth factors as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and TGF-β, which have been reported to promote angiogenesis in tumors such as gliomas, squamous cell carcinomas of the esophagus, and breast, bladder, and prostate carcinomas [59–63]. Importantly, they also release chemokines like Ccl2, Ccl5, Cxcl9, and Cxcl16 that contribute to angiogenesis [64]. Moreover, TAM-derived proteases, such as matrix metalloproteases (MMP-1, MMP-2, MMP-3, MMP-9, and MMP-12) are also beneficial to angiogenesis [65–67].

Additionally, TAMs have been found to accumulate in hypoxic regions of human and experimental tumors (including human endometrial, breast, prostate, and ovarian carcinomas) [68]. TAMs respond to the hypoxic microenvironment by upregulating the hypoxia-inducible transcription factors HIF-1 and HIF-2 that induce the expression of proangiogenic genes, such as VEGF, Cxcl8, and Cxcl12.

4.3. TAMs and Tumor Growth. Macrophage depletion studies have proven that TAMs are essential for tumor growth [69] and TAMs infiltration has been observed in several tumors such as breast cancer, endometrial cancer, and renal cell cancer [70], demonstrating a positive correlation between proliferation of tumor cells and TAMs infiltration. Several molecules/factors secreted by TAMs such as MMP-9, IL-23, IL-10 facilitate tumor cell proliferation thereby limiting the cytotoxicity of the microenvironment.

4.4. TAMs and Metastasis. Another process in which TAMs have been involved is in the regulation of metastasis. Indeed, a correspondence between the number of macrophages in metastatic sites and the metastatic potential of the tumor has been observed [71], and systemic depletion of macrophages results in reduced formation of lung metastases [72]. These findings are in line with clinical studies showing that increased numbers of macrophages in regional lymph node metastases correlates with poor patient survival [73].

TAMs appear to influence the microenvironment to facilitate migration of tumor cells [74, 75] by the release of MMPs, for example, MMP-2, MMP-7, and MMP-9. Those MMPs contribute to transform the proteins of the extracellular matrix and induce the expression of lymphatic endothelial growth factor (VEGF-C), events that promote dissemination of tumor cells by stimulating the formation of lymphatic vessels in tumors [76].

5. Tumor Suppressors and Immune System

Tumor suppressor genes act as sensors of multiple forms of cellular stress, being regulated to induce cell-cycle arrest, senescence, or apoptosis. Nevertheless, in last decades, a growing body of evidence indicates that tumor suppressors play a key role in the modulation of innate immune system, a function more relevant even than their activity as cancer inhibitors.

For instance, innate immune response in the metazoan *Caenorhabditis elegans* has been described to be dependent on p53 function [77]. A clear function in antiviral defense has been reported for the tumor suppressors p53, the promyelocytic leukemia (PML) protein, and the alternative reading frame (ARF) [78–82]. Activation of PML, p53, and ARF has been described after IFN treatment, expression of viral proteins and viral infection [79, 82–87].

In addition, a molecular link between p53, ARF, retinoblastoma protein (Rb), and Toll-like receptors (TLRs) has been shown [87–89] and recent studies have demonstrated that ARF regulates inflammatory response [87].

5.1. The Retinoblastoma Protein Rb. Rb was identified as the protein responsible for the congenital tumor retinoblastoma [90] and plays pivotal roles in cell cycle control, differentiation, and inhibition of oncogenic transformation. Rb regulates cellular proliferation by directly binding to E2F transcription factors [91, 92], a family of transcription factors that regulates cellular proliferation, growth, and differentiation.

Furthermore, additional functions of Rb in the control of immune response have been described including a novel role in viral infection surveillance. Thus Rb is required for the activation of the NF-κB pathway in response to virus infection [93]. In addition, a recent report has shown that Rb positively regulates expression of TLR3, the sensing receptor for viral double-stranded RNA [89]. The mechanism involves modulation of the transcription factor E2F1, which directly binds to the proximal promoter of TLR3.

5.2. The Promyelocytic Leukemia (PML) Protein. The PML gene was originally identified in acute promyelocytic leukemia (APL), being implicated in numerous cellular functions including oncogenesis, DNA damage, senescence, apoptosis, and protein degradation. In addition, accumulating reports have also demonstrated the role of PML in host antiviral defense [78]. PML functions as the organizer of PML nuclear bodies (NBs) that contains some proteins recruited in a transient manner and two permanent NB-associated proteins, the IFN-stimulated gene product Speckled protein of 100 kDa (Sp100) and death-associated dead protein (Daxx) [94]. PML is induced by IFN leading to a marked increase in the expression of several PML isoforms (PMLI-PMLVII) and NBs. PML confers resistance to numerous virus including foamy virus (HFV), vesicular stomatitis virus (VSV), influenza virus, poliovirus, rabies virus, lymphocytic choriomeningitis virus (LCMV), and encephalomyocarditis virus [78, 95–99]. Interestingly, viruses inhibited by PML

have developed various strategies to counteract the antiviral defense mechanisms by altering PML expression and/or localization on nuclear bodies [100].

5.3. Tumor Suppressor p53. The tumor suppressor p53 also known as "the guardian of genome" is activated in response to several types of cellular stress, including DNA damage and oncogene expression. Under normal conditions, p53 is maintained at very low levels through regulation by murine double minute 2 (Mdm2) protein. Mdm2 inhibits p53 transactivation and prompts p53 for proteasomal degradation by promoting its ubiquitination [101, 102]. However, in response to cellular stress, such as DNA damage, heat shock, or hypoxia, p53 levels rise as a consequence of activation of the tumor suppressor ARF that binds to Mdm2 and inhibit the ubiquitination, nuclear export, and subsequent degradation of p53 [103].

p53 has been implicated in multiple functions that play key roles in health and disease, including ribosome biogenesis, control of aging, cell cycle arrest, and apoptosis, having a clear importance in tumor suppression [104]. Interestingly, several lines of evidence also indicate that p53 may have a broader function in antiviral defense. Activation of p53 by IFN has been reported [80, 81] and p53-deficient mice are more permissive to viral infection [82]. This p53-mediated protection against viral infection is related with an induction of apoptosis, which is associated with reduced viral replication [82]. Moreover, in addition to activation of p53 by IFN, several genes involved in innate immunity have been described to be p53 direct transcriptional targets. IFN regulatory factors (IRFs) such as IRF-9 and IRF-5 have been described to be modulated by p53 [80]. Several mechanisms have been proposed for regulation of IRF-9 by p53 including upregulation at the transcriptional level [80], transactivation in response to influenza virus infection [105], and direct p53-IRF9 protein interaction upon Hepatitis C virus (HCV) infection [106]. Regarding IRF-5, an increase in IRF-5 levels in cancer cell lines has been shown through p53 binding and transactivation of the IRF-5 promoter [107].

Pattern recognition receptors such as TLR3 have also been reported to be regulated by p53 [88]. TLR3 plays a major role in the recognition of virus infection leading to the induction of the IFN pathway [108]. p53 activates TLR3 transcription by binding to the p53 consensus site in the TLR3 promoter. Moreover, TLR3 expression was downregulated in liver and intestine of $p53^{-/-}$ mice and HCT116 $p53^{-/-}$ cells, leading to a dysfunction in both NF-κB and IRF-3 signaling pathways [88]. Upregulation of TLR3 activity by p53 may also be responsible for the activation of interferon-stimulated gene 15 (ISG15). ISG15 is strongly induced by type I interferons and displays antiviral activity. Although a functional p53 binding site adjacent to the core ISRE site of ISG15 has been reported, upregulation of ISG15 has been observed after dsRNA stimulation rather than in response to IFN treatment or virus infection, suggesting that the observed effects on ISG15 could be mediated through p53-dependent upregulation of TLR3 activity [109].

Finally, important proinflammatory chemokines such as monocyte-chemoattractant protein (MCP)-1 have also been reported to be transcriptionally regulated by p53 [110]. MCP-1, also known as Ccl2, triggers the infiltration and activation of cells of the monocyte-macrophage lineage and has been linked with antitumor immunity [111] and cervical cancer [112].

Thus, in addition to the well-established function of p53 as a tumor suppressor through regulation of apoptosis or cell cycle, p53 exerts essential roles in the expression of key molecules of the innate immune response. Indeed, the loss of p53 function during carcinogenesis might affect the recognition of tumor cells by the immune system through interfering with inflammatory mediators expression.

5.4. The Tumor Suppressor ARF. Tumor suppressor ARF (p14ARF in human, p19ARF in mouse) is among the most frequent genes mutated in human cancer [113]. ARF is encoded by the *INK4a/ARF* locus (Cdkn2a) that generates two unrelated proteins, the cyclin-dependent kinase inhibitor p16INK4a and ARF, which, respectively, regulate the activity of Rb and the p53 transcription factor [114, 115]. ARF activates p53 by sequestering Mdm2, an E3 ubiquitin ligase, to the nucleolus, thereby inhibiting the Mdm2-mediated proteasomal degradation of p53. p53 subsequently activates p21 (CIP1/WAF1), which inhibits the cell cycle [103, 116]. Although tumor suppressor activity of ARF was initially attributed to p53 regulation, several p53-independent actions for ARF have been described [117]. Thus, ARF inhibits ribosomal RNA processing [118] and transcriptional factors that induce proliferation such as E2F1, Myc, and Forkhead box M1 (Foxm1b) [119–121]. In addition, ARF interacts with the protein related with cell proliferation nucleophosmin (NPM) [122].

Recent studies have shown that the tumor suppressor ARF is more than a simple tumor suppressor and acts a general sensor for different situation of cellular stress. In this context, a regulatory network between Heat shock protein 70 (Hsp70), ARF, and β-catenin has been shown after oxidative stress that leads to the induction of apoptosis [123]. ARF deficiency has been reported to aggravate atherosclerosis through the reduction of macrophage and vascular smooth muscle cell apoptosis [124]. ARF is also expressed transiently during mouse male germ cell and eye development and its inactivation compromises spermatogenesis as mice age and leads to aberrant postnatal proliferation of cells in the vitreous of the eye, resulting in blindness [125].

Furthermore, ARF plays an important role in the regulation of innate immunity and inflammatory processes. Several reports have described an antiviral action of ARF as well as its activation after the expression of viral proteins, viral infection, or type I IFN treatment [79, 85–87]. Indeed, the analysis of the ARF promoter revealed the presence of IFN response elements such as IRF-3 and interferon-sensitive response element (ISRE). The protective effect of ARF against viral infection seems to be a general feature for IFN-sensitive viruses as demonstrated the studies using VSV and Sindbis virus or vaccinia virus (VV) [79]. Mechanism involved in this protection is due, at least in part, to interaction with NPM and activation of the double-stranded RNA-dependent protein kinase, PKR [79].

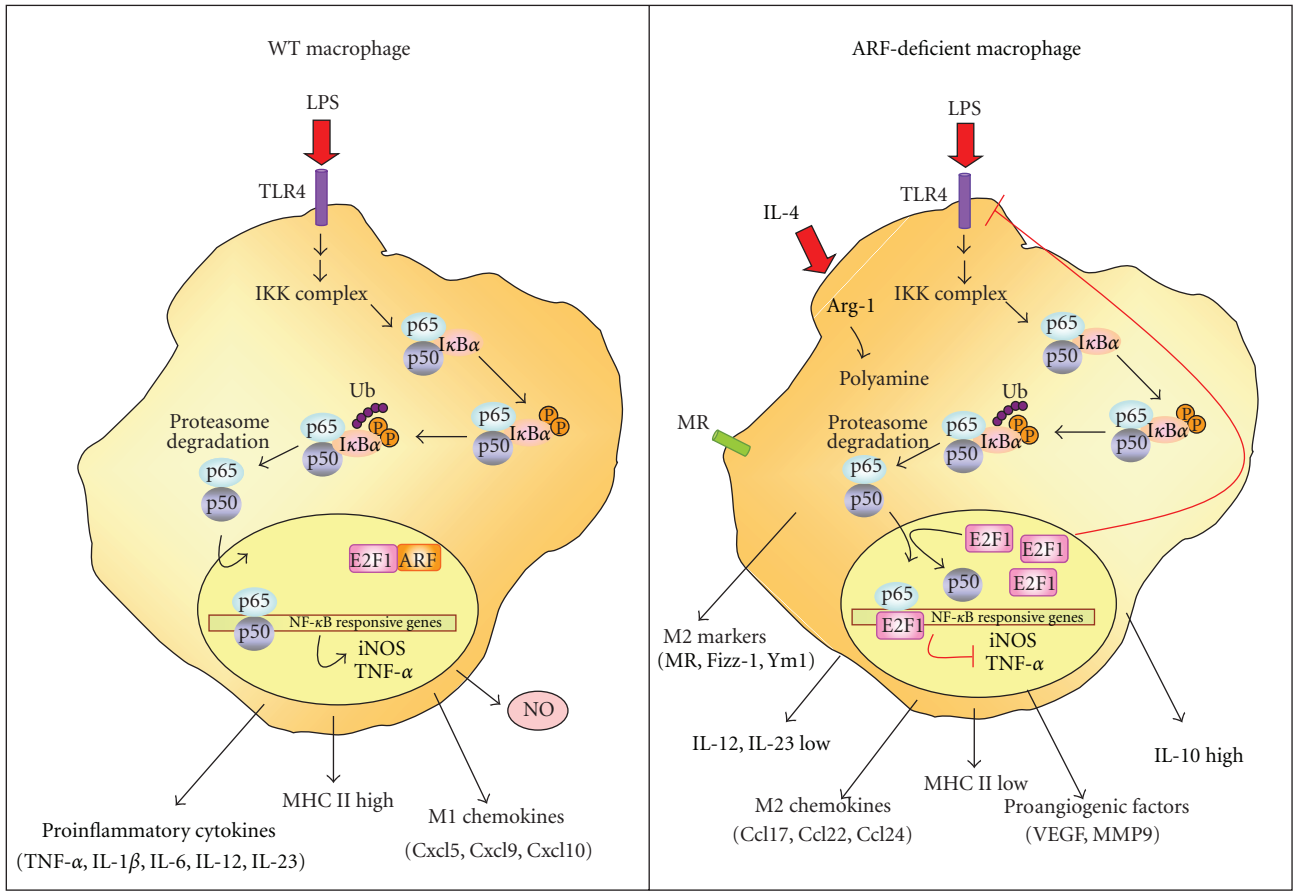

Figure 2: Alternative activation in ARF-deficient macrophages. In WT macrophages, a balance between M1 and M2 phenotype is established, depending on stimuli. Inflammatory stimuli induce NF-κB signaling pathways through the phosphorylation and subsequent ubiquitin-dependent degradation of IκBα by the 26S proteasome. Then, NF-κB translocates to the nucleus inducing target gene expression. ARF present in the nucleus displays physical and functional interaction with E2F1 resulting in destabilization of E2F1 protein and activation of NF-κB. However, ARF-deficient macrophages establish an immunosuppressive and tolerant microenvironment via impairment of M1 signals. When NF-κB translocates to the nucleus, excessive E2F1 inhibits NF-κB by binding to its subunit p65 in competition with the heterodimeric partner p50. Moreover, excessive E2F1 may inhibit transcriptional expression of TLRs. This leads to secretion of M2 chemokines Ccl17 and Ccl22, release of the anti-inflammatory cytokine IL-10, and stimulation of angiogenesis through expression of VEGF and MMP-9.

Interestingly, it has been recently described that ARF is a critical modulator of the inflammatory response and macrophage activation. It has also been reported a molecular link between ARF and TLRs [87]. Additionally, a manuscript by Herranz et al. [126] suggests that ARF might modulate the M1/M2 polarization and functional plasticity of macrophages (Figure 2).

Mice lacking the ARF gene are resistant to LPS-endotoxic shock, and a significant reduction of leukocyte recruitment in a model of thioglicollate-induced peritonitis was also reported [87]. Moreover, ARF-deficient macrophages present an impaired ability to develop proinflammatory properties showing a relevant downregulation of genes involved in M1 macrophage phenotype and inhibition of the antimicrobial and antitumoral responses, including expression of proinflammatory cytokines (TNF-α, IL-1β), chemokines (Cxcl10, Cxcl1, Ccl4), and inflammatory mediators (iNOS/PGE_2). Mechanisms involved in this inhibitory effect have not been fully explored, although a decrease in NF-κB and Mitogen-activated protein kinases (MAPK) activation have been described in ARF−/− macrophages after stimulation with LPS [87], as well as inhibition of IκB degradation in ARF-deficient macrophages stimulated with VSV [79]. Furthermore, an increase of the transcription factor E2F1 at basal state and after LPS-stimulation has been shown in absence of ARF [87]. E2F1 has been proposed to be an anti-inflammatory and immunosuppressive transcription factor since it represses NF-κB-dependent inflammatory signaling [127, 128]. Therefore, it has been proposed that in normal cells, ARF interacts with E2F1, resulting in destabilization of E2F1 protein and activation of NF-κB. In contrast, in the absence of ARF, E2F1 is overexpressed, and although NF-κB translocates to the nucleus, excessive E2F1 inhibits its activity by binding to p65 and thereby suppressing NF-κB-dependent genes (iNOS, COX-2, chemokines, etc.).

Loss of ARF gene can abrogate tumor surveillance mechanisms and increase cancer susceptibility. Indeed, mice lacking p19ARF are highly prone to tumor development

[129, 130] and deletion of ARF has been described in a variety of malignancies, including glioblastoma, melanoma, pancreatic adenocarcinoma, and non-small-cell lung cancer. Interestingly, M2-polarized TAMs have been demonstrated to be associated with poor prognosis and progression in many of these tumors.

However, the link between ARF and immune response in the tumoral context remains an open question. A manuscript by Herranz et al. [126] goes deep into the immune role of ARF, evaluating its possible contribution to the M1/M2 polarization of macrophages. In this study, authors demonstrate that ARF deficiency switches macrophages to a M2-like phenotype. Thus, in addition to downregulation of proinflammatory mediators, typical hallmarks of an anti-inflammatory M2-activation state were increased in both resting and IL-4-treated ARF−/− macrophages, as exemplified by the upregulation of Arg-1, Fizz-1 and Ym1. Moreover, the cytokine/chemokine pattern induced by IL-4 stimulation appeared upregulated in macrophages isolated from ARF−/− mice showing an M2-phenotype with higher levels of IL-10, Ccl22, Ccl5, Ccr3, or Ccr5. Together with these M2 markers, important proangiogenic factors such as VEGF and MMP-9 were also increased, suggesting the potential protumoral action of ARF−/− macrophages. Consistent with this notion, recent studies have demonstrated a role for ARF in suppressing tumor angiogenesis via modulation of VEGF expression [131] and the activity of its transactivator HIF [132]. Notably, ARF has also been described to inhibit angiogenesis by up-regulating the expression of TIMP3, an inhibitor of MMPs activity [133].

Taken together, it is tempting to speculate that ARF has a profound influence in regulating the polarization of macrophages. Thus, ARF deficiency might modify the immune tumor microenvironment as a result of the induction of multiple activities including (a) immune suppression, through the production of the anti-inflammatory cytokine IL-10 and the secretion of Ccl22 and Ccl2 that attracts Tregs, (b) stimulation of angiogenesis through expression of VEGF and MMP-9, and (c) induction of matrix remodeling through the production of MMP-9, Fizz-1, and Ym1.

6. Conclusions

More and more evidence indicates that tumor suppressors play an essential role in host immunity, placing them as general sensors and modulators of innate immune response. Mechanisms involved in this process remain unclear, although transcriptional regulation of several inflammatory mediators has been reported. In addition, M1/M2 polarization and functional plasticity of macrophages have been shown to be modulated by tumor suppressors. These studies reveal positive effects of tumor suppressors on cancer immunosurveillance that will pave the way for new targeted therapies.

Therefore, identification of the role of tumor suppressors in the pathways responsible for the skewing of macrophage function as well as in the regulation of inflammatory mediators will remain an important area of investigation in the years to come.

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Clinical Study

Alterations in Monocyte CD16 in Association with Diabetes Complications

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Monocytes express many cell surface markers indicative of their inflammatory and activation status. Whether these markers are affected by diabetes and its complications is not known and was investigated in this study. Blood was obtained from 22 nondiabetic and 43 diabetic subjects with a duration of diabetes >10 years, including 25 without and 18 with clinically significant complications. The number of $CD45^+CD14^+$ monocytes and the percentage expressing the proinflammatory marker CD16 were determined by flow cytometry. Other markers of monocyte activation and expression of chemokine receptors were also examined. The relationship between monocyte CD16 and clinical data, selected cytokines, and chemokines was also investigated. Diabetes had no effect on total white cell number but increased monocyte number. Diabetes also significantly decreased the number of $CD16^+$ monocytes but only in those with diabetic complications. Other markers of monocyte activation status and chemokine receptors were not affected by diabetes or complications status. Diabetes induced plasma proinflammatory cytokines and they were lower in diabetic subjects with complications compared to those without complications. These results suggest that the circulating monocyte phenotype is altered by diabetic complications status. These changes may be causally related to and could potentially be used to predict susceptibility to diabetic complications.

1. Introduction

The complications of diabetes are responsible for much of its associated morbidity and mortality. Landmark studies have shown that the risk for development of diabetic complications increases with poor glycemic control and disease duration [1–3], although other factors such as inflammation are also likely to be important [4–7]. The levels of circulating proinflammatory cytokines and chemokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and monocyte chemoattractant protein-1 (MCP-1) are commonly used as markers of inflammation, and they have been shown to be increased in diabetes [8–10]. Whether monocytes, a cell type central to the inflammatory response, are affected by the diabetic milieu and provide complementary information is not known.

Circulating monocytes are identified by flow cytometry as $CD45^+CD14^+$ cells. They are further characterized according to the presence or absence of expression of CD16, the low-affinity Fc receptor, FcγRIII [11]. The $CD16^-$ monocytes constitute a "classically activated" subset of monocytes which account for 80–90% of circulating monocytes in normal healthy individuals. This population is increased in acute inflammation and is rapidly recruited to sites of infection [11–13]. The $CD16^+$ monocytes constitute the "nonclassically activated" subset which makes up the remainder of the monocyte population. This $CD16^+$ population has a patrolling function to sense tissue injury, and

is increased in ageing and chronic inflammatory disorders [11–16]. In acute conditions, these subsets express different levels of chemokine receptors, the $CD16^-$ subset expresses CCR2 (chemokine receptor 2), the receptor for MCP-1, and the $CD16^+$ subsets expresses CCR5 (chemokine receptor 5) which binds macrophage inflammatory protein-1β (MIP-1β) [11, 17]. The $CD16^+$ subset typically responds to bacterial endotoxin by secretion of proinflammatory cytokines, in particular TNF-α and IL-1 [11, 15, 18]. Monocytes also express other cell surface proteins which can provide information regarding their inflammatory activation status. For example, in response to inflammatory stimuli 27E10 is expressed in the acute phase whilst 25F9 is increased at later stages [19–21]. Additionally, CD68 is a marker for tissue macrophages, and CD11b is expressed by activated monocytes [6, 22, 23].

Diabetes has been shown to alter the circulating monocyte populations [24–26], but the relationship between these changes and the presence of diabetic complications has not been investigated. In the current study, we used flow cytometry to examine the circulating monocytes in particular $CD16^+$ monocytes and other markers of monocyte activation such as 27E10, 25F9, CD68, and CD11b, in two groups of diabetic subjects with an equally long duration of diabetes but which differ by the presence or absence of clinically significant diabetic complications. Results were compared with nondiabetic control subjects. The relationship between the expression of these markers and circulating proinflammatory cytokines and chemokines was also investigated.

2. Materials and Methods

2.1. Study Participants. Patients with no micro- or macrovascular complications (D^{-Comps}), despite a duration of diabetes greater than ten years, were identified from the clinical database of the Diabetes Centre, Royal Prince Alfred Hospital and Central Sydney Eye Surgeons. Patients with similarly long durations of diabetes, but with diabetic complications (D^{+Comps}), were also recruited from the same database. In total, 43 patients (24 males and 19 females; mean age 62.7 ± 11.7 years) with type 1 ($n = 7$) and type 2 ($n = 36$) diabetes were studied. Based on standard screening, 18 subjects with complications and 25 without clinically significant microvascular and/or macrovascular disease were recruited. The presence of retinopathy was confirmed by fundal examination and/or photography. The absence of diabetic nephropathy was confirmed by normal serum creatinine and urinary albumin/creatinine ratio (U Alb/Cr) <2.5 mg/mmol for males and <3.5 mg/mmol for females. Study participants were considered to have macrovascular disease if they had any relevant symptoms of vascular disease or had reported a history of abnormal investigation or prior macrovascular event. In addition, a total of 22 nondiabetic participants (6 males and 16 females; age 49.7 ± 9.0 years) were recruited as controls from the general community or staff members of the hospital. The study has the approval of the Ethics Review Committee of the institution and was carried out in accordance with the principles of the Declaration of Helsinki as revised in 2000. All study participants gave their written informed consent.

2.2. Multicolor Flow Cytometry. Multicolor flow cytometry was used to characterize the monocyte subsets under investigation, and the following monoclonal antibodies conjugated with the fluorochromes fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC), peridinin chlorophyll protein-cyanine5.5 (PerCP-Cy5.5), and allophycocyanin-cyanine7 (APC-Cy7) were used. They are anti-CD45 (PerCP-Cy5.5 and APC), anti-CD14 (FITC and PerCP-Cy5.5), anti-CD16 (PE), anti-CD68 (PE), anti-27E10 (PE), anti-25F9 (FITC), anti-CD11b (APC-Cy7 and PE), anti-CCR2 (APC), and anti-CCR5 (APC). Appropriate mouse IgG subclasses (FITC, PE, APC, PerCP-Cy5.5, and APC-Cy7) were used as negative controls. The antibodies were obtained from Becton Dickinson (BD, San Jose, CA), R&D Systems (Minneapolis, MN, USA), Abcam (Cambridge, UK), and Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively.

For analysis, venous blood was collected into EDTA-treated tubes. Whole blood (100 μL) was then incubated with fluorescent-conjugated monoclonal antibodies to CD45 and CD14 in combination with a fluorescent conjugated antibody to CD16, 27E10, 25F9, CD68, CD11b, CCR2, or CCR5. After a 60-minute incubation at 4°C, the erythrocytes were lysed by the addition of 0.4% saponin (Sigma, Australia). The cells were washed immediately with Phosphate Buffered Saline (PBS) and collected by centrifugation at 300 g for 5 minutes. The supernatant was discarded, and the cell pellet was resuspended and washed in FACS buffer (PBS containing 0.5% w/v BSA, 0.1% w/v NaN_3, and 2 mM EDTA). Cell surface marker expression was then determined by flow cytometry using either the BD FACSCanto or BD FACSAria (Becton Dickinson, San Jose, CA, USA). A minimum of 30,000 events were counted for each CD marker set. All samples were analyzed in parallel. Conjugated isotype controls, single antibody-stained controls, and fluorescence-minus-one (FMO) controls were also included.

The data was analyzed using FlowJo version 8.1.1 (Ashland, OR, USA). Leukocytes were initially identified and separated into subgroups based on cell size (forward scatter) and granularity (side scatter) with dead cells excluded. The sequential gating strategy is shown in detail in Figure 1. The primary gating focused on the $CD45^+$ population, the secondary gating on the $CD14^+$ cells, and the final gating was based on the CD^+ marker of interest within the $CD45^+CD14^+$ cell population. The results were then expressed as the percentage of CD^+ cells within the $CD45^+CD14^+$ population.

2.3. Measurement of Plasma Cytokine Levels. The circulating concentrations of proinflammatory cytokines and chemokines IL-6, IL-8, TNF-α, interferon-gamma (IFN-γ), interferon gamma-induced protein 10 kDa (IP-10), MCP-1, and MIP-1β were measured in plasma using a Bio-Plex Pro Assay (Bio-Rad, Hercules, CA, USA). The concentration of IL-10 was quantified using a commercially available ELISA kit from R&D Systems (Minneapolis, MN, USA) according to the manufacturer's instructions.

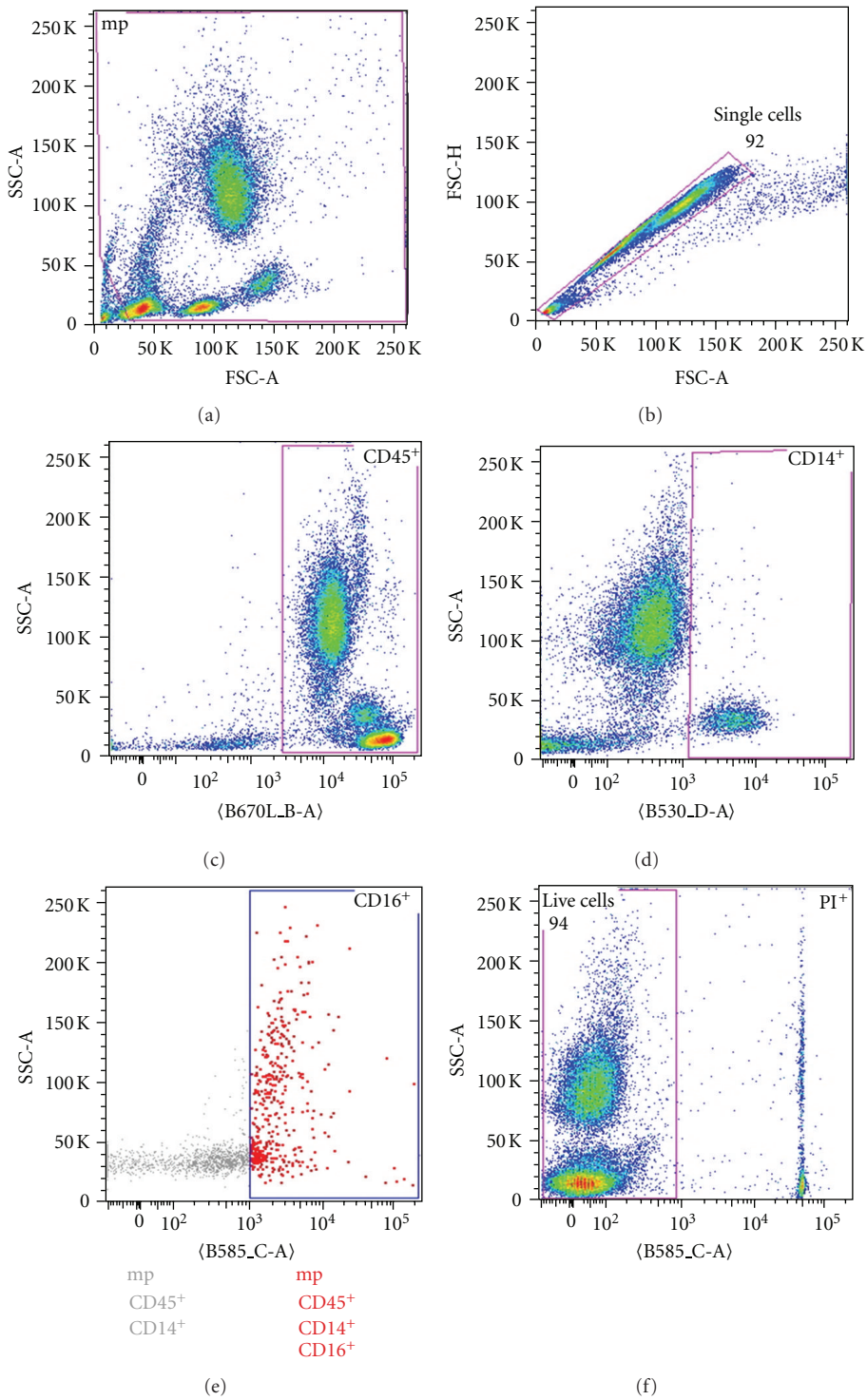

Figure 1: Flow cytometry gating strategy. Flow cytometry data obtained from a representative sample is shown. (a) Peripheral blood cells were first gated based on forward scatter (FSC-A)/side scatter (SSC-A), with exclusion of dead cells. (b) The events were then visualized using FSC-A/FSC-H dot plot, and the singlets (single cells) were gated. (c) Leukocytes were identified by their positive staining with CD45. (d) Monocytes were then defined as the $CD14^+$ cells within the $CD45^+$ leukocyte population. (e) The final gating was based on the CD marker of interest within the $CD45^+CD14^+$ cell population. (f) Necrotic cells in the whole blood sample were detected by Propidium Iodide (PI) staining.

2.4. Statistical Methods. Data analysis was performed using the NCSS 2004 statistical package. Continuous data were checked for normality and are presented as mean ± standard deviation or median and interquartile range. Grouped data were compared by *t*-test. Linear regression and correlation analysis was used to verify the significance of the relationship between monocyte subsets and the concentrations of proinflammatory cytokines, chemokines, and clinical variables, respectively. Statistical significance was accepted at $P < 0.05$.

3. Results

3.1. Clinical Profile. Demographic and clinical parameters of study participants are shown in Table 1 (individual patient data is shown in Supplementary Table 1 available online at doi:10.1155/2012/649083). The age of the control subjects ranged between 34.6 and 65.4 years whilst the diabetes' group ranged between 30.7 and 78.8 years. The groups of diabetic patients were well matched for diabetes type (D^{-Comps}: $n = 3$ T1DM versus D^{+Comps}: $n = 4$ T1DM), age (D^{-Comps}: 63.3 ± 8.6 versus D^{+Comps}: 61.8 ± 15.2), and duration of diabetes (D^{-Comps}: 17.8 ± 6.7 versus D^{+Comps}: 21.8 ± 8.1). The group with complications comprised subjects with microvascular disease alone ($n = 14$) or both micro- and macrovascular disease ($n = 4$) and had slightly poorer glycemic control and worse renal parameters. Between the groups, there were no significant differences in treatment with statins (D^{-Comps}: 21/25, D^{+Comps}: 14/18), antihypertensive agents (D^{-Comps}: 19/25, D^{+Comps}: 15/18), or aspirin (D^{-Comps}: 11/25, D^{+Comps}: 12/18).

3.2. The Effects of Diabetes on Monocyte Number and Morphology. There was no effect of diabetes on the number of circulating white cells ($CD45^+$ cells), but the monocyte component ($CD45^+CD14^+$) was significantly increased (diabetic: 8.3 ± 2.6% versus control: 7.2 ± 1.6%, $P < 0.05$) (Figures 2(A) and 2(B), resp.). This increase was not affected by diabetes type (T1DM: 7.2 ± 2.4% versus T2DM: 8.5 ± 2.6%, $P = 0.22$) or complications status (D^{-Comps}: 8.2 ± 2.7% versus D^{+Comps}: 8.5 ± 2.5%, $P = 0.72$). As shown in the representative flow cytometry scatter plots from control and diabetic subjects (Figure 2(C)), the monocytes were morphologically heterogeneous, showing a wide scatter in size (forward scatter) and granularity (side scatter). Based on these characteristics, three populations were identified as a smaller size and less granular population (a), an intermediate population (b), and a population of monocytes which is larger in size and more granular in group (c). These larger more granular monocytes (group (c)) were responsible for the 1% increase in monocyte number seen in diabetes (diabetic: 3.8 ± 2.3% versus control: 2.7 ± 1.4%, $P < 0.05$).

3.3. The Effects of Diabetes and Complications Status on Monocyte CD16 Expression. Representative flow cytometry plots of $CD45^+CD14^+CD16^+$ monocytes from a nondiabetic and a diabetic subject are shown in Figure 3(A). Overall, there was no significant difference in the percentage $CD16^+$ monocytes between nondiabetic control and diabetic subjects (control: 8.5 ± 3.4% versus diabetic: 11.4 ± 8.8%). However, within the diabetic cohort, the percentage of $CD16^+$ monocytes was lower in those subjects with diabetic complications compared to those without complications (Figure 3(B)). This result was also seen when only those with T2DM were analyzed (D^{-Comps}: 14.9 ± 9.4% versus D^{+Comps}: 8.2 ± 7.5%, $P < 0.05$). The increase in the percentage of $CD16^+$ cells observed in the diabetic cohort without complications was substantially due to an increase in the larger size and more granular monocytes (population (c) in Figure 3(C)). Regression analysis showed a relationship between cholesterol and CD16 expression ($r = 0.306$, $P = 0.05$) accounting for approximately 9.3% of the variance. No such relationship was observed for HbA1c.

3.4. The Effects of Diabetes on Other Monocyte Markers of Inflammation and Differentiation. Diabetes and its complications status had no effect on monocyte expression of chemokine receptors CCR2 and CCR5, or any of the differentiation markers 27E10, 25F9, CD68, and CD11b. Shown in Figure 4, there were significant correlations between the percentage of $CD16^+$ monocytes and the percentage of monocytes expressing the differentiation markers $CD68^+$ and $27E10^+$.

3.5. Plasma Levels of Inflammatory Cytokines and Chemokines. The plasma concentration of cytokines and chemokines in the control and diabetic subjects are shown in Table 2. Diabetic patients with complications had lower levels of the proinflammatory cytokines IL-6, IL-8, TNF-α, and IFN-γ but a higher level of the anti-inflammatory cytokine IL-10 when compared with their counterparts without complications. By contrast the complications group had higher chemokine levels with MIP-1β reaching statistical significance. No correlation between percentage of $CD16^+$ monocytes and circulating inflammatory cytokines and chemokines was observed.

4. Discussion

Inflammation plays a central role in the development of diabetic complications, and macrophages in the tissue are known to be an important cell type in this regard [5, 27, 28]. However, studies on macrophages are hampered by the difficulty of obtaining appropriate tissue samples. In this regard, circulating monocytes may provide a valuable alternative source of material to test various hypotheses. Monocytes are derived from myelomonocytic stem cells in the bone marrow where they mature to monocytes. Once in the blood, they develop further and migrate in response to chemokines to the tissue where they differentiate into functionally and phenotypically distinct macrophage types. This heterogeneity of tissue macrophages is well established. Their phenotype and function are known to be altered in response to multiple factors, including bacterial endotoxins, tissue injury and inflammatory signals [11, 29]. On the other hand, little is known regarding monocyte heterogeneity in diabetes. Further, whether any changes reflect clinical and complication status of this condition has not been studied. In this study, we used flow cytometry to identify and

Table 1: Demographic and clinical parameters of control and diabetic subjects with or without complications.

	Control ($n = 22$)	Diabetic ($n = 43$)	Diabetic	
			D^{-Comps} ($n = 25$)	D^{+Comps} ($n = 18$)
Age (yrs)	49.7 ± 9.0	62.7 ± 11.7*	63.3 ± 8.6	61.8 ± 15.2
Duration (yrs)	n.a.	19.5 ± 7.5	17.8 ± 6.7	21.8 ± 8.1
Weight (kg)	76.3 ± 18.7	79.9 ± 14.1	79.3 ± 14.8	80.7 ± 13.5
BMI (kg/m^2)	28.5 ± 7.8	28.7 ± 4.9	29.1 ± 5.1	28.2 ± 4.7
HbA_{1c} (%)	n.a.	7.5 ± 1.1	7.1 ± 0.6	8.2 ± 1.2†
Serum creatinine (μmol/L)#	66.0 (62.3–80.0)	80.0 (70.0–95.0)*	70.0 (63.5–83.5)	89.5 (79.8–103.8)†
U Alb/Cr ratio (mg/mmol) #	0.7 (0.6–1.2)	1.5 (0.8–3.0)*	0.9 (0.6–1.5)	3.2 (1.9–8.9)†
eGFR (mL/min)	91.4 ± 16.9	80.8 ± 24.4	86.6 ± 23.0	72.6 ± 24.6
Triglycerides (mmol/L)	1.2 ± 0.5	1.5 ± 0.8	1.6 ± 0.8	1.5 ± 0.8
HDL cholesterol (mmol/L)	1.8 ± 0.5	1.3 ± 0.4*	1.4 ± 0.3	1.2 ± 0.4
LDL cholesterol (mmol/L)	3.0 ± 0.6	2.1 ± 0.7*	2.2 ± 0.8	1.9 ± 0.6
Sys BP (mmHg)	116 ± 14	127 ± 16*	124 ± 16	132 ± 15
Dias BP (mmHg)	77 ± 8	74 ± 7	74 ± 7	74 ± 7

Results are expressed as mean ± SD, except (#) shown as median with interquartile range (IQR).
Significantly different if $P < 0.05$ indicated as (*) for controls versus diabetes and (†) for D^{-Comps} versus D^{+Comps}.

Table 2: Plasma levels of cytokines and chemokines in control and diabetic subjects.

(pg/mL)		Control ($n = 22$)	Diabetic ($n = 43$)	Diabetic	
				D^{-Comps} ($n = 25$)	D^{+Comps} ($n = 18$)
Cytokines	IL-6#	3.2 (2.1–5.3)	3.8 (2.7–4.8)	4.5 (3.2–7.1)	3.6 (2.6–4.2)
	IL-8#	3.1 (1.8–4.4)	4.5 (3.3–5.5)*	5.1 (3.7–6.4)	3.7 (2.9–4.6)†
	TNF-α#	10.4 (7.6–20.1)	12.2 (8.5–19.8)	12.8 (10.4–23.8)	10.0 (5.5–12.8)†
	IFN-γ#	61.6 (46.6–85.5)	56.8 (49.3–76.9)	68.4 (53.5–113.4)	50.3 (45.6–59.7)†
	IL-10#	0.03 (0.00–0.43)	0.52 (0.07–0.94)*	0.46 (0.00–0.82)	0.83 (0.20–1.08)
Chemokines	IP-10	449.4 ± 134.6	483.7 ± 161.4	463.4 ± 159.2	511.9 ± 164.7
	MCP-1#	14.7 (12.0–22.1)	21.7 (15.0–35.7)*	19.2 (14.8–35.1)	23.6 (15.7–43.4)
	MIP-1β	20.4 ± 8.8	23.7 ± 12.4	20.3 ± 11.0	28.5 ± 12.9†

Results are expressed as mean ± SD, except (#) shown as median with interquartile range (IQR).
Significantly different if $P < 0.05$ indicated as (*) for controls versus diabetes and (†) for D^{-Comps} versus D^{+Comps}.

examine the expression of a variety of cell surface markers on monocytes obtained from peripheral blood.

Similar to the study of Corrales et al., we observed an increase in circulating monocytes in diabetic subjects compared with nondiabetic controls [30]. It is of interest that the increase can be substantially attributed to the population with larger cell size and higher granularity (subset (c)), phenotypic features more like that of a macrophage. When analyzed as a single cohort, there was little difference between normal and diabetic monocyte expression of CD16. However, this masked the higher expression of these markers in the diabetic cohort with no complications despite a long duration of diabetes. These results are similar to those published by Mysliwska et al. which showed increased $CD16^+$ monocytes in young T1DM subjects without retinopathy compared to those with retinopathy [31]. Unlike our study, this study showed an increase in the $CD16^+$ monocytes in the diabetic cohort compared with nondiabetic controls. Why this difference occurs is not certain, but factors such as age and BMI of the cohort studied are likely to be important [24]. Interestingly, in our study, the main difference in the monocyte morphology appeared to be due to shape of the monocyte with the biggest changes being in the larger and more granular monocytes. Perhaps due to related mechanisms, there were strong correlations between CD16 expression and $CD68^+$ and $27E10^+$, both markers of monocyte activation and differentiation.

The results of plasma cytokines and chemokines showed some interesting parallels with the monocyte cell surface marker studies. The diabetic subjects with no complications again were different from those with complications in that they have higher proinflammatory cytokine levels. However, IL-10, known to be more anti-inflammatory in action, did not follow this trend. Whilst it is not possible to distinguish the monocyte subset contributing to the circulating cytokine levels, this increase maybe due to the increased percentage of IL-10 secreting $CD16^-$ monocytes in those diabetic subjects with complications [25]. This finding of higher proinflammatory cytokine levels in those without complications was somewhat unexpected. There are variable reports regarding proinflammatory cytokine levels and their association with diabetic complications [32–34]. Most studies have focused on the presence or absence of cardiovascular disease or diabetic nephropathy [32–34]. Our diabetic subjects had

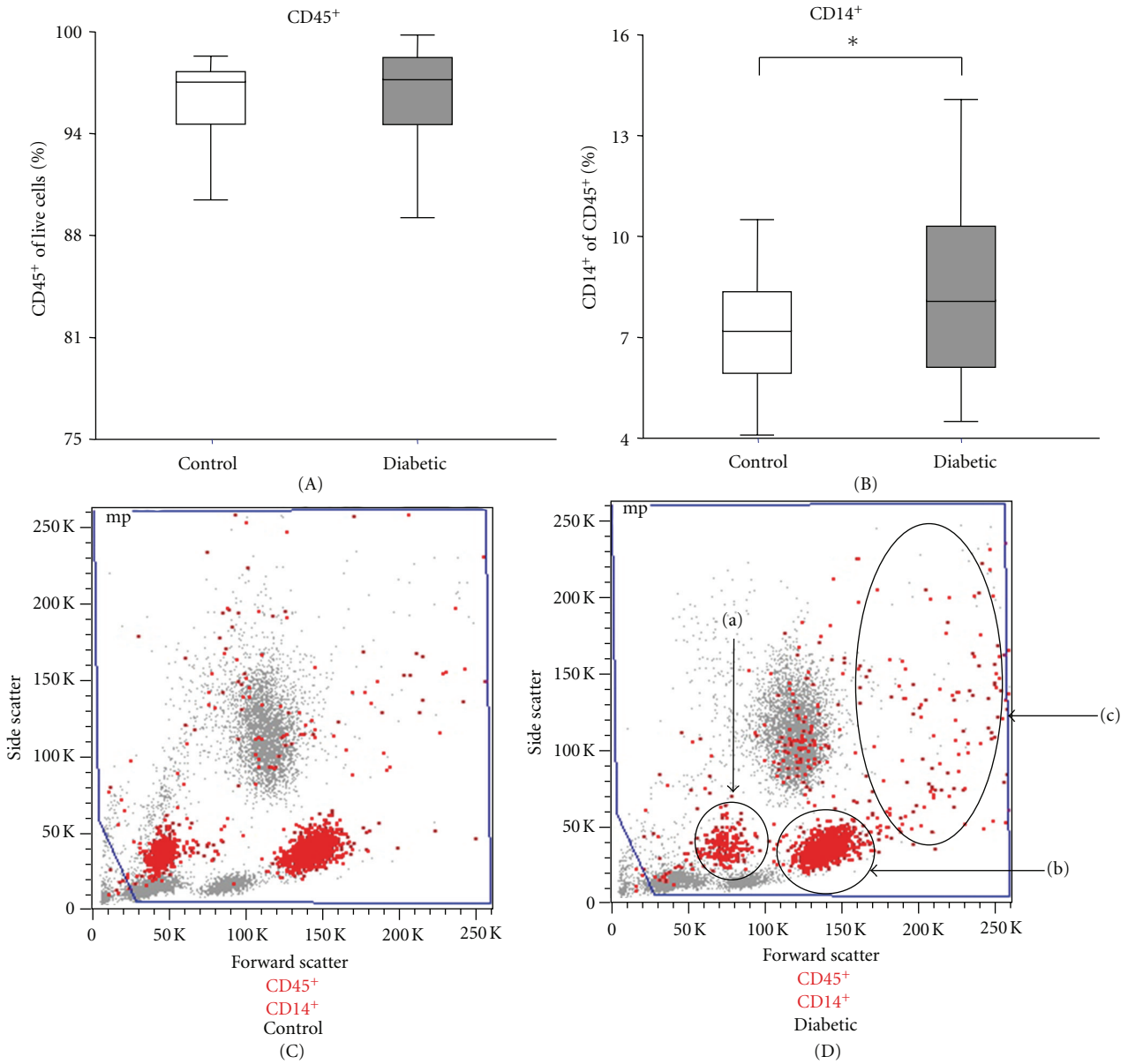

FIGURE 2: The effect of diabetes on circulating monocytes. Leukocytes and monocytes were identified by flow cytometry. Shown in control and diabetic subjects are (A) the $CD45^+$ leukocyte population and (B) the $CD45^+CD14^+$ monocyte population. (C) Representative forward scatter and side scatter plots from a control and diabetic subject showing $CD45^+CD14^+$ monocytes (red dots). The three subsets of monocytes grouped according to size and granularity are shown in the diabetic sample as (a), (b), and (c). $^*P < 0.05$ different between groups.

long durations of disease and were mostly T2DM with the majority having diabetic retinopathy in the earlier stages (only one with proliferative disease). The data regarding proinflammatory cytokine levels in subjects with retinopathy is less clear. There are few studies, and both increased and decreased levels have been described [31, 35–37]. For example, in T1DM subjects with nonproliferative diabetic retinopathy, TNF-α has been reported to be higher than the level seen in subjects without diabetic retinopathy [31]. In contrast, in a T2DM cohort, TNF-α level was shown to change depending on the stage of disease. In their study, the decreased TNF-α and IL-6 levels were observed in the subjects with nonproliferative diabetic retinopathy, whilst the presence of proliferative diabetic retinopathy was associated with the increased TNF-α and IL-6 levels [35]. How factors such as complications type and severity, disease duration, age, and type of diabetes affect proinflammatory cytokine levels remains to be systematically studied.

Together these studies of monocytes and plasma suggest that pro- and anti-inflammatory markers are altered in diabetes in a manner reflecting different status of diabetic complications. As mentioned somewhat counterintuitively, compared to those with complications, those diabetic subjects with no complications have higher circulating proinflammatory markers ($CD16^+$, IL-6, IL-8, TNF-α, and IFN-γ). Despite this pattern, we cannot conclude directly that the

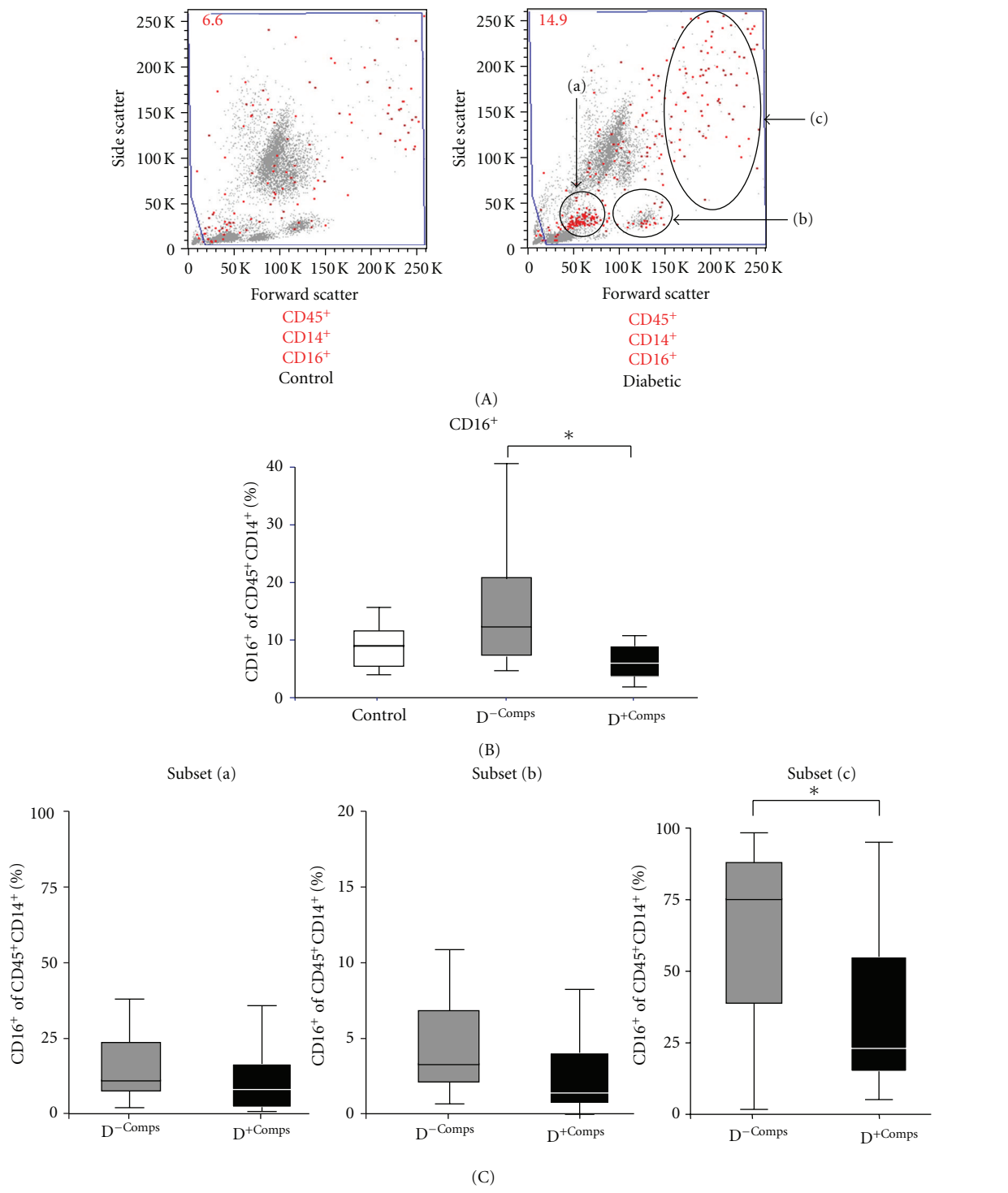

FIGURE 3: The effect of diabetes and its complications on percentage of CD16 monocytes. (A) Representative forward scatter and side scatter plots data from a control and diabetic subject showing the $CD16^+$ cells (red dots) within the $CD45^+CD14^+$ monocyte population. (B) The percentage of $CD16^+$ monocytes in control and diabetic subjects with and without complications. (C) The percentage of $CD16^+$ monocytes in each monocyte subset grouped according to size and granularity. $^*P < 0.05$ different between groups.

monocytes are overall more anti-inflammatory because the relationship between function and surface marker expression may not be a proportional one. It also remains to be proven in animal studies that the change in the profile of circulating monocytes in diabetes observed in the present study has any relationship to those occurring in tissue macrophages. In addition, there are other monocyte subsets and surface markers associated with different inflammatory states which

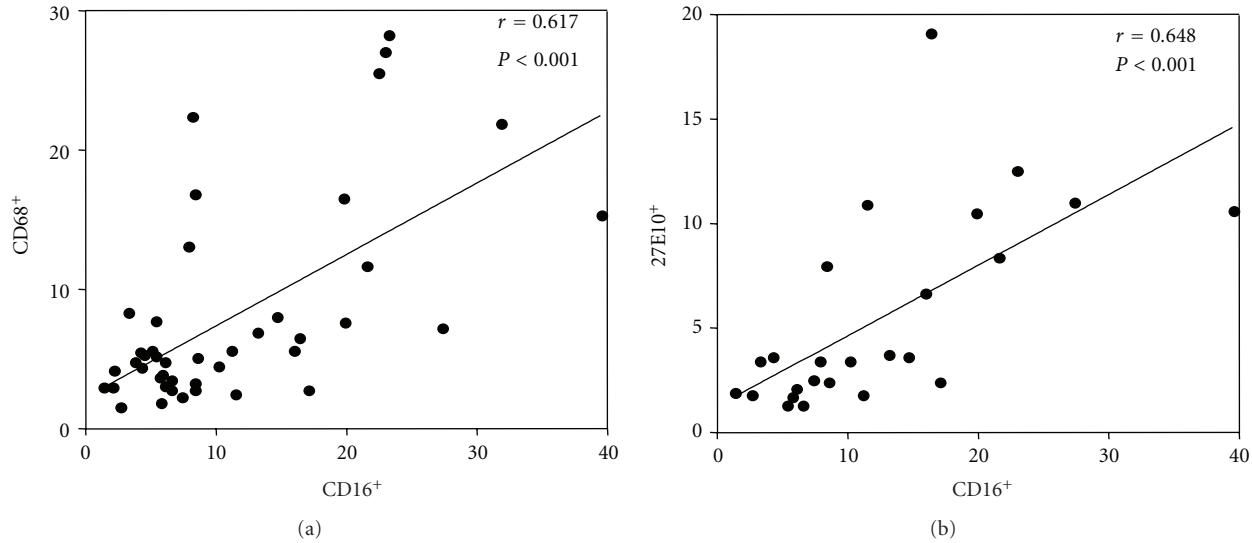

Figure 4: The relationship between $CD16^+$ monocyte and other CD markers. The relationship between the $CD16^+$ monocyte and (a) macrophage marker $CD68^+$ and (b) $27E10^+$, a marker of acute inflammatory response.

remain to be studied. For example, a recent report by Gonzalez et al. has shown that high glucose concentration can downregulate CD33, a membrane receptor, but increase secretion of monocyte proinflammatory cytokines [26]. However, the expression of this receptor in diabetic subjects with complications was not examined. Additionally, to assess the relationship between the percentage of monocytes expressing the surface marker and the net inflammatory status of monocytes, functional studies would need to be performed on purified monocytes and on their various subsets. The observed pattern of cells of different size and granularity suggests altered activation status of the monocytes and adds another level of complexity which may need to be studied separately. In addition, it is known that *in vivo*, a large pool of monocytes adheres to the endothelial cell surface [38]. Our results showed that diabetic subjects with complications may have higher chemokine levels. As such, their pool of adhered monocytes may be greater and differently constituted. This may also have a bearing on the results of experiments.

How the changes we have observed are mediated mechanistically needs to be answered with further investigations. Whether the changes of monocyte surface markers with diabetic complications is a causal one cannot be determined from this study. The slight differences in glycemic control and renal function between the two groups of diabetic subjects do not seem to play a significant role. In animal studies, some of these questions can be answered by observing the effects of suppressing individual subtypes of monocytes. In human studies, further insight can be obtained by monitoring longitudinally monocyte and plasma markers early in the natural history of diabetes before development of diabetic complications. Modalities of treatment proven to impart benefit on the microvascular or macrovascular complications (e.g., treatment with statin) can also be evaluated in this setting. Our diabetic cohorts are selected from a background of longstanding diabetes according to the presence or absence of complications using a composite criteria and assessed comprehensively at our Diabetes Complications Assessment Service [39]. Although the cohorts studied are heterogeneous, the grouping used in our study represents patterns commonly seen clinically but which cannot be readily explained. Future studies could further examine each complication separately. However, clinical complications tend to coexist, and it would be difficult to recruit individuals affected by only one type of complication. Although many questions remain unanswered, our study opens the possibility that peripheral blood monocytes can be used to investigate the pathophysiology of diabetic complications.

Abbreviations

CCR: C-C Chemokine receptor
IP-10: Interferon gamma-induced protein 10 kDa
MCP-1: Monocyte chemoattractant protein-1
MIP-1β: Macrophage inflammatory protein-1β
U Alb: Urinary albumin
U Alb/Cr: Urinary albumin/creatinine ratio.

Conflict of Interests

No conflict of interests relevant to this paper was reported.

Authors' Contribution

D. Min researched, analyzed, interpreted the data, and wrote the paper. B. Brooks and B. Harrisberg collected the clinical samples and clinical data and revised the paper. R. Salomon and W. Bao provided technical assistance. S. Twigg and J. Wong contributed to discussions and reviewed the paper. S. V. McLennan and D. K. Yue contributed to discussions and assisted with analysis, interpretation of the data, paper writing and review.

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Clinical Study

Increased Expression of Visfatin in Monocytes and Macrophages in Male Acute Myocardial Infarction Patients

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We demonstrated that visfatin expressed in monocytes and neutrophils and increased their reactivity in male acute ST-segment elevation myocardial infarction patients. Furthermore, visfatin was strongly appeared in lipid rich coronary rupture plaques and macrophages. These results suggest another explanation about leukocytes mediated visfatin that may play a pathogenesis role in coronary vulnerable plaques rupture.

1. Introduction

As we know, acute myocardial infarction (AMI) constitutes the major cause of death in most countries and remains substantial in the years thereafter. Inflammation is implicated in the development and rupture of atheromatous plaques, and there is considerable evidence supporting the involvement of adipocytokines in this inflammatory process [1–3]. The recruitment of circulating monocytes into the arterial wall, followed by their differentiations into tissue macrophages, is one of the earliest events in atherosclerotic plaque formation [4–8]. Increasing evidence suggests that monocytes control vascular smooth muscle cells proliferation and migration [9–12], lipid metabolism [13], and inflammation [14–17] within the vessel wall. Monocytes have thus been proposed to serve as markers, initiators and promoters of arterial occlusive diseases [18, 19]. Hence, the phenomenon of leukocytosis might contribute to the development of chronic heart disease via multiple pathophysiological mechanisms, including vascular inflammation, proteolytic, and oxidative damage to the endothelial cells, plugging the microvasculature hypercoagulability, and promoting infarct expansion [20].

Visfatin, also known as pre-B-cell colony-enhancing factor, is a 52- to 55-kDa protein that is suggested to be highly prevalent in visceral fat cells. Researchers demonstrated that

visfatin is a harmful, proinflammatory agent in obesity-induced metabolic and cardiovascular diseases [21, 22]. Clinical and basic reports have suggested that visfatin is an inflammatory protein associated with plaque destabilization and acute coronary syndrome (ACS) [23, 24]. Our recent studies also showed that plasma visfatin levels are associated with infarct-related artery (IRA) occlusion and that increased plasma visfatin may be closely related to the degree of myocardial damage [25, 26]. In addition, vulnerable lesions with a tendency to rupture are rich in activated macrophages, suggesting the macrophage as a key regulator of atherosclerotic plaque stability [27, 28]. Interestingly, visfatin has also been shown to be produced by immune cells (e.g., neutrophils and macrophages) [29, 30]. However, the expression of visfatin in monocytes and macrophage in circulation and coronary rupture plaques in patients with acute ST-segment elevation myocardial infarction (STEMI) has not been well elucidated.

In view of the possible association of visfatin with inflammation and pathogenesis of AMI, we thus studied the expression of visfatin in circulating leukocytes in male patients with STEMI by immunohistochemical (IHC) staining, flow-cytometry analysis, and real-time PCR. Furthermore, we also studied immunoreactivity of visfatin expression in macrophages in human coronary rupture plaques.

2. Materials and Methods

2.1. Study Population. We initially enrolled 7 consecutive male patients (age, 63 ± 14 years) admitted with a diagnosis of STEMI within 12 h of symptom onset, between June 2011 and June 2012. STEMI was indicated by prolonged chest pain (>30 min), typical rise increase in the levels of biochemical markers (Troponin-I and CK-MB/CPK) with ischemic symptoms lasting for ≥30 minutes, and an ST-segment elevation of ≥2.0 mm in ≥2 contiguous electrocardiographic leads. Six male non-CAD control subjects (age, 58 ± 13 years) had undergone a coronary angiography examination, and documented insignificant coronary stenosis was documented. Written informed consents were obtained from the patients before enrollment. The study was conducted in agreement with the guidelines approved by the Human Research Ethics Committee at our hospital.

2.2. Immunohistochemistry in Circulating Leukocytes. An avidin-biotin-peroxidase complex commercial method (DAKO Co., Carpinteria, CA) was used for IHC analysis. Approximately 100 μL heparin-containing bloods were incubated with 2 mL of red blood cell (RBC) lysing solution (BD Biosciences, San Jose, CA) at room temperature for 15 min to remove RBCs. After washing with 2 mL of DPBS (Gibco, Grand Island, NY) containing 5% fetal bovine serum, the cells were resuspended in 0.1 mL of the same buffer and were centrifuged at 250 rpm for 8 min to the poly-L-lysine (Sigma chemical Co., St. Louis, MO), precoated slide by cytospin (Kubota-5920, Kubota Co., Tokyo). The cells on the slide were fixed with 95% methanal for 3 min at room temperature. Endogenous peroxidase activity was blocked by incubation of the sections in 0.3% H_2O_2 in phosphate-buffered saline (PBS) for 30 min and incubated with 1% bovine serum albumin (BSA) for 30 min to block nonspecific staining. Slides were drained and incubated overnight at room temperature in a humidity chamber with the respective rabbit anti-human primary antibody for visfatin (1 : 200) diluted with antibody diluent (DAKO Corp., Glostrup, Denmark). The primary antibody was purchased from Phoenix Pharmaceuticals Inc. (Belmont, CA). The avidin-biotin-peroxidase complex (ABC complex; DAKO, Carpinteria, CA, USA) was applied on the sections after they were incubated with biotinylated secondary antibody (Dako Corporation, Carpinteria, CA, USA). The slides were incubated with DAB substrate-chromogen solution (Dako Corporation, Carpinteria, CA, USA), counterstained with hematoxylin, and mounted in an aqueous mounting medium. Negative control studies were performed with replacement of the primary antibody by nonimmune antiserum and counterstaining with hematoxylin.

2.3. Intracellular Staining and Flow-Cytometry. For double immunofluorescence staining, 100 μL of heparin-containing blood was incubated with PE/CY5-conjugated mouse anti-human CD3 for T lymphocyte, PE-conjugated mouse anti-human CD13 for neutrophil, PE-conjugated mouse anti-human CD14 for monocyte, and PE/CY5 conjugated mouse anti-human CD19 for B lymphocyte, in the dark room for 30 min at room temperature. All the antibodies using for double immunofluorescence staining were purchased from BD Biosciences. Then, 2 mL of RBC lysing solution was added and incubated for 15 min at room temperature. After washing with 2 mL of DPBS containing 1% fetal bovine serum and 2% sodium azide, the cells were fixed with 3% paraformaldehyde in DPBS for 20 min at room temperature. The cells were washed two times with DPBS containing 0.05% saponin (Sigma), and then incubated with the primary rabbit anti-human PBEF/visfatin serum (Phoenix), which is diluted 100-folds with DPBS-saponin, for 20 min at room temperature. After washed by DPBS-saponin, cells were incubated with Alexa fluor 488 goat anti-rabbit IgG (Invitrogen/Molecular Probes) for 20 min at room temperature. Cells were gated based on forward angle light scatter and side angle light scatter and further analyzed using the Cell Quest Pro software (Becton Dickinson) for the expression of visfatin on peripheral blood cells.

2.4. Visfatin and CD68 Immunohistochemistry Stain of Coronary Rupture Plaques. The coronary rupture plaques were available from 7 of STEMI study patients who underwent primary percutaneous coronary intervention (PCI) and were aspirated by Medtronic Guard Wire and Aspiration System device as previously described [24]. An avidin-biotin-peroxidase complex commercial method (ABC complex; Dako Corporation, Carpinteria, CA, USA) was used for IHC analysis. In human coronary rupture plaques were 4-μm-thick paraffin sections mounted on slides, dried for 30 min in an oven (60–70°C), and deparaffinized in xylene. The slides were pretreated with microwave heating as described

previously [31]. After microwave treatment, the sections were washed in PBS. After this, endogenous peroxidase activity was blocked by incubation of the sections in 0.3% H_2O_2 in PBS for 20 min and incubated with 1% BSA for 30 min to block nonspecific staining. Sections were drained and incubated overnight at room temperature in a humidity chamber with the respective rabbit anti-human primary antibody for visfatin (1 : 200) and mouse anti-human primary antibody for CD68 (1 : 50) diluted with antibody diluent (DAKO Corp., Glostrup, Denmark). The ABC complex was applied on the sections after they were incubated with biotinylated secondary antibody. The sections were incubated with DAB substrate-chromogen solution (Dako Corporation, Carpinteria, CA, USA), counterstained with hematoxylin, and mounted in an aqueous mounting medium. Negative control studies were performed with replacement of the primary antibody by nonimmune antiserum and counterstaining with hematoxylin.

2.5. Circulating Leukocytes Visfatin mRNA Expression. Total circulating leukocytes RNA were isolated from the blood of 7 randomized AMI study patients and 6 non-CAD controls using TRIzol reagent. One microgram of each total-tissue RNA was diluted in water in a final volume of 50 μL, and the RNA was reverse transcribed using the high-capacity cDNA archive kit (Applied Biosystems) in a 50 μL reaction mixture. The RT reaction was carried out in the GeneAmp PCR System 9700 thermal cycler in two incubation steps, an initial 25°C incubation for 10 min followed by a final 37°C incubation for 2 h. Real-time PCR analysis was performed using a lightCycler1.5 Instrument (Roche, Mannheim, Germany). PCR was performed in a LightCycler capillary in a 10 μL reaction volume that contained 1x DNA Master SYBR Green I, 2.5 mM $MgCl_2$, 1 μL cDNA, and 0.4 μM primers. The PCR protocol was as follows: initial denaturation for 2 minutes at 95°C, 45 cycles at 95°C for 10 seconds, 60°C for 5 seconds, and 72°C for 12 seconds. Results were analyzed with LightCycler Software, version 3.5.3. Sequence-specific primers for Visfatin were a forward primer, CATAGGAGCATCTGCTCACTT and a reverse primer, GCTGCTGGAACAGAATAGCC. Sequence-specific primers for β-actin were a forward primer, TCCTTCCTGGGCATGGAGTC and a reverse primer, 5′-TTCTGCATCCTGTCGGCAATG-3′.

2.6. Statistical Analysis. The differences of visfatin mRNA expression between acute STEMI and control subjects were analyzed using Nonparametric Wilcoxon test. A P value less than 0.05 was considered statistically significant. The statistical analyses were performed using SAS statistical software, version 8.2 (SAS Institute Inc.; Cary, NC).

3. Results

IHC analysis of visfatin protein in white blood cells (WBCs) showed positive brown staining evident of monocyte (Figure 1(A)) and neutrophil (Figure 1(B)) (magnification, ×1000). Staining was absent in the control section, in which the primary antibody was replaced with nonimmune antiserum (Figure 1(C); magnification, ×400). Figure 2 showed that the intracellular staining for differentiating the different WBC counts by gating based on forward angle light scatter and side angle light scatter analysis. The strong staining on most neutrophil (CD13+) and monocyte (CD14+) from all subjects, but near no staining on T (CD3+) or B (CD19+) lymphocyte.

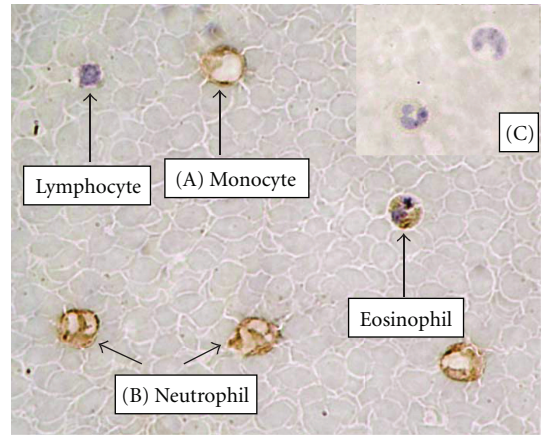


Figure 1: Immunohistochemical analysis of visfatin protein in WBC cells showed positive brown staining evident of monocyte (A) and neutrophil (B) (magnification, ×1000). Staining was absent in the control section, in which the primary antibody was replaced with nonimmune antiserum (C; magnification, ×400).

Furthermore, CD68 immunoreactivity of macrophages in coronary rupture plaques were noted (Figure 3(a); magnification, ×20), and strong visfatin immunoreactivity in atherosclerotic coronary rupture plaques from 7 patients with STEMI were found (Figure 3(c); magnification, ×20). Absence of staining was found in the control section when the primary antibody was replaced with nonimmune antiserum (Figures 3(b) and 3(d)). Furthermore, to further explore the potential role of visfatin in the pathogenesis of acute STEMI, circulating leukocytes with real-time PCR was conducted. Significant visfatin mRNA expression was observed in circulating leukocytes of acute STEMI patients than non-CAD controls ($P = 0.003$) (Figure 4).

4. Discussion

The predictive value of WBC counts in patients with AMI has been extensively reported in recent years [32–34] but rare research has been made regarding any specific subtype of leukocytes with adipocytokines that could be responsible for this detailed association. The present study indicates two major findings. First, we demonstrated that visfatin expressed in monocytes and neutrophils and increased their reactivity in male acute STEMI patients. Second, visfatin strongly appeared in lipid rich coronary rupture plaques and macrophages. These results suggest another explanation about how leukocytes mediated visfatin that may play a pathogenesis role in coronary vulnerable plaques rupture.

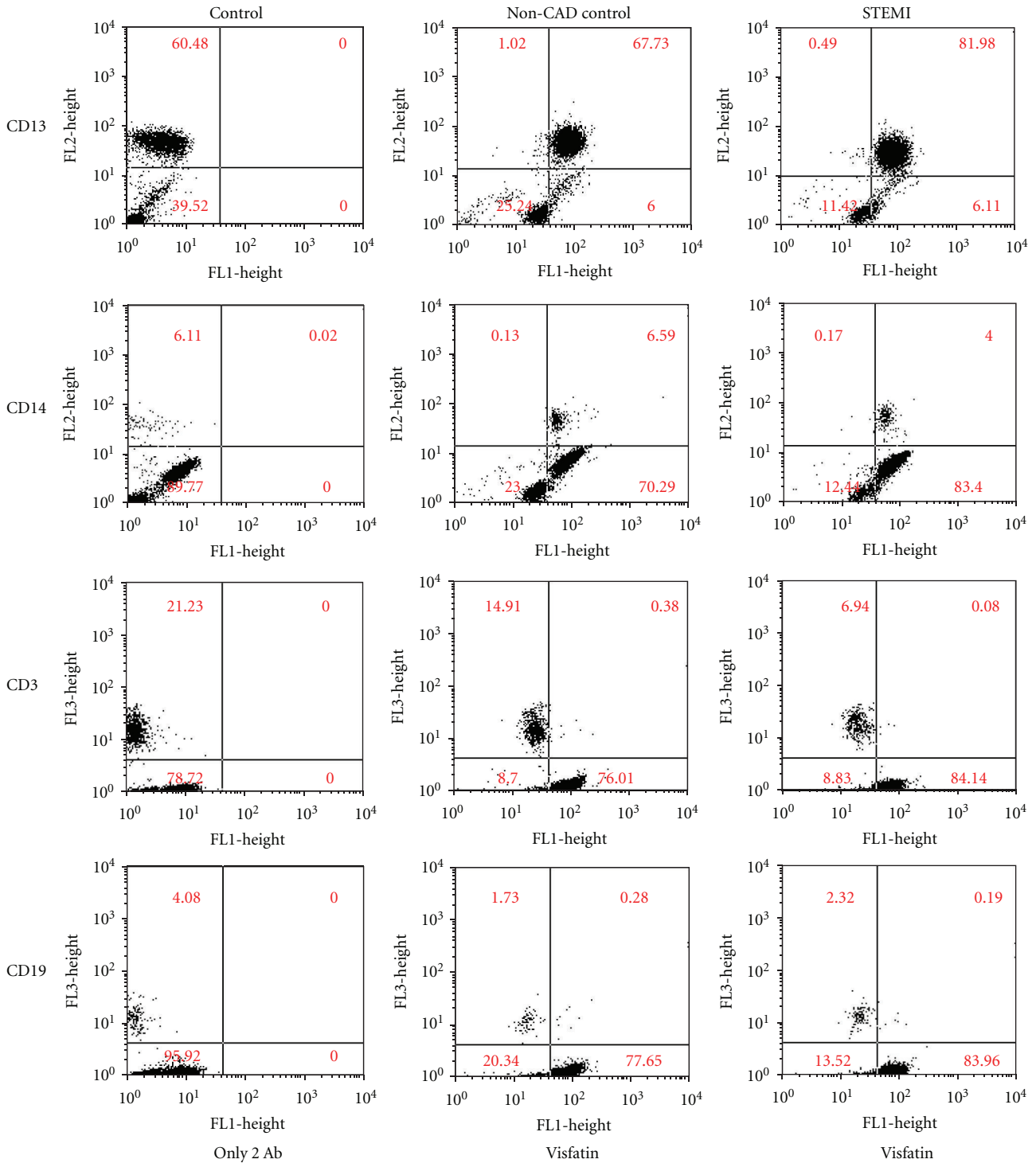
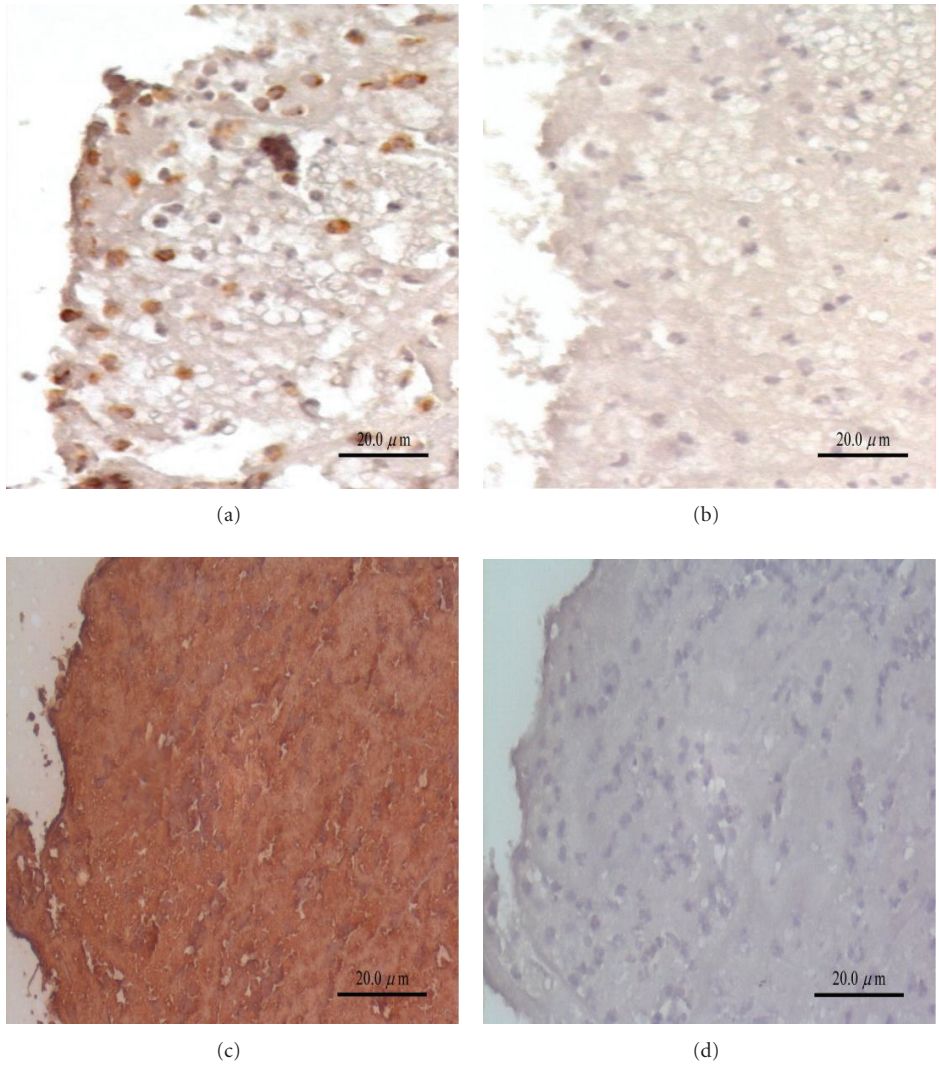


Figure 2: The intracellular staining for differentiating the different WBC counts by gating based on forward angle light scatter and side angle light scatter analysis. The strong staining on most neutrophil (CD13+) and monocyte (CD14+) from all subjects, but near no staining on T (CD3+) or B (CD19+) lymphocyte.

One large study has reported that a high monocyte count in middle-aged healthy men was a predictor of coronary events during the followup [35]. Among the WBC fractions evaluated, only the monocyte count predicted the risk of coronary events. The inflammatory infiltrate process should be considered an independent expression of coronary disease severity in all plaques of AMI. Plaque inflammation has emerged as an obligatory feature in events leading to plaque vulnerability and rupture [36]. Plaque rupture coexists with numerous inflammatory cells and chemokines, mainly from



(a) (b)

(c) (d)

Figure 3: CD68 immunoreactivity of macrophages in coronary rupture plaques were noted (a; magnification, 20x), and strong visfatin immunoreactivity in atherosclerotic coronary rupture plaques in patients with STEMI was found (c; magnification, 20x). Absence of staining was found in the control section when the primary antibody was replaced with nonimmune antiserum (b and d).

macrophage foam cells [37]. Macrophages synthesize and release multiple growth factors, and also secrete metalloproteinases that weaken the fibrous cap and predispose it to rupture which may be the important mechanism of AMI [38]. Recent studies suggest that visfatin may be one of the clinically important cytokines associated with inflammation, atherosclerosis, and the role of plaque destabilization in ACS [23, 24]. It has been demonstrated that visfatin could activate human leukocytes expression of IL-1β, TNF-α, IL-6 [39] and increase monocyte matrix metalloproteinase-9 activity in monocytic THP-1 cells [24]. These mechanisms may partially explain our previous reports about plasma visfatin levels which are associated with IRA occlusion and closely related to the degree of myocardial damage in acute STEMI patients [25, 26]. However, the biological mechanisms involving intraleukocyte visfatin expression in the pathogenesis of STEMI are not well understood.

Previous study demonstrated that visfatin induces the expression of the costimulatory molecules CD80 (B7-1) and CD40 in human monocytes and observed a significant induction of intercellular adhesion molecule-1 (CD54) [39], another costimulatory ligand that binds to lymphocyte function- associated antigen-1, thereby promoting the activation of T-cells [40]. In addition, evidence showed that visfatin affects primary lymphocyte responses was demonstrated by an increased dose-dependent proliferative response after preincubating monocytes with visfatin [41]. In the present study, IHC stain qualified visfatin location and mRNA real-time PCR quantified visfatin expression reveals the partial mechanism of visfatin elevation in acute STEMI. We have demonstrated visfatin is dominantly localized in neutrophil and monocyte by IHC stain and enhanced expression by real-time PCR. Hence, in acute stage of AMI patients, serum visfatin levels elevation, one of the major

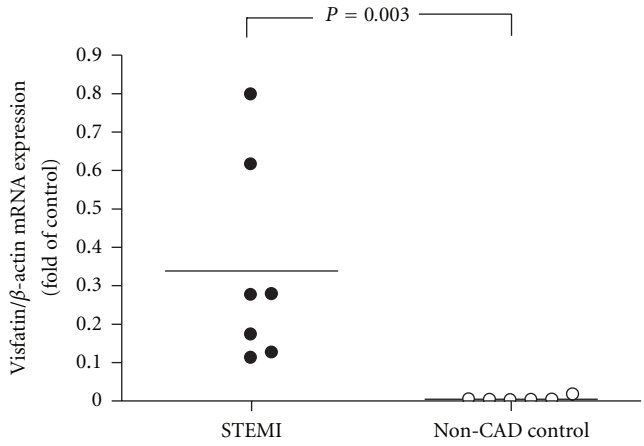


Figure 4: Individual values of visfatin mRNA expression according to disease status, the subjects were classified as having STEMI or being non-CAD controls. Data are expressed as the individual point values and median in relative fold. The horizontal line across individual values represents the median.

sources is from neutrophil and monocyte. It can be explained that in the pre-PCI acute phase of the AMI group subjects, rapid neutrophil and monocyte counts production is correlated with visfatin levels elevated than those of non-AMI controls.

In addition, our results also showed that visfatin was diffusely expressed in the coronary rupture plaques including in lipid core and many infiltrated macrophages besides the destruct endothelium and it was concurred to the previous study [24]. Furthermore, we also demonstrated that the visfatin mRNA expression in circulating leukocytes was significantly increased in acute STEMI patients than non-CAD control subjects. Maden et al. [42] and Barron et al. [43] found that elevated WBC count and activated leucocytes were associated with reducing epicardial blood flow and myocardial perfusion as well as thromboresistance, and playing a role in the pathophysiological process leading to IRA occlusion. On the basis of these observations, we think that inflammatory cells overexpression of visfatin in circulation and coronary rupture plaques may explain the growing body of literature that links inflammation and acute STEMI. We believe that further studies are required to ascertain the role of visfatin in patients presenting with AMI.

The limitation of our study, whether elevated visfatin levels was found with STEMI can be translated directly from intracellular signal over-expression into endothelial dysfunction, vascular inflammation, plaque dysabilization, oxidative stress, coagulant activity increase, still need further efforts to be elucidated.

In conclusion, male patients with STEMI showed increased visfatin expression in leukocytes, which may aggravate the development of instability of atherosclerotic plaques. Therefore, the leukocytes mediated visfatin expression may be a valuable marker for coronary vulnerable plaques rupture and may play a potentially pathological role in STEMI.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

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Review Article

Macrophage-Mediated Inflammation and Disease: A Focus on the Lung

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The lung is exposed to a vast array of inhaled antigens, particulate matter, and pollution. Cells present in the airways must therefore be maintained in a generally suppressive phenotype so that excessive responses to nonserious irritants do not occur; these result in bystander damage to lung architecture, influx of immune cells to the airways, and consequent impairment of gas exchange. To this end, the resident cells of the lung, which are predominantly macrophages, are kept in a dampened state. However, on occasion the suppression fails and these macrophages overreact to antigenic challenge, resulting in release of inflammatory mediators, induction of death of lung epithelial cells, deposition of extracellular matrix, and development of immunopathology. In this paper, we discuss the mechanisms behind this macrophage-mediated pathology, in the context of a number of inflammatory pulmonary disorders.

1. Pulmonary Macrophage Populations

The distinct environment of the lung, with high oxygen tension [1] and constant exposure to inhaled antigen, both harmful and harmless, presents challenges for the immune cells which patrol the airways. The inhaled matter must mostly be ignored, in order to prevent overreaction and subsequent bystander tissue damage in response to nonserious challenges; such a response would fill the alveoli with immune cells and disrupt the delicate gas-exchange process. However, immune cells must be able to respond rapidly to a genuine threat and, once it is dealt with, resolve any resulting inflammation and remodel any damage to the lung tissue. This is a complex list of requirements and so it is not surprising that on occasion the balance between ignorance, response, and resolution tips in the wrong direction, resulting in immunopathology.

In the noninflamed airway, very few haematopoietic cells are present. Of the cells in the fluid from bronchoalveolar lavage (BAL) of naive tissue, alveolar macrophages (AM) (identified as $CD11c^{+}CD11b^{-}$MHC II^{low} autofluorescent cells) constitute >90% [2–4]. As the first cell type to encounter inhaled antigen, AMs are superb phagocytes, rapidly clearing bacteria from the airways [5]. They also help to maintain the dampened immune characteristics of the airways by producing IL-10 [6] and directly suppressing both dendritic cells (DC) and T cells [7–9]. After infection has resolved, they clear the cellular debris remaining [10] and aid in the remodelling of the lung parenchyma. The unique environment of the lung and the constraints on AM result in these pulmonary macrophage populations expressing different surface molecules to those elsewhere in the body; for example, AMs express high CD11c, owing to the high GM-CSF and surfactant protein D levels in the alveoli [11], which is not seen elsewhere, and which may aid the AM in their phagocytic function.

The second and third most common cell types in the airways (and the dominant immune cells in the lung tissue) are DC ($CD11c^{+}$ MHC II^{high}) and inflammatory monocytes ($CD11c^{-}MHCII^{-}CD11b^{+}$) [3]; cells of lymphoid origin are sparse as monocyte-derived cells dominate. In addition to the resident steady-state lung populations, monocytes move

in rapidly after the onset of inflammation. Both resident and immigrating macrophages are implicated in the development of pulmonary immunopathology.

2. Macrophages as Mediators of Lung Pathology

Macrophages are critical in the clearance of pulmonary pathogens [5, 12, 13]. However, in the balance between responding to dangerous inhaled pathogens and in maintaining a healthy airway free of immune cells, macrophages occasionally tip into immunopathology. To avoid this, AMs are kept in check by a variety of mediators in the lung. Surfactant proteins A and D bind to the negative regulator SIRP-α on the AM surface [1]; its signalling induces repression of activation and of their phagocytic function. Alveolar epithelial cells also produce IL-10 [14], which suppresses costimulatory molecule expression by the AM.

The accumulation of myeloid populations in the lung is firmly linked to the development of disease. During early influenza infection, airway epithelial cells produce CCL2 [15] (MCP-1), which attracts $CCR2^+$ monocytes into the lung tissue from the blood [3]. Monocyte numbers peak on day 5 after influenza infection as the cells upregulate CD11c and MHC class II, before differentiating into either macrophages or monocyte-derived DC [3]. During this influenza infection, the majority of immigrating inflammatory cells are from $CCR2^+$ monocytic parents [3]; mice lacking CCR2 not only have decreased accumulation of monocytes after influenza infection but also decreased mortality and lung damage [3, 16, 17], with $CCR2^{-/-}$ mice exhibiting decreased lactate dehydrogenase (LDH, a measure of damaged and therefore leaky epithelium) in the BAL fluid. Ablation of CCR2 signalling also lessens lung damage in mouse models of pulmonary fibrosis and *Mycobacterium tuberculosis* [18, 19].

It is, therefore, clear that monocyte-derived cell types are critical mediators of inflammatory damage to lung tissue. In this paper, we discuss the mechanisms behind this damage in the context of a series of pulmonary inflammatory disorders.

3. Bacterial Infection and Cystic Fibrosis: Inflammatory Cytokine Storm

AMs are critical for the clearance of inhaled bacterial pathogens, which they achieve through phagocytosis, reactive oxygen species production, and the secretion of inflammatory cytokines and chemokines to attract other immune cells to the airways. However, the clearance of these pathogens comes at a heavy price as these inflammatory mediators can themselves lead to bystander damage of lung tissue.

In cystic fibrosis (CF), this inflammatory damage is a severe problem, with 85% of deaths as a result of persistent inflammation triggered by recurrent rounds of bacterial infection, clearance, inflammation, and remodelling [20–22]. AM switching from producing IL-10 to instead secreting a range of inflammatory cytokines (including TNF, IL-1β, IL-6, and IL-8) is well known to be key in the development of CF lung disease [23].

A recent study investigated this inflammatory cytokine production by AM in the response to *Burkholderia cenocepacia* and *Burkholderia multivorans*, which are key pathogens suffered by CF patients that initiate infections which result in overwhelming inflammation, cell death, and sepsis [24]. *B. cenocepacia* can infect AM as well as lung epithelial cells [25] and indeed both AMs of CF patients and of $CFTR^{-/-}$ mice are more permissive to infection and persistence of the bacterium, with the mouse $CFTR^{-/-}$ cells showing delayed phagolysosomal clearance compared to controls [26, 27].

Infection with *B. cenocepacia* results in overwhelming cytokine production by macrophages, in particular TNF [28], IL-8 [29], and IL-1β [2, 30]. This is a result of PI3K/Akt signalling inactivating GSK3β, a downstream repressor of NF-κB, thus allowing enhanced NF-κB activity, and consequent significantly higher cytokine production [26]. This macrophage production of IL-1β is enhanced in the absence of a functional CFTR channel [24] and is caspase-1 and TLR4-dependent. It is this hyperreaction by pulmonary macrophages which triggers the inflammatory storm and induces pathology and severe lung damage.

4. Influenza Infection: TRAIL and Death of Alveolar Epithelial Cells

In 2008, Lohmeyer and colleagues proposed an interesting new mechanism by which macrophages contribute to pathology during influenza infection [16]. It had been noted previously that patients with highly pathogenic influenza virus infection suffered widespread destruction of the respiratory epithelium [31, 32], but how this occurred was not clear. Lohmeyer's group showed that during infection of mice with PR/8 influenza, $CCR2^+$ monocytes which were recruited to the lung and became exudate macrophages were mediating this cell death [16, 33].

On moving into the lung, CCR2-recruited exudate macrophages upregulate TRAIL (TNF-related apoptosis-inducing ligand), with mRNA levels fourfold higher than peripheral blood monocytes in the same infected mice [16]. Correspondingly, although all alveolar epithelial cells express a low level of DR5, the TRAIL receptor, it too was upregulated following influenza infection (Figure 1). Blockade of TRAIL on these cells resulted in lessened alveolar epithelial cell death and alveolar leakage, and improved survival. Interestingly, this TRAIL-induced death was seen in the highly pathogenic PR/8 but not the milder X31 infection, indicating this may be an additional mechanism by which pandemic flu strains damage the lung.

Following these interesting results, a more recent paper showed blockade of CCL2 following influenza infection led to an increase in epithelial damage [34], with this being explained by the reduced numbers of cells leaving the AEC more open to infection, ascribing a protective role only to AM, and indeed a role in repairing the influenza-infected epithelium following clearance of the infection. Indeed, anti-CCL2 treatment resulted in a decrease in hepatocyte growth factor (HGF) present in the lung [34]; HGF augments resolution and repair of damaged epithelium. In this paper, however, the virus used was the sublethal Aichi strain,

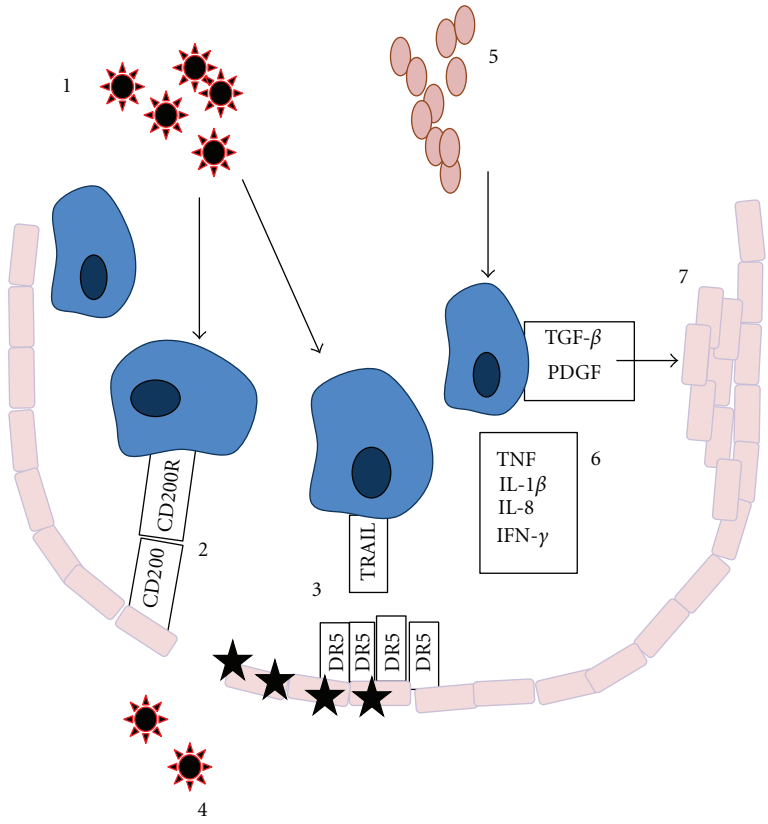

Figure 1: Alveolar macrophage-mediated disease in the lung. AMs are the dominant cell type in the uninfected airway. Following virus infection (1), AMs are restrained from overreacting and inducing bystander damage to tissues by the CD200R on their surface (2) which induces a negative signalling cascade. (3) Pandemic influenza strains induce the upregulation of TRAIL on the AM surface and of its receptor DR5 on the alveolar epithelial cells, which leads to increased apoptosis in the epithelium and consequent morbidity. Both the presence of TRAIL and the absence of CD200R can induce epithelial damage and spread of virus out of the alveolus (4). Bacterial infection (5) induces AM to produce inflammatory cytokines (6), leading to activation of bystander cells and tissue damage. AMs are also triggered to produce growth factors (7) which leads to epithelial hyperplasia, deposition of ECM and fibrosis.

supporting the theory that this AM-mediated cell death is only a factor in highly pathogenic strains.

5. Secondary Bacterial Infections: CD200 and CD200R

We [35] and others [36] have previously described the role of the CD200R on macrophages in the context of influenza infection. CD200 is expressed on airway epithelial cells, T cells, B cells, and some DC [35, 37–40], but has no intracellular signalling motif; its function is to bind to CD200R on myeloid-origin cells and induce a negative signalling cascade. AM express a high basal level of the receptor, allowing them to maintain a strong threshold for response in the normal lung, and prevent bystander tissue damage in the context of inhaled, but nonpathogenic, antigen. $CD200^{-/-}$ mice develop severe immune-mediated lung damage and morbidity following influenza infection [35] as a result of the loss of this "dampening" of the pulmonary macrophage population (Figure 1).

More recent studies with these mice have linked the CD200/R pathway to the development of bacterial pneumonia following a primary influenza infection [41]. Secondary bacterial infections following influenza, particularly pandemic strains, are common and are responsible for sudden development of pneumonia. In the 1918 influenza pandemic, pneumococcal cultures could be grown from the majority of patients [42] and in the decades since then, the link between severe influenza-related pneumonia and secondary bacterial infections has grown stronger.

The role of macrophages and their negative regulation in an influenza—*Streptococcus pneumoniae* coinfection model was investigated [41]. In the absence of CD200R on AM, outgrowth of *S. pneumoniae*, sepsis and death were reduced. It was proposed that following an initial viral infection, an upregulation of CD200 on pulmonary immune cells and production of IL-10 means that the negative signals received by both alveolar and exudate macrophages are greatly increased. This is presumably to allow the lung a period of grace to clear dead cells and to remodel post-infection. However, the "immune rheostat" can swing too far towards repression, resulting in the lung macrophage

populations not reacting to bacterial infection as they ought, and consequent outgrowth and sepsis [41]. As a majority of clinical influenza cases are thought to involve a secondary bacterial infection, this pathway is of significant therapeutic concern.

6. IPF: Production of Arginase and Proinflammatory Cytokines

Idiopathic pulmonary fibrosis (IPF) is a progressive interstitial lung disease, which is proposed to develop as a result of overexuberant remodelling following pulmonary epithelial damage [43], and which is characterised by chronic inflammation, alveolar epithelial hyperplasia, and deposition of extracellular matrix leading to development of a permanent "scar" [19, 44]. There has been much interest in the possible role of viruses in the development of the inflammation; in a mouse model, mice defective in IFN-γR signalling are unable to clear murine herpesvirus infection, and the ensuing chronic infection leads to symptoms very similar to IPF [45, 46].

Recently, a role for macrophages has been delineated in this pathological process. In a model of IPF induced by intratracheal FITC deposition, lung damage was lymphocyte-independent and reduced in $CCR2^{-/-}$ mice [19]. Macrophages are also dominant in the γ-herpesvirus infected mouse lung, and they localise to areas of epithelial hyperplasia and remodelling [47]. CCL2 and CCL3 are produced in the lung in the early stages of inflammatory fibrotic disease [47], attracting macrophages so that they can phagocytose debris, produce matrix metalloproteinases (MMPs) to alter the ECM degradation or alternatively produce ECM components themselves. These useful roles for macrophages are a result of their alternative activation; macrophages activated by Th2 cytokines upregulate a number of genes linked to wound healing, proliferation, and angiogenesis, including the secretion of a number of growth factors and of fibronectin (reviewed in [48]).

The mechanism by which macrophages are pathological in IPF is a result of this alternative activation. Lung fibrosis mediated by γ-herpesvirus is associated with recruitment of macrophages to the lung, their exposure to Th2 cytokines and subsequent alternative activation. Both the damaged lungs and the macrophages within stained positive for arginase 1, in both a mouse model and in patient samples [47]. AAMs have increased arginase activity; its role in converting arginine to ornithine, proline, and polyamine induces proliferation of fibroblasts, collagen production and, subsequently, the development of fibrosis [49].

In addition to arginase, macrophages are a critical factor in the initiation of fibrosis through their production of TNF, IL-6, IL-1β, and TGFβ and of platelet-derived growth factor (PDGF) ([50], reviewed in [44]). These, in particular PDGF and TGFβ, induce the proliferation of myofibroblasts, which secrete collagen [51]. Pulmonary macrophages also secrete MMPs, which degrade the extracellular matrix and so attract more immune cells to the site, perpetuating the inflammatory and remodelling cycle and exacerbating the formation of scar tissue.

7. Summary

In the absence of infection, cautiously reactive alveolar macrophages are the perfect guardians of the immune response in the lung, preventing overreaction to inhaled antigen and maintaining a generally "suppressive" environment. In addition, both alveolar and exudate macrophages are important in the early clearance of pathogens. However, following antigenic challenge, macrophages can tip from protection into immunopathology, with the best features that make them ideal to patrol the airways—cytokine and chemokine production, killing and phagocytosis of infected cells, clearance of debris, and remodelling of the lung—becoming damaging. The untethering of AM constraints and their resulting inflammatory cytokine storm in response to bacterial and viral infection can lead to development of epithelial cell death and consequent septic spread of bacteria to the blood, inappropriate migration of other cell types into the lung, clogging of the airways, deposition of ECM, and dysregulated repair of the damaged tissue. It is for these reasons that the constraining of AM responses during disease remains an attractive therapeutic target.

Abbreviations

AM: Alveolar macrophages
AAM: Alternatively activated macrophages
BAL: Bronchoalveolar lavage
CF: Cystic fibrosis
DC: Dendritic cells
GMCSF: Granulocyte/macrophage colony stimulating factor
HGF: Hepatocyte growth factor
IPF: Idiopathic pulmonary fibrosis
PDGF: Platelet-derived growth factor
MMP: Matrix metalloproteinase
TNF: Tumour necrosis factor
TRAIL: TNF-related apoptosis-inducing ligand.

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Review Article

Tissues Use Resident Dendritic Cells and Macrophages to Maintain Homeostasis and to Regain Homeostasis upon Tissue Injury: The Immunoregulatory Role of Changing Tissue Environments

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Most tissues harbor resident mononuclear phagocytes, that is, dendritic cells and macrophages. A classification that sufficiently covers their phenotypic heterogeneity and plasticity during homeostasis and disease does not yet exist because cell culture-based phenotypes often do not match those found *in vivo*. The plasticity of mononuclear phagocytes becomes obvious during dynamic or complex disease processes. Different data interpretation also originates from different conceptual perspectives. An immune-centric view assumes that a particular priming of phagocytes then causes a particular type of pathology in target tissues, conceptually similar to antigen-specific T-cell priming. A tissue-centric view assumes that changing tissue microenvironments shape the phenotypes of their resident and infiltrating mononuclear phagocytes to fulfill the tissue's need to maintain or regain homeostasis. Here we discuss the latter concept, for example, why different organs host different types of mononuclear phagocytes during homeostasis. We further discuss how injuries alter tissue environments and how this primes mononuclear phagocytes to enforce this particular environment, for example, to support host defense and pathogen clearance, to support the resolution of inflammation, to support epithelial and mesenchymal healing, and to support the resolution of fibrosis to the smallest possible scar. Thus, organ- and disease phase-specific microenvironments determine macrophage and dendritic cell heterogeneity in a temporal and spatial manner, which assures their support to maintain and regain homeostasis in whatever condition. Mononuclear phagocytes contributions to tissue pathologies relate to their central roles in orchestrating all stages of host defense and wound healing, which often become maladaptive processes, especially in sterile and/or diffuse tissue injuries.

1. Introduction

Mononuclear phagocytes are a group of phenotypic distinct members, often referred to as either macrophages or dendritic cells (DC), that derive from myeloid precursors and that contribute to the functions of peripheral tissues [1]. During the last decades, research has focused on the cell-type-specific properties of these cells in culture, which then led to an *immunocentric* view of their role in disease like if they were primed like T cells to infiltrate target organs to cause tissue damage and drive progressive scaring [2, 3]. A more *tissue-centric* view of these processes, claiming that the tissues define phenotype and function of resident and infiltrating immune cells to meet tissues needs during homeostasis and disease, seems provocative [4, 5]. In this paper we apply the *tissue-centric* perspective to discuss the role of resident and infiltrating macrophages and dendritic cells in different organs. We examine tissue needs to maintain homeostasis and how to regain homeostasis upon tissue injury. Furthermore, we discuss how published data supports the view that changing tissue environments induce the well-known different phenotypes of mononuclear phagocytes, a process that not only enforces each of the different environments but also explains the contribution of these cells to the different tissue pathologies. This slightly different perspective may somewhat shape our understanding of macrophage heterogeneity and tissue pathology but certainly also raise new questions for future research.

2. Tissues Need Mononuclear Phagocytes to Maintain Homeostasis

All solid organs and most other tissues harbor a network of DC or macrophages (Table 1). Due to their considerable plasticity and heterogeneity, the tissue-based DC and macrophage populations have been defined as mononuclear phagocytes [1, 6, 7]. These cells provide several important physiological functions during homeostasis (Figure 1). For example, organs like the lung and the liver are exposed to pathogen components from the air or from the gut barrier, respectively, which explains the predominance of a macrophage phenotype that has a higher capacity for phagocytic clearance of pathogen components. The same applies to the bone marrow that requires macrophages for the clearance of the nuclei that get expelled from erythroblasts during their maturation towards erythrocytes [8]. In contrast, the gut mucosa hosts dendritic cells that turn signals from the intestinal flora into the secretion of mitogenic mediators that assist in maintaining an intact epithelial lining of the gut as an important component of the intestinal barrier function [2]. Sterile organs rather harbor dendritic cells. During homeostasis, dendritic cells are sensors and guardians of peripheral tolerance due to their capacity to process self-antigens and signal tolerance to the T-cell pool upon evading the peripheral organs via the lymphatics to reach regional lymph nodes [9]. This functional property constantly assures the quiescence of the immune system in homeostasis. Dendritic cells share certain functions with tissue macrophages such as particle phagocytosis and danger recognition/signaling upon the recognition of pathogens, hence these cells taken together are now referred to as the mononuclear phagocyte system.

Table 1: Resident mononuclear phagocytes in various organs and tissues.

Tissue	Macrophages	Dendritic cells
Skin	Dermal macrophages [10]	Dermal DCs, Langerhans cells [10]
Bone	Osteoclasts [10]	
Bone marrow	Bone marrow macrophages [11]	
Ovary/testis	Ovarian macrophages [12]	
Kidney		Interstitial DCs [7, 13]
Pancreas		Dendritic cell precursors [14]
Spleen	Marginal zone macrophages, red pulp macrophages [10]	iDCs, follicular DCs [15]
Liver	Kupffer cells [10]	Plasmacytoid DCs, cDCs [16]
Colon	Intestinal macrophages [17]	Lamina propria DCs [17]
Ileum	Intestinal macrophages [17]	Lamina propria DCs [17]
Stomach	Intestinal macrophages [17]	Lamina propria DCs [17]
Lung	Alveolar macrophages [10]	
Brain	Microglia [10]	

DCs: dendritic cells.

3. Tissues Need Mononuclear Phagocytes to Fight Threats to Homeostasis

Tissue injury can be traumatic, infectious, toxic, ischemic, or autoimmune to which the tissue responds by a set of evolutionary conserved danger response programs (Figure 2) [18]. Traumatic injury usually involves vascular injury, which immediately activates clotting to control the danger of potentially fatal bleeding. Inflammation is the second danger response program that is needed to avoid pathogen entry to control infections [2]. Pathogens release pathogen-associated molecular patterns (PAMPs), and damaged tissue cells release damage-associated molecular patterns (DAMPs). PAMPs and DAMPs have an identical capacity to ligate Toll-like receptors (TLR) and other pattern recognition receptors on immune and nonimmune cells in the tissue to secrete proinflammatory cytokines and chemokines [19–21] (Figure 2). DAMPs may originate from intracellular sources that get released by cell necrosis, such as histones [22], HMBG1 [23], ATP [24], or uric acid [25]. Furthermore, proinflammatory macrophages release matrix metalloproteinases (MMPs) and hyaluronidase that digest extracellular matrix (ECM) proteins and thereby reduce the ECM viscosity. This process, together with increased vascular permeability, induces tissue swelling, promotes leukocyte migration, and increases the accessibility of surface receptors to their PAMP and DAMP agonists, that is, inflammation. It is of note that ECM digestion produces small ECM peptides and glycosaminoglycans, which can turn into immunostimulatory DAMPs that enhance the local proinflammatory microenvironment [26]. Tamm-Horsfall protein/uromodulin is another example for a compartment-specific DAMP. It is exclusively secreted at the luminal membrane of distal tubular epithelial cells into the urinary compartment of the tubular lumen. During tubular injury, it may lack into the renal interstitium, where it has the capacity to activate intarenal mononuclear phagocytes via TLR4 and the NLRP3 inflammasome [27, 28]. This way, traumatic and infectious injuries induce a PAMP- and DAMP-rich tissue environment that gets reenforced by dendritic cell and macrophage activation (Figures 2 and 3) [29, 30]. Activation of innate immunity subsequently involves the recruitment of additional leukocytes from the circulation including monocytes as well as IFN-γ-secreting NK cells to the injured tissue. When, upon arrival, the infiltrating macrophages get exposed to the PAMP- and/or DAMP-rich environment, hence, this will lead to their full activation towards the proinflammatory macrophage phenotype [20, 31–33]. Polarization to classically activated macrophages requires interferon-related factor (IRF)5 [34]. Such macrophages secrete IL-1, IL-12, IL-23, TNF-α, and ROS and induce iNOS, MHCII^{hi}, and IL1-R, an expression

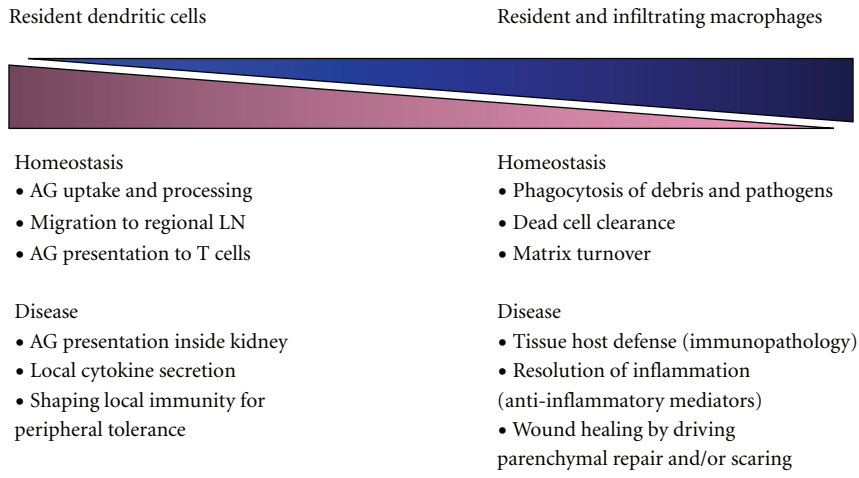


Figure 1: Roles of resident dendritic cells and tissue macrophages in homeostasis and disease. AG: antigen; LN: lymph nodes.

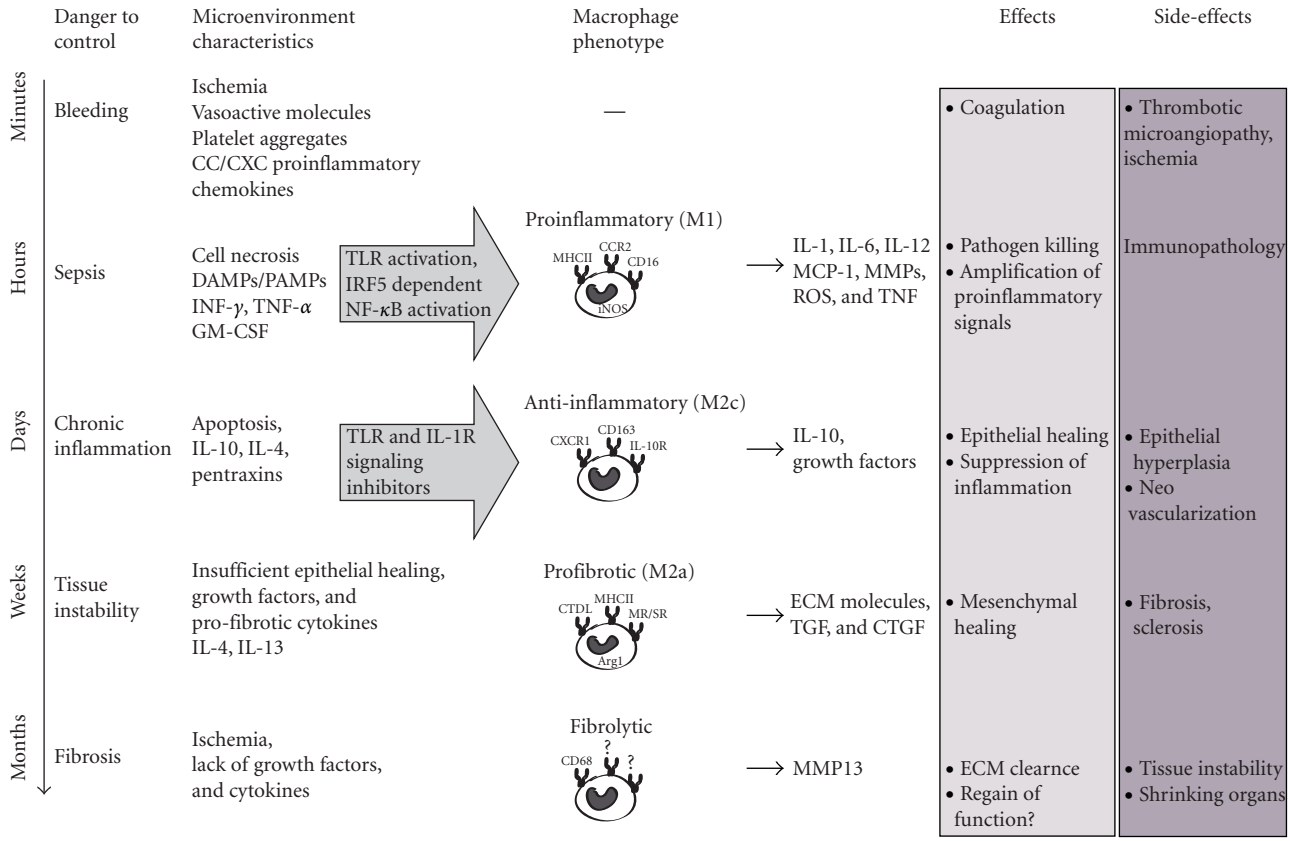
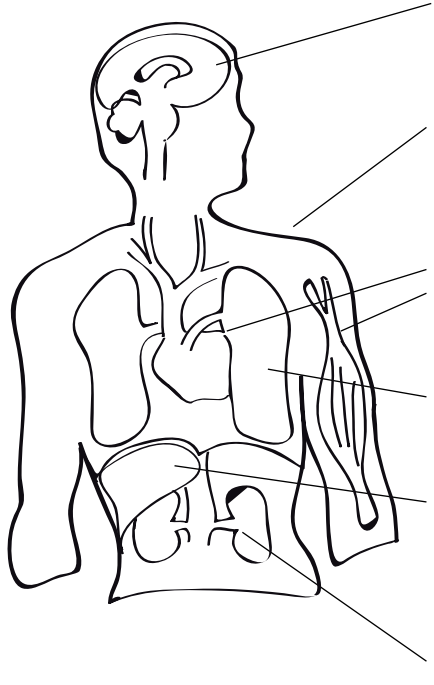


Figure 2: Tissue microenvironments and predominant macrophage phenotypes. Danger control involves several response programs that operate from seconds to months after injury. In each of these phases, the tissue environment shapes the phenotype of resident and infiltrating mononuclear phagocytes, which then enforce the particular environment in a feed-forward loop. Their potential to amplify inflammation, healing, or scaring has consequences on the tissue that may be beneficial or unfortunate in terms of rapidly regaining homeostasis and full function of the organ. This illustrates that the evolutionary programs of danger control are not perfect in all settings, but the fact that they were positively selected during evolution allows only one interpretation: they obviously represented the best compromise between the different needs of multicellular organisms. Where these programs cause malfunction, also mononuclear phagocytes contribute to the "disease" process. TLR: Toll-like receptor, ROS: reactive oxygen species, and ECM: extracellular matrix.



	Environment rich in DAMPs/PAMPs	Environment rich in apoptotic cells and ant inflammatory mediators	Environment rich in growth factors	Environment rich in profibrotic cytokines	Scar tissue
CNS	CCR2+ - M promotes EAE [63, 64], and microglia promotes inflammation after SCI [161]	Ms promote recovery after SCI [108]	CD11b+ cells promote astrogliosis [162], less astrogliosis with M-GSF deficiency [163], IL6-KO [164], microglia blockade [165, 166], and after steroids [167]	?	Ms reverse astrogliosis [168, 169]
Skin	Ms depletion ameliorates inflammation [43]; Ms promote inflammation via osteopontin [170]; iron-overloaded Ms promote inflammation [45]	Prolonged TNF-α signaling after M depletion [171]	Ms promote fibrosis [43, 145, 146, 155]	Fibrocytes promote fibrosis in wounds [172] and after sunburn [173]	TGFβ3 promotes scarless healing
Heart/ muscle	Inflammatory Ms are recruited to skeletal [103] and heart [174] muscle, and blocking CCL2/ CCR2 axis reduces inflammation [175, 176]	CXCR4+ cells reduce myocardial infarct area [100], and Ms suppress inflammation [103] and promote myogenesis after muscle injury [176]	Ms promote fibrosis [174, 177, 178]	Fibrocytes promote fibrosis [129, 178, 179]	Ms promote myocardial remodeling [180]
Lung	Blockade of CCR2 [181] or CXCR3 [182] ameliorates inflammation	?	CCR2-KO ameliorates fibrosis [152], and Ms promote fibrosis via TGFβ	Fibrocytes promote IPF [128], and bleomycin-induced fibrosis [184]	CXCL 10 blocks fibroblast proliferation [160]; CXCL 11 attenuates bleomycin-induced fibrosis [185]
Liver	CCR2+-Ms [148, 186] or iron loaded Ms [187] promote inflammation, Ms are PAMP sensors [188]	After phagocytosis of apoptotic cells [104, 189], Ms block inflammation; Ms limit IRI via IL-10 [105]; Ms limit schistosomiasis [106]	Suppression of macrophage infiltration inhibits fibrogenesis [147]; CCR2+ Ms promote fibrosis [140, 190]; Ms promote fibrosis in response to gadolinium [191] or CCL 4 [148]	Fibrocytes promote fibrosis [127, 192]	CCR2 [190] or Arg1+ [193] Ms reverse fibrosis [157,194], e.g. via MMP-13 [158] or after phagocytosis of apoptotic cells [195]; Ms recruit neutrophils to remove ECM [160]
Kidney	Ms promote inflammation [47, 51, 55, 57, 62, 83, 196]	Ms promote repair [57, 78, 87 90, 95, 99, 101, 197]	Ms promote fibrosis [83, 134, 137, 138, 142, 198, 199]	Fibrocytes promote fibrosis [125, 126, 131, 200, 201]	Ms reverse fibrosis [202–204]

Figure 3: Macrophages in different phases of solid organ pathologies.

profile that was classified as "M1" classically activated macrophage by *in vitro* stimulation with IFN-γ, TNF-α, LPS, or GM-CSF [31]. Polarization towards this bactericidal macrophage type provides the tissue with efficient support for local host defense against pathogens. This potentially life-saving effector functions outweigh the unspecific toxicity of the secreted mediators that can cause significant immunopathology and even transient organ dysfunction (Figure 2) [31, 35].

The danger response program of classically-activated mononuclear phagocyte-driven tissue inflammation for host defense remained evolutionally conserved in sterile solid organ injuries [36–38]. However, DAMP-driven proinflammatory macrophage effects are not needed to kill pathogens and mostly cause unnecessary immunopathology ("collateral damage"). In DAMP-rich but pathogen-free sterile inflammation (ischemic, toxic, or autoimmune injuries), however, this otherwise beneficial response turns into a maladaptive process, with immunopathology that is not balanced by any significant benefit for the tissue [39]. In sterile injuries, the inflammatory phase can be short-lasting, for example, after a transient insult such as a transient ischemia or toxin exposure (Figure 4) [40]. By contrast, inflammation persists upon repetitive or ongoing ischemia or toxin exposure. For example, proton pump inhibitors accelerate gastric and duodenal ulcer healing, also because they reduce persistent acidic damage of the gastric or duodenal mucosa, a process that is required for the resolution of the inflammatory response and for the completion of the wound healing process [41]. As another example, fetal dermal wound healing takes place in a sterile environment without PAMP exposure to the wound. Therefore, much less proinflammatory macrophages are recruited to the site of injury, which, together with the higher regenerative capacity of fetal tissues, explains why fetal wounds heal faster and without scaring [42]. During the early phase of injury, proinflammatory macrophages are entirely dispensable in sterile wounds as their depletion limits the inflammatory response and fastens the healing process [43]. That is why sterile (PAMP-free) wound care is a validated therapeutic strategy to limit the inflammatory response and to enforce healing of surgical wounds or other skin injuries [44]. In addition, in wounds with vascular lesions and subcutaneous bleeding, erythrocyte-derived iron serves as a DAMP that induces the inflammatory macrophage phenotype, which then again suppresses the wound healing process [45, 46].

The uselessness of inflammation in sterile injuries provides the rationale for anti-inflammatory and immunosuppressive treatments. For example, inhibiting the recruitment or activation of proinflammatory mononuclear phagocytes drastically reduces immunopathology and organ malfunction in acute and chronic tissue injuries, for example,

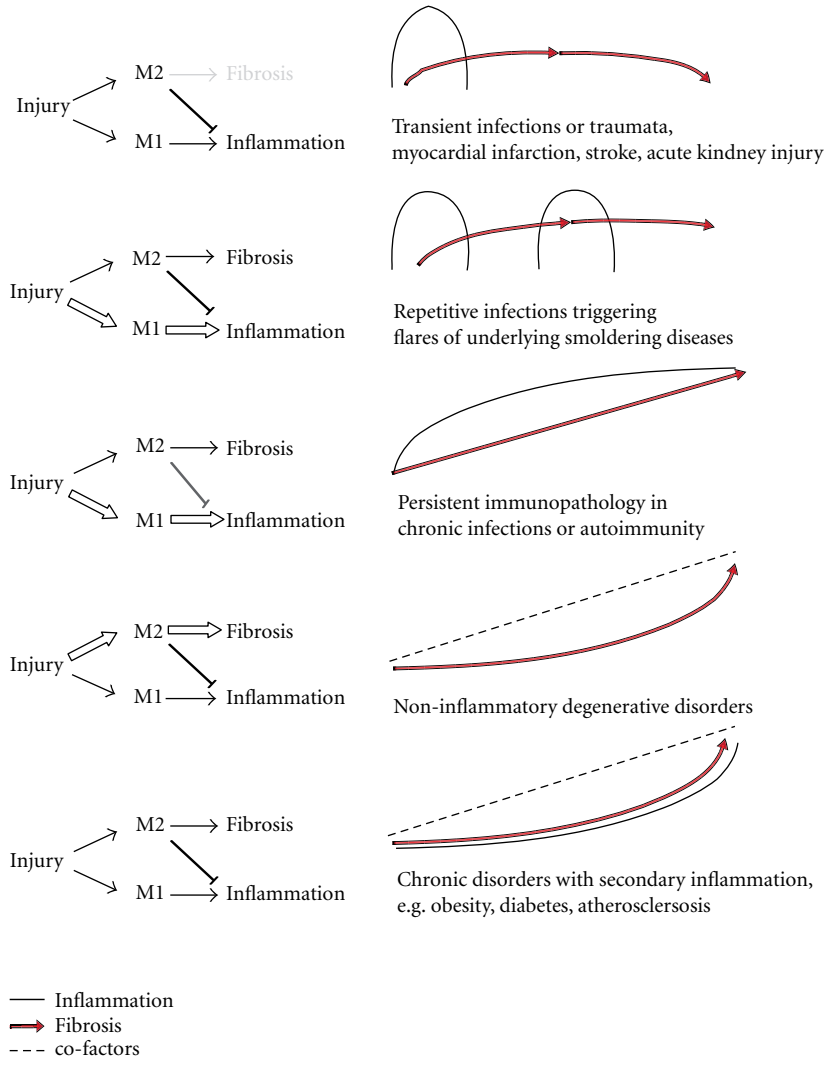


Figure 4: Translating the paradigm of classically activated (M1) and alternatively activated (M2) macrophage into clinical contexts. Classically activated (M1) macrophages promote tissue inflammation and immunopathology based on their role in host defense against intracellular pathogens. Extracellular pathogens are mostly attached by humoral factors such as complement, but when they persist, alternatively activated (M2) macrophages provide means of host defence that involve anti-inflammatory, progenerative, and profibrotic elements. The balance of inflammation and fibrosis varies over time and is different in different disease states and often operates in parallel. For this reasons tissue biopsies often become difficult to read and display a mixture of all these elements. The figure provides examples of common disease entities to illustrate how changing tissue environments involve M1- and M2-macrophages-mediated pathology either in a sequential manner, in an intermittent manner, or in a parallel manner, which largely depends on the associated underlying disease processes and cofactors. We propose that the sequential pattern shown at the top was the one that dominated during the evolution of wound healing from the stage of the first multicellular organisms, for example, healing of mechanical trauma in nonsterile environments. We further propose that all other mixtures that doctors often get to see in pathology textbooks and in their clinics originate from that and represent maladaptive variants of this underlying danger response program that was otherwise extremely successful during evolution.

in a variety of kidney diseases such as in anti-GBM glomerulonephritis [47], lupus nephritis [48–53], antigen-induced immune complex glomerulonephritis [54], renal allograft injury [55], ischemia reperfusion injury [40, 56–58], and adriamycin nephropathy [59]. In addition, environmental factors can aggravate tissue injury by activating mononuclear phagocytes towards a classically activated phenotype [60]. These can be circulating PAMPs, for example, during transient infections (Figures 2 and 4), vaccines or other drugs with distinct immunostimulatory properties. For example, the chemokine antagonists Met-RANTES and AOP-RANTES block monocyte recruitment but still activate resident tissue macrophages, which is sufficient to aggravate preexisting immune complex glomerulonephritis [54]. In contrast, chemokine antagonists without this immunostimulatory side effect were shown to substantially reduce the related immunopathology in multiple disease models of the kidney [61, 62], the CNS [63, 64], the liver, and other noninfectious types of solid organ inflammation as listed in Figure 3.

Dendritic cells support host defense by rather leaving the tissue via the lymphatics to carry foreign antigens to the regional lymph node, which then trigger antigen-specific immune responses and the influx of antigen-specific effector cells that contribute to tissue inflammation. The PAMP-driven innate immunity strongly activates this process in an adjuvant-like manner. Hence, PAMP exposure, for example, during transient infections, can induce the onset or flares of subclinical or chronic autoimmune disorders (Figure 4) [49–54, 65–69].

Together, injuries change the homeostatic tissue towards DAMP- and or PAMP-rich environments which activate resident and infiltrating mononuclear phagocytes. These produce additional immunostimulatory mediators that setup local inflammation, a process that is evolutionally conserved to control invading pathogens. This danger response program is often associated with significant immunopathology, especially in sterile inflammation. This causes unnecessary tissue injury and becomes a maladaptive disease pathomechanism, which provides the rationale for immunosuppressive and anti-inflammatory therapies.

4. Tissues Need Mononuclear Phagocytes to Avoid Excessive Immunopathology and to Orchestrate Repair

Overshooting systemic immune activation holds the risk of death like in early sepsis [70]. Similarly, overshooting organ inflammation holds the risk of acute organ dysfunction like in stroke, myocardial infarction, acute kidney injury, or severe pneumonia. As a consequence, numerous anti-inflammatory mediators provide a balance to immunostimulatory factors, a process that also allows the resolution of inflammation upon pathogen clearance [71–73]. Resolution of inflammation is initiated by a shift in the tissue microenvironment. For example, the early neutrophil influx into a PAMP-rich environment and DAMP release from necrotic cells can change once pathogen control is achieved, so that tissue environments display less PAMPs and DAMPs but become dominated by increasing numbers of apoptotic neutrophils. Macrophage clearance of apoptotic cells is already an important element of peripheral tolerance during homeostasis in healthy tissues, but it becomes an element of the resolution of inflammation in disease [71, 72]. Neutrophil phagocytosis triggers macrophage deactivation and the expression of anti-inflammatory mediators and growth factors that have the potential to stimulate tissue healing [74, 75]. In fact, apoptosis of activated neutrophils and T cells is a mechanism that prevents inappropriate or persistent immunopathology [74]. This also applies to the postinflammatory phase of sterile injuries (Figure 3). For example, transient ischemia reperfusion is associated with cell necrosis and DAMP release followed by the influx of neutrophils and classically activated macrophages for 1–3 days [40]. The excessive phagocytosis of apoptotic neutrophils activates the monocytic phagocytes to release TGF-β and IL-10 [76]. Serum amyloid-P, also named pentraxin-2, opsonizes apoptotic cells which further promotes the anti-inflammatory macrophage phenotype [77]. Infiltrating regulatory T cells also produce IL-10 and TGF-β, which further supports the polarization towards anti-inflammatory macrophages and also suppresses of T effector cells [78]. This deactivation of proinflammatory macrophages involves the transcription factor IRF4, which competes with IRF5, a nonredundant element of TLR and IL-1R signaling [79–82]. IRF4-deficiency does not allow this phenotype switch [80], hence, persistently activated macrophages contribute to ongoing immunopathology [83]. As another mechanism that promotes resolution of tissue injury, tissue dendritic cells produce pentraxin-3, which then blocks P selectin on the luminal surface of vascular endothelial cells, which blocks further immune cell recruitment [84–86].

The current macrophage classifications are derived from decent *in vitro* study conditions that have not yet integrated apoptotic cells as a stimulus of differentiation [31, 87–92]. However, the M2c phenotype of macrophages stimulated with IL-10 and TGF-β display certain characteristics of anti-inflammatory tissue macrophages (Figure 3) [31, 87–92]. The fact that M2c macrophages themselves produce large amounts of IL-10 illustrates how macrophages can amplify their surrounding environments by secreting similar cytokines in a feed-forward loop [93]. These cells are needed to enforce the resolution of inflammation, which is required to tip the balance of host defense and repair towards tissue regeneration (Figure 4). To enhance the regeneration process, anti-inflammatory macrophages acquire a phenotype of growth factor-producing cells that now actively drive epithelial or parenchymal repair. For example, macrophage depletion during the postinflammatory phase of sterile wounds delays wound healing and supports hemorrhage because of a persistent apoptosis of endothelial cells and detachment of the neuroepithelium [43, 94]. In addition, postischemic acute kidney injury involves the phenotypic switch from proinflammatory towards anti-inflammatory macrophages, a process driven by factors released by dying tubular epithelial cells and by the phagocytosis of apoptotic neutrophils [57, 95]. IRF4 or IRAK-M deficiency prevents this phenotype switch, which supports ongoing disease activity in a number of acute and chronic disease states [80, 83, 96–98]. In addition, treatment with recombinant IL-4/IL-10 or genetically modified or transfused IL-10-stimulated macrophages helps to resolve renal inflammation [87–90, 99]. The same phenomenon improves cardiac remodeling after myocardial infarction [100]. Glucocorticoids suppress tissue inflammation by inducing the anti-inflammatory phenotype of tissue macrophages [101, 102]. Monocyte recruitment to skeletal muscle may initially result in a proinflammatory macrophages phenotype, which then rapidly change their phenotype into anti-inflammatory macrophages that assist myogenesis and macrophage depletion that leads to a significantly reduced diameter of regenerating muscle fibers [103]. Toxic liver disease is another example of sterile organ dysfunction. CCl_4 induces hepatocyte apoptosis and subsequent phagocytic clearance by Kupffer cells, a mechanism that suppresses liver inflammation [104]. Ischemia-reperfusion injury of the liver is associated with significant IL-10 expression, which was found to be crucial for the

anti-inflammatory capacity of Kupffer cells [105]. In experimental schistosomiasis, IL-4Rα-deficiency of macrophages was sufficient to cause a lethal septic phenotype [106], which demonstrates the role of anti-inflammatory cytokines produced by alternatively activated macrophages in the gut and the liver, respectively [107]. Finally, axonal regeneration after spinal cord injury depends on the recruitment of IL-10-producing macrophages to the CNS [108].

The anti-inflammatory macrophage phenotype does not only contribute to the resolution of inflammation and the healing phase upon tissue injuries. Also non-necrotic environments of solid tumors induce alternative macrophage activation which then enforces tumor growth [109]. The same applies to degenerative tissue lesions or tissue damage upon slowly accumulating toxins dominated by apoptotic cell death [75].

5. Tissues Need Mononuclear Phagocytes for Effective Scaring When Epithelial or Parenchymal Healing Remains Incomplete

Evolution has maintained tissue scaring for its benefits for the function and survival of organisms. Scaring is necessary in more complex multicellular organisms when traumatic amputation or otherwise significant loss of tissue cannot be rapidly regenerated, a process that requires sealing and mechanical stabilization to assure function and survival. For example, a limited pericyte proliferation can assist vascular stability during regeneration upon injury [110]. However, myofibroblast proliferation and extensive fibrosis offer structural benefits only upon focal wounding and strongly depend on the site or compartment of injury. In diffuse fibrosis of the skin, like in progressive scleroderma, holds the potential to destroy the organ, a functional consequence that applies especially to organs that are commonly affected by diffuse injuries such as the lung, the liver, and the kidney [18, 111, 112]. But instead of taking fibrogenesis as a mechanism of progressive organ, destruction fibrous tissue mainly replaces lost parenchyma; therefore, inhibiting fibrogenesis may not necessarily be able to restore tissue function unless being accompanied by significant regeneration of the parenchyma. Therefore, apart from the healing of tendons, bones, and fasciae, only insufficient healing of epithelial and vascular structures is commonly associated with mesenchymal healing, that is, fibrosis when (1) the damage goes beyond epithelial layer injury, which can occur in some organs like skin, intestinal tract, pancreas and other glands or kidney. Damage to mesenchymal cell structures is more complex and requires more time, for example, in bone, tendons, heart, and skeletal muscle. (2) Local progenitor cells do not survive the injury phase. If at all terminally differentiated cells can divide is questionable and the concept of their dedifferentiation for mitotic repair remains under debate [113–120]. The evolving concept that terminally differentiated cells mostly regenerate from the division of committed local progenitor cells in all organs is appealing and could explain why regeneration remains insufficient when these cells get lost during a severe injury phase or undergo senescence, for example, during aging. (3) Repair is compromised by ongoing PAMP or DAMP exposure like during local infection or by persistent or remitting injuries that impair the repair process by persistent inflammation (Figure 4) [103].

An insufficient repair creates a microenvironment that becomes dominated by the persistent expression of multiple profibrotic cytokines [44, 94, 121]. In such environments, mononuclear phagocytes become a major source of profibrotic cytokines [3]. *In vitro*, IL-4 and IL-13 induce STAT6 signaling, which induces a macrophage phenotype that predominately releases fibronectin and other ECM molecules and that expresses mannose and scavenger receptors, IL-1R11, FIZZ, and YM-1, that is, M2a macrophages [31]. It remains to be determined whether anti-inflammatory and profibrotic macrophages clearly represent two different types of cells also *in vivo*, because macrophage plasticity usually creates a mixture or continuous variant shifts during tissue remodeling (Figure 4) [35]. However, a pro-fibrogenic phenotype of myeloid cells already exists at the level of circulating monocytes, that is, the fibrocyte that shares phenotypic similarities with monocytes and fibroblasts and that can produce large amounts of collagen [122–126], for example, in the liver [127], the lung [128], the heart [129], and in the kidney [60, 125] (Figure 3). However, their quantitative contribution to tissue scaring has been questioned by GFP lineage tracing of collagen 1α1-producing cells, that found only a minor contribution of fibrocytes to renal fibrogenesis and scaring [112, 130, 131].

Chemokine receptor CCR1 seems to be essential for profibrotic macrophage- and fibrocyte-mediated fibrosis because lack of CCR1 or CCR1 antagonism prevents progressive tissue scaring in many different organs and various types of injuries [12, 132–144]. Macrophages that contribute to dermal fibrosis express CXCR3 [145]. Insufficient macrophage activation in chronic diabetic leg ulcers delays scar formation, which can be restored by administering GM-CSF [146]. Similar mechanisms apply to progressive fibrosis of solid organs (Figure 3). Targeting the MCP-1/CCR2 axis [147, 148] or deficiency in CCR1/CCR5 blocked the recruitment of profibrotic macrophages, which was associated with less liver fibrosis [140] and renal fibrosis [132, 134, 137, 138, 149–151]. In the lung, CCR2 deficiency attenuated bleomycin-induced scaring [152], which was shown to be mediated by IL-13 signaling via IL-13-Rα1 and IL-13-Rα2 to stimulate TGFβ secretion in macrophages [153]. Together, tissues use their resident and infiltrating mononuclear phagocytes to fill the gaps of lost parenchyma, which stabilizes the tissue integrity. This is helpful upon focal injuries but may contribute to tissue loss in diffuse injuries, thus this evolutionally conserved danger control program often becomes a maladaptive disease process, especially when epithelial healing remains insufficient.

6. Tissues Need Fibrolytic Mononuclear Phagocytes to Clear Excess Extracellular Matrix

Progressive lung fibrosis, renal interstitial fibrosis, or liver cirrhosis is characterized by parenchymal cell loss which

gets partially replaced by fibrous tissue. Whether fibrogenesis itself contributes to the loss of parenchymal cells remains under debate [112, 154]. However, it is a matter of fact that even though fibrosis is often associated with advanced disease, it does not always progress to end-stage organ failure [155]. In fact, fibrosis can be a transient process that stabilizes tissue integrity during repair and almost entirely resolves later [44]. For example, dermal wound healing ends in the smallest possible scar, after a skin cut. Evidence for inducible fibrolysis in the skin comes from recombinant TGFβ-3 application in humans as well as preclinical models [156]. TGFβ-3 application prevented excessive proliferation of myofibroblasts and scar formation similar to fetal wound healing [156].

Macrophages are capable of clearing ECM via the secretion of selected MMPs, a process that limits and potentially reverses fibrosis [156]. For example, scar-associated macrophages remove fibrous tissue that accumulates after toxic liver injury by secretion of MMP13 and by recruiting neutrophils to the scar tissue [157–159]. In addition, such "fibrolytic" macrophages secrete CXCL10, which blocks the proliferation of fibroblasts in bleomycin-induced pulmonary fibrosis [160]. Excessive scaring, obviously, increases the physiological capacity of tissue macrophages to break down ECM during homeostasis into a scar tissue-reducing phenotype. Hence fibrolytic macrophages need to be added to the list of functionally important macrophage phenotypes (Figures 2 and 3). Surface markers that clearly identify fibrolytic macrophages remain to be described. One should keep in mind that MMP-secreting macrophages have been reported to contribute to tissue degradation by chopping up basement membranes [160]. Therefore, the fibrolytic macrophage may also rather contribute to tissue atrophy and further reduce the size and function of a shrunken organ, if its presence is not associated with extensive regeneration of de novo parenchyma. In fibrotic livers, however, transfer of bone marrow-derived macrophages was shown to reverse hepatic fibrosis and to improve regeneration and function of the liver [160].

7. Summary and Conclusions

Most tissues host mononuclear phagocytes because they help them to maintain peripheral tolerance. Mononuclear phagocytes provide this support by processing "self" into tolerogenic signals to the immune system (all organs), by removing cell debris (e.g., bone marrow) and incoming pathogen components (e.g., liver), and by turning PAMP recognition into epithelial growth to maintain barriers (e.g., gut). As different tissues have different needs to maintain tolerance, mononuclear phagocytes display very heterogeneous phenotypes already during homeostasis. These phenotypes are a result of the specific environment, which is provided in each organ. Similarly, when tissue injuries alter the organ-specific tissue environment, also the resident as well as the infiltrating myeloid cells will be affected as a result of their plasticity to polarize to different phenotypes in different environments. PAMP- and DAMP-rich (necrotic) environments [161–204] prime proinflammatory monocytic phagocytes for host defense, which, however, involves immunopathology, especially during sterile inflammation. Postinflammatory environments including tumor stroma are dominated by apoptotic cell bodies, which trigger polarization towards anti-inflammatory or tumor-associated macrophages that suppress immunity and support cell growth, which could mean epithelial healing but also tumor growth. A healing tissue environment, especially during insufficient epithelial healing, is dominated by growth factors that prime macrophages towards a profibrotic phenotype secreting profibrotic cytokines and ECM components. Scar tissue is hypoxic and lacks growth factors, which enable fibrolytic macrophages to predominantly secrete proteases that remove ECM. Together, mononuclear phagocytes are amplifiers of their surrounding environments because the tissue primes macrophages according to its needs to stabilize and to reenforce the current environment. Thus, organ- and disease-phase-specific environments determine the associated macrophage and dendritic cell heterogeneity, which assures their support to maintain and regain tissue homeostasis in whatever condition. Pathogenic roles of these cells in diffuse tissue injuries are related to maladaptive wound healing programs.

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Research Article

Changes in the Monocytic Subsets $CD14^{dim}CD16^{+}$ and $CD14^{++}CD16^{-}$ in Chronic Systolic Heart Failure Patients

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Different monocytic subsets are important in inflammation and tissue remodelling, but although heart failure (HF) is associated with local and systemic inflammation, their roles in HF are yet unknown. We recruited 59 chronic systolic HF patients (aged 58 ± 13 years, 45 males and 14 females) and 29 age-matched controls with no pervious heart disease. Compared to the controls, we found no change in the distribution of the $CD14^{+}CD16^{+}$ monocytic subset, whereas the classical $CD14^{++}CD16^{-}$ subset was decreased by 11% ($P < 0.001$), and the nonclassical $CD14^{dim}CD16^{+}$ subset was expanded by 4% ($P < 0.001$) in HF patients and was inversely associated with severe HF ($P = 0.015$), as assessed by increased end-diastolic dimension (EDD). Compared to the control group, serum TNFα, IL-1β, IL-10, and IL-13 levels were significantly elevated in the HF patients. Specifically, IL-13 levels were positively correlated to the $CD1CD14^{dim}CD16^{+}$ monocytic subset ($r = 0.277$, $P = 0.017$), and intracellular staining of IL-13 demonstrated that some of these monocytes produce the cytokine in HF patients, but not in the controls. We suggest that the inverse association between EDD values and the expansion of $CD14^{dim}CD16^{+}$ monocytes that can produce IL-13 could be explained as a measure to counterbalance adverse remodelling, which is a central process in HF.

1. Introduction

Inflammation plays an important role in the pathogenesis of heart failure (HF) and exerts an effect on its prognosis. Involvement of inflammatory mediators (e.g., cytokines), inflammatory markers [1], and oxidative stress [2–4] is known in HF and is associated with immune/inflammatory activation, myocardial hypertrophy, adverse myocardial remodelling, and increased mortality [5, 6]. Increased circulatory levels of both proinflammatory and anti-inflammatory cytokines, such as IL-6, TNFα, and IL-10, correlate with HF progression, severity, and increased mortality [6, 7]. Among leukocytes, activated monocytes and macrophages are considered a major source of both pro- and anti-inflammatory cytokines.

Although monocytes represent only 5–10% of peripheral blood leukocytes in humans, they play a major role in inflammatory processes and in tissue remodelling. This is due to their ability to phagocytose microorganisms, their products, or endogenous danger-associated molecular patterns (DAMPs) and process and present them to T cells to initiate an adaptive immune response. They also produce reactive oxygen and nitrogen species and secrete myriad of cytokines and growth factors in response to the stimulation. In fact, monocytes are main producers of proinflammatory and some anti-inflammatory cytokines, including TNFα, IL-6, and IL-10 [8]. Since it is not likely that so many diverse functions are carried out by the same cell, it was suggested that monocytes are a heterogeneous population of cells, each with distinct phenotypes and functions [8, 9].

The main markers used to distinguish between humans monocyte subsets are CD14 (part of the lipopolysaccharide receptor) and CD16 (FcγRIII). These markers define three distinct subsets: classical monocytes that express high levels of CD14 and no CD16 ($CD14^{++}CD16^{-}$, also termed $CD14^{+}CD16^{-}$), intermediate monocytes that express intermediate levels of CD14 and CD16 ($CD14^{++}CD16^{+}$ or $CD14^{+}CD16^{+}$), and nonclassical monocytes that express very low levels of CD14 and high levels of CD16 ($CD14^{dim}CD16^{+}$ or $CD14^{-}CD16^{+}$) [8, 10]. The two $CD16^{+}$ subsets were shown to expand in many inflammatory conditions (e.g., cancer, sepsis, and stroke), whereas the $CD14^{++}CD16^{-}$ subsets remained unchanged or even decreased [8, 11–13]. However, the function of all these subsets as cells secreting either pro- or anti-inflammatory cytokines is still controversial. For example, sorted monocytes subsets that were stimulated *ex vivo* with LPS or zymosan, but not with *S. aureus*, increased IL-10 secretion from $CD14^{+}CD16^{+}$ cells, whereas LPS-stimulated $CD14^{dim}CD16^{+}$ cells showed increased secretion of TNFα [11]. In a different study, $CD14^{dim}CD16^{+}$ cells showed patrolling characteristics with weak ability for phagocytosis and low production of ROS and cytokines when challenged by bacterial ligands of toll-like receptors but secreted high amounts of the proinflammatory TNFα and IL-1β cytokines upon stimulation with viral ligands or nucleic acids [14]. Thus, it seems that the monocytic subsets cannot be simply defined as pro- or anti-inflammatory, and their functions may depend on the nature of the stimulus.

Involvement of different monocytes and macrophages subsets has already been described in acute myocardial infarction (AMI), in humans [15, 16], and in a mouse model [17], and the different kinetics of their recruitment to the heart as well as their different receptor expression suggested that they have different roles in healing and remodelling of the myocardium. Accordingly, the purpose of the current study was to characterize possible changes in the distribution of monocyte subsets in patients with chronic systolic HF, and to further explore the potential impact of these subsets on specific key inflammatory cytokines as well as on clinical parameters of HF.

2. Materials and Methods

2.1. Patients. We recruited 59 patients with systolic HF from our out patients clinic: 45 males and 14 females. For comparison, we also recruited a group of 29 age-matched controls: 15 males and 14 females.

Inclusion criteria for HF patients were stage C, New York Heart Association class (NYHA) of 2–4, chronic systolic HF (left ventricular ejection fraction <40% per echocardiogram), and ages 18–90 years. On recruitment, HF patients had to be in their usual clinical stable status with no recent cardiac decompensation. All patients were treated according to the AHA/ACC guidelines, and their characteristics are summarized in Table 1.

Exclusion criteria for the HF group were recent ($\leq$1 month) admission for acute heart failure or acute coronary syndrome, or haemodialysis treatment or known systemic inflammatory disease or recent (<1 month) febrile illness.

The control group consisted of volunteers that were individually evaluated by a board certified cardiologist based on a detailed personal interview of medical history, review of available medical records, and medical treatments. Exclusion criteria for these volunteers were prior history of coronary/structural myocardial disease, systemic inflammatory disease, or a recent febrile illness (<1 month). Of note, history and/or treatment for diabetes mellitus/hypertension were not considered as exclusion criteria for the control group.

In all study participants, (59 HF patients and 29 controls), a single blood sample was drawn in the morning hours for analysis of both monocytes subsets (whole blood) and cytokines (serum sample).

The study conforms to the principles outlined in the Declaration of Helsinki and was approved by the local Helsinki committee of Lady Davis Carmel Medical Centre, and all participants signed a written informed consent prior to their inclusion in the study.

2.2. Monocyte Phenotyping. EDTA anticoagulated whole blood was collected from controls and HF patients. To avoid the activation of monocytes whole blood was used, and red blood cells were lysed with Uti-Lyse reagent (DAKO, Carpinteria, CA, USA) followed by two washes with PBS. Cells were resuspended in RPMI 1640 with 1% FCS and stained with fluorescently labelled monoclonal antibodies (PerCP anti-human CD16 clone 3G8, BioLegend, San Diego, CA, USA; Allophycocyanin (APC)-Alexa Fluor 750 anti-human CD14 clone 61D3, APC anti-human HLA-DR clone LN3, and appropriate isotype controls, eBioscience, San Diego, CA, USA) for 15 minutes at room temperature followed by an additional wash with PBS. In some samples, monocyte subsets were stained for intracellular IL-13 expression using permeabilization buffer and PE-anti-human IL-13 clone 32007 or its isotype control (R&D systems, Minneapolis, MN, USA). After washing, cells were fixed in PBS with 0.1% formaldehyde and were analysed using a LSR-II flow cytometer (BD, Bedford, MA). We used both compensation beads and isotype controls to determine the nonexpressing CD14 and CD16 cells. We first gated on all monocytes and granulocytes by their side and forward scattering and then further gated on the $HLA\text{-}DR^{+}$ monocytes, as described in [18] to ensure the exclusion of $CD16^{+}$ NK cells. The three different monocyte subpopulations were defined according to their expression of CD14 and CD16, as was described before [9, 11, 14, 18].

2.3. Cytokines. Serum cytokines from both patients and controls were measured with commercial ELISA kits for TNFα, IL-1β, IL-10, IL-13, and TGFβ (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions.

2.4. Statistical Analyses. Data were analysed by both the GraphPad Prism 5 program and the SPSS statistical package (version 18). Comparisons of two experimental groups

were carried out using the nonparametric Mann-Whitney test. As many of the cytokines showed very low levels, we transformed the data into categorical variables according to the calculated median values of both controls and HF patients, and then the Pearson chi-square test was used to determine the association between different cytokines and HF. Logistic regression models were used to assess the association between HF and monocyte subsets and specific cytokines, controlled by age and gender. Odds ratios and 95% confidence interval were estimated from the models. Receiver operating characteristic (ROC) curves were used to evaluate the performance of each of the cytokines in classified HF patients. To calculate the correlation between monocyte subsets and the cytokines we used the Pearson or Spearman correlation analyses as appropriate. All P values were two-sided, and statistical significance was defined as $P < 0.05$.

3. Results

3.1. Monocyte Subsets. There was no significant difference in total monocyte percentage between the HF and the control groups (Figure 1(b)). In order to detect more specific changes, we measured differences in specific monocytes subsets, by using flow cytometry analysis based on the recently accepted division of CD14 and CD16 expressing monocytes [8, 10]. Monocytes were separated into three subsets (Figure 1(a)): classical $CD14^{++}CD16^{-}$ (gated in R1 in red), intermediate $CD14^{+}CD16^{+}$ (R2 in green), and nonclassical $CD14^{dim}CD16^{+}$ (R3 in blue) subsets. The majority of circulating monocytes were $CD14^{++}CD16^{-}$ (above 70% of all monocytes), but this subset was reduced in HF patients relative to the healthy controls by 11%, (84.3 ± 1.9, median 86.9 versus 73.5 ± 1.8, median 77.5, $P < 0.0001$, Figure 1(d)). In contrast, the nonclassical $CD14^{dim}CD16^{+}$ subset was significantly expanded in HF patients (mean $9.3 \pm 0.5\%$, median 9.28 versus mean $6.5 \pm 0.98\%$, median 5.26, $P < 0.0002$, Figure 1(e)). The $CD14^{+}CD16^{+}$ subset, however, consisted of only 3-4% of the circulating monocytes and showed no difference between controls and HF patients (3.9 ± 0.76, median 2.58 versus 3.6 ± 0.55, median 2.52, Figure 1(c)). Association of each monocyte subset to the presence of HF disease was evaluated using a logistic regression model and was found significant only for the expansion of the $CD14^{dim}CD16^{+}$ and reduction of the $CD14^{++}CD16^{-}$ subsets (Table 2).

Looking at the HF group, we further investigated the possible association between the expanded $CD14^{dim}CD16^{+}$ or the reduced $CD14^{++}CD16^{-}$ subsets and several parameters of HF severity, including left ventricular end-diastolic dimension (EDD), left ventricular ejection fraction (LVEF), and New York Heart Association (NYHA) class on the day of recruitment (Table 3). Because of the obvious homogeneity of the HF group (symptomatic, systolic, HF patients), we divided the monocytic subsets and the clinical parameters according to their medians. The $CD14^{dim}CD16^{+}$ subset was significantly associated only with inverse EDD values, consistent with less-adverse myocardial remodelling, whereas the $CD14^{++}CD16^{-}$ subset was not significantly associated with any of the clinical parameters. Similar analysis performed on the $CD14^{++}CD16^{-}$ subset revealed no significant association with these HF parameters. In order to further investigate whether the presence of $CD14^{++}CD16^{-}$ impacts the association between $CD14^{dim}CD16^{+}$ and cardiomegaly, we performed chi-square multiple comparisons tests (where a significant P value is considered only <0.01), by dividing the patients into four subgroups, based on combinations of low and high median values for $CD14^{++}CD16^{-}$ and $CD14^{dim}CD16^{+}$. The only significant difference in the association to decreased EDD values was found in the high $CD14^{++}CD16^{-}$/high $CD14^{dim}CD16^{+}$ group, compared to the high $CD14^{++}CD16^{-}$/low $CD14^{dim}CD16^{+}$ ($P = 0.001$). All other comparisons were not significant, including the comparison between low $CD14^{++}CD16^{-}$/high $CD14^{dim}CD16^{+}$ and high $CD14^{++}CD16^{-}$/high $CD14^{dim}CD16^{+}$ subsets ($P = 0.07$). Additionally, we performed a chi-square goodness of fit test, in which we compared the distribution of high $CD14^{++}CD16^{-}$/high $CD14^{dim}CD16^{+}$ to the distribution of the low and high EDD in the high $CD14^{dim}CD16^{+}$ group (without $CD14^{++}CD16^{-}$ subgroup division, Table 3), but no difference was found ($P = 0.16$). Collectively, this means that the only important parameter that affects EDD values is the $CD14^{dim}CD16^{+}$ subset, and the $CD14^{++}CD16^{-}$ values have no effect on the associative protective effect of $CD^{dim}CD16^{+}$ on the heart size.

3.2. Cytokine Expression. We compared the concentrations of 5 key cytokines (TNFα, IL-1β, IL-10, IL-13, and TGFβ) in the sera of controls and HF patients (Table 4). As these cytokines do not have clear cutoff values, we determined cutoff values for each cytokine according to its median value and receiver-operator characteristics (ROC) curve analysis. As demonstrated in Table 4, with the exception of TGFβ, all 4 cytokines were significantly increased in the HF group, compared to the control group.

3.3. Association between Monocyte Subsets and Cytokines. Since monocytes may influence the inflammatory response by secreting both pro- and anti-inflammatory cytokines, we assessed the possible correlation between the two monocytic subsets ($CD14^{dim}CD16^{+}$ and $CD14^{++}CD16^{-}$) and the investigated serum cytokines (TNFα, IL-1β, IL-10, TGFβ, and IL-13) (Table 5). TNFα, IL-1β, and IL-13 were significantly but negatively linked with the classically activated $CD14^{++}CD16^{-}$. In contrast, only IL-13 was positively correlated with the $CD14^{dim}CD16^{+}$ subset. To further explore the relationship between the nonclassical $CD14^{dim}CD16^{+}$ and IL-13, we performed intracellular staining of the monocytes with anti-IL-13 and gated each of the three monocyte subsets defined in Figure 1 to observe their respective ability to produce IL-13. Figure 2 shows that healthy controls did not produce IL-13 (only $1.1 \pm 0.5\%$-positive cells), whereas in HF patients some of the $CD14^{dim}CD16^{+}$ monocytes ($17.7 \pm 2.3\%$ positive cells, the blue histogram) shifted to the right and clearly expressed intracellular IL-13.

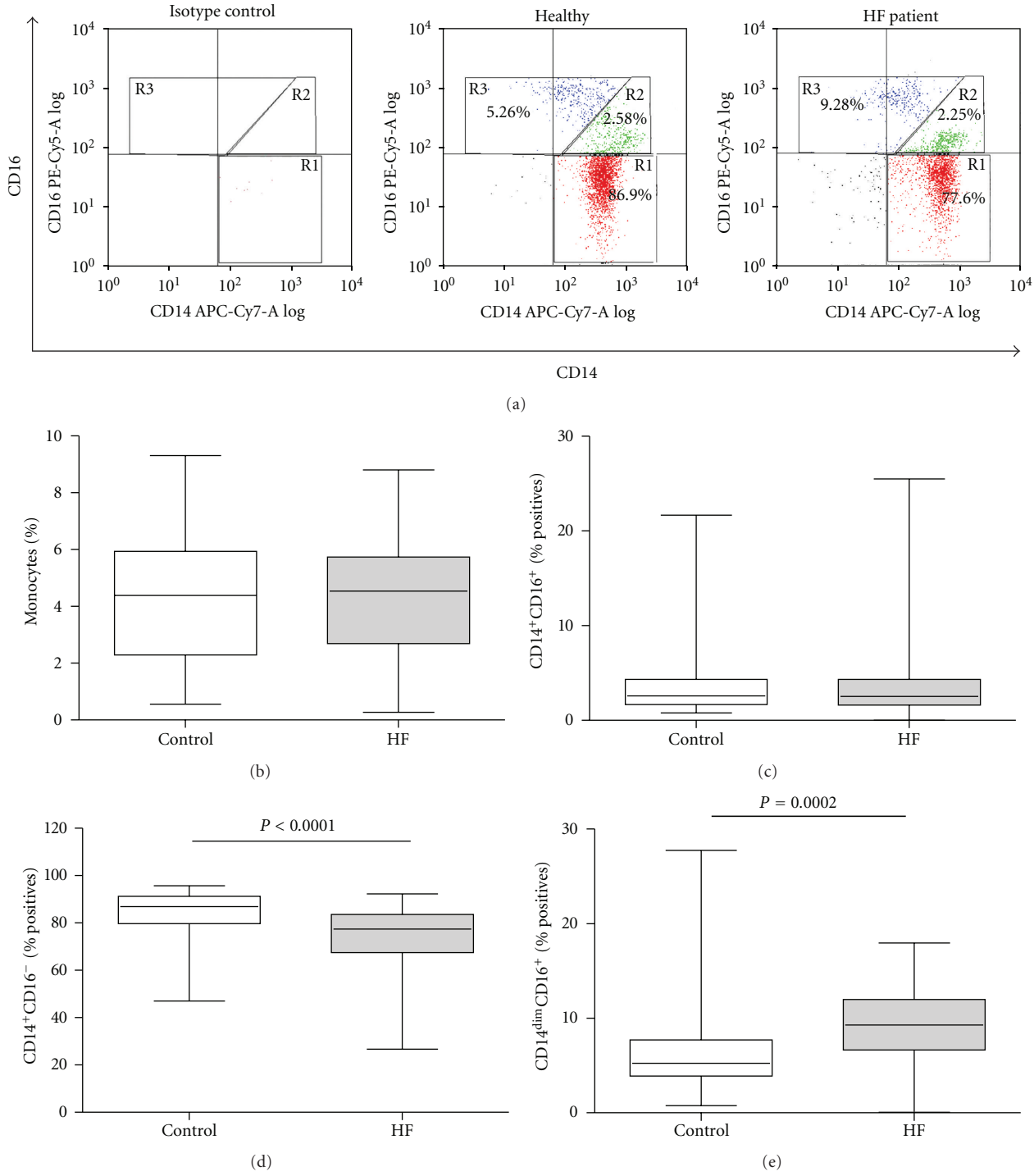

Figure 1: Characterization of monocyte subsets in HF patients and controls. (a) Representative flow cytometry dot plots of monocyte subsets in a healthy control and an HF patient, including the median values of each subset. Monocytes were gated by their side and forward scattering, and (b) their percentage from total blood leukocytes was determined; monocytes that were positive for HLA-DR expression were separated into three subsets according to their expression of CD14 and CD16, and their percentage of the total monocytes was determined in (c) $CD14^{+}CD16^{+}$ monocytes (gated R2 in (a), the green subpopulation), (d) $CD14^{++}CD16^{-}$ monocytes (gated R1 in (a), the red subpopulation), and (e) $CD14^{dim}CD16^{+}$ monocytes (gated R3 in (a), the blue subpopulation).

Table 1: Clinical characteristics of heart failure patients and controls.

Clinical characteristics	HF patients ($n = 59$)	Control ($n = 29$)
Age (years; mean ± sd)	58.1 ± 13.9	59.7 ± 6.4
Sex (male/female)	45/14	15/14
NYHA 1	7	N/A
NYHA 2	39	N/A
NYHA 3	11	N/A
NYHA 4	2	N/A
Ischemic etiology	30 (51%)	N/A
Diabetes mellitus	21 (35.6%)	4 (14%)
β-blockers (n/%)	59 (100%)	2 (7%)
ACE-I +/or ARB (n/%)	56 (95%)	2 (7%)
Aldosterone antagonist (n/%)	18 (30.5%)	None
Statins (n/%)	44 (75%)	N/A
LV ejection fraction (%, SD)	26.29 ± 8.63	N/A
Mean hemoglobin (g/dL, mean ± sd)	*12.9 ± 1.8*	*N/A*
Mean creatinine (mg/dL, mean ± sd)	*1.18 ± 0.06*	*N/A*
Creatinine clearance (CCT, mean ± sd)	*81 ± 4.4 cm/min*	*N/A*

Table 2: Association between the three monocyte subsets and HF.

Monocyte subset	Control mean ± SD (median)	HF patients mean ± SD (median)	O.R.[a]	95% CI	P value
$CD14^{++}CD16^{-}$	84.3 ± 10.63 (86.9)	73.5 ± 14.5 (77.4)	0.894	0.834–0.958	0.001***
$CD14^{+}CD16^{+}$	3.9 ± 4.11 (2.6)	3.6 ± 4.3 (2.5)	1.008	0.902–1.125	0.891^{ns}
$CD14^{dim}CD16^{+}$	6.5 ± 5.3 (5.3)	9.3 ± 4.0 (9.3)	1.179	1.038–1.339	0.011*

[a] Each odds ratio calculated by the logistic regression model is adjusted for age and gender.
*,**,*** Significance, ^{ns}non-significance.

4. Discussion

We found in our current work that, comparing to noncardiac volunteers, chronic systolic HF patients demonstrated significant changes in the distribution of their monocyte subsets. These changes lead to higher serum IL-13 levels and were inversely linked with increased size of the failing heart.

Inflammation has long been associated with HF, with disease progression and adverse outcome [7, 19]. However, the role of the cells responsible for these effects has not yet been fully uncovered. Since CD14 and CD16 expressions were first used to identify different monocyte subsets [20], several reports described a clinically relevant contribution of specific subsets to inflammation and repair, in noncardiac (e.g., asthma [21], infection by the human immunodeficiency virus [22]), and cardiovascular diseases (e.g., AMI and atherosclerosis). The involvement of specific monocyte subsets in tissue repair after AMI and the correct timing of their recruitment to the myocardium, which occurs in two phases, have recently been demonstrated to be critical for successful healing and regaining of normal function, both in humans [16] and mice [17]. After AMI, proinflammatory $CD14^{++}CD16^{-}$ monocytes (or their $Ly6C^{high}$ mouse equivalents) are recruited to the damaged tissue by proinflammatory cytokines and chemokines (e.g., TNFα, IL-1β, and IL-6) during the first phase which lasts about 4 days. This monocyte subset is responsible for the removal of apoptotic myocytes, inflammatory cells, and necrotic cellular debris by phagocytosis, and for the release of proteases (e.g., MMPs, cathepsins, and urokinase plasminogen activator) that degrade the extracellular matrix and facilitate cell movement. The second phase depends on $CD16^{+}$ monocytes or their $Ly6C^{low}$ mouse equivalents (with no clear distinction between human $CD14^{+}CD16^{+}$ and $CD14^{dim}CD16^{+}$ monocytes), which promote angiogenesis through the secretion of VEGF and FGF, recruit myofibroblasts, and deposit collagen and other ECM proteins to form granulation and scar tissues. Thus, insufficient or exaggerated presence of monocyte in

Table 3: Association between the $CD14^{dim}CD16^{+}$ monocytic subset and parameters of HF severity.

Parameter	Cutoff value	$CD14^{dim}CD16^{+} \leq 7.8$ *N*, %	$CD14^{dim}CD16^{+} > 7.8$ *N*, %	O.R.	95% CI	*P* value
EDD	<6	6, 26.1%	21, 58.3%	0.2521	0.0804–0.790	0.015*
	≥6	17, 73.9%	15, 41.7%			
LVEF	<25	9, 15.2%	13, 22.0%	1.137	0.3866–3.346	0.8151
	≥25	14, 23.7%	23, 39.0%			
NYHA	<2	2, 3.3%	5, 8.5%	0.5905	0.1045–3.335	0.5474
	≥2	21, 35.6%	31, 52.5%			

Table 4: Association between serum cytokines and HF.

Cytokine	Cutoff value	Control *N*, (%)	Range (pg/mL)	HF patients *N*, (%)	Range (pg/mL)	O.R.[a]	95% CI	*P* value	AUC (95% CI)
TNFα (pg/mL)	<0	20, 87%	0–2.7	30, 61%	0–276	4.175	1.045–16.68	0.043*	0.720
	≥1	3, 13%		19, 39%					(0.597–0.843)
IL-1β (pg/mL)	<9.8	16, 70%	0–51	21, 41%	0–362	3.390	1.133–10.14	0.029*	0.692
	≥9.8	7, 30%		30, 59%					(0.573–0.811)
IL-10 (pg/mL)	<10	16, 70%	0–18.9	20, 41%	0–236	3.751	1.237–11.38	0.020*	0.733
	≥10	7, 30%		29, 59%					(0.609–0.857)
TGFβ (pg/mL)	<100	10, 43%	28–280	27, 53%	27–619	0.694	0.252–1.91	0.479^{ns}	0.657
	≥100	13, 56%		24, 47%					(0.527–0.788)
IL-13 (pg/mL)	<9	20, 87%	0–34	17, 33%	0–371	14.393	3.48–59.5	<0.001***	0.797
	≥9	3, 13%		34, 64%					(0.685–0.908)

[a] Odds ratio calculated by the logistic regression models is adjusted for age and gender.

the heart, during the first or second phases, may contribute to impaired healing after myocardial damage, leading to myocardial remodelling and eventually to HF [23].

To the best of our knowledge, only two previous studies described changes in the distribution of monocytic subsets in HF patients, with conflicting results. Our finding of increased levels of the nonclassical $CD14^{dim}CD16^{+}$ and reduced levels of the classical $CD14^{++}CD16^{-}$ monocyte subsets in the peripheral blood of HF patients relative to healthy controls is consistent with one of the studies [18], but contradicts the other [24] that demonstrated the expansion of the $CD14^{+}CD16^{+}$ in HF patients, rather than the $CD14^{dim}CD16^{+}$ subset. This indicates that the role monocytes play during HF is only beginning to be explored and that the two subsets that make up the $CD16^{+}$ monocytes population are not homogenous and may have different roles. The possible role of $CD16^{+}$ monocytes was scarcely studied, and only few studies showed the secretion of proinflammatory cytokines from these monocytes, mostly in sepsis [25] or viral stimulation [14]. To the best of our knowledge, the role that $CD14^{dim}CD16^{+}$ subsets play in HF was not yet evaluated. HF is associated with inflammation, endothelial dysfunction, and oxidative stress [26, 27]. However, the inflammatory versus anti-inflammatory roles of both $CD16^{+}$ monocytes (including $CD14^{dim}CD16^{+}$) and the IL-13 cytokine are highly controversial. We observed in our patients significantly increased levels of $CD14^{dim}CD16^{+}$ and reduced levels of $CD14^{++}CD16^{-}$. These $CD14^{dim}CD16^{+}$ cells, a part of the $CD16^{+}$ population ($Ly6C^{low}$ in mice), were associated with beneficial wound healing and decreased adverse remodelling processes in both human and animal models [17, 23]. Moreover, in contrast to $CD14^{++}CD16^{-}$, $CD14^{dim}CD16^{+}$ were shown to express less PSGL-1 and higher CX_3CR1 [18]. PSGL-1 is associated with inflammation and endothelial dysfunction in atherosclerosis [28], which could be prevented by its deficiency [29]. $CD14^{dim}CD16^{+}$, as a part of the $CD16^{+}$ cells, express higher CX_3CR1 than $CD14^{++}CD16^{-}$ cells, which is associated with wound healing and limitation of oxidative stress and inflammation [30, 31]. Thus, the expanded $CD14^{dim}CD16^{+}$ subset can potentially reduce inflammation and endothelial dysfunction. We have further shown that HF patients have increased levels of IL-13 and that this subset can produce the cytokine. IL-13 by itself has anti-inflammatory properties [32, 33]. Collectively, it seems that the increase in $CD14^{dim}CD16^{+}$ subset combined with its ability to produce IL-13 may act as a counterbalance mechanism, designed to slow down the active remodelling process. This may explain the inverse correlation between the increased $CD14^{dim}CD16^{+}$ subset and reduced left heart dilatation as manifested in smaller EDD measurements. Of note, the $CD14^{++}CD16^{-}$ subset had no additional impact on the protective effect of $CD^{dim}CD16^{+}$ cells on the heart size.

Interestingly, previous results [24] showed inverse correlation between the expansion of the $CD14^{+}CD16^{+}$ subset and left ventricular ejection fraction (LVEF) in HF patients. This is compatible with our notion that the two $CD16^{+}$ monocytic subsets may participate in the remodelling process in HF, either to enhance or limit it. The lack of correlation between the $CD14^{dim}CD16^{+}$ subset and LVEF or NYHA in our study may be explained by the relative homogeneous patient population that we recruited to our study, which consisted

Table 5: Correlation between the $CD14^{dim}CD16^{+}$ and $CD14^{++}CD16^{-}$ monocytic subsets and serum cytokines in controls and HF patients.

	TNFα	IL-1β	IL-10	TGFβ	IL-13
$CD14^{++}CD16^{-}$					
r	−0.307	−0.370	0.019	−0.104	−0.412
P	0.009**	0.001***	0.875^{ns}	0.377^{ns}	<0.0001***
$CD14^{dim}CD16^{+}$					
r	−0.037	−0.085	−0.059	−0.096	0.277
P	0.756^{ns}	0.472^{ns}	0.622^{ns}	0.418^{ns}	0.017*

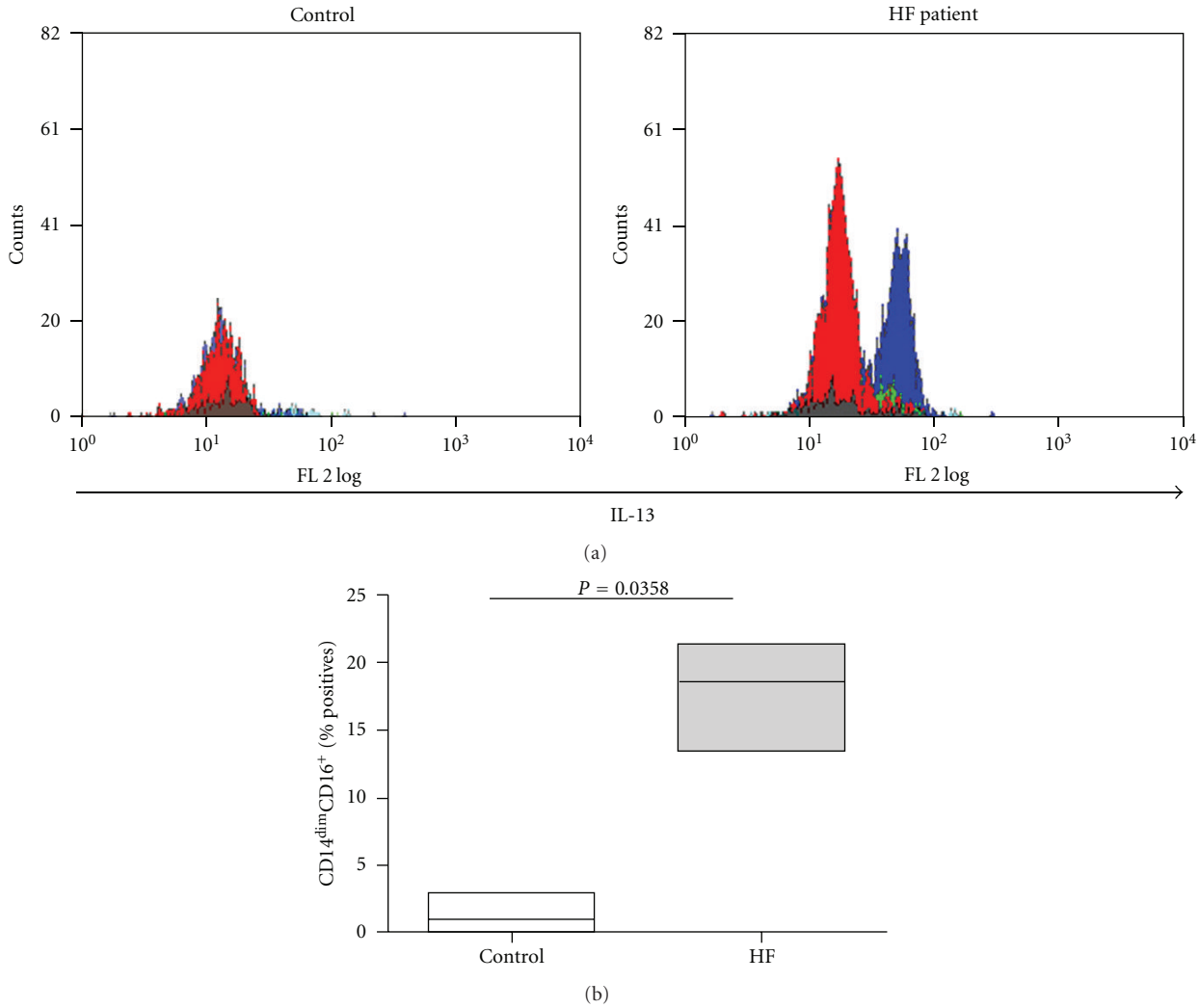

Figure 2: Production of IL-13 by each of the monocyte subsets. (a) Representative flow cytometry histograms of IL-13 producing monocyte subsets in a healthy control and an HF patient. Each of the three monocytes subsets was gated as described in Figure 1, and their respective ability to express IL-13 was evaluated by intracellular staining for the cytokine ($n = 5$). Grey histogram, isotype control for IL-13; red histogram, the $CD14^{++}CD16^{-}$ classical subset; green histogram, the $CD14^{+}CD16^{+}$ subset; blue histogram, the $CD14^{dim}CD16^{+}$ nonclassical subset. (b) Their percentage (median values) from the $CD14^{dim}CD16^{+}$ monocytes was determined.

only of symptomatic, systolic HF patients defined as low LVEF (NYHA 2–4 and LVEF < 40%, resp.) [34].

Monocytes exert their effects partly through the secretion of both pro- and anti-inflammatory cytokines and chemokines, and elevated levels of these were described in HF sera previously [5, 6]. The role of some of these cytokines and chemokines in the development of HF has been described in details elsewhere [35–37]. In accordance with previous studies, we found elevated levels of TNFα, IL-1β, IL-10, and IL-13 in serum of HF patients [38–41], whereas levels of TGFβ remained unchanged. We showed a significant positive correlation between $CD14^{dim}CD16^{+}$ and IL-13, suggesting

a causal link between this anti-inflammatory cytokine and the monocyte subset. To further explore this premise, we stained the monocyte subsets for intracellular expression of IL-13 and demonstrated directly that the nonclassical subset can produce IL-13 in HF patients, but not in healthy controls. This finding is in agreement with a recent study that also showed increased plasma levels of IL-13 in HF patients, but did not link IL-13 levels to the expansion of one specific monocytic subset. The role of IL-13 in HF was hardly investigated, and only one study showed a negative correlation between IL-13 levels and LVEF values [39]. Furthermore, IL-13 knockout mice exhibit severe cardiomyopathy, impaired cardiac function, and HF [42]. These data imply that although IL-13 is a known mediator of tissue fibrosis and remodelling in several diseases [43, 44], its role in the remodelling process in HF may be more complex and even protective.

To the best of our knowledge, our study is the first to suggest that IL-13 production may be directly linked to the specific $CD14^{dim}CD16^{+}$ monocyte subset in HF patients. We suggest that this cytokine and the nonclassical $CD14^{dim}CD16^{-}$ monocyte subset that produces it may be important in the counterbalance systems aiming to slow the remodelling process which is so central in the pathogenesis of HF. This premise, however, requires further experimental support and merits further investigation. The hallmark of HF pathogenesis is the remodeling process. Thus, interference with the process of remodeling could potentially affect HF disease onset and progression. Our research now identifies two new targets, the $CD14^{dim}CD16^{+}$ subset and the IL-13 cytokine they produce, whose manipulation could potentially slow down the remodeling process and its deleterious clinical consequences. In addition, these two parameters (i.e., $CD14^{dim}CD16^{+}$ levels and IL-13 concentrations) may be used as novel biomarkers for HF patients' restratification according to the severity of their disease. However, all these potential clinical implications require further investigation.

Conflict of Interests

The authors declare that there is no conflict of interests.

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Clinical Study

Increased Toll-Like Receptor 2 Expression in Peptidoglycan-Treated Blood Monocytes Is Associated with Insulin Resistance in Patients with Nondiabetic Rheumatoid Arthritis

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The close relationship between increased TLR-2 expression in blood monocytes and insulin resistance in RA patients is shown in this study. Traditional risk factors for metabolic disorders, including the waist circumstance, body mass index (BMI), triglyceride (TG), and ratio of TG to high density lipoprotein (HDL) cholesterol, were closely correlated with HOMA (homoeostasis model assessment) index in patients with nondiabetic RA. Expressions of TLR2 in peripheral blood monocytes, following stimulation with peptidoglycan which is known as a TLR2 agonist, were closely correlated with the HOMA index, TNF-α, and IL-6 concentrations. Accordingly, TLR-2 receptor and its related inflammatory cytokines could be potential therapeutic targets in managing insulin resistance in RA patients.

1. Introduction

Rheumatoid arthritis (RA) is a complex disease whose pathogenesis remains unknown. Patients with RA have systemic inflammation, as well as increased morbidity and mortality from cardiovascular disease [1, 2]. Various risk factors have been identified that contribute to atherosclerosis in RA patients [3, 4]. Insulin resistance is the most important of these risk factors. Recent years have seen increased attention devoted to inflammation associated insulin resistance [5]. Increasing numbers of research studies illuminate several possible mechanisms, including activation of innate system.

Toll-like receptors (TLRs), as key molecular components of the innate immune system, are of a central interest in innate system associated insulin resistance. Twelve members of TLRs have been identified in mammals [6].

The predominant site of TLRs expression is on cells of the innate system, particularly on monocytes [7]. Monocytes are involved in inflammation and the development of insulin resistance [8–10]. The TLRs recognize numerous ligands; for example, TLR2 and TLR4 were hypothesized to recognize components of the bacterial cell wall such as peptidoglycan and lipopolysaccharide, respectively, and to interact with lipid-containing molecules [11]. TLRs participate in the pathogenesis of insulin resistance in animal models [12, 13] and mediate vascular inflammation and insulin resistance in diet-induced obesity [14]. Additionally, TLRs can link innate immunity and fatty acid-induced insulin resistance [12]. Activation of TLRs in adipocyte has been implicated in the onset of insulin resistance in obesity and type-2 diabetes [15]. Raised TLR expression and signaling were also observed in muscles of insulin resistant individuals [16]. High glucose can induce TLR2 and TLR4 expression, activity, and inflammation via nuclear factor (NF)-κB [17]. Dasu and Jialal further reported that free fatty acids in the presence of high glucose may amplify monocyte inflammation through TLRs [18]. Saturated fatty acid may serve as a ligand for several members of the TLR family [19, 20]. Furthermore, TLR2, following ligation with specific ligands, can activate signal transduction, promote interleukin (IL)-6 production, and mediate initial events related to fatty acid-induced insulin resistance in muscle [21]. Tumor necrosis factor-alpha (TNF-α) stimulation was also suggested to disrupt insulin signal transduction and induce insulin resistance [22]. Additionally, the inflammatory kinase I-kappa-B kinase-β (Ik$\kappa\beta$) contributes to insulin resistance by activating NF-κB and induces production of various inflammatory cytokines, including TNF-α and IL-6 [23, 24]. TLRs thus might modulate inflammation and insulin resistance following ligation with specific ligands. Although numerous studies have demonstrated that TLR2- and TLR4-dependent signaling are involved in the development of insulin resistance, data on a similar mechanism in the pathogenesis of insulin resistance in RA patients are still lacking.

TLRs activation has been described as being involved in the pathogenesis of RA, and both TLR2 and TLR4 are potentially important receptors in the initiation and perpetuation of the inflammatory cycle in arthritis [25]. TLRs are present on tissue synoviocytes and blood monocytes which are recruited to the site of inflammation and involved in the pathogenesis of synovial inflammation [26–29]. Thus, TLRs-inducing inflammatory cascades potentially can contribute to the pathogenesis of insulin resistance in RA patients. However, the evidence of the relationship between TLRs and insulin resistance in RA patients remains rare. This investigation tests the hypothesis that expressions of TLR2 or TLR4 on monocytes and related inflammatory cytokines, such as TNF-α and IL-6, might be associated with insulin resistance in patients with RA. Notably, this study excluded RA patients with diabetes mellitus. TLR2 still plays a role in the development of insulin resistance in patients with RA even in the absence of hyperglycemia, which is a well-known risk factor for metabolic syndrome.

2. Materials and Methods

2.1. Study Design and Subjects. The study population included 30 consecutive RA patients that fulfilled the American College of Rheumatology (ACR) 1987 classification criteria [30] and 10 healthy volunteers. Patients or normal controls with diabetes mellitus were excluded. Written informed consents were obtained from the patients before enrollment. The study was in agreement with the guidelines approved by the Human Research Ethics Committee at our hospital. Demographic data, clinical characteristics, and current medications of the patients were recorded by two independent observers.

2.2. Assessments. Waist circumference was measured at the umbilical level. Overnight fasting blood samples were taken to determine blood glucose, serum insulin level, triglyceride (TG), and cholesterol profiles including total cholesterol, LDL cholesterol, and HDL cholesterol. High sensitivity C-reactive protein and Westergren erythrocyte sedimentation rates were determined at the E-DA Hospital Clinical Laboratory. RA disease activity was measured using the Disease Activity Score in 28 joints. To measure the insulin resistance, the homoeostasis model assessment (HOMA), as described by Matthews et al. [31], was calculated using the formula: ([fasting plasma glucose (mmol/L) $\times$ fasting plasma insulin (μU/mL)]/22.5). Monocyte staining for TLR2 and TLR4 expression was performed on the whole blood of RA patients and healthy controls before and stimulation with peptidoglycan from *Staphylococcus aureus* (10 μg/mL, Sigma-Aldrich, MO, USA) or lipopolysaccharide from *E. coli* O26 : B6 (10 ng/mL, Sigma-Aldrich) as ligands for TLR2 and TLR4. Whole blood specimens were incubated with antibodies against TLR2 (eBioscience, CA, USA) or TLR4 (PE labeled, BioLegend) and anti-CD14 conjugated with FITC (BD Biosciences, CA, USA) for 30 min at room temperature in the Polystyrene Round-Bottom Falcon Tube (BD Biosciences). Appropriate isotype controls were also used. Two mL working 1X BD FACSTM Lysing Solution was added to the reaction tube for 10 min at room temperature to lysis red blood cells, and the excess unbound antibody was then washed with PBS (phosphate-buffer saline). Cells were finally resuspended in PBS and analyzed with a flow cytometer (Becton-Dickinson Immunocytometry Systems, San Jose, CA, USA) by counting 10,000 cells. Expressions of TLRs were calculated as mean fluorescence intensity (MFI) and percentage of $CD14^+$ monocytes expressing TLR2 or TLR4 using WinMDI98 software (BD Biosciences). Whole blood samples were collected from both patients with RA and healthy volunteers via venipuncture into heparin containing tubes and diluted with 1X HBSS buffer at a 1 : 1 ratio. The blood samples were incubated in the presence of 5% CO_2 at 37°C in 24-well plates using 10 μg/mL peptidoglycan or 1 ng/mL lipopolysaccharide or medium alone. The cells were then pelleted via centrifugation (400 $\times$g for 2 min), and the cell-free supernatants were stored at -70°C for cytokine determination. Concentrations of IL-6 and TNF-α of cell supernatants were determined using ELISA kits (Bender MedSystems, CA, USA).

Table 1: Demographic and clinical characteristics of normal controls and RA patients.

Characteristics	Normal controls[b] ($n = 10$)	RA patients[b] ($n = 30$)	P[c]
Demographics			
Age (years)	57 (51–64)	57 (36–82)	0.778
Sex (percentage of females)	90	83	0.632
Cardiovascular risk factors			
Systolic blood pressure (mm Hg)	135 (91–170)	132 (89–170)	0.815
Diastolic blood pressure (mm Hg)	82 (60–105)	80 (51–107)	0.790
BMI[a] (kg/m^2)	24.9 (20.7–31.2)	24.3 (19.3–38.7)	0.373
Cholesterol (mg/dL)	197 (145–297)	196 (122–270)	0.755
Low-density lipoprotein (mg/dL)	111 (63–180)	104 (32–164)	0.719
High-density lipoprotein (mg/dL)	60 (36–80)	62 (35–104)	0.751
Triglycerides (mg/dL)	91 (36–178)	134 (48–566)	0.199
TG to HDL	1.7 (0.6–4.8)	2.6 (0.5–15.3)	0.325
TC to HDL	3.4 (2.3–4.8)	3.3 (2.2–4.8)	0.876
Glucose (mg/dL)	92 (82–104)	90 (66–115)	0.406
HOMA[a]	0.9 (0.3–2.2)	2.1 (0.3–13.0)	0.017
Measures of disease activity			
Disease activity (DAS28[a])	NA[a]	4.9 (3.1–7.4)	NA[a]
Current use of corticosteroids, number (%)	NA[a]	25 (83)	NA[a]
Cumulative corticosteroids dose (gm)	NA[a]	2.7 (0.14–9.6)	NA[a]
Other markers of inflammation			
ESR[a] (mm/h)	14 (3–29)	35 (5–80)	0.002
HSCRP[a] (mg/L)	1.2 (0.2–3.4)	9.0 (1.0–57.5)	0.008

[a]Abbreviations: BMI: body mass index; DAS28: Disease Activity Score in 28 joints; ESR: erythrocyte sedimentation rate; HOMA: homeostasis model assessment; HSCRP: high sensitivity C-reactive protein; NA: not applicable; RA: rheumatoid arthritis.
[b]Data are expressed as mean and range.
[c]$P < 0.05$ was considered statistically significant.

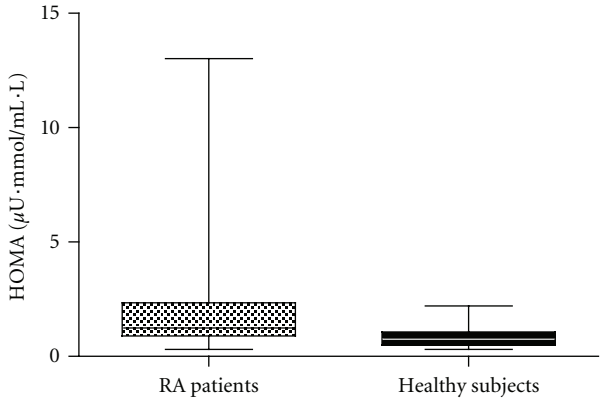

Figure 1: Homeostasis model assessment (HOMA) index in serum samples. Box plot graphs show HOMA index in serum samples from the 30 patients with rheumatoid arthritis (RA) and 10 healthy subjects. The P value was 0.017.

2.3. Statistical Analysis. All analyses were performed using the SPSS statistical software (version 15.0; SPSS Inc., Chicago, IL, USA). Chi-square test or Fisher's exact test, when necessary, was used for categorical values and Wilcoxon's rank sum tests for continuous variables. Spearman's rank correlation was calculated to assess relationships between variables. Only P values less than 0.05 were considered significant.

3. Results

3.1. Demographic and Clinical Characteristics of Normal Controls and RA Patients. A total of 30 patients with RA (25 females and 5 males) aged 36 to 82 years were enrolled and 10 healthy controls aged 51 to 64 years were also recruited. Demographic characteristics, lipid profiles, cardiovascular risk factors, and the HOMA index for RA patients and normal controls are presented in Table 1. Age, sex, systolic blood pressure, diastolic blood pressure, total cholesterol, low-density lipoprotein, high-density lipoprotein, triglycerides, BMI, and blood glucose between groups showed no significant differences. HOMA index was significantly higher in RA patients than in healthy subjects (RA patients versus healthy subjects $P = 0.017$, Figure 1). Eighty-three percent of RA patients are current users of corticosteroid, but the cumulative dose of steroid showed no significant correlation with insulin resistance of these patients ($r = 0.081$, $P = 0.675$). The datum suggests that perhaps

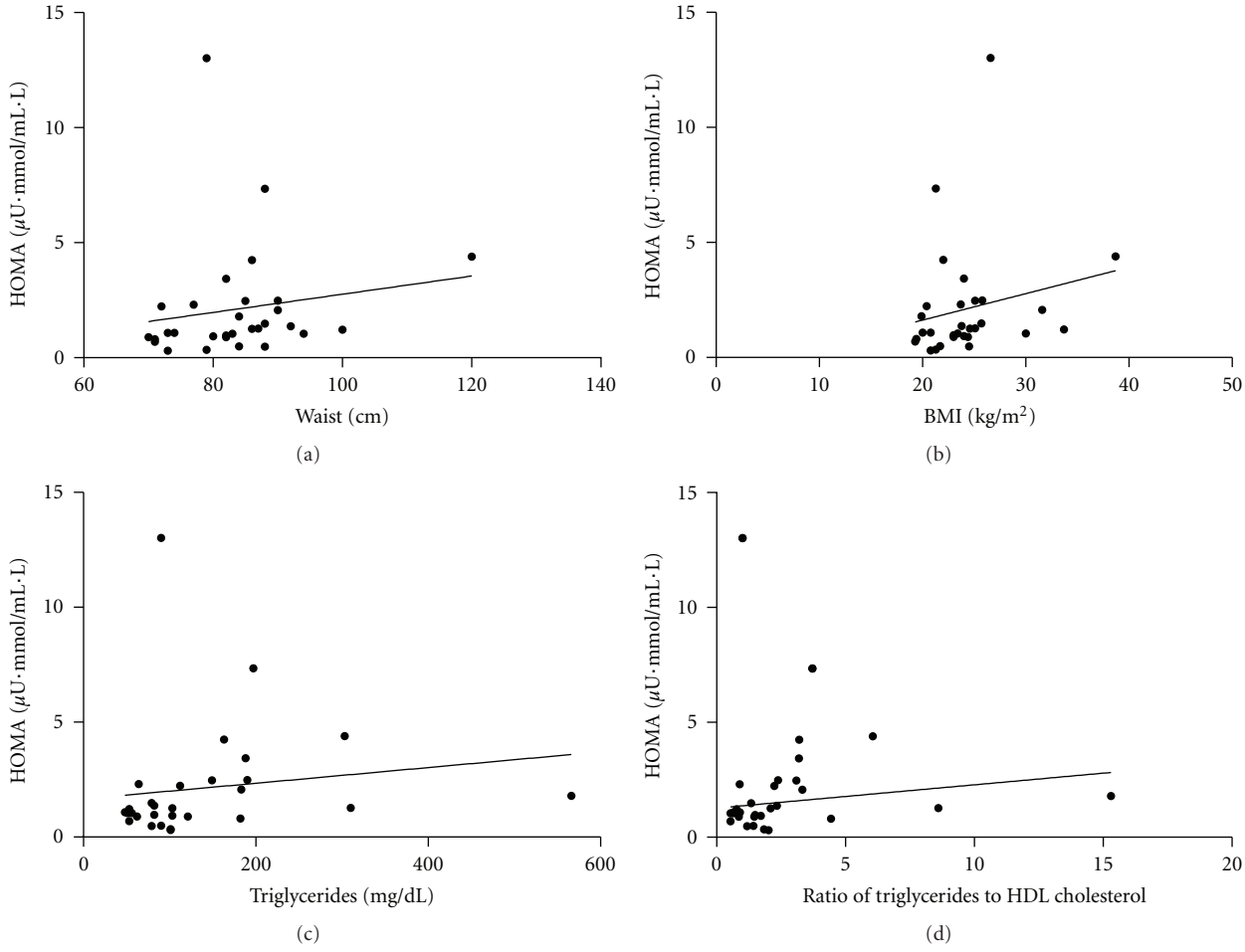

Figure 2: Relationship between HOMA index and waist (a), BMI (b), TG (c), and ratio of TG to HDL cholesterol (d) in patients with nondiabetic RA. In nondiabetic RA patients, HOMA index was significantly correlated with (a) waist ($r = 0.381$, $P = 0.038$), (b) BMI ($r = 0.374$, $P = 0.042$), (c) TG ($r = 0.444$, $P = 0.014$), and (d) ratio of TG to HDL cholesterol ($r = 0.423$, $P = 0.020$).

better control of inflammation after receiving treatment with steroid may counterbalance the deleterious effect of corticosteroids on glucose metabolism. Additionally, as would be expected, HSCRP (high sensitivity C-reactive protein) and ESR (erythrocyte sedimentation rate) revealed significantly higher concentrations in RA patients compared with normal controls ($P = 0.008$ and $P = 0.002$, resp.).

3.2. Relationship between HOMA Index and Waist, BMI, TG, and Ratio of TG to HDL Cholesterol in Patients with Nondiabetic RA. HOMA index was significantly correlated with waist ($r = 0.381$, $P = 0.038$), BMI ($r = 0.374$, $P = 0.042$), TG ($r = 0.444$, $P = 0.014$), and ratio of TG to HDL cholesterol ($r = 0.423$, $P = 0.020$) in nondiabetic RA patients (Figure 2). However, HOMA index was not correlated with total cholesterol ($r = 0.076$, $P = 0.689$) and LDL cholesterol ($r = 0.093$, $P = 0.626$) in RA patients. These results imply that monitoring traditional risk factors might also be important in managing insulin resistance in RA patients, even in the absence of hyperglycemia.

3.3. Relationship between HOMA Index and TLR2 Expression in Monocytes of Normal Controls and RA Patients. As shown in Figure 3, HOMA index was significantly correlated with TLR2 expression (calculated as percentage of $CD14^+$ monocytes expressing TLR2) after stimulation with 10 μg/mL of peptidoglycan ($r = 0.514$, $P = 0.009$, Figure 3(a)), but not with TLR2 expressions before stimulation by peptidoglycan ($r = 0.387$, $P = 0.056$). In contrast, HOMA index was not significantly correlated with TLR2 expression in monocytes after stimulation with 10 μg/mL of peptidoglycan in normal controls ($r = 0.150$, $P = 0.682$, Figure 3(b)). The experimental results revealed no significant correlation between HOMA index and TLR4 expression in monocytes before ($r = 0.288$, $P = 0.162$) or stimulation with 10 ng/mL lipopolysaccharide ($r = 0.230$, $P = 0.268$, Figure 3(c)).

As shown in Figure 4, HOMA index significantly correlated with fold increase of TLR2 expression (calculated as MFI) in monocytes of RA patients after stimulation with 10 μg/mL peptidoglycan ($r = 0.441$, $P = 0.027$, Figure 4(a)), while failing to reveal significant correlation in normal controls ($r = 0.249$, $P = 0.492$, Figure 4(b)). Additionally,

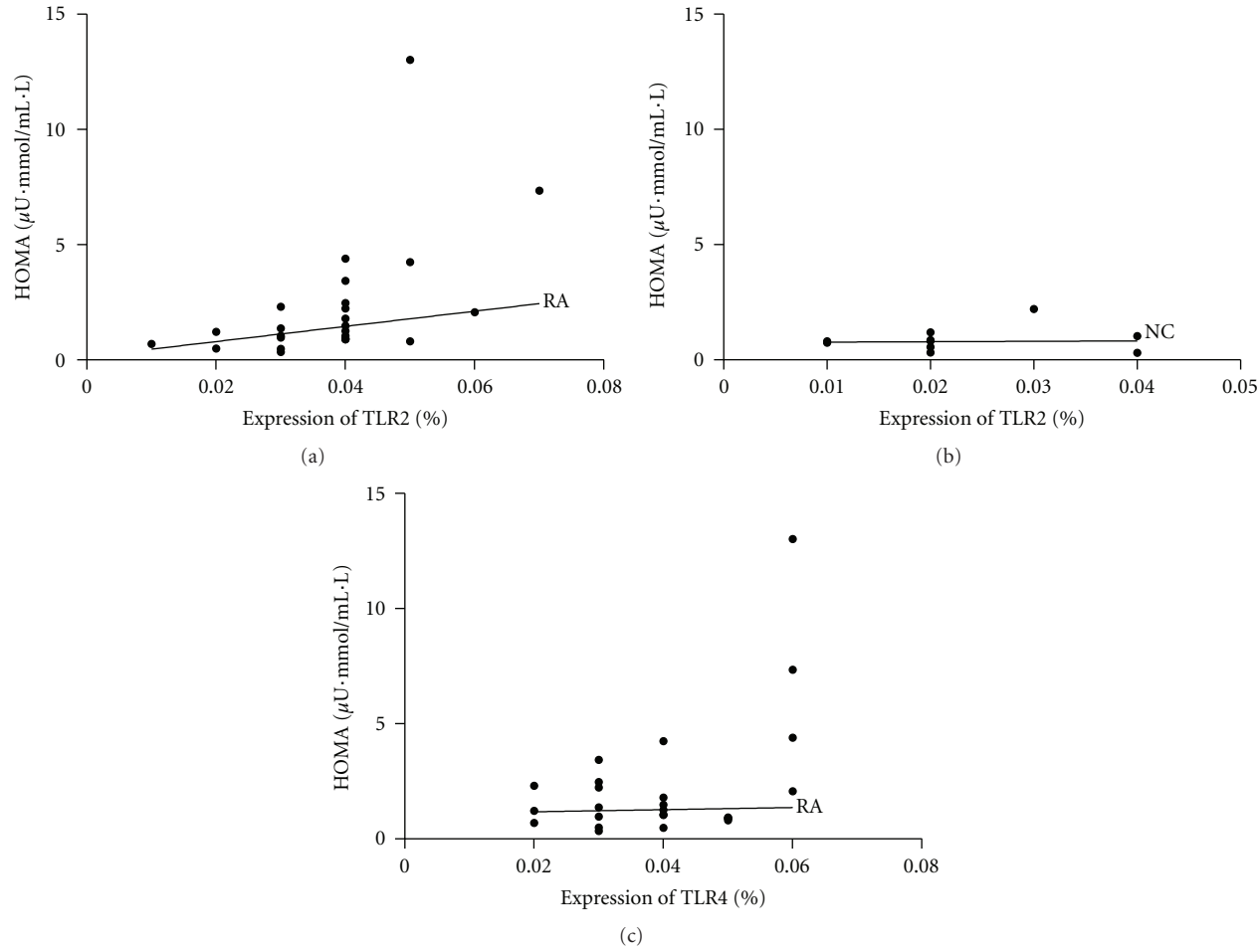

Figure 3: Relationship between HOMA index and TLR2 or TLR4 expression (calculated as percentage of $CD14^+$ monocytes expressing TLR2 or TLR4) in monocytes after stimulation with peptidoglycan or lipopolysaccharide in RA patients and normal controls (NC). (a) Positive correlation between HOMA index and TLR2 expression after stimulation with 10 μg/mL of peptidoglycan in RA patients ($r = 0.514$, $P = 0.009$); (b) no significant correlation between HOMA index and TLR2 expression in normal controls ($r = 0.150$, $P = 0.682$); (c) no significant correlation between HOMA index and TLR4 expression after stimulation with 10 ng/mL of lipopolysaccharide in RA patients ($r = 0.230$, $P = 0.268$).

there was no significant correlation between HOMA index and TLR4 expression (calculated as MFI) after stimulation with 10 ng/mL lipopolysaccharide ($r = 0.057$, $P = 0.787$, Figure 4(c)).

3.4. Relationship between TNF-α Concentration and TLR2 Expression in Monocytes after Stimulation with Peptidoglycan in Normal Controls and RA Patients. As shown in Figure 5, TLR2 expression (calculated as percentage of $CD14^+$ monocytes expressing TLR2) significantly correlated with TNF-α concentration following stimulation with 10 μg/mL of peptidoglycan ($r = 0.484$, $P = 0.014$), while failing to reveal significant correlation in normal controls ($r = 0.469$, $P = 0.172$). In addition, HOMA index was significantly correlated with concentration of TNF-α ($r = 0.433$, $P = 0.019$) in RA patients.

3.5. Relationship between IL-6 Concentration and TLR2 Expression in Monocytes following Stimulation with Peptidoglycan in Normal Controls and RA Patients. As shown in Figure 6, TLR2 expression (calculated as percentage of $CD14^+$ monocytes expressing TLR2) was significantly correlated with IL-6 concentration following stimulation with 10 μg/mL of peptidoglycan ($r = 0.611$, $P = 0.001$) in RA patients, but not significant in healthy volunteers ($r = 0.449$, $P = 0.193$). In addition, HOMA index was significantly correlated with concentration of IL-6 ($r = 0.468$, $P = 0.009$) in RA patients.

4. Discussion

The major finding of the present study is that TLR2 expression, after stimulation with peptidoglycan, showed significant correlation with insulin resistance in patients with

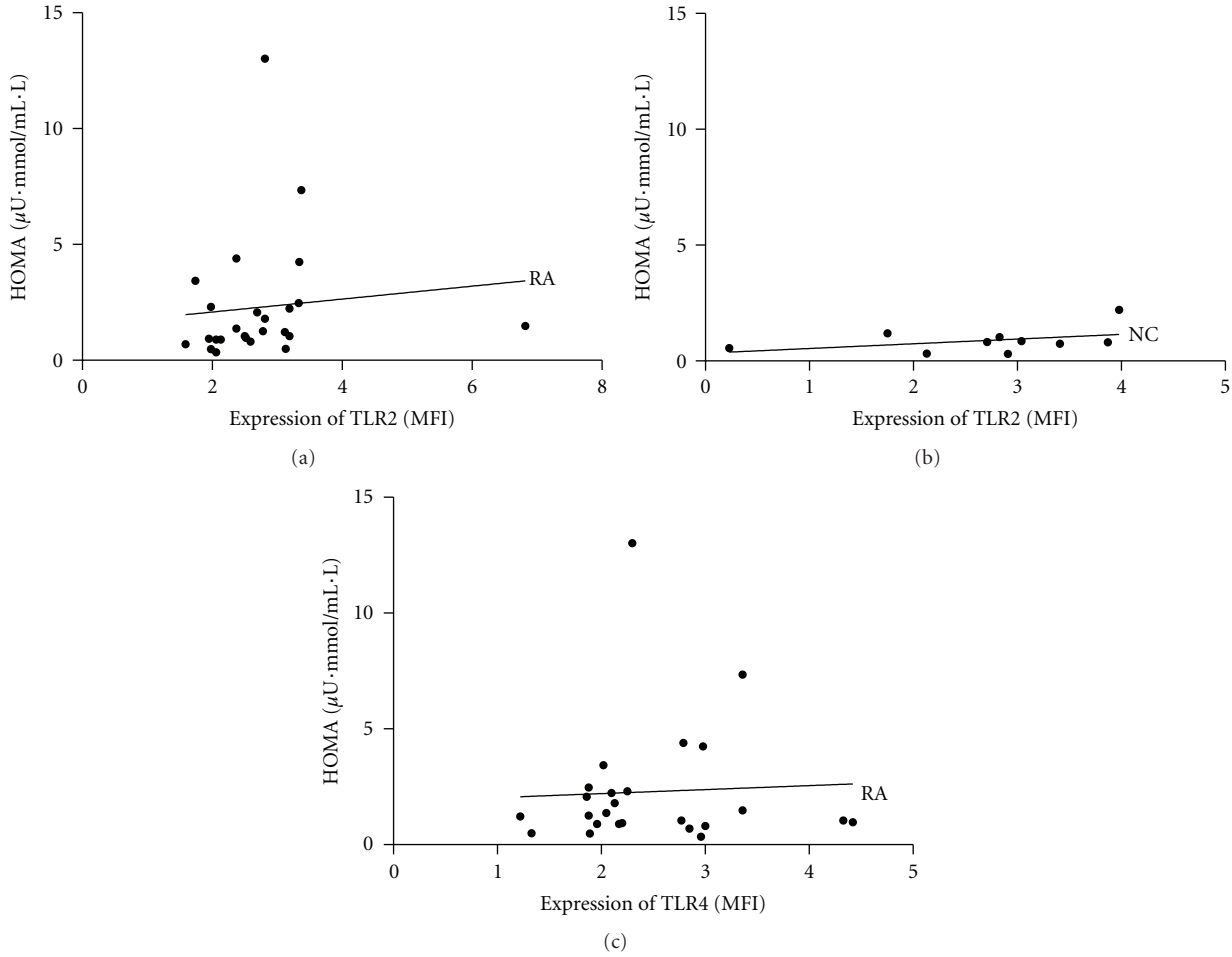

Figure 4: Relationship between HOMA index and fold increase of TLR2 or TLR4 expression (MFI) in monocytes after stimulation with peptidoglycan or lipopolysaccharide in RA patients and normal controls (NC). (a) HOMA index significantly correlated with TLR2 expression after stimulation with 10 μg/mL of peptidoglycan in RA patients ($r = 0.441$, $P = 0.027$); (b) no significant correlation between HOMA index and TLR2 expression in normal controls ($r = 0.249$, $P = 0.492$); (c) no significant correlation between HOMA index and TLR4 expression after stimulation with 10 ng/mL of lipopolysaccharide in RA patients ($r = 0.057$, $P = 0.787$).

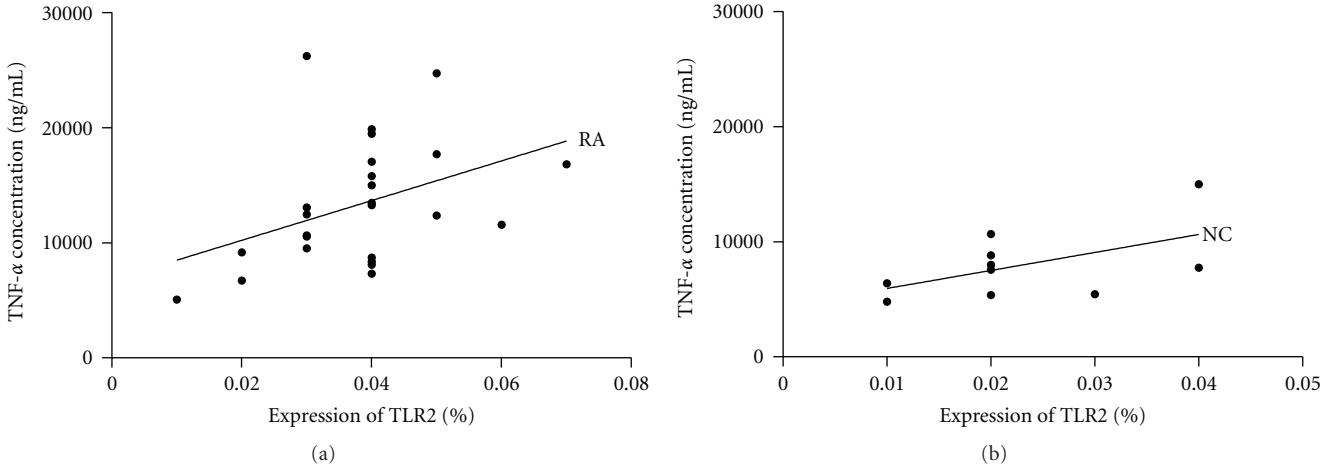

Figure 5: Relationship between TNF-α concentration and TLR2 expression (calculated as percentage of $CD14^+$ monocytes expressing TLR2) in monocytes after stimulation with 10 μg/mL of peptidoglycan in RA patients and normal controls (NC). (a) TLR2 expression significantly correlated with TNF-α concentration in RA patients ($r = 0.484$, $P = 0.014$); (b) no significant correlation in normal controls ($r = 0.469$, $P = 0.172$).

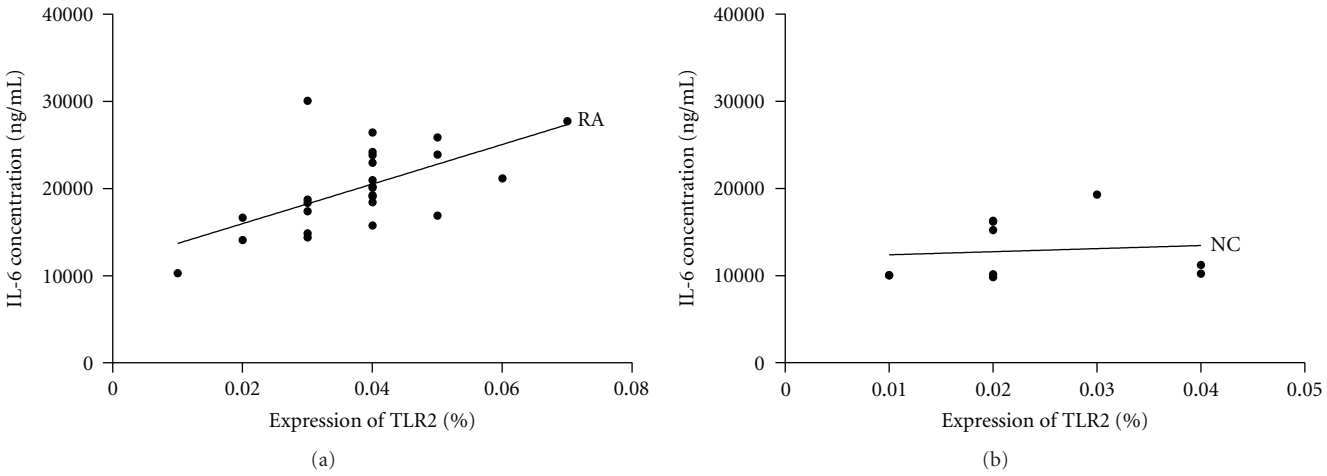

Figure 6: Relationship between IL-6 concentration and TLR2 expression (calculated as percentage of $CD14^+$ monocytes expressing TLR2) in monocytes after stimulation with 10 μg/mL of peptidoglycan in RA patients and normal controls (NC). (a) In RA patients, TLR2 expression was significantly correlated with IL-6 concentration ($r = 0.611$, $P = 0.001$); (b) no correlation in normal controls ($r = 0.449$, $P = 0.193$).

RA in the absence of hyperglycemia. RA is one of the most prevalent autoimmune diseases and affects about 0.5–1% of the adult population. The hallmark of RA is persistent polyarticular synovitis mainly affecting the small joints. Patients with RA have increased morbidity and mortality from cardiovascular disease such as atherosclerosis compared to patients without RA [1]. Both systemic inflammation and insulin resistance are reported to be important players in the development of atherosclerosis [32]. Insulin resistance was considered to contribute to the increased cardiovascular risk in the general population [33, 34]. Inflammation has been identified as fundamental in insulin resistance in patients with RA [5, 35]. The inflammatory pathways can be integrated to cause insulin resistance by activating membrane receptors such as TLRs [12, 36, 37]. TLRs are critical in the recognition of invading pathogens and activation of subsequent immune responses against them. Upon stimulation, TLRs induce the activation of NF-κB and mitogen-activated protein kinases (MAPK) and the expression of inflammatory cytokines [36, 38]. Palmitate treatment of differentiated C2C12 myotubes, through TLR2 activation, led to a time-dependent inhibition of insulin-activated signal transduction [21]. The inhibition of TLR2 expression can rescue cells from the activation of MAPK8 and improve insulin resistance [39]. TLR2 is crucial for diet-induced metabolic syndrome because mice lacking TLR2 are substantially protected from diet-induced adiposity and insulin resistance [40]. Additionally, polymorphisms in the TLR2 receptor gene have been linked to populations at high risk of developing type 2 diabetes [41, 42]. Overall, TLR2 could be a key modulator between inflammatory pathways and metabolic disorders such as insulin resistance.

Numerous factors are involved in the expression and activation of TLR2. Obesity and type 2 diabetes are associated with increased expression of TLR2 [43]. Obesity can induce increased c-Jun N-terminal kinase (JNK) activity [44, 45] which is activated in response to inflammatory cytokines, free fatty acids [22], activated NF-κB, and inflammatory mediators, including TNF-α and IL-6, and may contribute to insulin resistance [23, 24]. Furthermore, raised free fatty acids and TG in obese individuals and animals can be important etiologies of insulin resistance [46]. High glucose induces inflammatory cytokines, chemokines, p38 MAPK, NF-κB activity [47–52], and TLR2 expression [17]. Recently, Dasu and Jialal further indicated that free fatty acids in the presence of high glucose amplify monocyte inflammation via TLRs [18]. Collectively, TLRs inducing inflammatory pathway may contribute significantly to insulin resistance, particularly in the presence of obesity related conditions or high blood sugar. However, rare data exist describing the roles of TLRs in the development of insulin resistance in the absence of raised blood sugar in RA patients. The present study found that TLR2 expression in circulating $CD14^+$ monocytes, after stimulation with peptidoglycan, correlated significantly with HOMA index in nondiabetic RA patients. Additionally, the expression of TLR2, upon stimulation with peptidoglycan, was correlated with levels of TNF-α and IL-6. HOMA index was also significantly correlated with concentration of IL-6 and TNF-α in RA patients. These data implied that TLR2 could contribute to the development of insulin resistance in patients with RA without concurrent hyperglycemia. Additionally, the significant correlations of the HOMA index with TG and ratio of TG to HDL in RA patients are consistent with the previous literature. Waist circumstance and BMI, as parameters measured while evaluating obesity, displayed a significant correlation with HOMA index in this study. Control of obesity thus may also be critical in treating RA patients at a high risk of insulin resistance. Further study is required to elucidate the detailed roles of peptidoglycan or other TLR2 ligands in the occurrence of insulin resistance. This study indicates that body mass index, waist, TG, and ratio of TG to HDL were significantly associated with HOMA index in nondiabetic RA patients. These findings are consistent with previous

investigations showing that insulin resistance is associated with low HDL cholesterol and high TG in patients with inflammatory arthritis [53, 54]. Therefore, the so-called traditional risk factors also need to be closely monitored and carefully treated.

5. Conclusion

The results demonstrating the close relationship between HOMA index and TLR2 expression in monocytes and inflammatory cytokines such as TNF-α and IL-6 could provide therapeutic interventions against insulin resistance in nondiabetic RA patients. Treating traditional factors such as BMI, waist, TG, and ratio of TG to HDL cholesterol is crucial in minimizing the development of insulin resistance in RA patients, even in the absence of hyperglycemia. This study may lead to a better understanding of the relationship between TLR2 expression and insulin resistance in patients with RA. Further investigation is necessary to elucidate the role of TLR2 *in vivo*.

Abbreviations

ESR: Erythrocyte sedimentation rate
HOMA: Homoeostasis model assessment
HSCRP: High sensitivity C-reactive protein
Ik$\kappa\beta$: Inflammatory kinase I-kappa-B kinase-β
IL-6: Interleukin-6
JNK: Jun N-terminal kinase
MAPK: Mitogen-activated protein kinases
MFI: Mean fluorescence intensity
NF-κB: Nuclear factor-κB
PBS: Phosphate-buffer saline
RA: Rheumatoid arthritis
TG: Triglyceride
TLR: Toll-like receptor
TNF-α: Tumor necrosis factor-alpha.

Conflict of Interests

The authors declare that they have no competing interests.

Authors' Contribution

S.-W. Wang conducted most of the experiments and analysis of data. T.-M. Lin and C.-H. Wang performed some of the experiments. H.-H. Liu participated in the study design. J.-Y. Houng supervised the research. All authors read and approved the final paper.

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