Journal of Immunology Research

Macrophage Functions and Regulation: Roles in Diseases and Implications in Therapeutics

Lead Guest Editor: Kebin Hu
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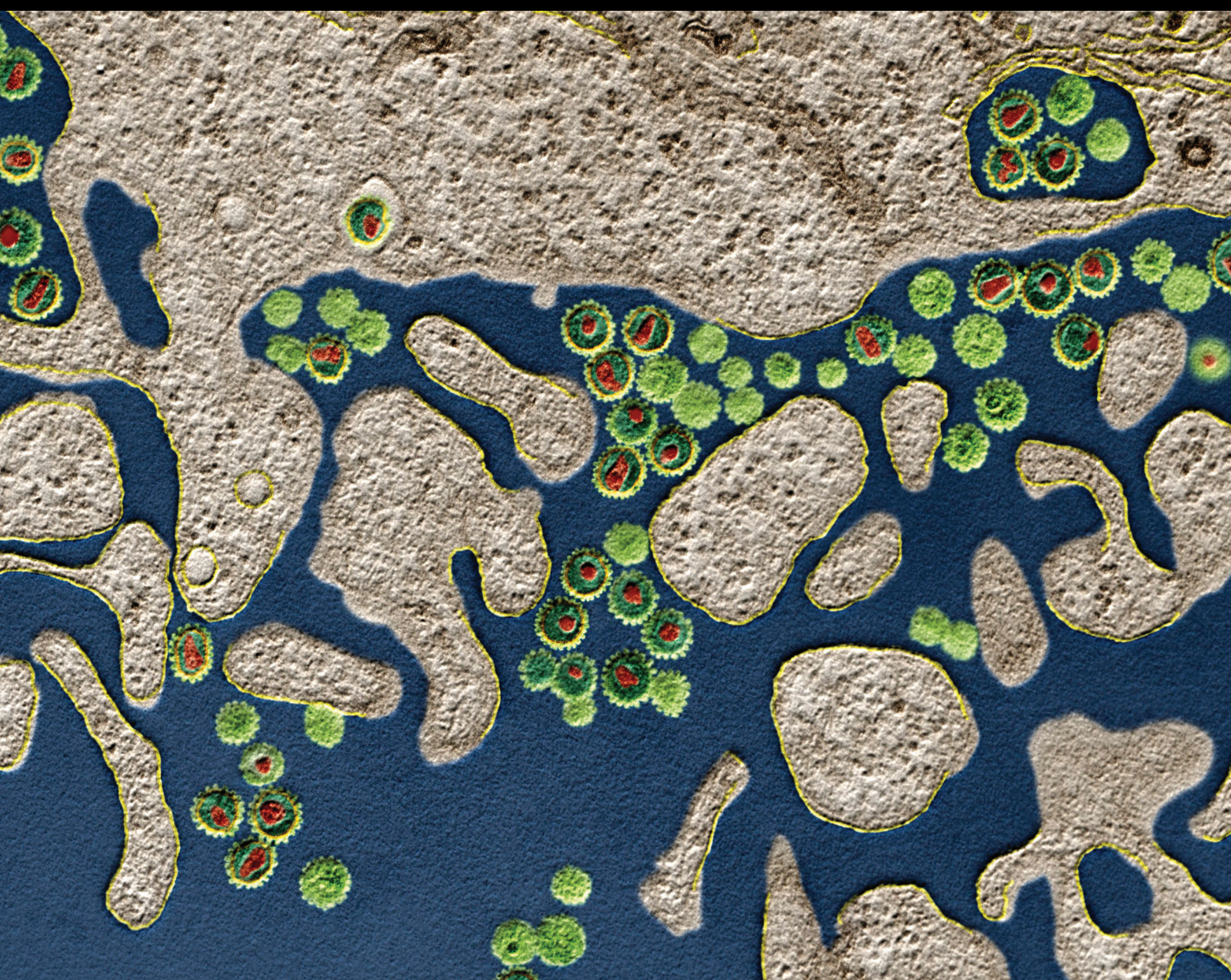

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Editorial Board

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Hindawi
Journal of Immunology Research
Volume 2018, Article ID 7590350, 2 pages
https://doi.org/10.1155/2018/7590350

Editorial

Macrophage Functions and Regulation: Roles in Diseases and Implications in Therapeutics

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Received 29 April 2018; Accepted 29 April 2018; Published 5 June 2018

Macrophages, as a key element in innate immunity, play an important role in the first-line defense against pathogens and modulating inflammatory responses. From the traditional point of view, tissue macrophages are differentiated from bone marrow myeloid progenitor-derived monocytes in the circulation and undergo a fine-regulated process of adaption to the local tissue microenvironment [1]. However, over the past decade, mounting evidence has demonstrated that macrophages are also derived from embryonic York sac and fetal liver and become a self-maintaining population residing locally and performing organ-specific functions [2].

Macrophages are not homogenous and consist of variably mixed populations, such as liver Kupffer cells and brain microglial cells that carry out specific functions in the local microenvironment [3]. In response to various physiological or pathological cues, macrophages display an extended life span and acquire different functional phenotypes through polarization that are generally categorized into two broad but distinct subsets as either classically activated (M1) or alternatively activated (M2). In general, M1 macrophages have high motility and promote inflammation and damage through a combination of transcription factors such as NF-κB, whereas M2 macrophages help to resolve inflammation and promote tissue remodeling [4]. Notably, M1 and M2 only represent two extremes of macrophage polarization, and most differentiated macrophages fall into a full spectrum of various polarization states between M1 and M2. In addition, macrophage polarization is a dynamic process and macrophages can switch their phenotypes between M1 and M2 in different pathological conditions [5]. Nevertheless, sustained macrophage infiltration in face of injury eventually becomes pathological and causes distorted repair and remodeling, leading to irreversible tissue destruction and disease progression and deterioration. Thus, better understanding of the regulation of macrophage differentiation and polarization, as well as their roles in disease pathogenesis, will contribute to the development of selective and effective therapies.

In this specific issue, fourteen quality manuscripts were selected for publication from a large number of submissions covering various topics of macrophage functions and regulation, as well as their roles in diseases and therapeutics. Ten of these publications are review articles reflecting the current status of knowledge and advances in understanding macrophage functions and regulation. L. Parisi et al. provided a comprehensive review regarding the role and regulation of M1-like (killers) and M2-like (builders) macrophages in various chronic diseases including cancers, type 2 diabetes, atherosclerosis, and periodontitis. They also discussed therapeutic approaches using cytokine antagonists and miRNAs. J. Yin et al. highlighted the current

understanding of microglia and macrophage functions and differentiation in CNS homeostasis, autoimmunity, and cancer. Other review articles are more focused with emphasis on an individual disease, molecule, or pathway. J. Shi et al. discussed the roles of macrophage subsets in bowel anastomotic leakage and healing. L. Shao et al. summarized the perspectives and potential targets of macrophage polarization in cerebral aneurysm, and L. Zhu et al. reviewed the roles of members of the phospholipase C family in macrophage-mediated inflammation. T.S. Kapellos et al. updated the current knowledge regarding macrophage dysfunction in chronic obstructive pulmonary disease with a focus on the well-known resident alveolar macrophages, whereas, J. Schyns et al. illuminated the important role of the less-studied lung interstitial macrophages. Of note, there are two review articles about macrophage tolerance and regulation from R. Huber et al. and R. Ocaña-Guzman et al. with focus on TNF and inhibitory receptors, respectively. H. Liao et al. provided an interesting retrospective literature analysis regarding the role of macrophage iNOS activity in the therapeutic effect of Huangqi, a traditional Chinese medicine, on diabetic nephropathy. The remaining four accepted manuscripts are research articles of translational significance using various patient samples or animal models to study macrophage functions and regulation. M. Yamashita et al. examined the expression pattern of CD163-positive macrophages in lung biopsy samples from patients with idiopathic interstitial pneumonias. I.A. da Silva et al. investigated the role of the platelet-activating factor in modulating the tumor-associated macrophage phenotype, and W.R. Shen et al. demonstrated the potential therapeutic role of targeting osteoclast formation in LPS-induced bone loss. Y.M. Flores-Martinez et al. established a rat model of Parkinson's disease with classical microglia activation, neuroinflammation, and degeneration. These research articles all highlighted the important role of macrophage functions and regulation in disease pathogenesis and therapeutics.

In summary, these articles illuminate the role and regulation of macrophage function and differentiation in the pathogenesis and therapeutics of various diseases, and provide guidance for future research on macrophage functions and development of selective and efficient therapeutics.

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Yang Jin
Zissis Chroneos
Xiaodong Han
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Ling Lin

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Hindawi
Journal of Immunology Research
Volume 2018, Article ID 1838921, 19 pages
https://doi.org/10.1155/2018/1838921

Research Article

Acute Neuroinflammatory Response in the Substantia Nigra Pars Compacta of Rats after a Local Injection of Lipopolysaccharide

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Received 26 October 2017; Revised 3 February 2018; Accepted 8 March 2018; Published 8 May 2018

Academic Editor: Hao Liu

Models of Parkinson's disease with neurotoxins have shown that microglial activation does not evoke a typical inflammatory response in the substantia nigra, questioning whether neuroinflammation leads to neurodegeneration. To address this issue, the archetypal inflammatory stimulus, lipopolysaccharide (LPS), was injected into the rat substantia nigra. LPS induced fever, sickness behavior, and microglial activation (OX42 immunoreactivity), followed by astrocyte activation and leukocyte infiltration (GFAP and CD45 immunoreactivities). During the acute phase of neuroinflammation, pro- and anti-inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-4, and IL-10) responded differentially at mRNA and protein level. Increased NO production and lipid peroxidation occurred at 168 h after LPS injection. At this time, evidence of neurodegeneration could be seen, entailing decreased tyrosine hydroxylase (TH) immunoreactivity, irregular body contour, and prolongation discontinuity of TH^+ cells, as well as apparent phagocytosis of TH^+ cells by $OX42^+$ cells. Altogether, these results show that LPS evokes a typical inflammatory response in the substantia nigra that is followed by dopaminergic neurodegeneration.

1. Introduction

Neuroinflammation plays a critical role in Parkinson's disease and other neurodegenerative diseases [1, 2]. The main hallmark of neuroinflammation in Parkinson's disease is the presence of activated microglia in the substantia nigra of humans [3] and animal models of that disease [4–6]. Similar to macrophages, activated microglia can phagocytose, present antigens through the major histocompatibility complex (MHC) class II [2, 7], synthesize, and release humoral factors such as cytokines, chemokines, reactive oxygen-nitrogen species, complement cascade proteins, and

prostaglandins [8–11]. The tumor necrosis factor- (TNF-) α, interleukin- (IL-) 1, and IL-6 transform astrocytes into proliferative immunological cells, recruited in the inflamed brain area [12–15]. The participation of glial cells in the neuroinflammation of Parkinson's disease has been characterized to a large extent in animal models generated by neurotoxins such as 6-hydroxydopamine (6-OHDA), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), or rotenone [5, 16–19]. These potent neurotoxins primarily cause the death of dopaminergic neurons, so they have not favored the clarification whether neuroinflammation is the cause or consequence of dopaminergic neurodegeneration. Lipopolysaccharide (LPS) appears to be a neuroinflammatory stimulus more suitable to mimic the acute response of microglia that might also occur in the early stage of Parkinson's disease [20].

LPS is a major component of the outer membrane of gram-negative bacteria and a potent inducer of inflammation via activation of toll-like receptor 4 (TLR4) [21], not only in peripheral tissues and organs [22, 23] but also in the central nervous system (CNS) [24, 25]. Studies using systemic injection [26] or ventricular infusion of LPS [24] in rodents have shown accumulation of activated microglia in various brain nuclei mainly in the substantia nigra, thus suggesting that LPS can be useful to study neurodegeneration as a model of Parkinson's disease [24, 25]. LPS injected directly into the substantia nigra can elicit a strong macrophage/microglial local reaction that is followed by the specific death of nigral dopaminergic neurons, thus suggesting that LPS can cause neuronal cell death indirectly through the inflammatory reaction [25, 27]. A recent study has confirmed the microglial activation in the substantia nigra after the local injection of LPS at a dose of 5 μg/5 μL and demonstrated the mRNA expression of proinflammatory cytokines (TNF-α and IL-1β) after 7 days of injection, alterations in oxidative stress markers after 14 days postinjection, and apoptosis activation after 21 days of LPS injection [28]. However, those inflammatory variables were evaluated in the whole midbrain and not restrained to the substantia nigra [28]. In addition, the time course of neuroinflammation was studied after the end of acute neuroinflammation, in the same period where the specific neurotoxins also cause neuroinflammation [28]; then, the possibility that neuroinflammation would precede dopaminergic neurodegeneration has not been clarified. Nevertheless, microglial response evaluated through OX42 immunohistochemistry has been shown as early as 6 h after an intrastriatal injection of 22.5 μg of LPS, preceding the dopaminergic neurodegeneration [29]. However, this study did not evaluate any proinflammatory cytokines or astrocyte cell markers [29]. In addition, other studies have shown neither microglial activation in the substantia nigra nor nigrostriatal neurodegeneration, but only transient motor dysfunction, after an intrastriatal administration of 10 μg of LPS [30]. This background information shows that the role of microglia and astrocytes in LPS-induced neuroinflammation is not entirely understood in the substantia nigra [25].

The astrocyte is also another key player in human diseases and animal models of neuroinflammation [31]. Activated astrocytes (reactive astrogliosis) have been shown in different models of chronic demyelinating pathology [32] and of neurotoxin-induced Parkinson's disease in the rat [5, 19]. Cultured astrocytes from the cerebellum of rats with a natural demyelinating disease can produce high levels of nitric oxide (NO) and inducible NO synthase (iNOS) mRNA and protein and release TNF-α when stimulated with LPS; those responses are resistant to the inhibitory effect of TGF-β1 [33]. Genomic analysis in mice has also suggested that the reactive astrocytes induced by a systemic LPS (5 mg/kg of body weight) administration exhibit a phenotype that may be detrimental [26]. These results suggest that activated astrocytes produce hazardous molecules that can prolong and aggravate neuroinflammation, which eventually will lead to neuronal death. Whether activated astrocytes have a role in the model of intranigral administration of LPS remains unknown.

Dopaminergic neurons of the substantia nigra are particularly vulnerable to neuroinflammation due to internal and external factors that lead to a maintained, elevated mitochondrial oxidant stress [34]. An internal factor, for instance, is the decrease in glutathione levels and glutamylcysteine ligase activity that are the natural antioxidant defenses in neuronal cells [35]. This feature can account for the inefficient neutralization of the nonenzymatic oxidation products of dopamine and the powerful oxidants resulting from Fenton reaction in the presence of iron [36]. An external factor for the vulnerability of dopaminergic neurons is the relatively enriched microglial population in the substantia nigra as compared to other brain regions, which can mount a fast response to the minimum imbalance of oxidative stress [37, 38]. Therefore, the evaluation of the acute stage of neuroinflammation in the substantia nigra should provide insight into the physiopathology of dopaminergic neurodegeneration.

Here, we propose that a single dose of LPS in the substantia nigra will activate local microglia followed by astrocyte activation as a primary event of neuroinflammation and then followed by the dopaminergic neurodegeneration. To test this hypothesis, we injected a single dose of LPS (5 μg/2 μL of endotoxin-free physiological saline solution) into the substantia nigra. Then, we evaluated NO production; lipid peroxidation index; immunoreactivity of microglia (OX42), astrocyte (GFAP), and leucocyte (CD45) markers; and pro- and anti-inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-4, and IL-10) during the acute phase of neuroinflammation (0 to 96 h). We also evaluated the immunoreactivity to tyrosine hydroxylase (TH), a dopaminergic neuron marker in the substantia nigra. We evaluated the molecular and cellular markers at 168 h after LPS injection to determine the end of acute neuroinflammation. Although using an LPS animal model in the research of Parkinson's disease has been well documented [25], the results presented here emphasize the timing course within the substantia nigra, which add new evidence to support that inflammation is the cause of dopaminergic neurodegeneration. This acute neuroinflammation model will be useful in a fast screening of new anti-inflammatory drugs with potential for Parkinson's disease treatment.

2. Materials and Methods

2.1. Ethics Statement. The experimental protocol (Permit number 162-15) was approved by the Internal Committee for the Care and Use of Laboratory Animals of the Center for Research and Advanced Studies of the National Polytechnic Institute (Cinvestav-IPN) in accordance with the current Mexican legislation, NOM-062-ZOO-1999 and NOM-087-ECOL-1995 (Secretaría de Agricultura, Ganadería, Desarrollo Rural, Pesca y Alimentación (SAGARPA)). All efforts were made to minimize suffering, and the number of animals used was kept to a minimum by the experimental design.

2.2. Animals. Adult male Wistar rats weighing between 210 g and 230 g were used. Five rats per cage (acrylic; 34 cm × 44 cm × 20 cm) were housed at constant room temperature (22°C) and 12 h-12 h light-dark cycle with food and water ad libitum.

2.3. Stereotaxic Injection of LPS. The rats were anesthetized with a single dose of ketamine (70 mg/kg) and xylazine (6 mg/kg; intraperitoneally) and fixed in a stereotaxic apparatus (Stoelting, Wood Dale, IL, USA). A single dose of LPS from *Escherichia coli* 055:B5 (5 μg/2 μL of endotoxin-free physiological saline solution; Sigma-Aldrich, St. Louis, MO, USA) [39, 40] was injected into the left substantia nigra. We used the following coordinates: AP, +3.2 mm from the interaural midpoint; ML, +2.0 mm from the intraparietal suture; and DV, −6.5 mm from the dura mater [19]. A micropump Mod. 100 (Stoelting, Wood Dale, IL, USA) maintained the flow rate (0.2 μL/min). After the total dose was injected, the needle was allowed to remain in the brain for 7 min and then was withdrawn in 1 min steps. The mock group was injected with 2 μL of endotoxin-free physiological saline solution into the left substantia nigra. An additional control group was rats with no treatment (Untreated (Ut)).

2.4. Body Surface Temperature. A fine thermocouple thermometer (Hanna Instruments, Woonsocket, RI, USA) was attached to an adhesive tape and secured on the ventral surface of the chest to measure body surface temperature. The measurements were made at different times after intranigral injection of LPS (1, 2, 3, 5, 8, 24, 48, 96, and 168 h) in the experimental group and at 8 h in the negative control group (mock rats).

2.5. Sickness Behavior. Sickness signs consisting of absent exploration and locomotion, curled body posture, irregular fur, piloerection, and closed eyes were evaluated in the LPS-treated group and control (untreated and mock) groups over time after the intranigral injection of LPS [41, 42]. Measurements were performed while the rats were in transparent cages and scored on a four-point scale: 0 = no signs, 1 = one sign, 2 = two signs, and 3 = three or more signs. The experimenter quantifying the sickness signs were blind to experimental and control conditions. The overall agreement between two "blind" raters was 95%.

2.6. Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR). Each brain was obtained free of meninges and immediately rinsed with cold PBS. Six 0.5 mm coronal slices of the brain between the anterior border of the pituitary and the anterior border of the cerebellum were obtained using a cold metallic rat brain matrix (Stoelting, Wood Dale, IL, USA). The left substantia nigra was quickly dissected out from every coronal slice in cold conditions using a stereomicroscope (Leica ZOOM 2000, Buffalo, NY, USA) equipped with an especial metallic stage to contain ice. Each left substantia nigra was immediately stored in a respective Eppendorf tube at −70°C until use. Total RNA was isolated from the substantia nigra using Trizol (Invitrogen Corporation, Carlsbad, CA, USA), and then RNA treated with RNase-free DNase I. The reverse transcription was made with SuperScript III reverse transcriptase (200 U) using 3 μg of total RNA and 0.1 mg of oligo dT (Invitrogen Corporation, Carlsbad, CA, USA). The reverse-transcribed product was diluted 4 times with molecular biology-grade water. A 2.5 μL sample of the diluted cDNA was mixed with 2X TaqMan Universal Mastermix and 20X TaqMan gene-specific probe (Applied Biosystems, Foster City, CA, USA) in a final volume of 5 μL. cDNAs were amplified in 45 cycles using a 7900HT Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). The TaqMan gene-specific probes were Rn01525859_g1 for rat TNF-α, Rn00580432_m1 for rat IL-1β, Rn01410330_m1 for rat IL-6, Rn01456866_m1 for rat IL-4, Rn99999012_m1 for rat IL-10, and Rn00667869_m1 for rat β-actin, which were used as internal controls and for normalization. The cycle threshold (Ct) values for β-actin and rTNF-α, rIL-1β, rIL-6, rIL-4, and rIL-10 were measured and calculated by Sequence Detection System software (SDS 2.2; Applied Biosystems, Foster City, CA, USA). The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative transcript levels expressed as fold change for gene expression with respect to each of the probes used [5, 43, 44].

2.7. Enzyme-Linked Immunosorbent Assay (ELISA). The substantia nigra was homogenized with the protein extraction buffer containing 100 mM Tris-HCl (pH 7.4), 750 mM NaCl (sodium chloride), 10 mM EDTA (ethylenediaminetetraacetic acid), 5 mM EGTA (ethylene glycol tetraacetic acid), and protease inhibitors (Roche, Basel, Switzerland) [5, 45]. The samples were centrifuged at 1000g for 10 min at 4°C. The supernatant was collected and centrifuged again at 20,000g for 40 min at 4°C to remove remaining debris. ELISA was performed using a Milliplex MAP Rat cytokine/chemokine magnetic bead panel kit according to the provider's protocol (RECYTMAG_65K; Millipore, Temecula, CA, USA), and reading was made by using the LUMINEX MAGPIX detection system with xPONET software (Millipore Corporation, Billerica, MA, USA). The sensitivity ranges were 2.4 to 10,000 pg/mL for TNF-α and IL-1β, 73.2 to 300,000 pg/mL for IL-6, and 7.3 to 30,000 pg/mL to IL-4 and IL-10.

2.8. NO Production. The content of nitric oxide (NO) was determined through nitrite (NO_2^-) accumulation in the supernatant of homogenized substantia nigra samples using the Griess reagent assay [5, 33, 46]. Briefly, tissue samples were mechanically homogenized in PBS and centrifuged at 20,000g for 30 min at 4°C. The colorimetric reaction in

100 μL of the supernatant was initiated by adding 100 μL of the Griess reagent (equal volumes of 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride and 1.32% sulfanilamide in 60% acetic acid). The absorbance of the samples was read at 540 nm with a SmartSpec 3000 spectrophotometer (Bio-Rad, Hercules, CA, USA) and interpolated by using a standard curve of sodium nitrite ($NaNO_2$; 1 to 10 μM) to calculate the nitrite content.

2.9. Lipid Peroxidation Assay. Lipid peroxidation was measured through malondialdehyde (MDA) and 4-hydroxyalkenal (4-HAE) concentration in the supernatant of homogenized substantia nigra samples using the colorimetric method reported previously [5, 33, 46]. Briefly, the tissue samples were homogenized in PBS and centrifuged at 20,000g at 4°C for 40 min. Then, 325 μL of 10.3 mM N-methyl-2-phenylindole diluted in a mixture of acetonitrile : methanol (3 volume : 1 volume) was added to 100 μL of the supernatant. The colorimetric reaction was initiated by the addition of 75 μL of methanesulfonic acid. The reaction mixture was strongly shaken and incubated at 45°C for 1 h and then centrifuged at 1000g for 10 min. The absorbance in the supernatant was read at 586 nm with a SmartSpec 3000 spectrophotometer (Bio-Rad, Hercules, CA, USA). The absorbance values were compared to a standard curve from 0.5 to 5 μM of 1,1,3,3-tetramethoxypropane to calculate the content of MDA and 4-HAE in the samples.

2.10. Western Blot Analysis. Western blot analysis was performed in substantia nigra homogenates. Total protein was determined using the BCA protein kit (Pierce; Meridian, Rockford, USA). Fifty micrograms of protein per line was run on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto PVDF membranes (Bio-Rad Laboratories, Hercules, CA, USA). Blots were blocked with TBS containing 5% skim milk, 1% BSA, and 0.1% Tween 20 and incubated overnight at 4°C with a mouse monoclonal anti-CD11b/c (OX42), a marker for activated microglia (1 : 100; Abcam, Cambridge, UK), and a rabbit polyclonal anti-glial fibrillary acidic protein (GFAP), a marker for astrocytes (1 : 1000; DakoCytomation, Glostrup, Denmark). Membranes were washed and then incubated with the secondary antibodies conjugated with horseradish peroxidase (HRP), either goat anti-mouse IgG (1 : 5000; Zymed, San Francisco, CA, USA) or donkey anti-rabbit IgG (1 : 5000; Zymed, Cambridge, MA, USA) in blocking solution, for 1.5 h with continuous shaking at room temperature. Blots were washed, and the immunolabeled proteins were detected using the ECL Western blotting system and Hyperfilm ECL (Amersham, Buckinghamshire, UK). To normalize the total amount of protein per lane, membranes were stripped and incubated with a mouse monoclonal antibody against β-actin (1 : 500; Cinvestav, Mexico) [47], followed by a HRP-conjugated goat anti-mouse (1 : 6000; Zymed, San Francisco, CA, USA) following the same procedure of luminescence detection.

2.11. Immunostaining Techniques. The presence of microglia, astrocytes, and dopaminergic neurons was shown by double immunofluorescence techniques using the procedure described previously [5, 48]. The rats were deeply anesthetized with sodium pentobarbital (50 mg/kg intraperitoneally) and perfused through the ascending aorta with 30 mL of PBS, followed by 100 mL of 4% paraformaldehyde in PBS. The brain was then removed and maintained in the fixative for 24 h at 4°C. After an overnight incubation in PBS containing 30% sucrose at 4°C, the brain was frozen and then sectioned. Briefly, serial coronal sections of 30 μm thickness were cut using a sliding microtome with a freezing stage (Leica SM1100, Heidelberg, Germany) and consecutively collected in 6 wells, using only the slices in one well for the analysis. The slices were rinsed with PBS for 5 min, permeabilized with PBS-0.1% Triton for 20 min, and incubated with 1% BSA in PBS-0.1% Triton for 30 min to block unspecific binding sites. The primary antibodies were mouse monoclonal anti-tyrosine hydroxylase (TH) (clone TH-2) (1 : 1000; Sigma-Aldrich, St. Louis, MO, USA), rabbit polyclonal anti-TH (1 : 1000; Millipore, Temecula, CA, USA), mouse monoclonal anti-CD11b/c (OX42) (1 : 200; Abcam, Cambridge, UK), mouse anti-CD45 (BD Bioscience, USA), and rabbit polyclonal anti-GFAP (1 : 500; DakoCytomation, Glostrup, Denmark). The secondary antibodies were Alexa Fluor 488 chicken anti-mouse H+L IgG (1 : 300; Invitrogen Molecular Probes, Eugene, Oregon, USA), Alexa Fluor 488 chicken anti-rabbit H+L IgG (1 : 300; Invitrogen Molecular Probes, Eugene, Oregon), Texas red horse anti-mouse H+L IgG (1 : 900; Vector Laboratories, Burlingame, CA, USA), and Texas red goat anti-rabbit H+L IgG (1 : 900; Vector Laboratories, Burlingame, CA, USA). The slices were washed with PBS and mounted on glass slides using VECTASHIELD (Vector Laboratories, Burlingame, CA, USA). Fluorescence images were obtained with a Leica DMIRE2 microscope, using 20x and 40x objectives and filters K3 for Alexa Fluor 488 (green fluorescence) and TX2 for Texas red (red fluorescence). The images were digitized with a Leica DC300F camera (Nussloch, Germany). A multispectral confocal laser scanning microscope (TCS SPE; Leica, Heidelberg, Germany) was used to analyze through a 100x oil-immersion objective the double immunofluorescence against TH-OX42 and TH-GFAP at excitation-emission wavelengths of 488–522 nm (green channel) and 568–635 nm (red channel). Their consecutive 1 μm optical sections were also obtained in the Z-series (scanning rate 600 Hz). The images were acquired using LAS AF software (Leica Application Suite; Leica Microsystems, Nussloch, Germany).

TH immunohistochemistry was made after depletion of endogenous peroxidase using PBS-0.3% Triton X-100 solution containing 3% hydrogen peroxide and 10% methanol at room temperature. The primary antibody was a mouse monoclonal anti-TH clone TH-2 (1 : 1000; Sigma-Aldrich, St. Louis, MO, USA), and the secondary antibody was a horse biotinylated anti-mouse H+L IgG (1 : 200; Vector Laboratories, Burlingame, CA, USA). The immunohistochemical staining was developed using the avidin-biotin-peroxidase complex (1 : 10; ABC Kit; Vector Laboratories, Burlingame, CA, USA) and 393-diaminobenzidine (DAB; Sigma-Aldrich, St. Louis, MO, USA) [19]. After the immunohistochemistry, the slides were stained with hematoxylin-eosin (H&E)

and then were mounted on glass slides using Entellan (Merck KGaA, Darmstadt, Germany). Finally, the slides were then examined with a light microscope equipped with 5x and 63x oil-immersion objectives (Leica Microsystems, Nussloch, Germany).

2.12. Statistical Analysis. All results were expressed as the mean ± standard deviation (SD) values at least from 3 independent experiments ($n = 3$). The following statistical tests to analyze the difference among groups were used: repeated-measures two-way ANOVA and Bonferroni post hoc test for temperature, sickness behavior, nitrites, and lipid peroxidation qPCR and analysis of IF area density, repeated-measures one-way ANOVA, and Newman-Keuls post hoc test for OX42 and GFAP Western blot and ELISA results. GraphPad Prism 5.0 software (GraphPad Software Inc., La Jolla, CA, USA) was used for statistical analysis. The accepted significance was at $P < 0.05$.

3. Results

3.1. Time Course of Fever and Sickness. As systemic manifestations of LPS-induced neuroinflammation, the feverish reaction ($n = 45$ rats) and external signs of sickness ($n = 45$ rats) were measured over time (1, 2, 3, 5, 8, 24, 48, 96, and 168 h) after the intranigral injection of LPS. The untreated control rats maintained their body temperature at 32.65 ± 0.75°C, whereas the rats injected with LPS gradually increased their body temperature to a maximum of 38.25 ± 0.15°C detected at 8 h postinjection (Figure 1(a)). After 24 h, the body temperature was maintained at 32.7 ± 0.9°C until 168 h, the end of the experiment (Figure 1(a)). The mock rats (intranigrally injected with 2 μL of endotoxin-free physiological saline solution) showed a body temperature as low as 29.20 ± 0.82°C (1 h) and 31.16 ± 0.45°C (2 h) because of the anesthetic effect [49]. After that, the temperature value attained was 32.65 ± 0.85°C in the untreated control group (Figure 1(a)).

There were no sickness signs in the mock group ($n = 45$ rats) as compared with the untreated control rats, except for slightly irregular fur in 1% of mock rats (score = 1) at 8 h after the vehicle injection (Figure 1(b)). When compared with the untreated controls and mock groups, the rats intranigrally injected with LPS exhibited clear signs of sickness that followed the time course of fever. The maximum score was reached at 8 h postinjection when the rats presented adynamia (absence of locomotion and exploration), curled body posture, closed eyes, and piloerection (Figure 1(b)). The sickness signs varied during their time course, although their score was similar. At 5 h after LPS injection, the predominant signs were the absence of locomotion and exploration, curled body posture, and piloerection, and only closed eyes were observed in 1% of the animals (Figure 1(b)). At 2 and 24 h after LPS injection, the score was 2, but the signs were different in those times. At 2 h, there was no locomotion, exploration, and curled body posture, whereas at 24 h, the locomotion and exploration were recovered, but irregular fur with a slight degree of piloerection was seen in 1% of the rats. At 3 h after LPS injection, the obvious signs were adynamia and curled body posture (Figure 1(b)).

3.2. Time Course of Microglial Activation. OX42 immunodetection by Western blot ($n = 3$ rats for each time) and double immunofluorescence with TH ($n = 3$ rats for each time) was used to analyze the time course (0.2, 1, 5, 24, and 168 h) of microglial activation in the substantia nigra after LPS injection. The basal levels of OX42 immunoreactivity in the untreated control rats were low and normalized concerning β-actin in Western blot assays (Figures 2(a) and 2(b)). Immunofluorescence assays showed scarce OX42 immunoreactivity in the substantia nigra pars compacta of both untreated and mock groups (Figures 2(c) and 2(e) and Supplementary Figure 1). OX42 immunoreactivity increased immediately after the LPS injection, reached a maximum value at 24 h, and still was high at the end of the experiment (168 h) as shown by both immunodetection techniques (Figures 2(a)–2(e)). A significant increase in OX42 immunoreactivity was only detected in the mock condition when compared with the untreated condition at 168 h after the vehicle injection (Figure 2(e) and Supplementary Figure 1), but the increase in the LPS group was twice greater than that in the mock group and was statistically significant (Figure 2(e)). At this time, a significant 39% decrease in the number of TH-immunoreactive cells occurred with respect to the untreated control and mock condition (Figures 2(c) and 2(d) and Supplementary Figure 1), suggesting neurodegeneration of dopaminergic neurons (Figures 2(c) and 2(d)).

It is interesting to notice the morphological changes of microglia as the time elapses after LPS exposure (Figure 3). The absence of OX42-immunoreactive cells in the untreated condition suggests the resting or quiescent condition of microglia. At 12 minutes after LPS injection (0.2 h), the cells exhibited a strong OX42 immunoreactivity and a robust branched morphology, with long thick branches, as well as a regular and slightly enlarged soma (Figure 3). After 1 h, two types of morphology were observed (Figure 3). One type consists of long, thick branching and a well-delimited, wide soma and nucleus (Figure 3). The second type consists of short, stout branches and a larger soma and nucleus. From 5 to 24 h after LPS injection, the reactive-state, round-shape cells with retracted processes and enlarged body, also referred to as the amoeboid form, can be observed (Figure 3). At 168 h after LPS injection, the OX42-immunoreactive cells exhibited a round, irregular, and larger shape than the amoeboid cells suggestive of the phagocytic state (Figure 3).

3.3. Time Course of Astrocyte Activation. GFAP immunodetection by Western blot ($n = 3$ rats for each time) and double immunofluorescence with TH ($n = 3$ rats for each time) was used to analyze the time course (0.2, 1, 5, 24, and 168 h) of astrocyte activation in the substantia nigra after the LPS injection. Contrary to OX42 immunoreactivity, the Western blot analysis showed that the increase in GFAP immunoreactivity was belated and with statistical significance since 5 h following LPS injection with respect to the basal levels of untreated control rats (Figures 4(a) and 4(b)). GFAP immunoreactivity continued increasing until the end of the study

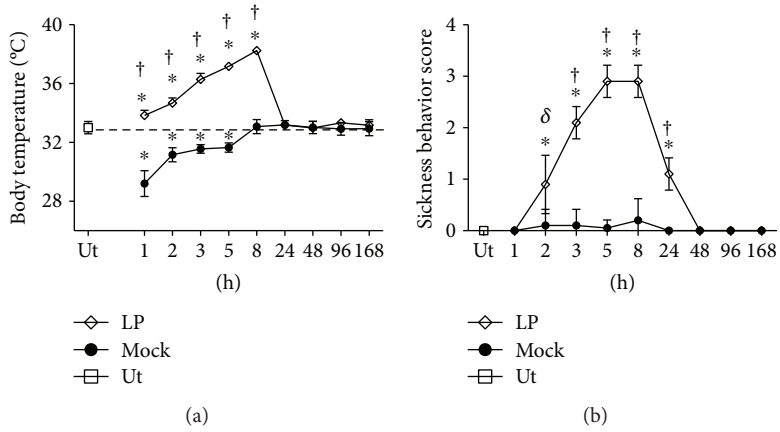

Figure 1: Clinical evolution after a single injection of LPS in the substantia nigra of the rat. (a) Fever. (b) Sickness behavior. Ut = untreated control rats. Mock = rats injected with the vehicle (2 μL of endotoxin-free physiological saline solution) in the left substantia nigra. All values represent the mean ± SD ($n = 45$). $^{*}P < 0.001$ when compared with the untreated control group. $^{\delta}P < 0.05$ or $^{\dagger}P < 0.001$ when compared with the respective mock. Repeated-measures two-way ANOVA and Bonferroni post hoc test.

(Figures 4(a) and 4(b)). The immunofluorescence assay agrees with the time course of GFAP immunoreactivity shown by Western blot analysis and revealed details on changes of localization of the GFAP-immunoreactive cells (Figures 4(c) and 4(e)). GFAP-immunoreactive cells were scarce in the substantia nigra of untreated control rats (Figures 4(c) and 4(e)). After LPS administration, GFAP-immunoreactive cells started appearing in the pars reticulata of the substantia nigra (1 h) and then in the pars compacta (24 h). At the end of the study, the GFAP-immunoreactive cells were abundant in both the pars compacta and pars reticulata of the substantia nigra (Figures 4(c) and 4(e)). These results suggest that the astrocytes of the substantia nigra are first activated in the pars reticulata and then recruited in the pars compacta where the dopaminergic neurons dwell. The vehicle injection increased GFAP-immunoreactive cells, which was significant with the untreated controls, but the increase in the LPS group was much greater than that in the mock group and was statistically significant (Figures 4(c) and 4(e) and Supplementary Figure 2). The significant decrease in TH-immunoreactive cells in the LPS group was also confirmed (Figures 4(c) and 4(d)).

The morphological changes in astrocytes were less dramatic than those in microglia (Figure 4). Because GFAP-immunoreactive cells were rarely observed in the substantia nigra pars compacta of untreated control rats, their morphological details were shown in GFAP-immunoreactive cells in the substantia nigra pars reticulata (Figure 5). In basal conditions, the GFAP-immunoreactive cells were scarce with small soma and few thin branches (Figure 5). The changes induced by LPS injection mainly consisted of an increase in the cell number, in the GFAP-immunoreactivity intensity, and in the number of branching, which was long and robust (Figure 5). These changes were present until the end of the study.

3.4. Nitrosative and Oxidative Stress. We evaluated nitrite concentration as a marker of nitrosative stress ($n = 5$ rats for each time) and MDA + 4-HAE levels as a marker of oxidative stress ($n = 5$ rats for each time). Mock values did not show statistical significance when compared with those of untreated group stress. As compared with the untreated control group, a significant 3.8-fold increase in nitrite levels was observed at 2 h after LPS injection and a second 2.0-fold increase from 8 h to 168 h (Figure 6(a)). The mock rats show a 2.0-fold increase in nitrite levels at 2 h after injection when compared with the untreated control group, but that increase was significantly lesser than that caused by LPS at the same time (Figure 6(a)). Different from nitrosative stress, lipid peroxidation was only significant at 168 h after LPS injection with respect to the untreated control group, suggesting that lipid peroxidation follows the acute neuroinflammation (Figure 6(b)).

3.5. Proinflammatory and Anti-Inflammatory Cytokines. Three proinflammatory cytokines (TNF-α, IL-1β, and IL-6) and two anti-inflammatory cytokines (IL-4 and IL-10) were evaluated in the substantia nigra through ELISA and qPCR ($n = 4$ rats for each time and each experimental condition; Figure 7). The LPS intranigral injection significantly increased mRNA levels of the three proinflammatory cytokines, but the onset and the peak were different for each proinflammatory cytokine (Figures 7(a)–7(c)). TNF-α and IL-1β mRNA levels were significant at early times and were maximum at 5 h (Figures 7(a) and 7(b)), followed by IL-6 mRNA levels that were maximum at 8 h (Figure 7(c)), when compared with those of the untreated and mock controls. After that, TNF-α and IL-1β mRNA levels decreased to reach the basal levels at 24 h; only IL-1β mRNA levels remained significantly increased up to 168 h (Figure 7(b)). mRNA levels of the two anti-inflammatory cytokines were significant only at late times when compared with those of the untreated controls: at 168 h, IL-4, and from 24 to 96 h, IL-10 (Figures 7(d) and 7(e)). We found that NO production precedes the increase in proinflammatory cytokine levels and that the clinical effect (fever and sickness behavior) was associated with the time course of proinflammatory cytokines (Figures 1, 6, and 8). Since the vehicle injection neither increased NO production nor elicited clinical manifestations

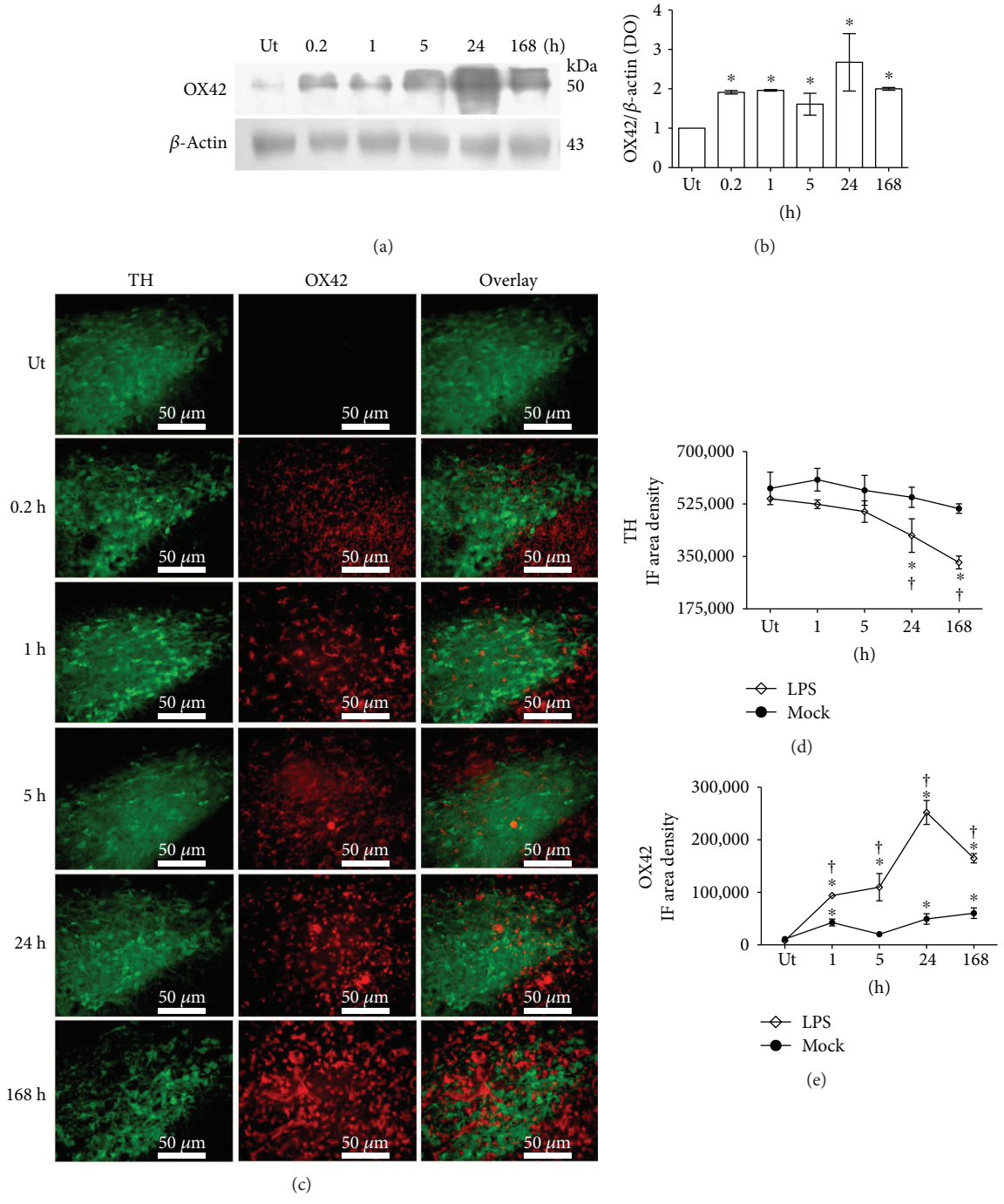

Figure 2: Time course of microglial activation. (a) A representative photograph of a Western blot membrane showing the electrophoretic fractionation of OX42 and β-actin from substantia nigra homogenates of LPS-treated rats and untreated (Ut) control rats. The numbers indicate the time of evaluation. (b) Graph of densitometry analysis showing the normalized values of OX42 bands concerning β-actin bands. The values represent the mean ± SD ($n = 3$ independent rats in each time of each experimental condition). $^{*}P < 0.001$ when compared with the untreated control group using repeated-measures one-way ANOVA and Newman-Keuls post hoc test. (c) Representative micrographs of the double immunofluorescence of TH and OX42 in the substantia nigra of untreated (Ut) control rats and rats at different times after LPS injection that were taken at 3.8 mm from the interaural midpoint on the dorsal-ventral axes of the rat brain atlas by Paxinos and Watson [50]. The numbers at the left side of micrographs indicate the time of evaluation. Immunofluorescence (IF) area density for TH (d) and OX42 (e) was determined using ImageJ software v.1.46r (National Institutes of Health, Bethesda, MD). The TH and OX42 values for the mock rats correspond to the quantification in Supplementary Figure 1. All values represent the mean ± SD ($n = 3$ independent rats in each time of each experimental condition). $^{*}P < 0.001$ when compared with the untreated control group of the respective immunostaining. $^{\dagger}P < 0.001$ when compared with the respective mock group. Repeated-measures two-way ANOVA and Bonferroni post hoc test.

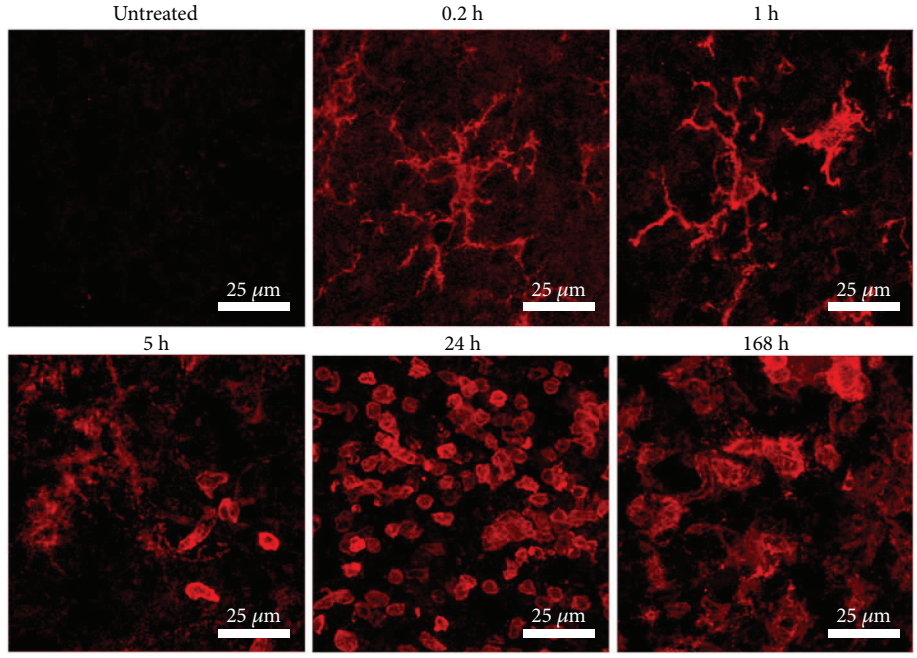

Figure 3: Morphological changes during activation of microglia in the substantia nigra after LPS exposure. Representative confocal micrographs of OX42 immunofluorescence in the substantia nigra of untreated control rats and rats at different times after LPS injection.

(i.e., fever and sickness behavior) over time, the effect of the vehicle on cytokine mRNA levels was only measured in the period of the maximum increase in mRNA levels in the LPS groups. There was no statistical difference from the untreated controls (Figures 7(a)–7(e)). The basal levels of the three proinflammatory cytokines and the two anti-inflammatory cytokines were, in pg/mL, 5.58 ± 0.80 (TNF-α), 22.14 ± 7.72 pg/mL (IL-1β), 1001.70 ± 144.01 (IL-6), 67.58 ± 12.80 pg/mL (IL-4), and 33.89 ± 11.21 (IL-10). The LPS intranigral injection significantly increased the basal levels of TNF-α, IL-1β, IL-6, and IL-10 with a time course similar to that of their respective transcripts (Figures 7(a)–7(d) and 7(f)–7(i)). IL-4 decreased at 24 and 48 h after LPS injection (Figure 7(f)), although its transcript levels were not significantly different over time (Figure 7(e), suggesting posttranscriptional regulation for this anti-inflammatory cytokine. Because the time course of protein levels was similar to that of transcript levels, the vehicle effect on cytokine protein levels was only determined at 3 h postinjection when qPCR showed the maximum increase in the mock group (Figures 7(a)–7(e)). A statistically different increase only occurred in TNF-α and IL-1β of the mock group with respect to the basal values, but such increase was statically different and 55% lower than that in the respective LPS group (Figures 7(f)–7(h)).

3.6. Apparent Microglial Phagocytosis of Damaged Dopaminergic Neurons. Confocal analysis with orthogonal projections was used to evaluate whether $OX42^+$ cells (microglia) might engulf damaged TH^+ cells (dopaminergic neurons) in the substantia nigra at 168 h after LPS local administration. The substantia nigra pars compacta of untreated control rats is characterized by the absence of active microglia and normal morphology of dopaminergic neurons with well-defined soma and continuous prolongations (Figure 9(a)). At 168 h after LPS administration, irregular and large $OX42^+$ cells are present in tight contact with harmed TH^+ cells (Figure 9(a)). At this time, evidence of neurodegeneration could be seen, entailing the decreased number of TH^+ cells (Figures 2(d), 4(d), and 9(a)), irregular body contour, unidentifiable nuclear area, and scarce and discontinuous prolongations. Also, there was evidence of apparent phagocytosis of TH^+ cells by $OX42^+$ cells (Figure 9(a)). This suggestion is further reinforced by the confocal orthogonal views that show TH^+ cell fragments being encircled by $OX42^+$ cell prolongations (Figure 9(b)). These results suggest that acute neuroinflammation by LPS local injection can lead to dopaminergic neurodegeneration in the substantia nigra.

3.7. Leukocyte Infiltration. The H&E staining in combination with TH immunohistochemistry of the untreated substantia nigra showed the presence of TH^+ and the absence of infiltrating cells (Figure 10). At 24 and 168 h after LPS injection, two kinds of infiltrating cells can be observed in the cerebral parenchyma: (1) macrophage-like cells with an elongated cytoplasm and a large, eccentric nucleus known as "rod cells" (Figure 10), a characteristic of macrophages and active microglia [51], and (2) leukocyte-like cells characterized by a small, regularly round basophilic cytoplasm and a well-defined large nucleus (Figure 10). The TH immunochemistry staining, besides its usefulness to delimit the substantia nigra compacta, confirmed the findings of the double immunofluorescence, that is, a decrease in TH immunoreactivity and irregular TH^+ cells suggesting damage of the dopaminergic neuron population

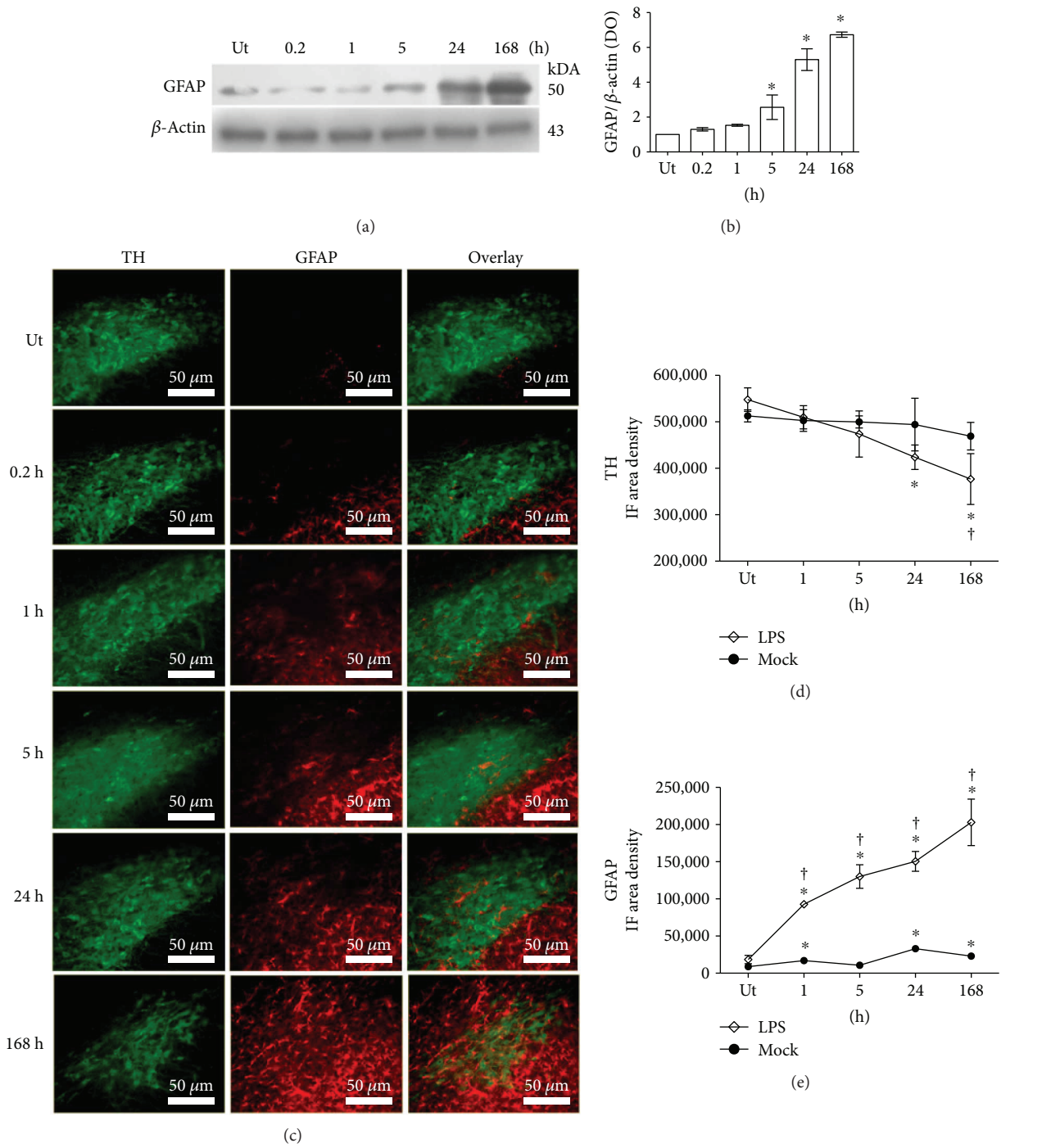

Figure 4: Time course of astrocyte activation. (a) A representative photograph of a Western blot membrane showing the electrophoretic fractionation of GFAP and β-actin from substantia nigra homogenates of LPS-treated rats and untreated (Ut) control rats. The numbers indicate the time of evaluation. (b) Graph of densitometry analysis showing the normalized values of GFAP bands with respect to β-actin bands. The values represent the mean $\pm$ SD ($n = 3$ independent rats for each time of each experimental condition). $^{*}P < 0.001$ when compared with the untreated control group using repeated-measures one-way ANOVA and Newman-Keuls post hoc test. (c) Representative micrographs of the double immunofluorescence of GFAP and TH in the substantia nigra of untreated (Ut) control rats and rats at different times after LPS injection that were taken at 3.7 mm from the interaural midpoint on the dorsal-ventral axis of the rat brain atlas by Paxinos and Watson [50]. The numbers at the left side of micrographs indicate the time of evaluation. Immunofluorescence (IF) area density for TH (d) and GFAP (e) was determined using ImageJ software v.1.46r (National Institutes of Health, Bethesda, MD). The TH and GFAP values for the mock rats correspond to the quantification in Supplementary Figure 2. All values represent the mean $\pm$ SD ($n = 3$ independent rats in each time and each experimental condition). $^{*}P < 0.001$ when compared with the untreated control group of the respective immunostaining. $^{\dagger}P < 0.001$ when compared with the respective mock group. Repeated-measures two-way ANOVA and Bonferroni post hoc test.

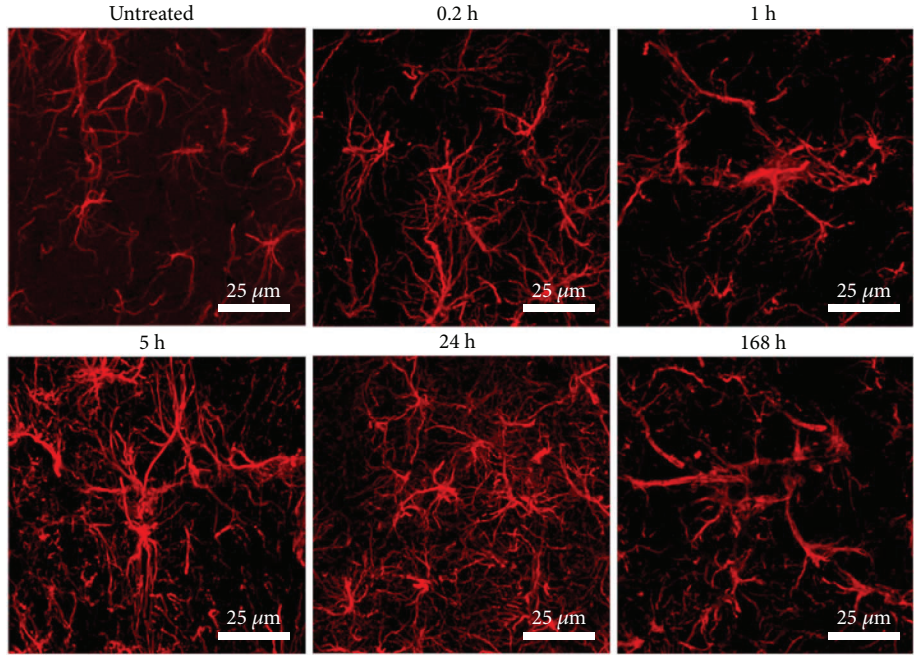

Figure 5: Morphological changes during activation of astrocytes in the substantia nigra after LPS exposure. Representative confocal micrographs of GFAP immunofluorescence in the substantia nigra of untreated control rats and rats at different times after LPS injection.

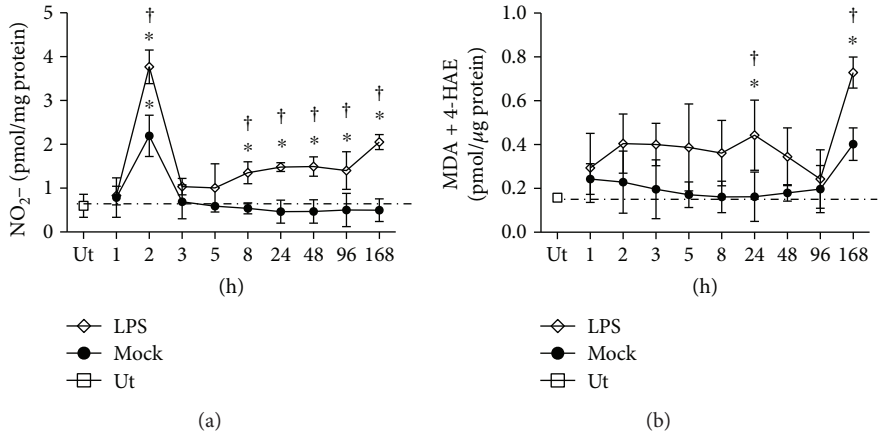

Figure 6: Nitrosative and oxidative stress in the substantia nigra after LPS local injection. (a) Nitrosative stress was evaluated through the levels of nitrites. (b) Oxidative stress (lipid peroxidation) was evaluated through the levels of malondialdehyde (MDA) + 4-hydroxyalkenals (4-HAE). Ut = untreated control rats. Mock = rats injected with the vehicle (2 μL of endotoxin-free physiological saline solution) in the left substantia nigra. The values represent the mean ± SD from 5 rats for each time and each experimental condition. $^*P < 0.001$ when compared with the untreated control group. $^\dagger P < 0.001$ when compared with the respective mock group. Repeated-measures one-way ANOVA and Bonferroni post hoc test.

(Figure 10). It is interesting to note that a large number of TH^+ neurons are clearly seen in the substantia nigra pars reticulata at 24 and 48 h after LPS injection, suggesting that those neurons are more resistant to the neuroinflammation elicited by LPS in comparison to those of the substantia nigra compacta (Figures 10(c) and 10(e)). The expression of calretinin in those neurons might explain the resistance to neuroinflammation as occurring for 6-OHDA [52].

To further support leukocyte infiltration, immunostaining of CD45, a leucocyte common antigen [53], was performed in the substantia nigra ($n = 3$ rats in each time and experimental condition). The results show the absence of $CD45^+$ cells in the untreated control and in the mock rats (Figure 11(c) and Supplementary Figure 3). In contrast, the presence of $CD45^+$ cells is abundant at 24 h and 48 h after LPS injection (Figures 11(a) and 11(c)), as compared with those of their respective mock and untreated controls (Figure 11(c) and Supplementary Figure 3). These results show that infiltration of immunological cells predominates in the late phase of acute neuroinflammation.

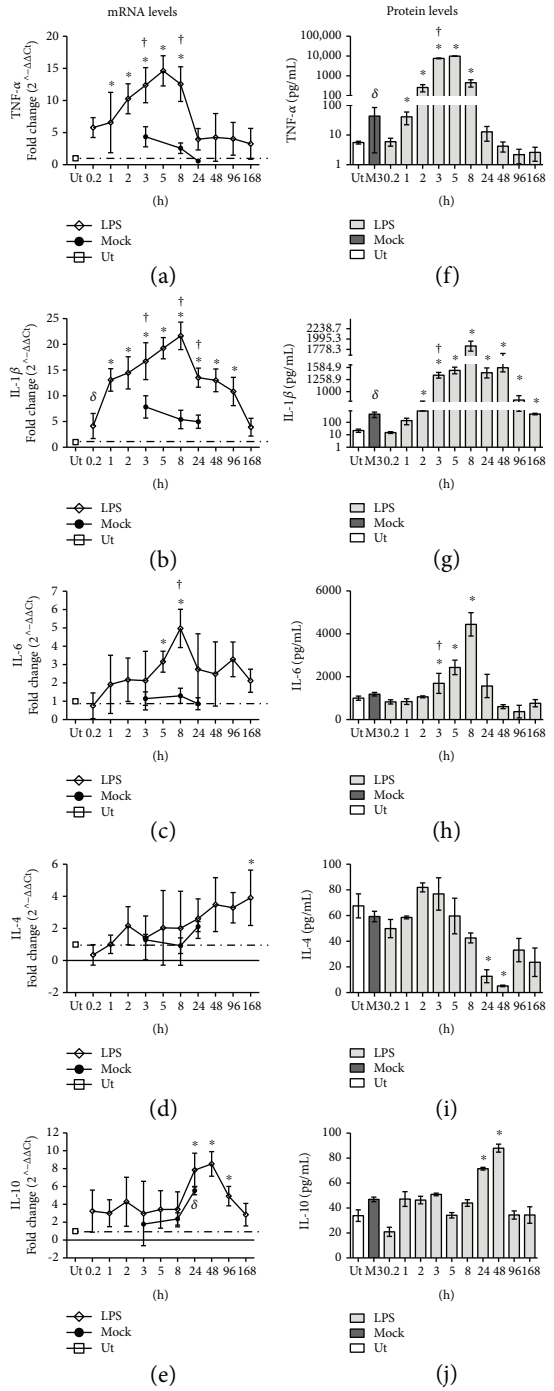

Figure 7: Levels of cytokines in the substantia nigra after LPS local injection. qPCR (a–e) and ELISA (f–j) were used to measure mRNA and protein levels, respectively, for TNF-α, IL-1β, and IL-6 (proinflammatory cytokines) and IL-4 and IL-10 (anti-inflammatory cytokines). Ut = untreated control rats. Mock = rats injected with the vehicle (2 μL of endotoxin-free physiological saline solution) in the left substantia nigra. All values represent the mean ± SD ($n = 4$ independent rats in each time of each experimental condition). $^{\delta}P < 0.05$ or $^{*}P < 0.001$ when compared with the untreated control group. $^{\dagger}P < 0.001$ when compared with the respective mock group. Repeated-measures one-way ANOVA and Newman-Keuls post hoc test for ELISA and repeated-measures two-way ANOVA and Bonferroni post hoc test for the qPCR assay.

4. Discussion

A leading line of research establishes that the microglial activation during neurodegeneration in the substantia nigra pars compacta is atypical [54]; that is, microglial activation leads to proinflammatory cytokine transcription but not translation [55]. This implies that microglial activation in certain conditions does not lead to an inflammatory response as

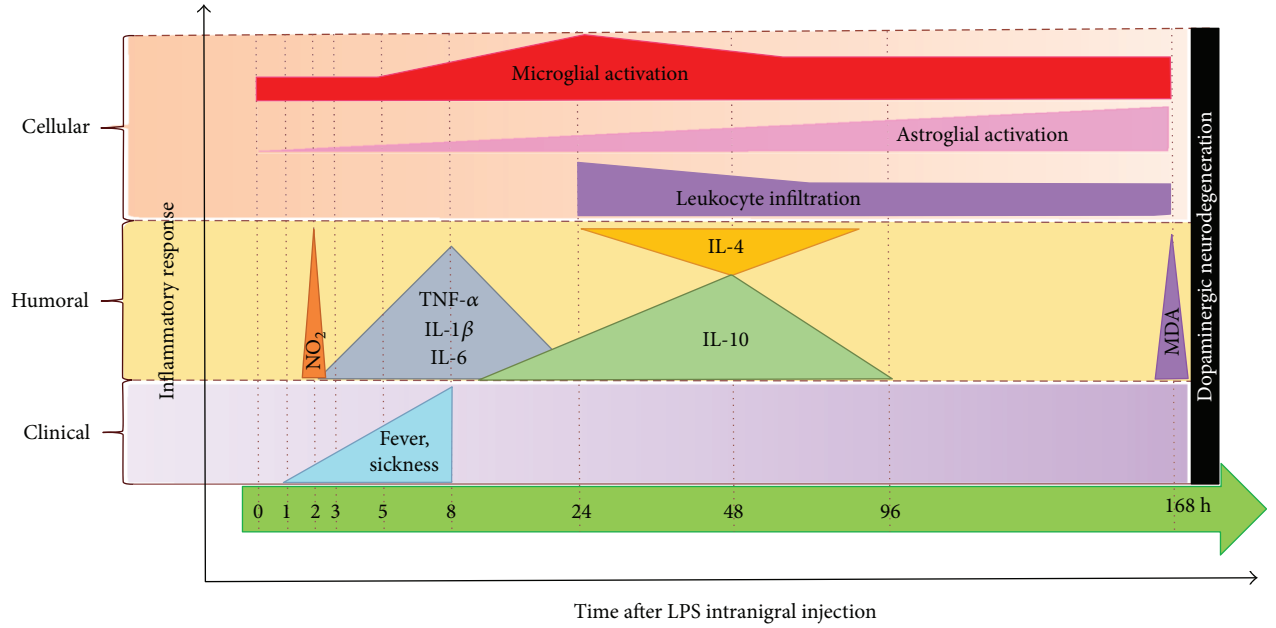

Figure 8: Schematic summary of LPS-induced acute neuroinflammation in the substantia nigra of the rat.

believed. Experiments in a 6-OHDA Parkinson's disease model support the asseveration and truly extend the knowledge that proinflammatory cytokines such as TNF-α instead of being detrimental are beneficial for neurotoxin-induced neurodegeneration [56]. The studies on neuroinflammation in neurotoxin-induced Parkinson's disease models have been addressed in the critical period of neurodegeneration (7–21 days after neurotoxin injection) where the apoptotic process predominates [5, 19]. During this period, it is possible that a modulatory mechanism might be exerted on activated microglia to restrain cytokine translation, but not transcription, thus preventing their participation in neuroinflammation. In contrast, our results show that microglial activation by the archetypal inflammatory stimulus LPS can lead to transcription and translation of proinflammatory cytokines in a similar timeframe as that observed in macrophages activated during the innate immune response (local and systemic) [57–59]. Consecutively, astrocyte activation takes place and continues increasing until the end of the study (168 h after LPS injection). At this time, increased NO and lipid peroxidation levels, apparent phagocytosis of TH^+ cells, and a significant decrease in TH immunoreactivity occur in the substantia nigra, thus suggesting the onset of neurodegeneration of dopaminergic neurons. The increase in NO already 2 h after the LPS stimulus suggests that NO production was independent of LPS-elicited proinflammatory cytokines. Based on these results, we propose that the alleged controversy on the involvement of activated microglia in neuroinflammation can be explained by the difference in the inflammatory stimulus used and the period where neuroinflammation variables are determined.

The increase in body temperature and sickness behavior induced by LPS show the systemic impact of pyrogenic cytokine production (TNF-α, IL-1β, and IL-6) in the substantia nigra. Our results agree with the results of previous studies showing that LPS-induced TLR4 signaling stimulates the synthesis of pyrogenic cytokines at the site of infection including the brain [42, 60]. The time course of fever and sickness behavior induced by the intranigral injection of LPS correlated with that of pyrogenic cytokines TNF-α, IL-1β, and IL-6 [42, 60]. It is interesting to note that the end of fever and sickness behavior coincides with the normalization of IL-6 levels, which is an important mediator of fever induction and a requisite in sustaining fever [60]. In this regard, the loss of IL-6 signaling is sufficient to abrogate fever in LPS- or IL-1-induced inflammatory models, even though TNF and IL-1 are increased in these settings [61–64]. Also, the time of defervescence of fever evoked by the intranigral injection of LPS coincided with the significant expression of IL-10, an antipyretic cytokine [65], in the substantia nigra. The mechanism of the anti-inflammatory effect of IL-10 is likely to be mediated through the inhibition of IL-1β which is locally produced [66]. This suggestion is supported by the finding that the increase in IL-10 expression accompanies the falling of IL-1β expression in the substantia nigra 24 h after LPS intranigral injection.

Microglial cells are resident macrophages of the CNS [67] and also bear TLR4, which can be activated by LPS to initiate an immune response entailing a wide range of immunomodulatory molecules such as proinflammatory cytokine and reactive oxygen species [68]. Since astrocytes are unresponsive to LPS [69], their activation depends on microglial NOX2-generated H_2O_2 that subsequently stimulates activation of transcription factors STAT1 and STAT3 [70]. This evidence indicates that microglial activation precedes astrocyte activation as supported by our results. We found that the peak of OX42 immunoreactivity was reached 24 h after LPS injection and was followed by a maximum increase in GFAP immunoreactivity 168 h after the LPS injection. At this latter time, a significant increase

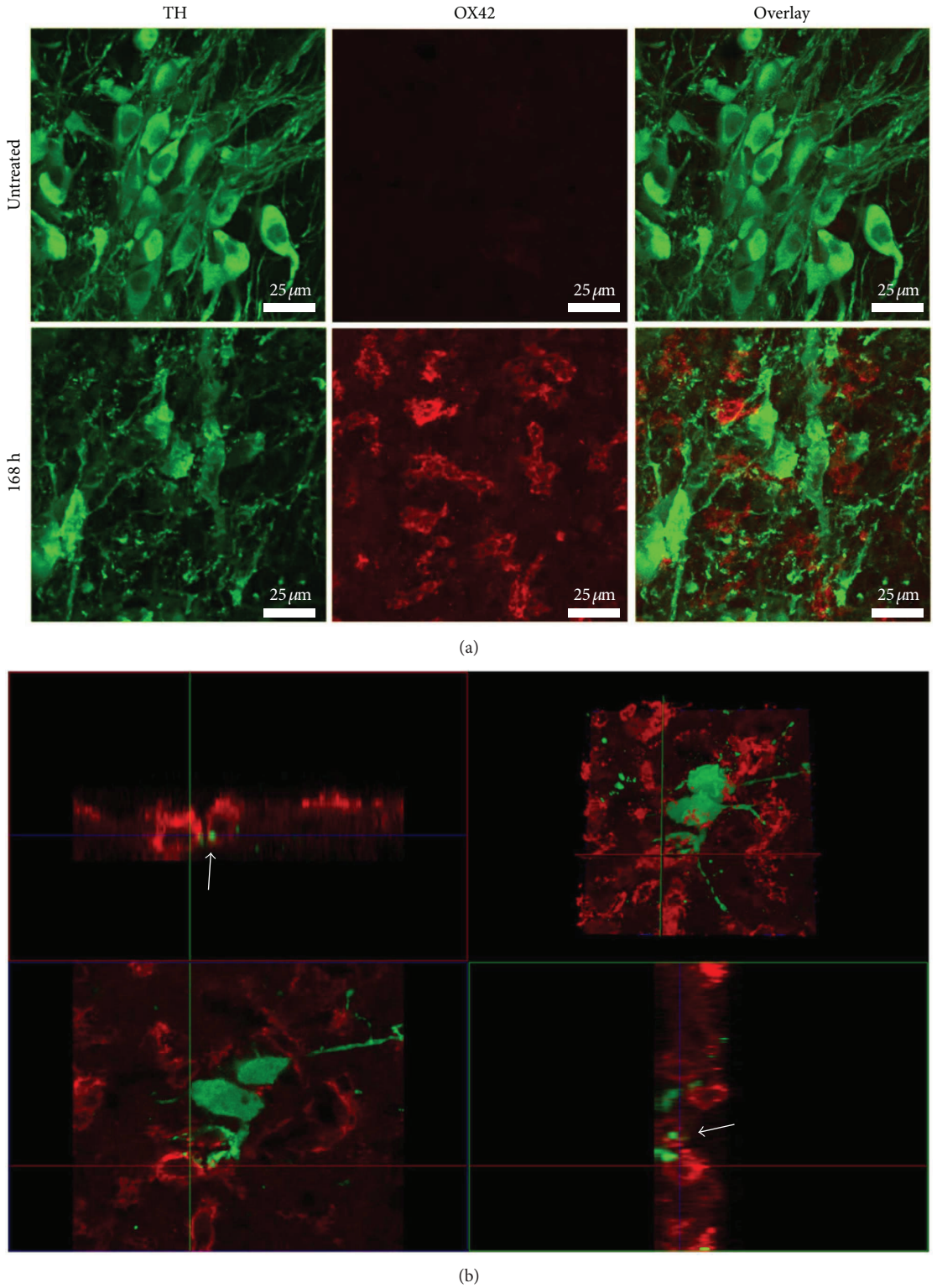

Figure 9: Apparent phagocytosis of damaged dopaminergic neurons by microglia. Representative confocal micrographs of TH and OX42 double immunofluorescence. (a) Integrated projections from x-y optical stacks. (b) Orthogonal projections from a 1 μm z-confocal optical section. The arrows show a green fluorescence dot (TH immunoreactivity) surrounded by a red fluorescence ring (OX42). The right top panel corresponds to the integrated image where the orthogonal analysis was performed.

in NO concentration and lipid peroxidation was also present coinciding with the increased GFAP immunoreactivity. These results suggest that the generation of free radicals, mainly the radical H_2O_2, might participate in astrocyte activation. This phenomenon can be seen through the increased GFAP immunoreactivity, thickening of branches,

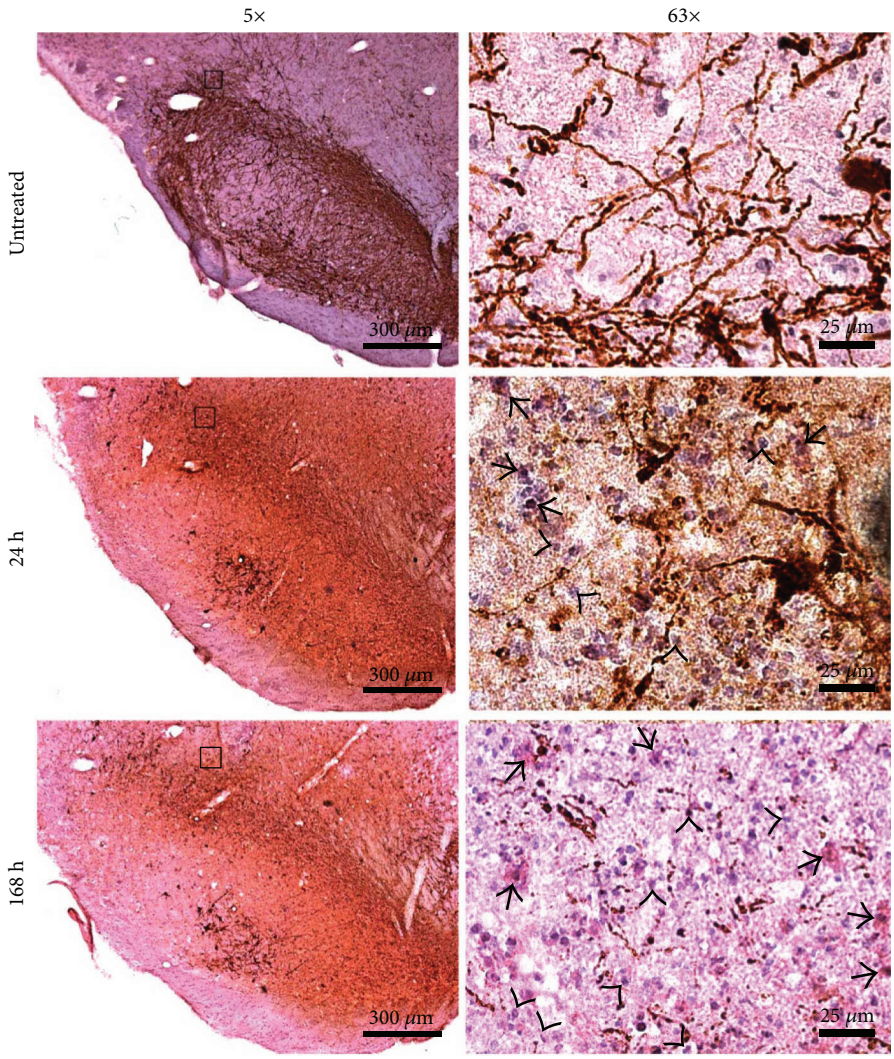

Figure 10: Leukocyte infiltration. Representative micrographs showing the H&E staining and TH immunohistochemistry of the substantia nigra of untreated control rats and experimental rats at 24 and 168 h after LPS injection. The micrographs were taken at 3.2 mm from the interaural midpoint on the dorsal-ventral axis of the rat brain atlas by Paxinos and Watson [50]. The black square on 5x micrographs indicates the area where 63x amplification was taken. Arrowheads indicate leukocyte infiltration, and arrows indicate microglia/macrophages.

and apparent mobility from the pars reticulata to the pars compacta of the substantia nigra shown by the detailed morphological analysis and the panoramic view of GFAP immunofluorescence.

The detailed morphological analysis also shows the prompt activation of microglia. The appearance of OX42 immunoreactivity and morphological changes is observed immediately after LPS injection. Five hours later, a combination of branched microglia and amoeboid microglia can be seen coinciding with the peak of proinflammatory cytokine production. These results suggest that at this time, the greatest transition state of active cells occurs. A predominant amount of amoeboid microglia can be seen 24 h after LPS injection, and it is possible that they also correspond to infiltrating macrophages attracted to the inflamed area by microglial chemokines as suggested by our CD45 immunostaining results at 24 h after LPS injection and the results of previous reports [8, 71]. This suggestion is further supported by the H&E staining that shows the presence of "rod" cells (characterized by the elongated and irregular nuclei and enlarged cytoplasm). Also, the H&E staining also supports leukocyte infiltration in the substantia nigra that can be attracted by microglial chemokines. At 168 h after LPS injection, the large and irregular OX42-immunoreactive cells resemble a phagocytic state of microglia. This suggestion is supported by the confocal orthogonal views that show microglial prolongations surrounding deteriorated dopaminergic neurons as if microglia were engulfing them for degradation. Also, previous studies have shown that this phenotype corresponds to phagocytic microglia when eliminating cellular debris [72].

Our results provide three pieces of evidence that sustain the degeneration of dopaminergic neurons in the substantia nigra at 168 h after LPS injection: (1) the decrease in TH immunoreactivity shown by TH immunofluorescence and immunohistochemistry assays that were performed together with glial markers (OX42 and GFAP) and H&E, respectively, (2) the irregular body contour and prolongation

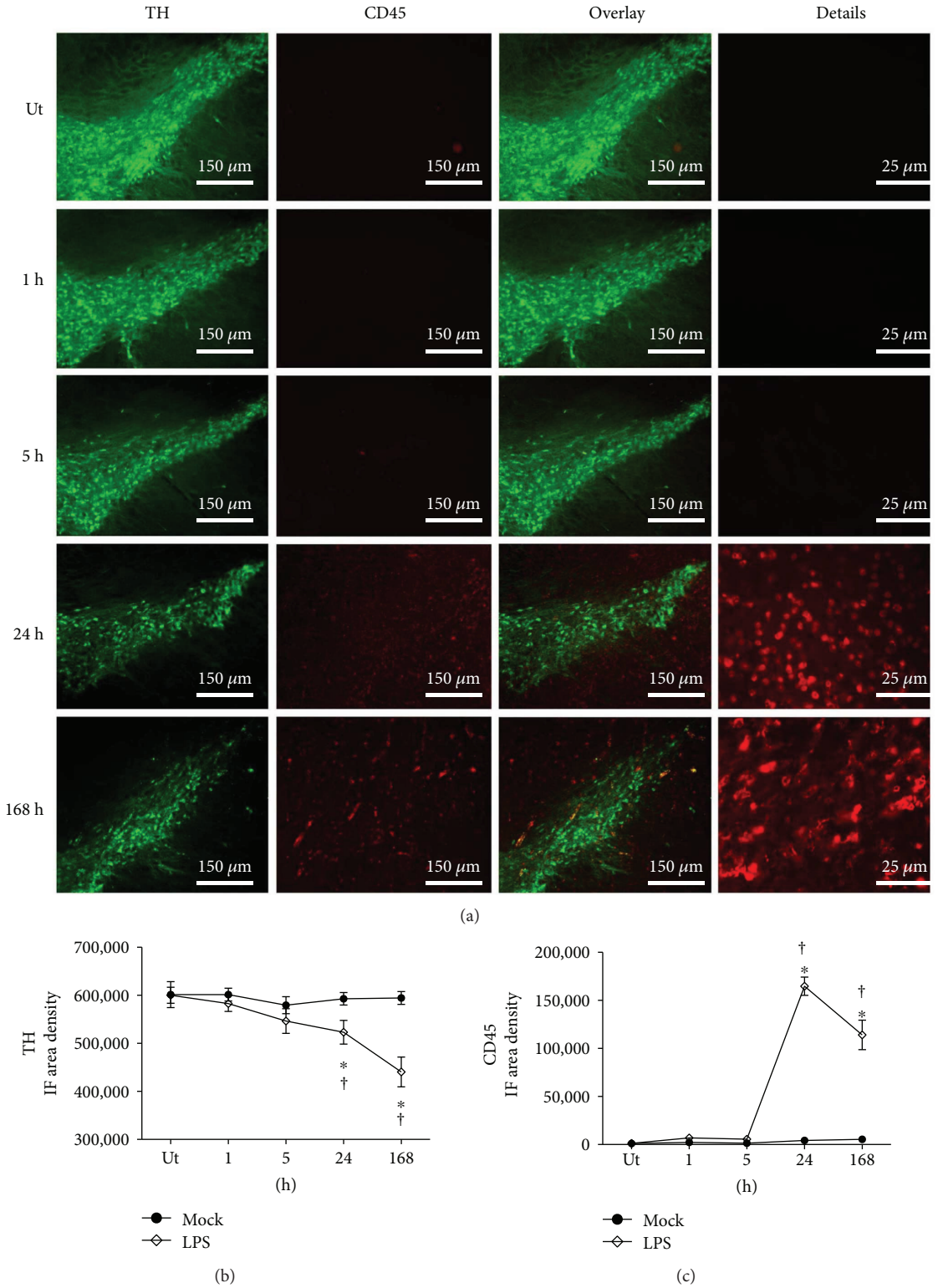

Figure 11: Time course of leucocyte infiltration. (a) Representative micrographs of the double immunofluorescence of CD45 and TH in the substantia nigra of untreated (Ut) control rats and rats at different times after LPS injection that were taken at 3.4 mm from the interaural midpoint on the dorsal-ventral axis of the rat brain atlas by Paxinos and Watson [50]. The numbers at the left side of micrographs indicate the time of evaluation. Immunofluorescence (IF) area density for TH (b) and CD45 (c) was determined using ImageJ software v.1.46r (National Institutes of Health, Bethesda, MD). The TH and CD45 values for the mock rats correspond to the quantification in Supplementary Figure 3. All values represent the mean ± SD ($n = 3$ rats for each time and for each experimental condition). $^{*}P < 0.001$ when compared with the untreated control group of the respective immunostaining. $^{\dagger}P < 0.001$ when compared with the respective mock group. Repeated-measures two-way ANOVA and Bonferroni post hoc test.

discontinuity of TH^+ cells displayed by confocal microscope analysis, and (3) the phagocytosis of TH^+ cells by $OX42^+$ cells. The induction of neurodegeneration cannot be explained by a direct effect of LPS because dopaminergic neurons lack TLR4. Since nigral dopaminergic neurons are particularly vulnerable to nitrosative-oxidative stress [34], we propose that LPS-induced neuroinflammation is the cause of neurodegeneration.

5. Conclusions

Our results show that LPS evokes a typical acute inflammatory response in the substantia nigra of the rat (Figure 8). In this model of acute neuroinflammation, the microglial activation is the first event induced by LPS that is followed by astrocyte activation and leukocyte infiltration (Figure 8). Contrary to the "atypical" response observed in neurotoxin models of dopaminergic neurodegeneration, LPS leads to transcription and translation of proinflammatory cytokines at the initial phase of acute neuroinflammation, from 3 to 8 h (Figure 8). During this period, the increase in proinflammatory cytokine levels is associated with fever and sickness behavior (Figure 8). The acute increase in nitrosative-oxidative stress at the end of the period studied can favor neurodegeneration of dopaminergic neurons because of their susceptibility to neuroinflammation (Figure 8). While neuroinflammation in Parkinson's disease is chronic, our results in acute neuroinflammation can be useful to understand the progression of this disease.

Conflicts of Interest

The authors have no financial, personal, or other relationships with other people or organizations in the past three years of the beginning of the submitted work that could inappropriately influence, or be perceived to influence, their work. The authors declared that no competing interests exist.

Acknowledgments

The authors would like to thank the Unit for Production and Experimentation of Laboratory Animals (UPEAL) of the Center for Research and Advanced Studies (Cinvestav); Rafael Leyva, BSc, Ricardo Gaxiola, BSc; and Mr. René Pánfilo Morales for animal handling. Yazmin M. Flores-Martinez, Manuel A. Fernandez-Parrilla, David Reyes-Corona, Luis O. Soto-Rojas, and Claudia Luna-Herrera were recipients of doctoral fellowships from CONACYT. This work was supported by Grant no. 254686 from CONACYT.

Supplementary Materials

Supplementary 1. Supplementary Figure 1: double immunofluorescence analysis against OX42 and TH in the substantia nigra of untreated (Ut) control rats and mock rats. The representative micrographs correspond to 3.8 mm from the interaural midpoint on the dorsal-ventral axis of the rat brain atlas by Paxinos and Watson [50]. The graph of immunofluorescence (IF) area density for TH and OX42 determined with ImageJ software v.1.46r (National Institutes of Health, Bethesda, MD) is shown in Figures 2(d) and 2(e), respectively. $n = 3$ independent rats in each time of each experimental condition.

Supplementary 2. Supplementary Figure 2: double immunofluorescence analysis against GFAP and TH in the substantia nigra of untreated (Ut) control rats and mock rats. The representative micrographs correspond to 3.8 mm from the interaural midpoint on the dorsal-ventral axis of the rat brain atlas by Paxinos and Watson [50]. The graph of immunofluorescence (IF) area density for TH and GFAP determined with ImageJ software v.1.46r (National Institutes of Health, Bethesda, MD) is shown in Figures 4(d) and 4(e), respectively. $n = 3$ independent rats in each time of each experimental condition.

Supplementary 3. Supplementary Figure 3: double immunofluorescence analysis against CD45 and TH in the substantia nigra of untreated (Ut) control rats and mock rats. The representative micrographs correspond to 3.4 mm from the interaural midpoint on the dorsal-ventral axis of the rat brain atlas by Paxinos and Watson [50]. The graph of immunofluorescence (IF) area density for TH and CD45 determined with ImageJ software v.1.46r (National Institutes of Health, Bethesda, MD) is shown in Figures 11(b) and 11(e), respectively. $n = 3$ independent rats in each time of each experimental condition.

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Hindawi
Journal of Immunology Research
Volume 2018, Article ID 5160794, 10 pages
https://doi.org/10.1155/2018/5160794

Review Article

Lung Interstitial Macrophages: Past, Present, and Future

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Received 25 October 2017; Revised 12 February 2018; Accepted 11 March 2018; Published 30 April 2018

Academic Editor: Zissis Chroneos

For a long time, investigations about the lung myeloid compartment have been mainly limited to the macrophages located within the airways, that is, the well-known alveolar macrophages specialized in recycling of surfactant molecules and removal of debris. However, a growing number of reports have highlighted the complexity of the lung myeloid compartment, which also encompass different subsets of dendritic cells, tissue monocytes, and nonalveolar macrophages, called interstitial macrophages (IM). Recent evidence supports that, in mice, IM perform important immune functions, including the maintenance of lung homeostasis and prevention of immune-mediated allergic airway inflammation. In this article, we describe lung IM from a historical perspective and we review current knowledge on their characteristics, ontogeny, and functions, mostly in rodents. Finally, we emphasize some important future challenges for the field.

1. From Septal Cells to Interstitial Macrophages

Phagocytic "septal cells" were observed by Kaplan and colleagues already in 1950 [1] and likely represented "nonalveolar" macrophages located in the alveolar wall. Nevertheless, the alveolar macrophages (AM) remained the main macrophage population investigated in the lung until the early 1970s. By that time, it was proposed by van Furth and Cohn that, like any other tissue-resident macrophages, AM originated from bone marrow promonocyte precursors, which then circulated in the blood as monocytes and could differentiate into macrophages within the alveoli [2]. As a corollary, an intermediate state of AM maturation, located in the pulmonary interstitium, presumably existed between the blood compartment and the airways. In 1972, "mononuclear interstitial cells" were first proposed as precursors of the AM lineage in cultured lung explants [3]. Since then, lung tissue macrophages were long merely considered as a transition state between circulating monocytes and AM [4–6].

The development of methods to harvest pulmonary macrophages using mechanical and enzymatic treatments allowed the comparison between AM (isolated by bronchoalveolar lavage (BAL)) and lung tissue macrophages (TM) in rodents, even though the latter were contaminated by residual AM [7, 8]. While both AM and TM displayed classical macrophage features such as a phagocytic potential and expression of Fc receptors, these features were reduced in TM as compared to AM [9–13]. Moreover, additional differences were underscored within TM. In mice, TM exhibited a higher percentage of cells positive for the complement receptor C3 [8, 9], a higher production of arachidonic acid metabolites following phagocytosis [14], and an increased spreading capacity when exposed to plasma [9] as compared to AM. In rats, TM were shown to have a higher peroxidase activity [15], a greater major histocompatibility complex class II (MHC-II) expression [16], and a greater number of filopodia [17]. Upon ex vivo stimulation with lipopolysaccharide (LPS), AM displayed greater cytotoxic and antimicrobial activities than TM, while TM secreted more interleukin-(IL-) 1 and IL-6, in mice [13] and rats [16]. Unlike AM, mouse TM were also very potent in promoting mitogen-stimulated spleen lymphocyte proliferation in mice [13]. Despite these morphological, phenotypical, and functional differences, many authors still interpreted them as being part of the transition process between blood-circulating monocytes and AM [3–6, 13], but others raised the possibility that lung TM (also

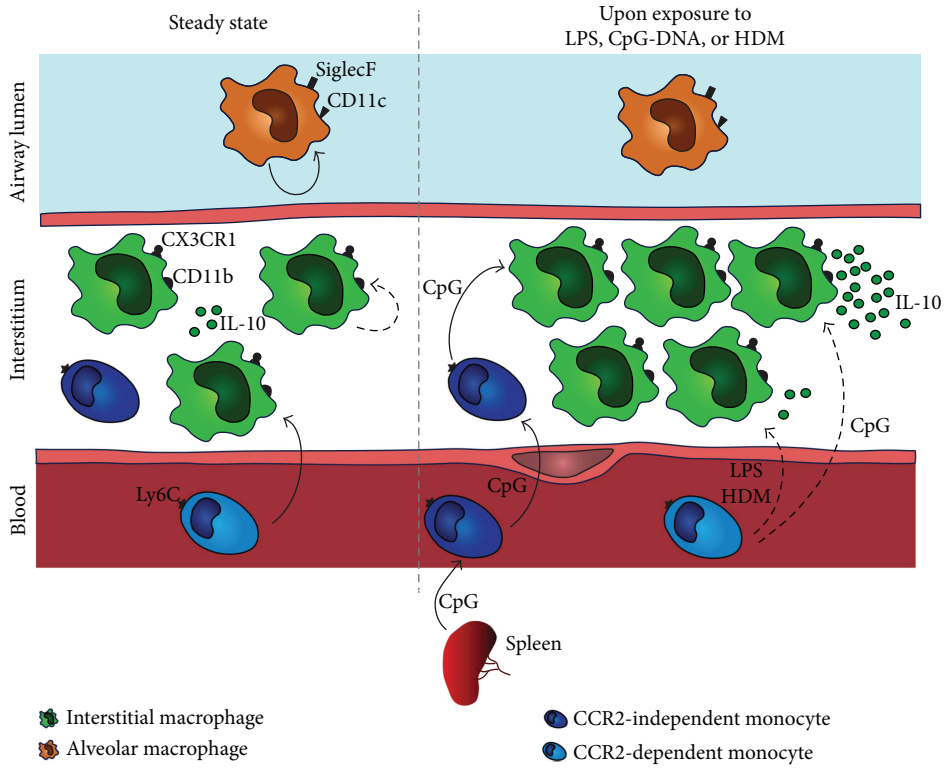

Figure 1: Mouse lung interstitial macrophage phenotype and origin at the steady state and upon exposure to LPS, CpG-DNA, or HDM. For clarity, the ratio between the numbers of depicted AM, IM, and monocytes does not reflect the reality. By definition, IM are located in the lung interstitium, while AM reside in the airway lumen. IM can produce IL-10 at baseline, a phenomenon that is potentiated by an exposure to LPS, CpG-DNA [26], or HDM [50]. Phenotypically, IM are non-autofluorescent SiglecF$^-$CD11b$^+$CX3CR1$^+$Ly6C$^-$ cells, while AM are autofluorescent SiglecF$^+$CD11c$^+$CD11b$^-$CX3CR1$^-$Ly6C$^-$ cells. Steady-state IM, as well as LPS- or HDM-induced IM, are thought to be maintained or expanded by the recruitment of CCR2-dependent Ly6C$^+$ classical blood monocytes, at least in part. Local proliferation may also account for the maintenance of steady-state IM. Following exposure to CpG-DNA, CCR2-independent lung-resident and splenic Ly6C$^+$ monocytes contribute to a large extent to the expansion of the IM pool endowed with enhanced immunoregulatory properties.

called interstitial macrophages (IM)) represented a distinct and fully competent macrophage population [11, 16, 18], a concept that is now well accepted in the field [19–21].

2. Morphological and Phenotypical Features

Most of the abovementioned studies have been performed ex vivo and have defined TM as the cells collected from enzymatically digested lungs and adherent to the culture plate in vitro. Obviously, such a technique did not allow a specific isolation of IM, and the resulting cells were likely contaminated with variable amounts of other mononuclear cell types, such as residual AM (despite extensive BAL [11, 22–24]), conventional dendritic cells (cDCs), or monocytes [25, 26]. In addition, accepting that macrophages, once extracted from their native microenvironment and cultured ex vivo, undergo rapid morphological and phenotypical changes [27], the conclusions drawn from ex vivo-cultured IM have to be interpreted with caution.

Morphologically, Sebring and Lehnert were the first, to our knowledge, to combine a Fc receptor-based affinity technique with a cytometric approach to sort IM from rat lungs and identified them as being smaller than AM, with a smoother surface and a more irregular and heterochromatin-containing nucleus [28]. More recently, freshly isolated mouse IM were shown to exhibit an irregularly shaped nucleus and numerous vacuoles in their cytoplasm, while mouse AM were larger cells [26] with more prominent pseudopodia [29].

The availability of technologies allowing analysis of freshly isolated single cells, such as multicolor flow or mass cytometry, substantially improved the phenotypic characterization of lung immune cells [20, 25, 26, 29–31]. The work of several investigators in the field has allowed, based on the levels of expression of several surface markers, a discrimination between each of the lung myeloid mononuclear cell populations in the steady-state lung, including IM (Figure 1). These markers are compiled in Table 1. Both IM and AM express the macrophage-specific markers CD64 and Mertk, as opposed to cDCs and monocytes. While AM are autofluorescent SiglecF$^+$CD11c$^+$CD11b$^-$CCR2$^-$CX3CR1$^-$ cells, IM are non-autofluorescent SiglecF$^-$CD11c$^{+/-}$CD11b$^+$CCR2$^{+/-}$CX3CR1$^+$ cells [26, 31] (Figure 1). Notably, a recent report has shown that a fraction of mouse IM, defined as

Table 1: Expression of the indicated surface markers between alveolar macrophages (AM), interstitial macrophages (IM), $CD11b^+$ and $CD103^+$ conventional dendritic cells (cDCs), and $Ly6C^+$ and $Ly6C^-$ monocytes (Mo) in the mouse lung at steady state.

Marker	AM	IM	$CD11b^+$ cDCs (cDC2)	$CD103^+$ cDCs (cDC1)	$Ly6C^+$ Mo	$Ly6C^-$ Mo
CCR2	− [26, 31]	+/− [26, 31]	− [31]	− [31]	+ [26, 31]	+/− [26]
CD103	− [29]	− [29]	− [29]	+ [29]	− [29]	− [29]
CD115	+/− [26, 31]	+/− [26, 31]	+ [31]	− [31]	+ [26, 31]	+ [26]
CD11b	− [10, 26, 29, 31, 50]	+ [10, 26, 29, 31, 50]	+ [29, 50]	− [29, 31]	+ [26, 29, 31]	+ [26, 29]
CD11c	+ [10, 26, 29, 31, 32, 50]	− [10, 32]; +/− [26, 31, 50]; + [29]	+ [10, 29, 31, 32]	+ [10, 29, 31, 32]	− [26, 29]; +/− [31]	+/− [26, 29]
CD14	+/− [29]	+ [29]	+/− [29]	− [29]	− [29]	− [29]
CD169	− [13]; + [26, 31]	+/− [13, 31]; + [26, 31]	− [31]	− [31]	− [26, 31]	− [26]
CD206	+ [29]	+/− [29]	+/− [29]	− [29, 31]	− [29, 31]	− [29]
CD24	− [29]	− [29]	+ [29]	+ [29]	− [29]	− [29]
CD36	+ [29, 31]	+ [29, 31]	− [31]; +/− [29]	+ [29, 31]	+/− [29]; + [31]	− [29]
CD64	+ [26, 29, 31]	+ [26, 29, 31]	− [29, 31]	− [29, 31]	− [26]; +/− [29, 31]	− [26]; +/− [29]
CD86	− [50]; +/− [26]	+ [26, 50]	− [50]		− [26]	− [26]
CX3CR1	− [26, 31]	+ [26, 31]	+/− [31]	− [31]	+/− [31]; + [26]	+ [26]
F4/80	+ [10, 26, 29, 31, 32, 50]	+ [10, 26, 29, 31, 32, 50]	− [10, 32, 50]; +/− [29]	− [10, 29, 31, 32]	+/− [31]; + [26, 29]	+ [26, 29]
Ly6C	− [26, 29]	− [26, 29]	+/− [29]	− [29]	+ [26, 29]	− [26, 29]
Lyve-1	− [31]	+/− [31]	− [31]	− [31]	− [31]	
Mertk	+ [26, 31]	+ [26, 31]	− [31]	− [31]	− [26, 31]	− [26]
MHC-II	+/− [13, 26, 29, 32]	+/− [31]; + [13, 26, 29, 32, 50]	+ [29, 31, 32, 50]	+ [26, 29, 31]	− [26, 29]; +/− [31]	− [26, 29]
SiglecF	+ [26, 29, 31]	− [26, 29, 31]	− [29, 31]	− [29, 31]	− [26, 29]	− [26, 29]
Zbtb46	− [31]	− [31]	+ [31]	+ [31]	− [31]	

−: absence of expression or low expression; +/−: intermediate or variable expression; +: high expression.

Mertk$^+$CD64$^+$CD11b$^+$SiglecF$^-$ cells, expressed CD11c and MHC-II [31], like cDCs, so that both cell types may potentially contaminate each other. Nevertheless, cDCs differ from IM by their low or absent expression of macrophage markers (e.g., CD64, Mertk, and F4/80). The situation may be more confusing when inflammation is present and monocyte-derived cells are infiltrating the lung, in which case IM may be included in inflammatory subtypes of monocytes or DCs. Nevertheless, IM may be discriminated from such cells by their low expression of the inflammatory/classical monocyte marker Ly6C.

3. Tissue Localization

At steady state, lung IM are primarily considered as "nonalveolar" macrophages and are therefore virtually absent in the airways, while AM represent the macrophages present in the airway lumen. To date, however, it must be noted that information about the exact localization of IM within the lung tissue remains scarce and is based on standard immunohistochemical procedures using nonspecific pan-macrophage markers [31, 32]. Earlier studies in mice using immunostainings against F4/80 and CD11c markers identified F4/80$^+$CD11c$^-$ cells, defined as IM, within the lung parenchyma, whereas AM, defined as F4/80$^+$CD11c$^+$ cells, were mostly located in the lumen [32]. Experiments using intravenous injection of clodronate-containing liposomes, which efficiently depleted blood monocytes, had no impact on IM numbers [26], supporting that steady-state IM were not associated with blood vessels but truly located in the lung tissue. More recently, Gibbings and colleagues have performed a staining for Mertk on mouse lung sections from CX3CR1-GFP reporter mice at steady state, allowing the visualization of Mertk$^+$CX3CR1$^+$ IM in the bronchial interstitium, in the vicinity of lymphatic vessels, but not in the lung parenchyma [31]. In the same report, no Mertk$^+$CX3CR1$^+$ cells were observed on the pleural surface nor in the blood vessels [31]. Given the complexity and dynamic regulation of the lung tissue macrophage compartment, the generation of novel transgenic tools allowing the specific tracking and visualization of IM in vivo will help solve the question of their localization and their spatiotemporal relationships with the local microenvironment, such as bronchial and alveolar epithelial cells, stromal cells, endothelial cells, or lymphoid tissues [33], at steady state and during inflammation. The fact that distinct subpopulations of IM exist, as reported recently [31], is consistent with the idea that they may reside in more than one anatomical site.

4. Origin, Maintenance, and Expansion

Most tissue-resident macrophages are thought to derive from embryonic precursors arising from different sources: the yolk sac (i.e., erythromyeloid progenitor- (EMP-) derived premacrophages), the fetal liver (i.e., EMP-derived monocytes or hematopoietic stem cell- (HSC-) derived monocytes), or the bone marrow (i.e., HSC-derived monocytes), as reviewed and discussed extensively elsewhere [34–36]. At steady state, the well-characterized AM have been shown, in mice, to originate from fetal monocytes that seed the airway lumen around birth [20]. On the contrary, IM ontogeny seems more complicated and less documented. In 2016, Tan and Krasnow investigated the development of lung macrophages by marker expression patterns and genetic lineage tracing [21]. The authors used *Runx1*^{CreER} transgenic mice expressing the tamoxifen-inducible Cre recombinase under the control of the *Runx1* promoter (*Runx1* being expressed in primitive hematopoietic cells located exclusively in the yolk sac between E7 and E8 [37, 38]) and found that a subset of yolk-sac-derived premacrophages seeded the lung starting at E10.5 and persisted as "primitive" IM at specific submesothelial and perivascular locations in adults [21]. In addition, they identified an additional wave that developed rapidly after birth to give rise to "definitive" IM located diffusely in the lung parenchyma and thought to originate from the bone marrow [21]. These results are consistent with the idea that IM have a mixed origin, both an embryonic yolk-sac-derived origin and a postnatal bone marrow-derived origin.

During homeostasis, most embryonically derived tissue-resident macrophages, like AM, can self-maintain throughout life with minimal contribution from circulating monocytes [20, 35, 39–41]. In the case of IM, parabiosis studies have suggested that they are, at least in part, replenished from blood monocytes for their maintenance in adults [21, 26] (Figure 1), like macrophages from the intestinal lamina propria [42], skin [43], and heart [44]. In the report of Tan and Krasnow, parabiotic wild-type (WT) mice were sutured together and exchanged their circulation with "donor" ubiquitous EGFP mice for 4 months. The lungs of WT mice were then examined for enrichment in EGFP$^+$ cells, and 17% of IM were EGFP$^+$, demonstrating that circulating precursors can maintain the IM pool in adults, as opposed to AM [21]. Further supporting this, our group has analyzed the lungs of parabiotic *Ccr2*$^{-/-}$ mice (in which the egress of monocytes from the bone marrow is compromised [45]) that were sutured together with a WT "donor" for 6 months and showed that 35% of IM derived from WT cells. The relatively low percentage of IM replacement by circulating "donor" cells in parabiotic studies is consistent with the mixed origin proposed by Tan and Krasnow [21] and with the idea that only one subpopulation of IM is maintained by circulating monocytes after birth, whereas another subpopulation is long-lived and may be able to self-renew in the tissue (Figure 1). This idea is further supported by the study of Gibbings and colleagues identifying, in the mouse steady-state lung, at least three IM subsets, with one subset displaying a higher turnover rate and replenishment by circulating precursors than the two others [31].

Which population is preponderant in young, adult, and aged animals, which consequences does it have on their biological functions, and how is it influenced by the numerous immune challenges to which the lung is exposed throughout life remain interesting open questions for future research. Emphasizing the complexity of IM ontogeny in response to environmental stimuli, our group reported that local exposure to unmethylated CpG-rich DNA (CpG-DNA) promoted a robust TLR-9-dependent expansion of IM unexpectedly originating from monocytes residing in the

lung or recruited from the spleen, independently of CCR2 [26] (Figure 1).

5. Heterogeneity and Plasticity

The existence of subpopulations of IM in rats was first proposed in 1986 by Chandler and colleagues [15] and further investigated by the same group [46, 47], based on density gradient fractionation. The fractions of lower density displayed greater functional capacities (e.g., Fc-mediated binding and phagocytic activity, production of prostaglandin and thromboxane, and migration upon exposure to chemotactic stimuli) as compared to the fractions of higher density [15, 46, 47]. However, it is unclear whether these differences may be attributed to a true heterogeneity within IM or to a contamination of the higher density fractions with granulocytes, as reported [15]. Nevertheless, several recent reports have provided experimental evidence that IM represented a heterogeneous population in the steady-state lung. First, the use of IL-10 reporter ITIB mice [48] supported that two subpopulations of IM exist in terms of IL-10 expression [26]. Second, IM have been shown to segregate in a phagocytic and a nonphagocytic compartment in vivo [49]. Third, as stated above, Gibbings and colleagues have recently described three distinct IM subpopulations, based on their relative surface expression of CD11c and MHC-II, namely, $CD11c^{low}MHC\text{-}II^{low}$ (IM1), $CD11c^{low}MHC\text{-}II^{high}$ (IM2), and $CD11c^{+}MHC\text{-}II^{high}$ (IM3) [31]. Phenotypically, IM1 and IM2 expressed higher levels of CD206, Lyve-1, and CD169 as compared to IM3, which expressed higher levels of CCR2 and CD11c. Functionally, IM1 and IM2 appeared to be more efficient than IM3 but less efficient than AM in the phagocytosis of latex microbeads or microbial bioparticles in vivo, whereas the three populations had similar phagocytic abilities when the experiment was performed ex vivo to provide a similar access to the beads for each subset [31].

These results highlight the potential diversity of mouse IM at steady state. It is very likely that the picture becomes even more complex when the lungs are exposed to endogenous or exogenous stress signals, such as following tissue damage or during inflammation or infection. Under these circumstances, IM may adapt their phenotype and function to respond to the needs of the lung tissue, and additional inflammatory monocytes may also be recruited into the lung and acquire features of IM. Supporting this, Kawano and colleagues have shown that the numbers of IL-10-producing IM were increased following local challenge with house dust mite extracts (HDM) in mice [50]. In addition, we have shown that local exposure of mice to LPS or CpG (i.e., ligands of the TLR-4 and TLR-9, resp.) induced increases in IM numbers as well as substantial phenotypical changes, while no change in IM numbers was detected in response to lung infection with influenza A virus or *Staphylococcus pneumoniae*, or following intranasal exposure to ligands of TLR-1/2, TLR-3, and TLR-2/6 [26]. Besides microbial products, IM may also be impacted by tissue damage and hypoxia [29, 51, 52]. Indeed, increases in IM numbers have also been observed in mouse models of acute lung injury (ALI) based on local instillations of bleomycin [29] or high doses of LPS [51]. Such IM expressed higher levels of classically activated "M1" macrophage markers (CD40, CD80, and CD86) as compared to basal IM. During the later stages of tissue repair, however, IM numbers and phenotype returned to baseline levels [51]. In response to low oxygen levels in mice, numbers of IM transiently increased and their transcriptome seemed to shift toward an anti-inflammatory gene profile at a later stage [52], consistent with a previous observation that the hypoxia-responsive transcription factor Hif1α promoted IM immunoregulatory activity in allergenic contexts [53].

6. Biological Functions *In Vivo*

Many putative functions of IM in vivo could be speculated based on their phenotypical and functional properties. Like AM, IM are phagocytic cells [14–16, 31, 32, 49, 54] and could thus be considered as a second line of defense against invading microorganisms. In addition, based on their expression of MHC-II [26, 31, 54], one can postulate that mouse IM could exhibit some antigen-presenting cell activity, as suggested by earlier reports [55]. So far, however, most of the functional studies on IM in mice focused on their potential immunoregulatory properties. Indeed, mouse and human IM have been shown to express the immunosuppressive cytokine IL-10 at steady state [24–26, 32, 56] (Figure 1). Such IL-10 expression increases in response to environmental stimuli such as LPS, CpG-DNA, or HDM [26, 50, 56] (Figure 1). Knowing that the lung mucosa is constantly exposed to a wide range of immunostimulatory molecules and allergens, we postulated that IM may contribute to lung homeostasis through the alteration of lung cDC functions, which are endowed with the ability to trigger an allergen-specific T helper type 2 (Th2) cell response orchestrating the development of allergic airway inflammation in mice exposed to LPS and allergens [57–60].

Using a coculture system between freshly isolated IM and LPS- and ovalbumin- (OVA-) pulsed bone marrow-derived DCs (BMDCs) in vitro, IM were found to impair the ability of BMDCs to migrate to the draining lymph node and to induce features of Th2-mediated airway allergy once reinjected in the trachea of recipient mice through TLR-4-, HIF1α-, and IL-10-dependent mechanisms [32]. Notably, while isolated and cocultured IM may have encompassed other cell types such as F4/80-expressing monocytes or resident eosinophils [26, 61], the "true" IM were the only cells able to secrete IL-10, and the ability of FACS-sorted pure IM to inhibit DC function has been confirmed later [26, 61]. In vivo, systemic treatment of WT mice with depleting antibodies directed against F4/80 induced a depletion of IM, but not AM, and triggered increased activation of lung cDCs and the development of overt Th2 and allergic airway inflammation when mice were exposed to low doses of an allergen/LPS mixture [32], further supporting a tolerogenic role for IM in maintaining lung homeostasis.

Mouse IM may be implicated in the control not only of allergic asthma in mice but also of other asthma phenotypes. Indeed, Kawano and colleagues have provided evidence that IM contributed to the prevention of Th17-mediated

neutrophilic airway inflammation by IL-10-dependent mechanisms [50]. They used a neutrophilic asthma model based on HDM instillations in *Il10*$^{-/-}$ mice, which dramatically increased the number of neutrophils in the BAL fluid and promoted lung neutrophilic infiltration and expression of Th17-related cytokines as compared to HDM-exposed WT mice. In this model, they showed that the transfer of WT IM in *Il10*$^{-/-}$ mice before the HDM challenge could inhibit the neutrophilic inflammation and mucus production, which was associated with a decrease of Th17-related cytokines and IL-13 [50].

The fact that IM respond to LPS and CpG-DNA, two bacterial products omnipresent in the environment [62, 63], suggests a link with the "hygiene hypothesis," which postulates that decreased exposure to environmental and commensal microbes or their products (PAMPs), partly because of changes associated with urban lifestyles, is responsible for the dramatic increase in the prevalence of allergies and asthma over the past decades [64, 65]. In line with this assumption, several epidemiological studies have demonstrated that growing up on a farm, where exposure to environmental and commensal PAMPs is high, reduces the risk of allergic sensitization [62, 65]. Exposure of humans or mice to CpG-DNA from bacteria reproduces these protective effects [66–71], suggesting a contribution of CpG-DNA to microbe-induced asthma resistance. In mouse models, local CpG-DNA exposure had the unique ability to amplify the IM pool from monocytes residing in the lung or recruited from the spleen, which acquired a hypersuppressive profile [26]. Importantly, such CpG-DNA-induced IM were suggested to mediate the protective effects of CpG-DNA on allergic airway sensitization and inflammation, since adoptive transfer of IM isolated from CpG-DNA-treated WT mice, unlike the *Il10*$^{-/-}$ counterparts, recapitulated the effects of CpG when administered before allergen sensitization or challenge [26]. While speculative at this point, these findings provide a possible mechanistic explanation for the reduced risk of asthma in a microbe-rich environment and for the immunotherapeutic effects of synthetic CpG-DNA in experimental models and human clinical trials.

7. IM in Human and Nonhuman Primates

IM were already observed more than three decades ago in lungs from healthy subjects [72] or diseased patients [73, 74]. By that time, the functional studies comparing human lung macrophages obtained from the BAL and from the whole lung revealed very few differences between BAL-derived AM and tissue IM, possibly because tissue macrophages were heavily contaminated by residual AM [74].

Like in rodents, IM isolated from minced and digested human lungs are smaller and more heterogeneous in shape [54, 56], displayed a lower phagocytic activity [54], and expressed more surface MHC-II (HLA-DR) [56, 75] as compared to AM. Lung IM, which were obtained from the uninvolved lung tissue of patients undergoing a surgical resection for lung carcinoma and put in contact with stimulated T cell membranes, produced higher levels of matrix metalloproteinases (MMPs) and of an inhibitor of MMP (TIMP-1), whereas AM did not significantly react under the same conditions [75], suggesting a possible contribution of human IM in the regulation of lung tissue remodeling.

Hoppstädter and colleagues showed that the secreted levels of IL-6, IL-10, and IL-1 receptor antagonist (IL-1Ra) were higher in IM than in AM, both at baseline and after stimulation with LPS [56]. *Il10* gene expression was also higher in IM at baseline and after stimulation with LPS or DNA from certain bacteria [56], reminiscent of what is observed in mice. On the contrary, AM secreted higher levels of proinflammatory cytokines, such as IL-1β, IFN-γ, IL-12p40, or IL-12p70, after LPS stimulation [56]. Altogether, these results supported a more pronounced anti-inflammatory phenotype of IM as compared to AM and are consistent with a potential role for IL-10-producing IM in the maintenance of lung homeostasis in humans. Interestingly, a recent study performed on bronchial biopsies of asthmatic patients and healthy subjects showed that asthmatic airways were characterized by less IL-10^{+} IM as compared with healthy airways, suggesting that IM may be functionally impaired in asthma [76].

Given the limited access to healthy human samples, Cai and colleagues performed some studies on rhesus macaques as a model to unravel the human lung macrophage identity and diversity [5]. IM were defined as HLA-DR^{high}CD206$^{-/int}$CD11b^{high} cells and were located in the peribronchovascular and subpleural regions, whereas AMs were defined as CD206^{+}CD11b^{int} larger cells that were located almost exclusively in the alveoli. Notably, the IM population had probably been confounded with tissue monocytes in this study, since IM and CD14^{+} blood monocytes resembled each other when analyzing the expression of 27 different markers, with the exception of CCR2 being highly expressed by blood monocytes and poorly by IM. This could also account for the fact that "IM" were found positive for BrdU as soon as 48 hours after its intravenous injection and were thought to contribute to the repopulation of AM after BAL-induced depletion [5].

Recent reports are aimed at identifying markers to discriminate IM from other lung monocyte and macrophage populations in human pulmonary tissue. In humans, AM and monocytes can be defined as highly autofluorescent SSC^{hi}CD169^{hi}CD206^{hi} and SSC^{lo}CD169^{-}CD206^{-}CD14^{+-} CD16$^{lo/hi}$ cells, respectively [77–79]. In addition, a population of HLA-DR^{+}CD169^{lo}CD206^{int} cells was identified in the human lung, whose size was intermediate between AM and monocytes [77–79] and which may correspond to human IM [77, 79]. Functional studies of these cells could help in determining their homology with murine IM. Human lung macrophages of COPD patients were also characterized recently [80]. In this report, "IM" were shown to be divided into two subpopulations: a scarce population of large macrophages, which expressed more CD206, and a population of small macrophages, potentially monocyte-derived, expressing more HLA-DR, CD14, CD38, CD36, and proinflammatory genes as compared to the other lung macrophages [80]. While this study has mainly focused on the analysis of pathological tissues, it emphasizes the complexity of the IM

pool in diseased patients. It also suggests that IM can exert either anti- or proinflammatory properties, depending on the physiological or pathological conditions to which they are exposed.

8. Future Challenges

While substantial progress has been made regarding the ontogeny, phenotype, and functions of IM in mice, it is only the beginning of the story. First, to date, IM or IM subsets have been only characterized as bulk populations, defined according to a limited number of markers, thus revealing average signatures and ignoring the true and unbiased heterogeneity and structure of the populations of interest. There is therefore a need for an unbiased characterization of the IM population, both in mice and humans. Recent development and availability of high-dimensional single-cell technologies [81, 82] should help study highly diverse and heterogeneous immune cells such as IM.

Second, the biological responses modulated by IM or IM subpopulations in vivo remain rudimentarily investigated but are likely highly diverse and complex. This is partly due to the current lack of selective tools to track, modulate, or deplete IM (or IM subsets) in animal models, which will be instrumental in deciphering the biological functions of IM in health and diseases. On the one hand, IM may contribute to important physiological processes during lung development, metabolism, or aging. On the other hand, IM may also modulate several aspects of the pathological responses observed in lung chronic inflammatory disorders such as chronic obstructive pulmonary diseases (COPD).

Third, translational studies aimed at defining lung IM identity, heterogeneity, and functions in humans will be essential to find novel therapeutic targets for the prevention or treatment of lung diseases in which IM (dys)functions are, or will be, implicated.

Conflicts of Interest

The authors declare that no conflict of interest exists. Joey Schyns is a research fellow of the FRIA (Fund for Research Training in Industry and Agriculture—Fonds De La Recherche Scientifique - FNRS). Thomas Marichal is a research associate of the Fonds De La Recherche Scientifique - FNRS.

Authors' Contributions

Fabrice Bureau and Thomas Marichal equally contributed to this work.

Acknowledgments

This publication is supported by the Fonds De La Recherche Scientifique - FNRS for the FRFS-WELBIO (Walloon Excellence in Life Sciences and Biotechnology). Fabrice Bureau is supported by the Fonds De La Recherche Scientifique - FNRS for the FRFS-WELBIO under Grant CR-2012s-01R. Thomas Marichal is supported by an "Incentive Grant for Scientific Research" of the Fonds De La Recherche Scientifique - FNRS (F.4508.18), by the FRFS-WELBIO under Grant CR-2017s-04, and by the Fondation Acteria. The authors thank Raja Fares, Cédric François, and Ilham Sbai for excellent secretarial and technical assistance.

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Hindawi
Journal of Immunology Research
Volume 2018, Article ID 5783639, 10 pages
https://doi.org/10.1155/2018/5783639

Research Article

The Glucagon-Like Peptide-1 Receptor Agonist Exendin-4 Inhibits Lipopolysaccharide-Induced Osteoclast Formation and Bone Resorption via Inhibition of TNF-α Expression in Macrophages

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Received 27 July 2017; Accepted 24 January 2018; Published 13 March 2018

Academic Editor: Hao Liu

Glucagon-like peptide-1 (GLP-1) receptor agonists are an effective treatment approach for type 2 diabetes. Recently, anti-inflammatory effects of GLP-1 receptor agonists have also been reported. Lipopolysaccharide (LPS) induces inflammation and osteoclast formation. In this study, we investigated the effect of exendin-4, a widely used GLP-1 receptor agonist, in LPS-induced osteoclast formation and bone resorption. LPS with or without exendin-4 was administered on mouse calvariae by daily subcutaneous injection. The number of osteoclasts, the ratio of bone resorption pits, and the level of C-terminal cross-linked telopeptide of type I collagen (CTX) were significantly lower in LPS- and exendin-4-coadministered mice than in mice administered with LPS alone. RANKL and TNF-α mRNA expression levels were lower in the exendin-4- and LPS-coadministered group than in the LPS-administered group. Our *in vitro* results showed no direct effects of exendin-4 on RANKL-induced osteoclast formation, TNF-α-induced osteoclast formation, or LPS-induced RANKL expression in stromal cells. Conversely, TNF-α mRNA expression was inhibited in the exendin-4- and LPS-cotreated macrophages compared with cells treated with LPS alone. These results indicate that the GLP-1 receptor agonist exendin-4 may inhibit LPS-induced osteoclast formation and bone resorption by inhibiting LPS-induced TNF-α production in macrophages.

1. Introduction

The prevalence of type 2 diabetes mellitus is increasing worldwide, and the condition has become a major public health problem. Individuals with type 2 diabetes have been shown to have a higher risk of bone fracture compared with individuals without type 2 diabetes [1]. This higher risk might be associated with the pathobiology of type 2 diabetes itself; however, the underlying mechanisms remain unclear [2]. Additionally, increased bone fracture risk is a consequence of therapeutic regimen used to treat hyperglycemia [3]. For example, patients treated with thiazolidinediones and human recombinant insulin have been shown to have an increased bone fracture risk [4–7]. Conversely, treatment with metformin is related to decreased bone fracture risk [8].

Osteoclast recruitment is crucial to the pathogenesis of diseases involving bone erosion, such as rheumatoid arthritis [9]. Osteoclasts derived from bone marrow cells are responsible for bone resorption and remodeling [10]. Receptor activator of NF-kB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) are two key factors required for osteoclast formation and activation [11]. Independent of RANKL, tumor necrosis factor- (TNF-) α has also been reported to induce osteoclast formation *in vitro* [12–14] and *in vivo* [15, 16].

Lipopolysaccharide (LPS) strongly induces inflammation and inflammatory bone loss [17–21]. LPS has also been found to induce production of proinflammatory cytokines, such as TNF-α, from macrophages or other cells at the site of inflammation [22, 23]. Such proinflammatory cytokines have been reported to be involved in LPS-induced osteoclast

formation and bone destruction in *in vivo* and *in vitro* studies [18, 24–27]. Additionally, LPS can stimulate osteoblasts to produce or secrete RANKL [28].

Glucagon-like peptide-1 (GLP-1), an intestinal hormone, plays important roles in blood glucose control and proliferation of pancreatic islet β-cells [29, 30]. GLP-1 receptor-deficient mice were reported to exhibit osteopenia and increased osteoclast formation, suggesting that the GLP-1 signaling has an inhibitory effect of bone resorption on bone metabolism [31]. An anabolic effect of GLP-1 on bone metabolism has also been proposed. GLP-1 receptor activation has been shown to induce bone formation in streptozotocin-induced diabetic and fructose-stimulated insulin-resistant rats [32].

It has been reported that patients with type 2 diabetes have high risk of bone fracture [1, 2]. Furthermore, antidiabetic medicines such as thiazolidinediones may further promote bone resorption and increase fracture risk [33–35]. However, a recent meta-analysis has reported that GLP-1 receptor agonist treatment does not affect fracture risk in type 2 diabetic patients [36, 37].

The anabolic and antiresorptive effects of GLP-1 receptor suggest that GLP-1 receptor signaling may be a promising therapeutic target for osteoporosis or other osteolytic bone diseases; such a therapeutic approach would be facilitated by the fact that the first commercially available GLP-1 receptor agonist, exendin-4, has already been approved for the treatment of diabetes for over 10 years [38]. Exendin-4 shares similar structural and functional properties to GLP-1 but is resistant to the degradation by dipeptidyl peptidase-IV, which can degrade GLP-1 immediately in the blood [39]. The extended half-life, improved pharmacokinetics, and high potency of exendin-4 make it suitable for clinical use [39–41].

In the present study, we investigated the effects of exendin-4 on LPS-induced osteoclast formation and bone remodeling in mice.

2. Materials and Methods

2.1. Animals and Reagents. Eight- to ten-week-old male C57BL6/J mice were obtained from CLEA Japan (Tokyo, Japan) and maintained at our animal facility. All animal care and experiments were conducted according to Tohoku University rules and regulations. Four mice were randomly assigned to each experimental group. Both *Escherichia coli* LPS and exendin-4 were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Histological Analysis. A previous *in vivo* study demonstrated that daily subcutaneous injections of 100 μg LPS to mouse calvariae for 5 days effectively induced osteoclast formation [42]. Therefore, we followed the same protocol, dose, and LPS administration period in this study. The mice were divided into four experimental groups and subjected to daily subcutaneous injections on the calvaria with phosphate-buffered saline (PBS, negative control group), LPS alone (100 μg/day, positive control group), LPS (100 μg/day) and exendin-4 (20 μg/day), and exendin-4 alone (20 μg/day) for 5 days. All mice calvariae were excised immediately after sacrifice on the sixth day. The calvariae were fixed in 4% PBS-buffered formaldehyde at 4°C overnight and then demineralized with 14% ethylenediaminetetraacetic acid (EDTA) at room temperature for three days. Each calvaria was cut into three pieces perpendicular to the sagittal suture. Samples were then embedded in paraffin and cut into 5 μm sections using a microtome. The paraffin sections were stained with tartrate-resistant acid phosphatase (TRAP) solution prepared by mixing acetate buffer (pH 5.0), naphthol AS-MX phosphate (Sigma Chemical, St. Louis, MO, USA), Fast Red Violet LB Salt (Sigma), and 50 mM sodium tartrate. The sections were counterstained with hematoxylin. Osteoclasts were defined in this study as TRAP-positive cells with three or more nuclei. We counted the number of osteoclasts only at the suture mesenchyme of the sagittal suture in all slides according to the method in our previous work [43].

2.3. Preparation of Osteoclast Precursors for Osteoclastogenesis. To isolate bone marrow cells from C57BL6/J mice, femora and tibiae were aseptically removed after sacrifice. The epiphyses of these long bones were removed, and the bone marrow was flushed into a sterile Petri dish with a 25-gauge needle and 10 ml syringe filled with culture medium. The bone marrow was then filtered with a 40 μm nylon cell strainer (Falcon, USA) and centrifuged. The harvested cells were incubated in a culture medium comprising alpha-modified minimal essential medium (α-MEM; Sigma) containing 10% fetal bovine serum (FBS), 100 IU/ml penicillin G (Meiji Seika, Tokyo, Japan), and 100 μg/ml streptomycin (Meiji Seika), with M-CSF added. Nonadherent cells were removed by washing with PBS, and adherent cells were harvested using trypsin-EDTA solution (Sigma-Aldrich). The harvested cells were seeded and further cultured in the presence of M-CSF. Adherent cells were used as osteoclast precursors in this study as previously reported [43]. Osteoclast precursors were seeded at 5×10^4 cells per 200 μl of medium in a 96-well plate and cultured in medium containing M-CSF alone (100 ng/ml), M-CSF (100 ng/ml) and RANKL (100 ng/ml) or TNF-α (100 ng/ml), M-CSF (100 ng/ml) and RANKL (100 ng/ml) or TNF-α (100 ng/ml) with exendin-4 (100 ng/ml), and M-CSF (100 ng/ml) with exendin-4 (100 ng/ml), for 5 days. The cultured cells were then fixed with 10% formalin for 30 min. After fixation, the cells were permeabilized with 0.2% Triton X-100 for 5 min at room temperature, then incubated in TRAP staining solution prepared as described above. TRAP-positive cells with three or more nuclei were considered to be osteoclasts and were counted under a light microscope.

2.4. Preparation of Bone Marrow Stromal Cells. Bone marrow cells were obtained by the method described above and cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma) containing 10% FBS, 100 IU/ml penicillin G (Life Technologies, Carlsbad, CA), and 100 μg/ml streptomycin (Life Technologies) for two weeks. Then the culture disks were washed vigorously with PBS to remove nonadherent

cells. Adherent cells were used as stromal cells in this study as previously reported [43].

2.5. Isolation of Murine Macrophages. Macrophages were obtained from the peritoneal cavity of mice. To obtain resident macrophages under resting conditions, we injected 5 ml of sterile ice-cold PBS (pH 7.4) into the peritoneal cavity and aspirated the fluid to harvest peritoneal cells. The cells were washed twice with α-MEM medium (Sigma) containing 10% FBS. After 1 hour of culture, nonadherent cells were removed, and after 24 hours of culture, adherent cells were harvested and used as macrophages.

2.6. Isolation of RNA and Real-Time RT-PCR Analysis. Calvariae from the *in vivo* experiments were frozen in liquid nitrogen and crushed by Micro Smash MS-100R (Tomy Seiko, Tokyo, Japan) in 800 μl TRIzol reagent (Invitrogen, Carlsbad, CA) for each sample. Total RNA was extracted with an RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. For the *in vitro* experiments, bone marrow stromal cells or macrophages were incubated in culture medium supplemented with PBS, LPS (100 ng/ml), LPS (100 ng/ml) and exendin-4 (100 ng/ml), and exendin-4 (100 ng/ml). After three days of culture, total RNA was isolated from adherent cells. Total RNA of stromal cells or peritoneal macrophages was isolated using an RNeasy mini kit (Qiagen). cDNA was synthesized for each sample from 2 μg total RNA with oligo-dT primers (Invitrogen) and reverse transcriptase in a total volume of 20 μl. The corresponding expression levels of RANKL and TNF-α mRNA were evaluated by real-time RT-PCR using a Thermal Cycler Dice Real Time System (Takara, Shiga, Japan). Each reaction comprised a total volume of 25 μl containing 2 μl cDNA and 23 μl of a mixture of SYBR Premix Ex Taq (Takara) and 50 pmol/μl primers. The PCR cycling conditions were as follows: 95°C for 10 s for initial denaturation followed by 45–60 amplification cycles, with each cycle comprising a denaturation step of 95°C for 5 s and then an annealing step of 60°C for 30 s. Relative expression levels of TNF-α and RANKL mRNAs were calculated by normalization to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA levels. The primer sequences used for cDNA amplification were as follows: 5′-GGTGGAGCCAAAAGGGTCA-3′ and 5′-GGGG GCTAAGCAGTTGGT-3′ for GAPDH; 5′-AGGCGGTGC TTGTTCCTCA-3′ and 5′-AGGCGAGAAGATGATCTGA CTGCC-3′ for TNF-α; and 5′-CCTGAGGCCAGCCATTT-3′ and 5′-CTTGGCCCAGCCTCGAT-3′ for RANKL as already reported [43].

2.7. Micro-CT Imaging and Analysis for Bone Destruction Area. We obtained mouse calvariae immediately after sacrifice. The calvariae were fixed in 4% PBS-buffered formaldehyde at 4°C for 3 days. To assess the bone resorption pits on the calvariae, samples were washed thoroughly with PBS and scanned with microfocus computed tomography (ScanXmate-E090, Comscan, Kanagawa, Japan). TRI/3D-BON64 software (RATOC System Engineering, Tokyo, Japan) was used to create three-dimensional images of the mouse calvariae, and the ratio of bone resorption area to total area was measured by ImageJ (NIH, Bethesda, MD) as previously reported [43].

2.8. Measurement of Serum CTX (C-Terminal Cross-Linked Telopeptide of Type I Collagen) Value. Blood was collected with microhematocrit tubes from the orbital sinuses of the mice after 5 days of daily administration of PBS, LPS with or without exendin-4, or exendin-4 alone. The levels of CTX were determined using a mouse C-terminal telopeptide of type I collagen assay kit (IDS, Tyne and Wear, UK). Levels of C-terminal telopeptide of type I collagen were assessed by measuring absorbance at 450 nm with a microplate reader (Remote Sunrise; Tecan, Japan), with 620 nm as the reference wavelength.

2.9. Cell Viability Assay for Osteoclast Precursors. Osteoclast precursors were seeded in a 96-well plate (1×10^4 cells in 200 μl medium per well) and incubated with M-CSF (100 ng/ml) with or without exendin-4 (100 ng/ml). After 5 days of incubation, the cells were washed with PBS and cultured in 100 μl culture medium of each well. Four replicates were assessed for each sample. Then, 10 μl cell counting kit-8 (Dojin, Kumamoto, Japan) solution was added to each well, and the plate was further incubated for 2 h at 37°C. Absorbance at 450 nm was measured by a microplate reader for each well as previously reported [43].

2.10. Statistical Analysis. Data are expressed as means ± standard deviation. The statistical significance of differences between groups was determined by Scheffe's test. $P < 0.05$ was considered significant.

3. Results

3.1. In Vivo Inhibitory Effect of Exendin-4 on LPS-Induced Osteoclast Formation. We injected LPS with or without exendin-4 on mouse calvariae to analyze the effect of exendin-4 on LPS-induced osteoclast formation *in vivo*. After LPS administration for 5 consecutive days, many large multinucleated osteoclasts were observed within the suture mesenchyme in the histological sections. However, the mean number of osteoclasts was significantly lower in the LPS- and exendin-4-coadministered group than in the group administered with LPS alone (Figures 1(a) and 1(b)).

3.2. In Vivo Inhibitory Effect of Exendin-4 on LPS-Induced Bone Resorption. The mouse calvariae were scanned with microfocus computed tomography, and the amount of bone resorption areas was compared between each group. Many bone destruction defects were noted in the LPS group. The ratio of the bone resorption area to the total area was significantly higher in the LPS-administered group than in the PBS-administered and exendin-4-administered groups. Moreover, the LPS- and exendin-4-coadministered groups demonstrated less bone destruction than the group administered with LPS alone (Figures 2(a) and 2(b)). Serum levels of C-terminal telopeptide of type I collagen (CTX), a marker of bone resorption, in mouse serum samples were analyzed by a mouse CTX assay kit. The serum CTX level in the LPS-alone-administered group was higher than PBS-administered

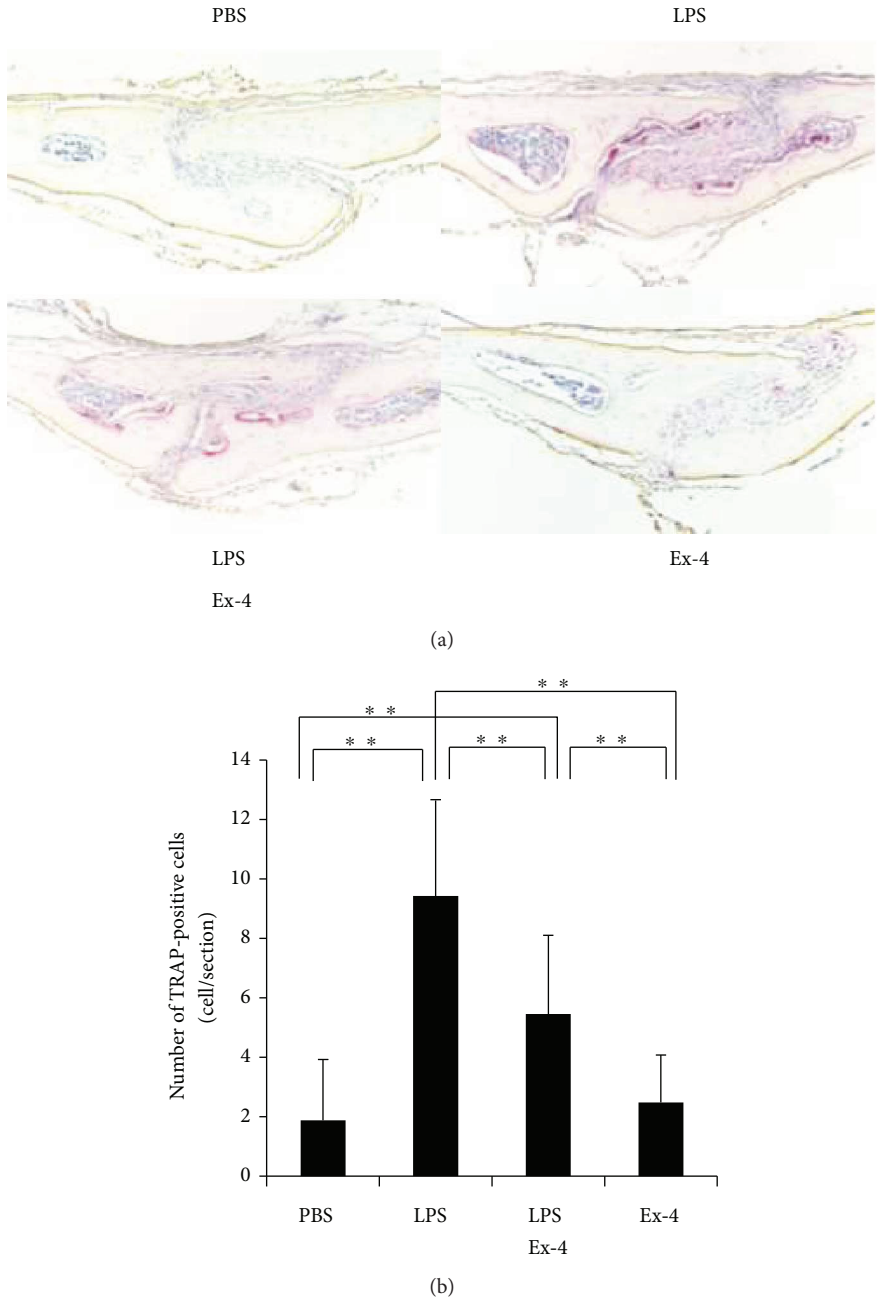

Figure 1: *In vivo* effect of exendin-4 on lipopolysaccharide- (LPS-) induced osteoclast formation. (a) Histological sections of mouse calvariae after 5-day daily supracalvarial injections with phosphate-buffered saline (PBS), LPS (100 μg/day), LPS (100 μg/day) with exendin-4 (20 μg/day), and exendin-4 (20 μg/day). Tartrate-resistant acid phosphatase (TRAP) staining and hematoxylin counterstaining were performed. TRAP-positive cells were stained dark red. (b) The numbers of TRAP-positive cells in the suture mesenchyme of calvaria from the mouse groups administered with PBS, LPS, LPS with exendin-4, and exendin-4, respectively. Data is expressed as means ± standard deviation (SD). Statistical significance were determined by Scheffe's test ($n = 4$; $^{**}p < 0.01$).

group. However, the serum CTX level in the LPS- and exendin-4-coadministered group was lower than that in the LPS-alone-administered group (Figure 2(c)).

3.3. In Vivo Inhibitory Effect of Exendin-4 on the Expression of LPS-Induced Osteoclast-Related Cytokines (TNF-α and RANKL). Bone chips from mouse calvariae were analyzed by real-time RT-PCR to measure expression levels of TNF-α and RANKL mRNA. TNF-α and RANKL mRNA levels were elevated in the LPS-administered group compared with the PBS-administered group. Conversely, TNF-α and RANKL mRNA expression levels were reduced in the exendin-4- and LPS-coadministered group compared with the LPS-administered group (Figure 3).

3.4. Exendin-4 Cannot Affect RANKL-Induced Osteoclast Formation, TNF-α-Induced Osteoclast Formation, Cell Viability of Osteoclast Precursor Cells, and LPS-Induced

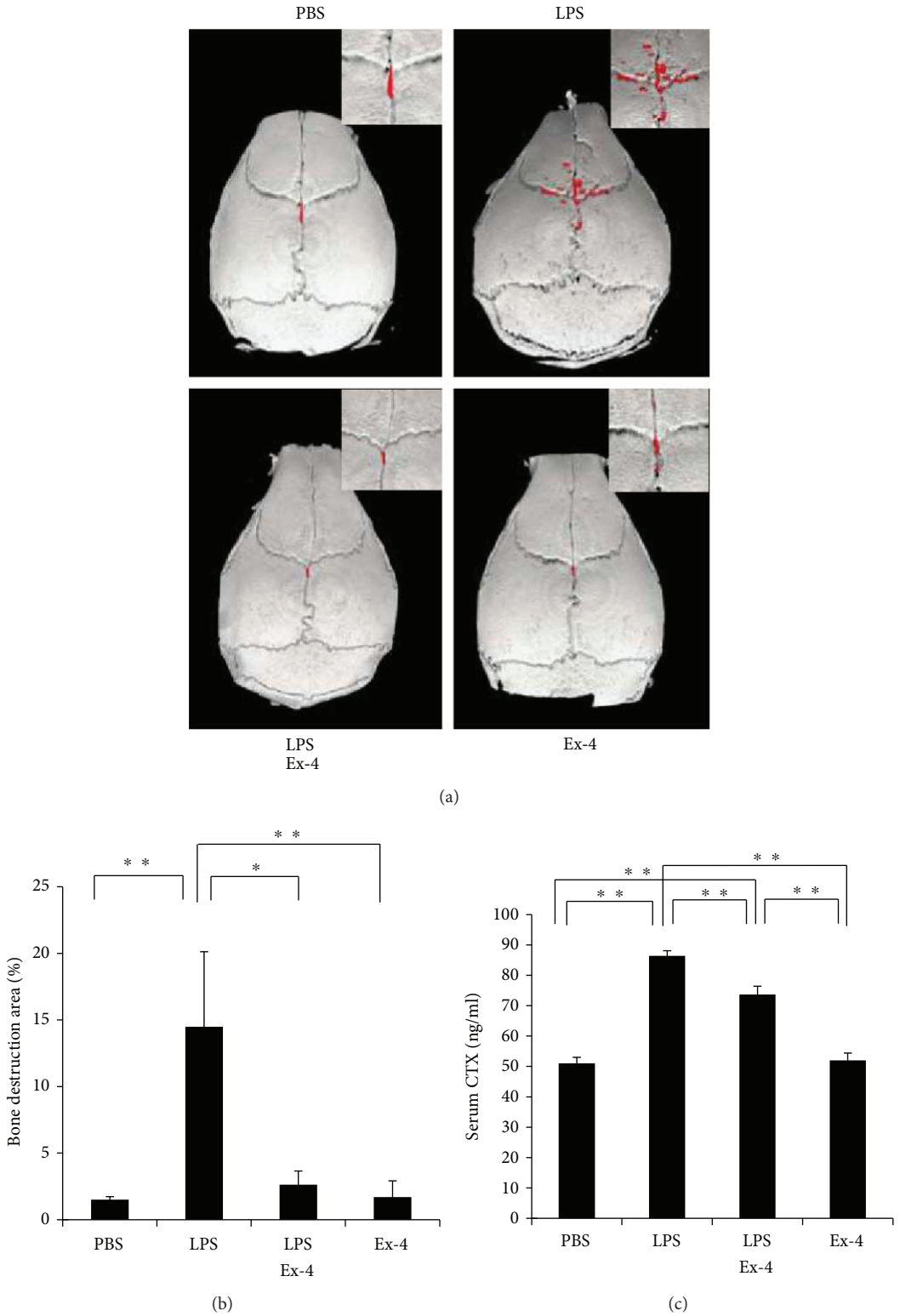

Figure 2: Exendin-4 inhibited LPS-induced bone resorption *in vivo*. (a) 3D reconstructed images of calvariae from micro-CT scanning. Mice were subjected to 5-day daily subcutaneous injections on the calvariae with PBS, LPS (100 μg/day) with or without exendin-4 (20 μg/day), and exendin-4 (20 μg/day), and calvariae were excised on the sixth day. The red dots indicate areas of bony destruction. (b) Ratio of bone destruction area to total bone area. Data is expressed as means ± SD ($n = 4$; $^{*}p < 0.05$, $^{**}p < 0.01$). The statistical significance of differences was determined by Scheffe's test. (c) Serum levels of C-terminal telopeptide of type I collagen (CTX) determined by a mouse CTX assay kit. Data is expressed as means ± SD. The statistical significance of differences was determined using Scheffe's test ($n = 4$; $^{**}p < 0.01$).

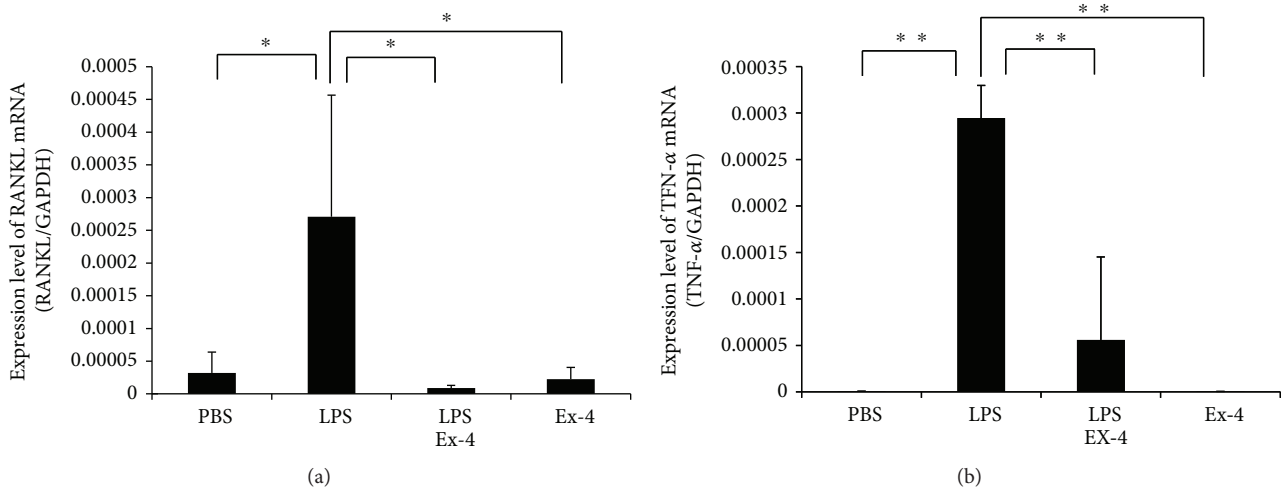

Figure 3: Exendin-4 suppressed expression of LPS-induced tumor necrosis factor- (TNF-) α and receptor activator of NF-kB ligand (RANKL) *in vivo*. TNF-α and RANKL mRNA levels in mouse calvariae were determined using real-time RT-PCR. Total RNA was isolated from mouse calvariae after 5-day daily supracalvarial injections with PBS, LPS (100 μg/day) with or without exendin-4 (20 μg/day), and exendin-4 alone (20 μg/day). TNF-α and RANKL mRNA levels were normalized to the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Data is expressed as means ± SD. The statistical significance of differences was determined using Scheffe's test ($n = 4$; $^{*}p < 0.05$, $^{**}p < 0.01$).

RANKL Expression in Stromal Cells. To investigate whether exendin-4 affects osteoclast precursor cells directly, we analyzed the effects of exendin-4 on RANKL-induced osteoclast formation, TNF-α-induced osteoclast formation, and viability of osteoclast precursors. There were large numbers of TRAP-positive cells among osteoclast precursor cells cultured with M-CSF and RANKL or TNF-α. Likewise, TRAP-positive cells were also observed among the osteoclast precursor cells cultured with M-CSF and RANKL or TNF-α in the presence of exendin-4 (Figures 4(a) and 4(b)). Additionally, there was no evident difference in cell viability between the two cultures after 5 days of culture (Figure 4(c)). These results indicate that the inhibitory effect of exendin-4 may not be related to a direct action of exendin-4 on the proliferation and differentiation of osteoclast precursors.

We next evaluated whether exendin-4 inhibited LPS-induced RANKL expression in stromal cells *in vitro*. RANKL mRNA expression levels were higher in LPS-treated stromal cells than in control and exendin-4-treated stromal cells. However, stromal cells treated with both LPS and exendin-4 demonstrated similar RANKL mRNA expression levels to those treated with LPS alone (Figure 4(d)). These results show that the inhibitory effect of exendin-4 may not be related to a direct action of exendin-4 on RANKL expression in stromal cells.

3.5. Exendin-4 Suppresses LPS-Induced TNF-α Expression in Macrophages. Real-time RT-PCR was performed to analyze TNF-α mRNA expression levels. TNF-α mRNA expression was elevated in macrophages treated with LPS alone compared with those treated with PBS. Conversely, TNF-α mRNA expression was inhibited in the exendin-4- and LPS-treated macrophages, compared with those treated with LPS alone (Figure 5).

4. Discussion

In the present study, we evaluated the effect of the GLP-1 receptor agonist exendin-4 on LPS-induced osteoclast formation and bone-resorption *in vivo*. We found that the GLP-1 receptor agonist inhibited LPS-induced osteoclast formation and bone resorption and also suppressed LPS-induced RANKL and TNF-α expression *in vivo*. Conversely, the GLP-1 receptor agonist did not directly inhibit RANKL-induced osteoclast formation, TNF-α-induced osteoclast formation, osteoclast precursor cell viability, or LPS-induced RANKL expression in stromal cells *in vitro*. However, the GLP-1 receptor agonist inhibited LPS-induced TNF-α expression in macrophages *in vitro*.

GLP-1 plays a crucial role in blood glucose control. To simulate the effect of GLP-1, many GLP-1 analogues and GLP-1 receptor agonists have been developed. The amino acid sequence of the GLP-1 receptor agonist exendin-4 is a modified version of the sequence of GLP-1. Exendin-4 is resistant to degradation by dipeptidyl peptidase-IV and has a much longer plasma half-life than GLP-1 [40], which has a half-life of less than two minutes [39, 41]. The extended half-life, improved pharmacokinetics, and high potency of exendin-4 make it suitable for clinical use [39, 40].

GLP-1 receptor-deficient mice have been reported to exhibit increased bone breakdown, which indicates that GLP-1 receptor signaling is essential to inhibition of osteoclast formation and bone resorption [31]. In the present study, exendin-4 inhibited LPS-induced osteoclast formation. Daily injections of 20 μg of exendin-4 for 5 days (a total of 100 μg) were sufficient to inhibit LPS-induced osteoclast formation *in vivo*. We also evaluated the inhibitory effect of exendin-4 on LPS-induced bone resorption. The extent of bone destruction was determined by the ratio of the destroyed bone area to total bone area, assessed by

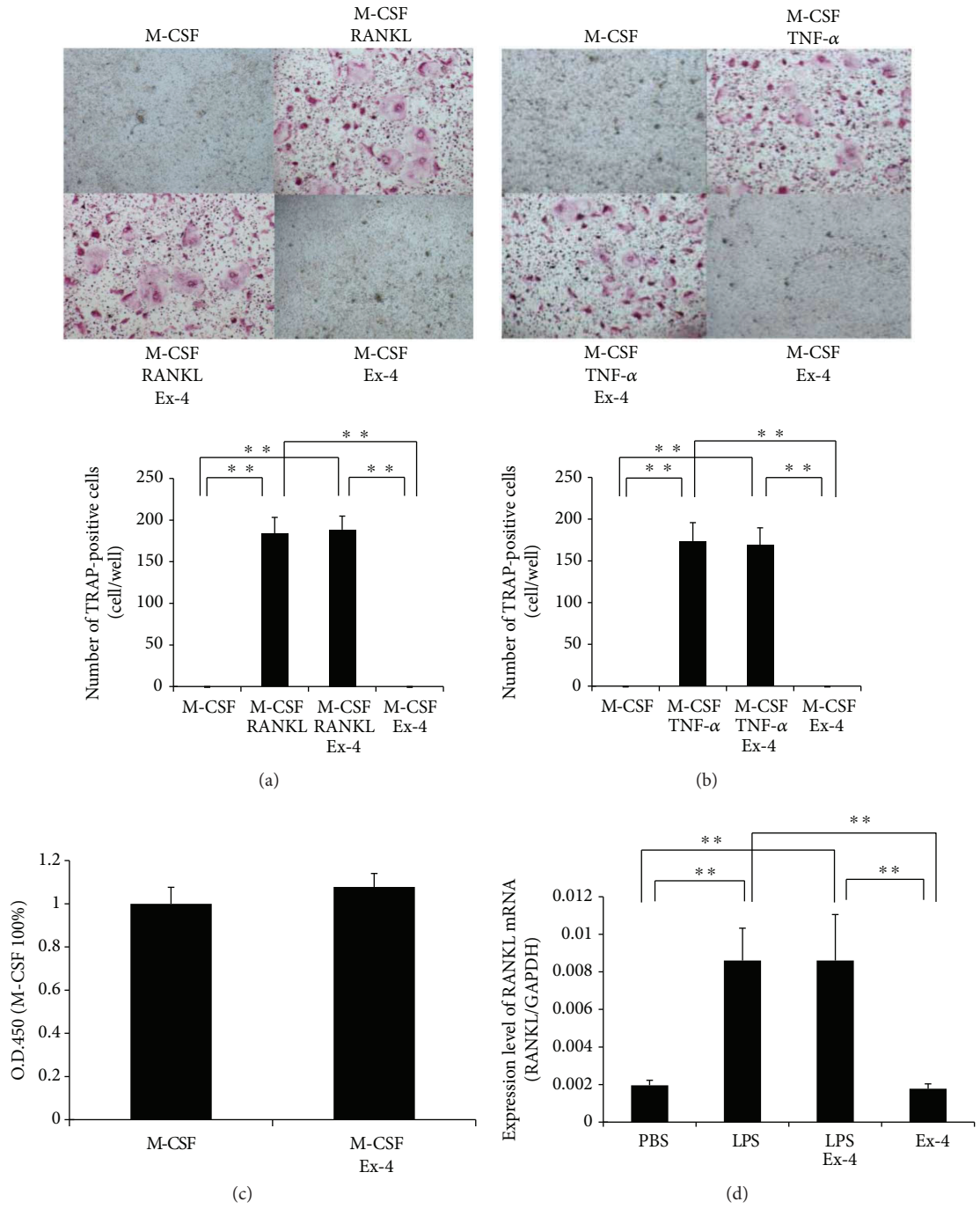

Figure 4: Exendin-4 had no effect on RANKL-induced osteoclast formation, TNF-α-induced osteoclast formation, osteoclast precursor cell viability, or LPS-induced RANKL expression in stromal cells *in vitro*. (a) Microscopic images and numbers of TRAP-positive cells. Osteoclast precursors were treated with macrophage colony-stimulating factor (M-CSF) alone, M-CSF with RANKL, M-CSF with RANKL and exendin-4, and M-CSF with exendin-4 for 5 days, then stained with TRAP solution. (b) Microscopic images and numbers of TRAP-positive cells. Osteoclast precursors were treated with M-CSF alone, M-CSF with TNF-α, M-CSF with TNF-α and exendin-4, and M-CSF with exendin-4 for 5 days, then stained with TRAP solution. (c) Cell viability of osteoclast precursor cells treated with M-CSF alone and M-CSF with exendin-4 for 5 days. Cell viability was determined by cell counting kit-8. Data is presented as percentage activity relative to the activity in the culture with M-CSF alone and is expressed as means ± SD. (d) RANKL mRNA expression levels in stromal cells determined by real-time RT-PCR method. Total RNA was extracted from stromal cells that were cultured with PBS, LPS with or without exendin-4, and exendin-4 alone, respectively. RANKL mRNA levels were normalized to that of GAPDH. Statistical significance of differences was determined by Scheffe's test ($n = 4$; $^{**}P < 0.01$).

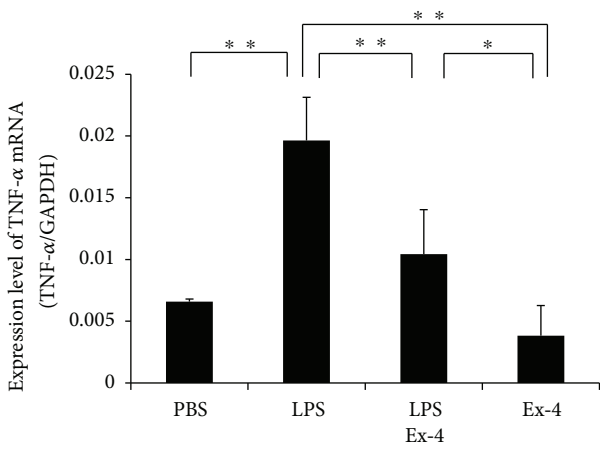

Figure 5: Exendin-4 inhibited LPS-induced expression of TNF-α in macrophages. TNF-α mRNA levels in macrophages were detected by real-time RT-PCR. Total RNA was isolated from macrophages cultured with PBS, LPS with or without exendin-4, and exendin-4 alone. TNF-α mRNA levels were normalized to the levels of GAPDH. Statistical significance of differences was determined by Scheffe's test ($n = 4$; $^{*}p < 0.05$, $^{**}P < 0.01$).

microfocus computed tomography imaging, and by the serum CTX value of each experimental group. We found that the extent of bone destruction was significantly lower in the LPS- and exendin-4-coadministered group than the group administered with LPS alone. Our results suggest that exendin-4 inhibited LPS-induced osteoclast formation and bone resorption *in vivo*.

In this study, we administered 20 μg/day exendin-4 for 5 days, injected into the supracalvaria. Although previous rodent studies used 20 μg/kg exendin-4 daily for 4 weeks [41, 44], we opted to use a higher dose to enhance the inhibitory effects of exendin-4. Further investigation using clinically relevant doses is needed.

Our findings prompted us to explore the mechanisms contributing to the inhibition of LPS-induced osteoclast formation and bone resorption. We considered two possible mechanisms. First, we considered whether exendin-4 inhibited LPS-induced expression of inflammatory cytokines related to osteoclast formation, such as TNF-α and RANKL. Many studies have indicated that LPS induces TNF-α and RANKL *in vivo* [28, 45]. RANKL is an essential cytokine for osteoclast formation [10], and it has been reported that TNF-α also can induce osteoclast formation *in vivo* [15, 16]. Therefore, it is reasonable to suspect that if levels of both of these cytokines are decreased, osteoclast formation will be inhibited. In the present study, TNF-α and RANKL mRNA levels were elevated in the LPS-administered mice. However, this LPS-induced increase in TNF-α and RANKL mRNA levels was inhibited in the exendin-4- and LPS-coadministered group, compared with the group administered LPS only. This suggests that one of the mechanisms underlying the inhibitory effect of exendin-4 on LPS-induced osteoclast formation is the inhibition of LPS-induced osteoclast-related cytokines. The other mechanism that we considered was that exendin-4 directly inhibited RANKL- and TNF-α-induced osteoclast formation. In the present study, we investigated whether exendin-4 exerted its inhibitory effect on osteoclasts by directly acting on osteoclast precursors. However, exendin-4 did not inhibit RANKL- or TNF-α-induced differentiation of osteoclast precursor cells into osteoclasts. Moreover, we investigated whether exendin-4 inhibited osteoclast precursor cell viability. We observed no difference in cell viability between the two groups after 5 days of culture. These results suggest that the inhibitory effect of exendin-4 on osteoclast formation is not due to a direct action of exendin-4 on osteoclast precursors. We then evaluated whether exendin-4 inhibited LPS-induced RANKL expression in stromal cells. Exendin-4 also failed to inhibit LPS-induced RANKL expression in stromal cells. This indicates that inhibition of RANKL expression by exendin-4 may not be due to a direct action of exendin-4 on stromal cells. Finally, we evaluated whether exendin-4 inhibited LPS-induced TNF-α expression in macrophages. In our study, exendin-4 inhibited LPS-induced TNF-α expression of macrophages. Because TNF-α induces osteoclast formation and promotes RANKL expression in stromal cells, our results suggest that the *in vivo* inhibition of LPS-induced osteoclast formation by exendin-4 may be the result of inhibition of LPS-induced TNF-α expression in macrophages and subsequent suppression of RANKL expression in stromal cells.

5. Conclusions

In conclusion, our results suggested that exendin-4 can inhibit LPS-induced osteoclast formation and bone resorption in vivo. The underlying mechanism may be related to its inhibition in the production of LPS-induced TNF-α in macrophages but not related to its direct effect on osteoclast precursors or RANKL expression in stromal cells.

Conflicts of Interest

The authors declare that there is no conflict of interest.

Authors' Contributions

Wei-Ren Shen contributed to conception, design, data acquisition, analysis, and interpretation and drafted the manuscript. Hideki Kitaura contributed to conception, design, data acquisition, data analysis, and interpretation and drafted and critically revised the manuscript. Keisuke Kimura, Masahiko Ishida, Haruki Sugisawa, Akiko Kishikawa, Kazuhiro Shima, Saika Ogawa, and Jiawei Qi contributed to data acquisition and data analysis and drafted the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

Acknowledgments

This work was supported in part by JSPS KAKENHI from the Japan Society for the Promotion of Science (no. 16K11776 to Hideki Kitaura).

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Hindawi
Journal of Immunology Research
Volume 2018, Article ID 2349045, 19 pages
https://doi.org/10.1155/2018/2349045

Review Article

Dysregulated Functions of Lung Macrophage Populations in COPD

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Received 30 July 2017; Accepted 29 November 2017; Published 18 February 2018

Academic Editor: Ethan M. Shevach

Chronic obstructive pulmonary disease (COPD) is a diverse respiratory disease characterised by bronchiolitis, small airway obstruction, and emphysema. Innate immune cells play a pivotal role in the disease's progression, and in particular, lung macrophages exploit their prevalence and strategic localisation to orchestrate immune responses. To date, alveolar and interstitial resident macrophages as well as blood monocytes have been described in the lungs of patients with COPD contributing to disease pathology by changes in their functional repertoire. In this review, we summarise recent evidence from human studies and work with animal models of COPD with regard to altered functions of each of these myeloid cell populations. We primarily focus on the dysregulated capacity of alveolar macrophages to secrete proinflammatory mediators and proteases, induce oxidative stress, engulf microbes and apoptotic cells, and express surface and intracellular markers in patients with COPD. In addition, we discuss the differences in the responses between alveolar macrophages and interstitial macrophages/monocytes in the disease and propose how the field should advance to better understand the implications of lung macrophage functions in COPD.

1. Lung Macrophage Populations in Mice and Humans

The lung is constantly exposed to the host's outer environment; therefore, constitutively active mechanisms are required to monitor for irritants and infections with pathogens. This pivotal sentinel function is assumed by lung-resident immune cell populations including macrophages, dendritic cells (DCs), and airway epithelial cells [1]. To date, three major myeloid cell populations have been identified in the lung which differ in their exact localisation in the tissue and their developmental origin (Figure 1): resident alveolar macrophages (AMs), resident interstitial macrophages (IMs), and blood monocytes [2–4].

AMs reside in the airspaces of the lung, whereas IMs are found in the interstitial space between the alveoli and blood vessels. Morphological observation of these two populations indicated that AMs are larger in size than IMs [5]. In addition, phenotypic characterisation of AMs and IMs in mice revealed differences in the expression levels of MHC class-II, CD11b, CD14, CD45, CD54, CD68, CD71, CD204, CD206, and Siglec-F [5–9]. Altogether, lung-resident macrophages have been characterized as $CD11c^{+}CD11b^{lo}$ cells and can be distinguished from recruited cells during endotoxin or viral-induced inflammation by the level of CD11b expression [10]. In humans, AMs are described as $CD45^{+}CD206^{+}CD14^{lo}CD71^{+}CD169^{+}$ cells, whereas IMs are reported as $CD45^{+}CD206^{+}CD14^{hi}CD71^{-}CD169^{-}$ cells [11]. However, recently a study suggested high expression of the mannose receptor (CD206) in both macrophage populations and revealed two AM subpopulations with differential expression of the hemoglobin-haptoglobin complex

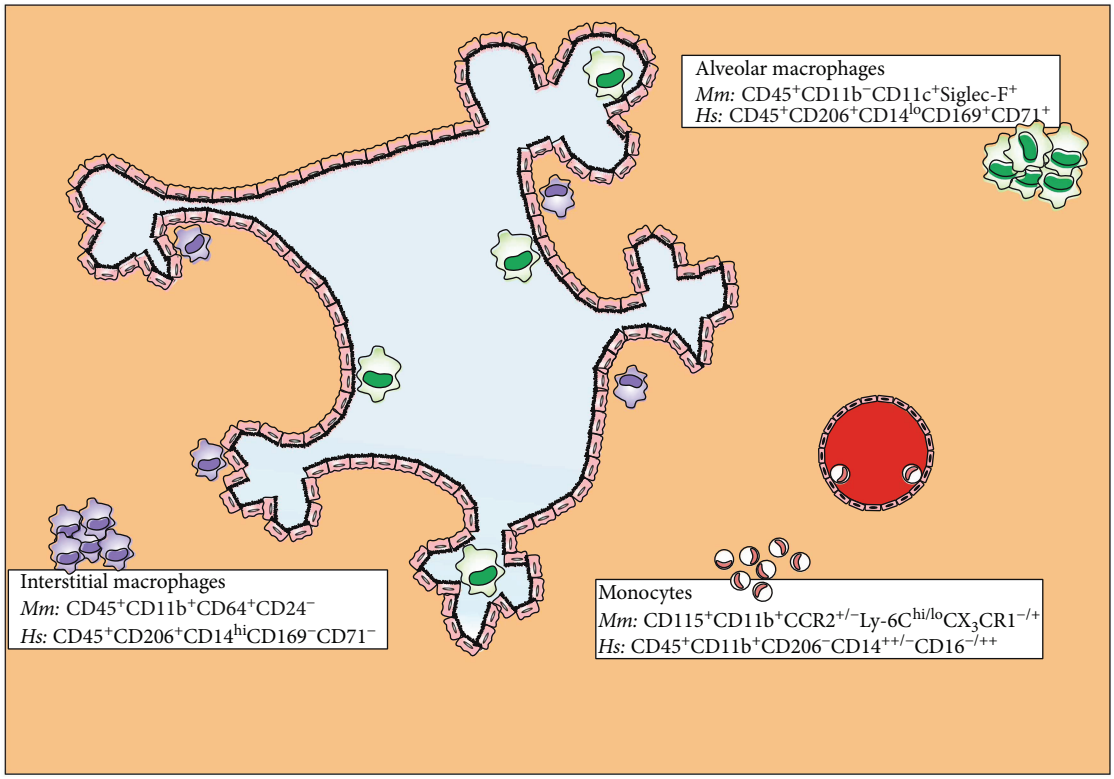

Figure 1: Murine and human lung macrophage populations under steady-state conditions. AMs reside at the airspaces of the lung, while IMs localise in the interstitial space between the alveoli and blood vessels. In both the murine and human lungs, there is also a monocyte population which enters the tissue from blood vessels. AMs are the biggest of all three lung macrophage populations, are potent phagocytes, and secrete a range of proinflammatory mediators. IMs are smaller than AMs but display comparable phagocytic capacity and ability to produce soluble factors. They are believed to serve as an intermediate step in monocyte differentiation towards AMs and demonstrate proliferative potential. Finally, monocytes are sensitive to migratory gradients and have been shown to exhibit proinflammatory mediator capacity, but no antigen presentation. The currently acceptable nomenclatures for AMs, IMs, and monocytes in mice (*Mm*) and humans (*Hs*) are indicated next to each population.

scavenger receptor CD163 [12]. Lastly, Desch et al. found that human AMs ($CD206^+CD14^{lo}HLA\text{-}DR^+CD64^+CD141^+$ cells) could be distinguished from lung tissue monocytes based on CD14 and CD16 surface expression [13].

Functionally, although a small fraction of AMs was shown to be present in lymph nodes in *S. pneumoniae*-infected mice [14], IMs are considered to be classical modulators of adaptive immunity in human and murine lungs [7, 15–18]. In humans and rodents, AMs have been reported to remove surfactants and debris [19], suppress adaptive immunity [20, 21], and regulate neutrophil and monocyte recruitment to the lung [22–24]. With regard to other typical macrophage functions, both populations display high phagocytic capacity [5, 25], but AMs are considered to be more potent phagocytes [17, 26–28] and they were shown to secrete proinflammatory mediators and reactive oxygen species (ROS) upon activation in animal studies [17, 27, 29, 30].

Research on both human and animal AMs challenged the homogeneity of this population [31, 32]. Instead, density-gradient centrifugation splits them into distinct subpopulations with differences in the expression of surface markers and intracellular enzymes as well as tumour lysis, migration, cytotoxicity, phagocytosis, lymphoproliferative response augmentation, soluble mediator release, and procoagulant activity [33–42].

Under steady-state conditions, the replenishment of AMs in humans and mice occurs mainly via self-renewal as recently demonstrated in long-term lung transplant, parabiosis, and fate-mapping studies [43–45]. During lung inflammation, a proportion of AMs dies by apoptosis and the cells are replenished in part by local proliferation of local stem cells, but also via the recruitment of blood mononuclear phagocytes [46–48]. IMs acquire proinflammatory markers upon activation, such as CD40, CD80, and CD86, and their numbers are increased in mice [6]. Between the two populations, AMs secrete more TNF-α, but less IL-6, IL-1ra, and IL-10 than IMs in rats [49]. Furthermore, in humans, the two populations exhibit differential sensitivity to pathogen recognition receptor (PRR) activation with IMs being less sensitive to TLR9 priming [5].

IMs are not a homogeneous population either, and in the rat lung interstitium, they are currently believed to be contaminated with up to 20% AMs [50]. Similar to AMs, several density-defined populations have been identified exhibiting differential prostaglandin secretion, migration, and phagocytosis capabilities [51–53]. It has long been considered that

IMs are an intermediate step in maturation of infiltrating blood monocytes towards AMs [54, 55] because they display blunt lamellipodia and fewer lamellar inclusions than AMs and are morphologically more closely related to blood monocytes [4, 56, 57]. Moreover, in mice, they seem to proliferate more than AMs [17]. However, considering more recent findings in macrophage ontogeny and the possibility to measure hundreds to thousands of genes at the single cell level, these observations need to be revisited.

Monocytes are divided into subpopulations in both humans and mice (reviewed in [58]). Fate-mapping experiments in mice unraveled a $CD115^+CD11b^+Ly\text{-}6C^{hi}CCR2^+$ and a $CD115^+CD11b^+Ly\text{-}6C^{lo}$ monocyte population [59, 60]. $Ly\text{-}6C^{lo}$ monocytes express high levels of the fractalkine receptor CX_3CR1, and they were shown to crawl inside blood vessels via lymphocyte function-associated antigen 1 interactions with the endothelial lining [60, 61]. Upon activation with an inflammatory stimulus, they rapidly respond by secreting TNF-α [62]. In contrast, $Ly\text{-}6C^{hi}CCR2^+CX_3CR1^-GR\text{-}1^+$ monocytes are actively recruited to inflamed tissues where they can differentiate into so-called inflammatory DCs or different flavours of macrophages [60, 63–65]. This subset was shown to express high levels of chemokine receptors, complement peptides, and annexins, while $Ly\text{-}6C^{lo}$ monocytes express more MHC class-II, growth factors, integrins, and scavenger receptors [66, 67].

In analogy to mice, human monocytes are divided into different subsets including $CD14^{++}CD16^-$ (classical), $CD14^+CD16^+$ (intermediate), and $CD14^-CD16^+$ (nonclassical) [58]. All subsets are $CD206^-CD64^+$ [13] and express CX_3CR1 and CXCR4 ($CD16^+$ monocytes express CX_3CR1 at higher levels which allows them to adhere firmly to vessel walls [58]). Classical monocytes also express several CC chemokine receptors [58, 60] and are characterised by an antimicrobial phenotype [68]. Intermediate monocytes express genes related to antigen processing and presentation, transendothelial migration, and angiogenesis and secrete higher amounts of cytokines and ROS than other subsets [68, 69]. Human classical monocytes resemble murine $Ly\text{-}6C^{hi}$ monocytes, whereas nonclassical monocytes were described to be the counterparts of $Ly\text{-}6C^{lo}$ monocytes (reviewed in [64]). The human blood monocyte population structure was recently challenged by Villani et al. who, by application of single cell RNA sequencing, suggested that peripheral blood monocytes can be further divided in four subsets [70]. Whether this also holds true for lung monocytes awaits further investigation.

2. Chronic Obstructive Pulmonary Disease (COPD): Epidemiology, Pathology, and the Role of the Immune System

COPD is a chronic disease of the lower respiratory tract and is characterised by irreversible airway obstruction, chronic bronchitis, and loss of alveolar parenchyma (emphysema) [71]. It affects almost equally men and women, has its onset in midlife, and progresses slowly during adulthood [72] resulting in airway obstruction by mucus exudates and lung tissue remodelling [71]. Patients with COPD are diagnosed as stage 1 (mild) to 4 (very severe) based on spirometric grading as well as group A to D based on clinical assessment of symptoms and exacerbation risk according to GOLD classification [73]. Besides the well-documented increase in patients' disability-adjusted life years, COPD is also a huge economic burden for countries due to its chronic nature, the exacerbations which lead to patient hospitalisation and the lack of effective drugs [74–76].

COPD ranked sixth globally as a leading cause of death in 1990 and is projected to rank third by 2020 accounting for 7% of total deaths worldwide [73, 77, 78]. There are several causative factors for the disease (reviewed in [79, 80]) including environmental factors, such as smoking (which is now accepted as the main causal factor of the disease), the use of biomass fuel, occupational exposure to toxic gases or dust, infections, outdoor pollution, genetic susceptibility as exemplified by the deficiency of α_1-antitrypsin (reviewed in [81]), and accelerated lung ageing [82, 83].

COPD is thought to be initiated when inhaled irritants activate innate immunity either directly by triggering common PRRs on immune and bronchial epithelial cells or indirectly by inducing the release of danger signals by epithelial and endothelial cells [84–86]. In fact, the subsequent recruitment of blood leukocytes and the destruction of lung tissue are TLR-dependent and macrophage activation occurs in an inflammasome-dependent manner [87]. Patients with COPD present with elevated levels of a broad range of proinflammatory mediators in their bronchial lavage, such as TNF-α, IL-8, CCL2, CCL3, LTB_4, myeloperoxidase, and eosinophilic cationic protein among others [88–94]. In parallel, the vasculature upregulates surface adhesion molecules [95] and becomes permeable to attract blood neutrophils, monocytes, and eosinophils to the lung. Secretion of the tissue remodelling cytokine TGF-β by epithelial cells has also been reported to relate to small airway obstruction in COPD [96].

Neutrophil percentages in COPD correlate with deterioration of lung function and airway obstruction [97] and, together with macrophages [98], they contribute to disease pathology via the production of extracellular matrix- (ECM-) degrading enzymes [99]. Disintegrated alveolar wall components can be readily detected in the biological fluids of patients with COPD and are significantly higher than in healthy smokers [100]. Neutrophil elastase (NE) and metalloproteinases (MMPs) cause lung tissue destruction and trigger mucus secretion which obstructs small airways [101]. The imbalance between proteases and protease inhibitors in the lungs of patients with COPD causes enhanced chemotactic factor secretion by macrophages and further amplification of neutrophil recruitment [102].

In the healthy lung, DC sample inhaled exogenous material or apoptotic cells to induce immune tolerance or initiate appropriate immune responses [1]. In COPD, DCs accumulate in the lung in an IL-1α-dependent manner following a CCL20-CCR6 axis [103, 104]. Recent reports have suggested that the numbers of the various DC subsets are differentially altered in the several lung compartments. For example, Langerhans-type DCs have been observed

selectively in small airways [105], whereas the numbers of bronchial mucosal DCs in the epithelium as well as the migratory $CD83^+$ and $CCR7^+$ DC subsets are reduced in patients with COPD [106, 107]. The dysregulated localisation of these immune cells comes together with altered immune responses regulated by the different subsets [108]; cigarette smoke and the lung inflammatory milieu decrease lung myeloid DC maturation [109, 110] and cause an imbalance to the costimulatory status of these cells [111]. In contrast, $CD1c^+$ DCs favour tolerogenic signalling and the induction of regulatory T cells [112].

DC-mediated $CD4^+$ T cell activation is predominantly skewed to a T_H1 phenotype [113], although T_H17 cells have also been found in the lungs of patients with COPD [114, 115]. However, in the epithelium, submucosa, and adventitia of peripheral airways of patients with COPD, CXCR3-expressing $CD8^+$ cells are the predominant T cell subtype [116]. $CD8^+$ lymphocytes contribute to tissue injury and cell death in the lung via the release of proteolytic enzymes, such as perforin and granzymes [117–120]. Finally, the numbers of regulatory T cells have been demonstrated to be in decline in patients with COPD in comparison with healthy smokers which highlights another causality factor for the chronicity of the disease [121, 122]. Regarding the factors responsible for the increase in T cell numbers, Di Stefano et al. showed that IL-27 secretion by $CD68^+$ cells in the BAL of patients with COPD may contribute to IFN-γ and granzyme B secretion by $CD8^+$ lymphocytes as well as the induction of regulatory T cells [123]. However, more studies are needed to clarify the role of T cells as part of an efficient acute or a dysregulated chronic response mounted by alterations in innate immunity.

In 2006, the presence of B cells was also described in lymphoid follicles in small airways and lung parenchyma of patients with COPD and animal models [124]. Supporting evidence came from the detection of elevated levels of B cell-activating factor in lymphoid follicles which inversely correlated with lung function [125]. Although the nature of the antigens that activate B cells is not fully known, it has been speculated that they range from cigarette smoke irritants [126] to cell death and ECM degradation by-products, microbial components, and autoantigens [127].

Finally, a frequent manifestation of COPD is the colonisation of the patients' lungs by bacteria and viruses (likely due to impaired phagocytosis by AMs [126]) which cause exacerbations diminishing the patients' quality of life [128, 129]. *H. influenzae*, *S. pneumoniae*, and *M. catarrhalis* are most usually detected in patients with frequent exacerbations, while *P. aeruginosa* infections account for exacerbations in patients with severe COPD [130–132]. Furthermore, in recent years, the role of viral infections in the worsening of patients' health has begun to be appreciated and research has focused on the identification of the immune cells and mechanisms that contribute to the loss of lung function. Rhinoviruses [133], picornavirus [134], adenoviruses, the respiratory syncytial virus, and influenza virus are the most common viruses found in the sputum of patients with COPD and are responsible for about half of all exacerbations observed (reviewed in [135]). Infections augment the innate immune responses and lung tissue remodelling in mice [136], while human patients present with dysregulated neutrophil and T cell mobilisation [89, 137], increased proinflammatory mediator levels [138, 139], and antibacterial humoral responses [140].

3. Why the Functions of Lung Macrophage Populations in COPD Warrant Further Investigation

The numbers of lung-resident macrophages in the lung have been reported to be dramatically increased in COPD due to the recruitment of blood leukocytes from the periphery [141, 142]. Macrophages are plastic cells and respond in several ways to accommodate changes in their microenvironment. For example, AMs from smokers present with increased expression of cytokines and chemokines, growth factors, proteases, antioxidant proteins, adhesion molecules, transcription regulators, and signalling pathway genes, whereas they reduce expression of genes related to neutrophil activation, serine protease inhibitors, and macrophage differentiation genes [143]. Consequently, in the constantly changing microenvironment of the COPD lung, resident macrophages will respond accordingly and shape their effector functions to orchestrate the immune responses. Hence, the study of the functions of lung macrophage populations as well as their interplay with other immune cells and the lung stroma has the potential to enhance our understanding of COPD pathology and provide with novel biomarkers and therapeutic targets.

4. AMs in COPD

Over the last decades, numerous studies have accumulated knowledge about the role and functions of AMs in COPD. Major aspects of change in cellular functions concern the secretion of proinflammatory mediators, the induction of oxidative stress, the deregulation of the protease-protease inhibitor balance, and the impairment of pathogen phagocytosis as well as changes in gene expression which we highlight next (Figure 2 and Table 1). Many of these studies have been performed in the pregenomic era and most of them prior to the era of single cell genomics. Therefore, as for every other field in life sciences, some of the previous findings might be challenged once we have applied cutting-edge technologies to better understand the basic unit of life—the cell—and its changed functionality in complex diseases like COPD. Nevertheless, we review the current knowledge which has often been obtained only at the population level, but not at the single cell level yet.

4.1. Altered Secretion of Proinflammatory Mediators. AMs from patients with COPD present with alterations in the secretion of cytokines and chemokines. In particular, the levels of TNF-α, IL-1β, IL-6, IL-10, IL-12, CCL2, CCL5, CCL7, CCL13, CCL22, IL-8, CXCL9, and CXCL10 in AM secretions from smokers were significantly different from healthy subjects [126, 144–152]. Similarly, the levels of the chemokine receptors CCR2 and CCR5 were found to be increased [153, 154]. Moreover, macrophages primed with

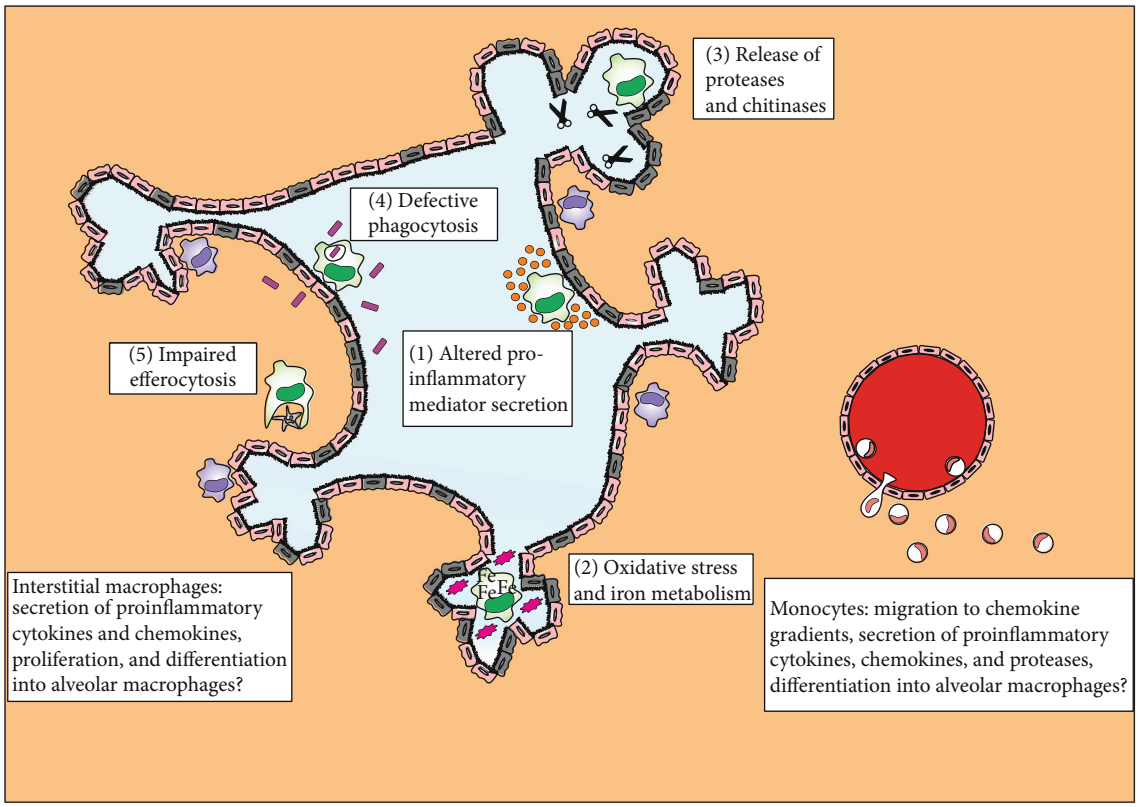

Figure 2: Lung macrophage population functions in COPD. AMs exhibit alterations in their physiological responses in COPD; the secretion of proinflammatory cytokines and chemokines is dysregulated (1). The cells undergo oxidative stress and secrete ROS and nitrite species into the lung micro-environment (2), they store intracellularly large amounts of iron (2), and they overexpress and release proteases which cause alveolar tissue destruction (3). In contrast, processes, such as phagocytosis of microbes (4) and apoptotic neutrophils or epithelial cells (5), are downregulated in AMs from patients with COPD, an observation which could explain the frequent colonisation of the lungs with bacteria and viruses in exacerbations. In the meantime, monocytes are recruited from blood vessels following chemokine gradients and contribute to disease pathology via the secretion of proinflammatory mediators and proteases. It is also believed that monocytes differentiate into macrophages via an intermediate step of IMs which morphologically and functionally resemble monocytes.

endotoxin and cigarette smoke presented with delayed IL-1β and IL-6 secretion in comparison with control endotoxin-treated cells and a subsequent increase in IL-8 levels [155]. Finally, sputum macrophages from patients with COPD were found to express more prostaglandin H synthases 1 and 2 than unaffected control subjects [156].

TLR signalling is pivotal for proinflammatory mediator secretion by macrophages in COPD as exemplified by the TLR4-dependent cigarette smoke-mediated activation of human macrophages [157]. Downstream of TLR activation, lung macrophages from patients with COPD also exhibit dysregulated signalling including p38, ERK1/2, JNK and IRAK-1 phosphorylation, IκBα expression, and NF-κB p65 activation compared to healthy individuals [145, 147, 155]. Finally, the importance of TLR signalling for macrophage proinflammatory mediator secretion in COPD is also illustrated by the downregulation of the chemokines CXCL9, CXCL10, and CXCL11 [147, 154, 158] as a result of the attenuation of TLR3 activation [158]. While all these findings are very informative, we still do not have an integrative, systemic, and causal model of the main regulatory mechanisms operative in AMs of patients with COPD.

Therefore, more light needs to be shed on the molecular programmers that drive these functional differences and conclude whether these are observed in a fraction of the AM population. To this end, microRNAs have been involved in the regulation of proinflammatory cytokine release by AMs [159], whereas recent investigation into the epigenetic networks active in macrophage populations of patients with COPD and healthy smokers revealed that the histone deacetylases HDAC2 and HDAC3 are downregulated in comparison with healthy individuals and correlate negatively with disease severity [160, 161]. Similarly, Yang et al. showed that oxidative stress induces posttranslational modifications on HDAC2 which are responsible for the loss of function of this enzyme's activity [162]. Taken together, it seems plausible to hypothesise that defects in the transcriptional and epigenetic regulation of proinflammatory genes in COPD cause dysregulated TLR signalling and effector biomolecule secretion by AMs.

4.2. Induced Oxidative Stress. Inhaled cigarette smoke and airborne pollutants induce oxidative stress in human lungs. In more detail, cigarette smoke contains approximately

Table 1: Molecules differentially expressed by AMs from animals or patients with COPD compared to healthy controls.

Molecule family	Encoded proteins	References
Cytokines	TNF-α ↓, IL-1B ↑↓, IL-6 ↓, IL-10 ↓, IL-12 ↑, Tnf-α ↓, Il-6 ↓	[126, 145, 147, 148, 150–152]
Chemokines	IL-8 ↓, CCL2 ↑, CCL5 ↓, CCL7 ↑, CCL13 ↑, CCL22 ↑, Cxcl10 ↓, CXCL9 ↓, CXCL10 ↓, CXCL11 ↓	[126, 145, 147–149, 151–154, 158, 165]
Chemokine receptors	CCR2 ↑, CCR5 ↑	[153, 154]
Prostaglandin metabolism	PTGS1 ↑, PTGS2 ↑	[156]
Oxidative stress	GSH ↓, Gsh ↓, iNOS ↑, HO-1 ↓	[147, 150, 155, 165, 167]
Iron metabolism	Hemosiderin ↑, *transferrin* ↑, transferrin receptor ↓, ferritin ↑	[172–175, 219]
Proteinases	MMP-1 ↑, MMP-2 ↑, MMP-7 ↑, *MMP-9* ↑ (SNPs), MMP-12 ↑, matriptase ↑	[154, 188–194, 196]
Neutrophil proteases and inhibitors	α_1-Antitrypsin	[185]
Chitinolytic activity	CHIT1 ↑, YKL-40 ↑	[199, 200]
Recognition markers	CD31 ↓, CD44 ↓, CD91 ↓, CR-3 ↑, CR-4 ↑, DC-SIGN ↑, MARCO ↓	[150, 219, 226]
Cytoskeletal rearrangements	RAC1 ↓, VAV1 ↓, RhoA ↑	[216, 229]
Mitochondrial stress	MCL-1 ↑	[230]
Integrins, scavenger receptors, and adhesion molecules	CD11a ↓, CD11c ↑, CD163 ↑, CD204 ↑, CD206 ↑, MSR-1 (SNPs), MERTK ↑	[220, 227, 234, 235]
Antigen presentation molecules	MHC-I ↓, MHC-II ↓, HLA-DR ↓, CD80 ↓	[150, 233]
Fc gamma receptors, PRRs	FcγR1 ↑, CD16 ↓, TLR2 ↓, TLR3 ↓, TLR4 ↓, TLR5 ↑, TLR9 (SNPs)	[126, 148, 150, 158, 165, 206, 233, 234, 236–238]

4000 chemicals including oxidants which impact lung physiology [163, 164]. On the contrary, the antioxidant protein glutathione (GSH) is heavily suppressed [150, 165] in macrophages by the actions of aldehydes in cigarette smoke [166] and biomolecules are modified (e.g., protein carbonylation) [147] leading to deleterious effects on living cells.

In response, AMs from patients with COPD have been demonstrated to express the nitrite synthase gene iNOS, but less heme oxygenase 1 (HO-1) than healthy smokers [167]. As mentioned above, other inflammation-related molecules, such as the histone deacetylases HDAC2 and SIRT1, are downregulated in AMs in an oxidative stress-dependent manner [165, 168, 169]. Eventually, cigarette smoke-induced oxidative stress and subsequent downstream gene expression changes in AMs result in Bak/Bax and cytochrome c-dependent apoptosis [170] increasing the cell debris pool that needs to be removed from the lung tissue to prevent secondary inflammation.

Finally, iron metabolism is dysregulated in the lungs of patients with COPD. Iron regulatory protein 2 and hemosiderin overexpression cause cellular and mitochondrial deposition of iron in alveolar tissue and resident macrophages which is associated with neutrophilia and infective exacerbations [171, 172]. Indeed, a recent report showcased the enhanced nutrient uptake and storage in AMs from patients with COPD. Philippot et al. found that these cells present with increased transferrin and ferritin expression important for iron uptake and storage [173]. Iron-loaded AMs from smokers also secrete higher amounts of ferritin than nonsmokers [174, 175] which could catalyse oxidative stress reactions in the alveolar tissue.

It has become apparent that exacerbated oxidative stress in AMs of patients with COPD impacts on other physiological pathways. For instance, oxidation of phospholipids in AMs impairs bacterial intracellular killing in mice [176]. To date, investigation of such concepts with available analytical tools is challenging. On the contrary, whole transcriptome analysis approaches complemented by bioinformatic coexpression network analysis would allow to link the expression patterns of dysregulated oxidative stress genes to the rest of the transcriptome in order to uncover overlooked interconnected biological pathways.

4.3. Deregulation of the Protease-Protease Inhibitor Balance. COPD progression correlates with the persistent activation of AMs and changes in the balance of secreted proteases and protease inhibitors (Figure 2). The importance of these molecules was illustrated in an experimental model of COPD where macrophage infiltration and the expression of proinflammatory mediators were induced in response to released mast cell-tryptases [177, 178].

ECM degradation enzymes, such as MMPs and cathepsins, are produced by macrophages and result in elastolysis and alveolar tissue damage [179–182]. Furthermore, these proteases have the potential to cleave small proteins and expose chemotactic fragments or they act as chemoattractants themselves and perpetuate macrophage accumulation in the lungs [183, 184]. On the contrary, cigarette smoking has been shown to induce the functional inactivation of α_1-antitrypsin, a NE inhibitor, which leaves smokers vulnerable to lung tissue destruction [185].

Monocytes and AMs are potent producers of several proteases; MMPs including MMP-1, MMP-2, MMP-7, MMP-9, and MMP-12, and cathepsins, such as K, L, B, and S, [180, 181, 184, 186, 187] and study have documented the overexpression of MMP-1, MMP-2, MMP-7, MMP-9, and MMP-12 in the lungs of smokers compared to healthy individuals [154, 188–194].

In patients with COPD, the expression of MMP-9 by AMs was shown to coincide with that of tissue inhibitor of metalloproteinases 1. The balance of these two mediators can be detrimental for the level of tissue damage in COPD lung and is controlled by the anti-inflammatory cytokine IL-10 [195]. Additional evidence for the current consensus of protease-protease inhibitor deregulation in macrophages from patients with COPD was provided by the fact that human patients with the most common α_1-antitrypsin mutation have greater proteolytic activity partially due to higher expression levels of the membrane-bound serine protease matriptase [196].

Furthermore, patients with COPD have more MMP-12-positive macrophages than healthy individuals in their lungs [193]. Macrophages are the main source of MMP-12 in the lungs of emphysematous mice [113, 182], and this MMP was shown to be important for connective tissue breakdown and neutrophil recruitment [99]. The mechanism MMP-12 utilises to promote inflammation was shown to involve the cleavage of the TNF precursor on the surface of macrophages and its release to the lung microenvironment [197].

Lastly, a perhaps not so well-documented function of AMs in COPD is their chitinolytic activity. Chitinases are released in the bronchoalveolar fluid of patients and are overexpressed by AMs from patients with COPD [198]. The presence of chitinase 1 and YKL-40, a chitin-binding protein, was found to correlate with airway obstruction and emphysema and to promote the production of proinflammatory mediators, such as cytokines, chemokines, and proteases by AMs from patients with COPD [198–200]. To date, we do not fully understand whether the upregulation in the expression of chitinases by AMs is a specific immune response against fungal opportunistic infection of patients with COPD and this warrants further investigation.

Given the significance of the protease activation pathway in irreversible tissue damage, it is necessary to understand how protease and protease inhibitor production is regulated in AMs aiming to fully characterise potentially defective molecular pathways that are responsible for the imbalance in the release of these mediators. Moreover, the literature is often contradicting with regard to the identity of protease members expressed by AMs. Currently available genomic techniques could settle the discrepancy noticed between older and more recent reports and show whether genetic polymorphisms account for the deregulation of protease-protease inhibitor imbalance in AMs.

4.4. Impaired Pathogen Phagocytosis. Due to their strategic localisation at the host-environment interface, AMs are key players in sensing microbes and irritants and initiating the phagocytosis process in order to remove and destroy them. Macrophage phagocytosis in patients with COPD has been extensively studied in humans and animal models, and our current understanding is that AMs present with a phagocytosis defect when treated with air pollutants (Figure 2) [201, 202].

AMs from patients with COPD and cigarette smoke-treated animals have been reported to display impaired phagocytosis of pathogens, such as *H. influenzae* [203–207], *C. albicans* [208, 209], *E. coli*, *M. catarrhalis* [206, 207], and *S. pneumoniae* [205, 206, 210] compared to controls. Interestingly, defective phagocytosis of latex particles has only been described for murine AMs which implies that data generated from different species should be taken with caution [211]. It is not entirely clear whether the inability of macrophages to efficiently uptake foreign material is tissue-specific or whether it is the result of a global genetic defect. For instance, in some studies, monocyte-mediated phagocytosis was comparable with that of AMs [204], whereas in others monocytes from patients with COPD demonstrated dysregulated phagocytic abilities [212], especially when the subjects were diagnosed with acute bronchopneumonia [213]. Therefore, further work is needed to determine whether the suppressed macrophage phagocytic capacity in patients with COPD is governed by lung-specific factors.

Besides phagocytosis of external stimuli, macrophages are also responsible for the clearance of accumulating apoptotic cells to avoid the release of toxic intracellular substances which can cause secondary inflammation and inhibit tissue repair [214]. This process, coined efferocytosis, has been suggested by some studies to be compromised in AMs from patients with COPD when coincubated with apoptotic neutrophils [215, 216], eosinophils [217], or epithelial cells [150, 218, 219]. Moreover, AMs from cigarette smokers upregulate the apoptotic cell removal tyrosine kinase MERTK, arguably in a compensation mechanism to restore endogenous efferocytosis levels [220]. Interestingly, macrophage efferocytosis index was reversed in AMs from animals and patients with COPD treated with native α_1-antitrypsin implying a relationship between the protease-protease inhibitor balance and apoptotic cell engulfment [221]. Moreover, mechanistic data provided by a number of groups support the idea that an increased expression of genes of the sphingosine-1 phosphate system can explain the defective efferocytic responses of AMs [222–225], although it is currently unclear whether other lipid metabolism pathways also play a role.

Studies designed to provide an insight into the molecular mechanisms that account for the suppressed AM efferocytosis showed that the expression of recognition receptors, such as CD31, CD44, CD91 [219], CR-3, CR-4, FcγR1, MARCO, and DC-SIGN, was significantly changed in AMs from patients with COPD [150, 226]. However, the expression of recognition molecules was found to be similar between smokers and patients with COPD in other reports contradicting the original findings [205]. In another report, the expression of the macrophage scavenger receptor 1 in monocyte-derived macrophages was associated with genetic variants which also controlled *in vitro* cell adhesion and survival in culture [227]. Finally, conflicting data have been published concerning the involvement of p38, ERK1/2, PI3K, ROCK, and p65 kinases and cytoskeletal changes in AM phagocytosis in COPD [147, 228].

Recently, Richens et al. showed that Rac1 activation inhibits RhoA kinase resulting in actin rearrangement and lamellipodia protrusion [229], while Minematsu et al. confirmed that RAC1 and VAV1 kinase levels are reduced in cigarette smoke-treated macrophages [216]. Therefore, it is possible that the compromised phagocytic/efferocytic

capacity of macrophages in COPD can be partially explained by impaired effector kinase signalling. Finally, Bewley et al. recently showed that the defective intracellular pathogen killing exhibited by AMs from patients with COPD is caused by a MCL-1-mediated failure to increase mitochondrial ROS production [230]. Collectively, while enormous progress has been made in understanding the molecular mechanisms of altered phagocytosis in COPD, we still do not have an integrated model of the pathophysiological changes operative in AMs in this disease.

4.5. Surface and Intracellular Marker Expression. To date, the assessment of AM surface marker expression in patients with COPD has focused on classical M1/M2 markers [231, 232], while our own work clearly indicated that this outdated classification cannot be applied to macrophages in COPD [144]. AMs from patients with COPD express less costimulatory molecules, such as the T cell activation and survival signalling molecule CD80, major histocompatibility antigens [150, 233], Fcγ receptors and integrins on their surface [234], more CD163, and carbohydrate and lipid scavenger receptors, such as CD206 and CD204 than non-COPD smokers and non-smokers [235].

Similarly, as already indicated above, the expression of surface PRRs is modulated in patients with COPD; TLR2, TLR4, and TLR5 are expressed at lower levels in macrophages from patients with COPD [126, 148, 236, 237]. However, there is contradicting evidence regarding the regulation of TLR2 expression which suggests that more work is needed to delineate whether this PRR and subsequent downstream signalling pathways play a role in the functional differences observed between macrophages from healthy individuals and patients with COPD. In contrast to the aforementioned receptors, TLR3 expression as well as downstream effector molecules, such as IL-8 and MMP-9, are overexpressed in macrophages in COPD [238]. Furthermore, polymorphisms in certain PRRs, such as TLR9, are associated with the compromised proinflammatory mediator secretion described above [206]. Lastly, patients with COPD have more $CD163^+$ macrophages in their lungs [239] which is most likely the consequence of lung microenvironment imprinting, as incubation of a human macrophage cell line with sputum from patients with acute exacerbation of COPD induced the expression of other anti-inflammatory genes, such as CD206 and arginase *in vitro* [240].

5. IMs and Monocytes in COPD

The literature has mainly focused on the role that AMs play in COPD. However, not much is known about the functions of IMs in the lung or monocytes in the blood (Figure 2 and Table 2). In mice, inhaled smoke causes an accumulation of CX_3CR1^+ monocytes and lung macrophages which associate with lung inflammation [241]. Monocytes infiltrate the lung and were shown to replace the dying resident macrophages [242]. In particular, $CX_3CR1^-GR\text{-}1^{hi}$ monocytes undergo a differentiation step into $CX_3CR1^+GR\text{-}1^{lo}$ cells before subsequently differentiating into lung macrophages after an inflammatory insult or the depletion of lung-resident macrophages [243]. Whether this is also the case for humans remains an open question.

Table 2: Molecules differentially expressed by monocytes or IMs from animals or patients with COPD compared to healthy controls.

Molecule family	Encoded proteins	References
Cytokines	TNF-α ↓, IL-6 ↑	[146, 245]
Chemokines	CCL2 ↑, IL-8 ↓	[146, 252]
Chemokine receptors	CCR2 ↑	[253]
Metalloproteinases	MMP-9 ↓, Mmp-12 ↑	[146, 251]
Antigen presentation molecules	CD86 ↓	[252]
Integrins, PRRs	CD11b ↓, CD14 ↓, CD54 ↓	[146, 252]
MicroRNAs	miR-24-3p ↑, miR-93-5p ↑, miR-320a ↑, miR-320b ↑, miR1273g-3p ↓	[254]

Monocytes are believed to develop into lung parenchyma macrophages which in mice have been identified as $CX3CR1^{hi}CD11b^+CD11c^{hi}MHC\text{-}II^{hi}$ macrophages and express TNF-α and IL-6 [244]. More evidence for the presence of monocytes in the human lung during inflammatory diseases came from the characterisation of a $CD14^+HLA\text{-}DR^+$ macrophage population in the sputum of patients with COPD capable to produce high levels of TNF-α [245]. In the lung, recruited monocytes have been shown to modulate neutrophil infiltration via the secretion of proinflammatory mediators [246].

Similar to AMs, monocyte activation in patients with COPD presents with gene expression signatures related to apoptosis, protease function, proliferation and differentiation, glycerol metabolism, and cytosolic transport as shown by a microarray study [247]. As a result of their activation state, monocytes display more prominent migration towards CCL5, CXCL1, CXCL7, or CXCR3 chemokine gradients [248, 249], production of IL-6 and CCL2, but less IL-8, MMP-9, and CD54 compared to controls [146]. In contrast, the literature on phagocytosis by monocytes from healthy individuals and patients with COPD is contradictory [205, 250]. With regard to MMP production, Pérez-Rial et al. showed that the recruited monocytes are responsible for the overall increase of macrophage numbers in a murine model of COPD [251]. Interestingly, monocyte/macrophage responses depend a lot on the causative agent of COPD as exemplified in a diesel exhaust particle-induced study where monocytes exhibited less CXCL8 and phagocytic responses due to dampened CD11b, CD14, and CD86 surface expression [252], while they overexpress CCR2 in smokers [253].

There have been various mechanistic lines of evidence to explain the augmented proinflammatory phenotype of monocytes; Dang et al., for example, found that miRNA expression, such as miR-24-3p and miR-93-5p, correlates with dysregulated downstream TLR and NOD-like receptor signalling proteins, such as IκBα [254]. On top of that, altered epigenetic cues as exemplified by the downregulation of HDAC levels cause an upregulation in proinflammatory gene expression and NF-κB-mediated inflammation [160, 255].

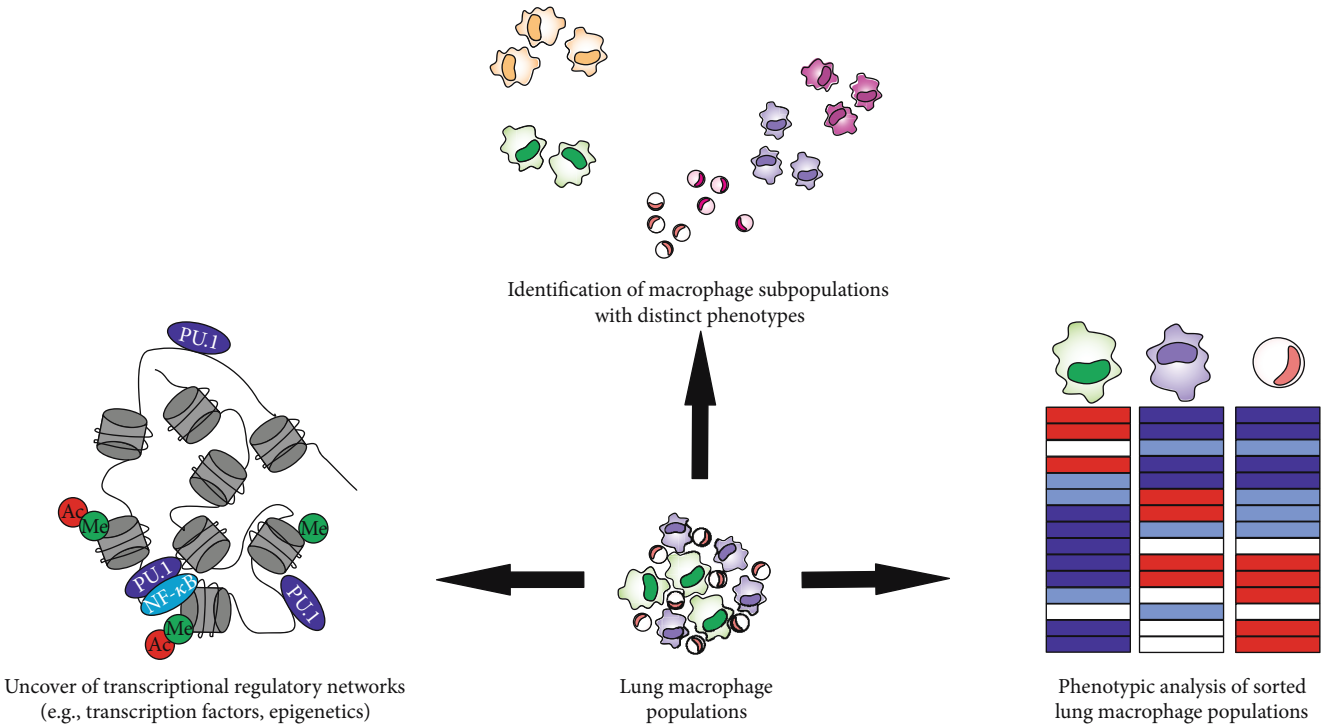

Figure 3: Future directions in COPD lung macrophage population research. Recent advances in Immunogenetics and Structural Biology make it possible to evaluate the heterogeneity of lung macrophage populations. In particular, single cell RNA sequencing can identify homogeneous macrophage subsets with distinct transcriptomes and functions. Mass cytometry can complement and validate initial findings establishing prognosis/diagnosis biomarkers for human patients with COPD. Moreover, analysis of the nuclear heterochromatin state with ATAC sequencing and subsequent validation with ChIP-sequencing can shed light on the epigenetic regulation of lung macrophage populations and highlight the molecular mechanisms responsible for their functions *in vivo*. Lastly, the role of AMs, IMs, and lung monocytes warrants further investigation in order to better understand the contributions of each macrophage population to COPD progression and severity. Transcriptome analysis will determine whether these populations are distinct or part of a differentiation continuum from the monocyte to the AM phenotype and will associate gene expression with unique biological processes.

6. Concluding Remarks

COPD affects around 328 million people worldwide, and it is projected to rank within the top four most fatal diseases by 2030 [77, 256]. Moreover, the chronic nature of the disease and the frequently observed exacerbations and comorbidities have major consequences on patients' lives and countries' economic status [256]. It is therefore important to advance our knowledge of immune system manifestations in COPD and uncover the molecular pathways responsible for the cross talk between immune cells and the lung stroma in order to provide the clinic with prognosis/diagnosis biomarkers and the pharmaceutical industry with novel testable genes/pathways for future drug development screenings.

Already in 1979, it had been suggested that the macrophage population, which comprises of lung-resident macrophages and blood monocytes, constitutes more than 97% of all cells in the human bronchoalveolar lavage [257], while two decades later, the severity in COPD was linked to the presence of macrophages, neutrophils, NK cells, and activated epithelial cells in the lung [258]. However, due to the lack of specific markers and respective technologies at that time, no further subset specifications or functional subdivision could be performed and these studies remained incomplete. This is also true for studies which suggested correlation between COPD severity or small airway infiltration and macrophages [259–261] and reports which demonstrated less apoptosis and more proliferation in AMs from smokers [262]. Taken together, many of the findings concerning the role of certain immune cells and their relation to disease state, severity, and outcome have been obtained more than two decades ago. While still of value, these findings are challenged by very recent findings concerning cellular classification and function of immune cells in general.

With regard to lung macrophage populations, the efforts to better appreciate their role in COPD remain elusive. AMs are the only lung-resident macrophage population that has been extensively investigated in the past, whereas IMs have long been considered solely as an intermediate step in monocyte differentiation mainly due to limitations associated with their harvest from human subjects. The field is missing out on valuable information about potentially existing homogeneous macrophage subsets with distinct phenotypes associated with a pathological feature or clinical subgroup of COPD. In addition, the molecular mechanisms that dictate the functions of lung macrophage populations remain poorly characterised; for example, although there is evidence that the dysfunctions

of lung macrophages in COPD are regulated epigenetically, an unbiased evaluation of the interplay between transcription factors and epigenetic networks active in lung macrophages in COPD is currently lacking.

To this end, latest advances in the fields of Immunogenomics and Systems Biology have been very encouraging and can help address these open questions (Figure 3). The deconvolution of the lung macrophage structure with high-dimensional single cell technologies, such as RNA sequencing, could identify lung-resident macrophage subpopulations with unique transcriptomes that reflect the niche, activation state, or interactions with other immune cells at the time of harvest [232]. Subset-specific genes could then be associated with a COPD subgroup and be validated with mass cytometry. Such an approach could stratify COPD patient cohorts according to new biomarkers and replace currently used symptom-based readouts [263].

Furthermore, the early discovery of HDAC downregulation in patients with COPD should be followed up by complementary assay for transposase-accessible chromatin (ATAC) sequencing to predict complex networks of histone-modifying enzymes and transcription factors that direct transcription in lung macrophages and link them to certain genes/biological functions [232]. Subsequent chromatin immunoprecipitation (ChIP) sequencing would validate these targets and lead to new hypothesis generation and potentially novel therapeutic interventions.

To conclude, there are many exciting research avenues to be followed, now supported by genetic and computational approaches made available in the last decade. The high level of macrophage plasticity *in vivo* implies that there are complex stimulatory and regulatory molecular circuits that act simultaneously and result in their physiological dynamics. Hence, to better understand the role lung macrophages play in COPD, we will need to take advantage of these novel tools and revisit older findings.

Abbreviations

AM: Alveolar macrophage
ATAC: Assay for transposase-accessible chromatin
COPD: Chronic obstructive pulmonary disease
ChIP: Chromatin immunoprecipitation
DC: Dendritic cell
ECM: Extracellular matrix
GSH: Glutathione
HO-1: Heme oxygenase 1
IM: Interstitial macrophage
MMP: Metalloproteinase
NE: Neutrophil elastase
PRR: Pathogen recognition receptor
ROS: Reactive oxygen species.

Conflicts of Interest

The authors declare no conflicts of interest. Joachim L. Schultze is a member of the Excellence Cluster ImmunoSensation.

Acknowledgments

This work was supported by the German Research Foundation to Joachim L. Schultze (SFB704, Excellence Cluster ImmunoSensation).

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Hindawi
Journal of Immunology Research
Volume 2018, Article ID 6827237, 8 pages
https://doi.org/10.1155/2018/6827237

Review Article

Roles of Macrophage Subtypes in Bowel Anastomotic Healing and Anastomotic Leakage

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Received 28 July 2017; Revised 21 December 2017; Accepted 2 January 2018; Published 18 February 2018

Academic Editor: Zissis Chroneos

Macrophages play an important role in host defense, in addition to the powerful ability to phagocytose pathogens or foreign matters. They fulfill a variety of roles in immune regulation, wound healing, and tissue homeostasis preservation. Macrophages are characterized by high heterogeneity, which can polarize into at least two major extremes, M1-type macrophages (classical activation) which are normally derived from monocytes and M2-type macrophages (alternative activation) which are mostly those tissue-resident macrophages. Based on the wound healing process in skin, the previous studies have documented how these different subtypes of macrophages participate in tissue repair and remodeling, while the mechanism of macrophages in bowel anastomotic healing has not yet been established. This review summarizes the currently available evidence regarding the different roles of polarized macrophages in the physiological course of anastomotic healing and their pathological roles in anastomotic leakage, the most dangerous complication after gastrointestinal surgery.

1. Introduction

Macrophages are myeloid immune cells that play a central role in inflammation and host defense [1, 2]. These cells are characterized by the powerful ability of phagocytosis and are credited with protecting the host from infection through a process so-called "innate immunity" [3]. In recent years, with the accumulation of evidence, macrophages have emerged as one of the most versatile cells. Their roles have shifted from immune effector cells which conduct host defense just as "trashmen" to predominant "directors" and "executors" for regulating inflammatory response, keeping tissue homeostasis, participating in wound healing and tissue remodeling [4].

Macrophages are actively involved in the wound healing process, while their role in a special surgical wound, also known as the anastomotic wound, has not yet been fully established. Anastomosis is constructed after removal of gastrointestinal tumor or bowel resection by surgeons to reconstruct the continuation of the gastrointestinal tract. Abnormal healing of anastomosis may develop into anastomotic leakage (AL), defined as luminal contents leaking from a surgical bowel connection [5]. It is the most dangerous complication after colorectal surgery [6–8], because it is responsible for up to 40% postoperative mortality rate, prolonged hospitalization, and an increase in the cost of healthcare due to the treatment of sepsis and the need for reoperation [9].

From a macroscopic point of view, the cause of AL mainly includes communication, infection, and healing disturbances [10]. However, a detailed mechanism on a cellular level is yet to be established due to the limited evidence. In this review, basing on heterogeneous populations of macrophages and their opposed tendencies of polarization, we tend to discuss the roles of different types of macrophages in an uneventful anastomotic healing and their pathological roles in anastomotic leakage.

1.1. Subtypes of Macrophages. Macrophages or mononuclear phagocytes had been long thought to originate from hematopoietic stem cells (HSCs). The prevailing dogma has stated that all macrophages derived from and were also replenished

by monocytes [11]. However, macrophage family cells (cells of the mononuclear phagocyte system) manifest remarkable heterogeneity, in both their morphology and biological functions [12, 13]. These recent data have challenged the long-held conception about "HSC-monocyte-macrophage." Evidence showed that tissue-resident macrophages like Kupffer cells of the liver, epidermal Langerhans cells of the skin, and microglia of the brain derived from a yolk sac and could persist in adult mice independent of HSCs [14–21]. Those tissue-resident macrophages can renew in situ, although they might also be replenished by blood monocytes in certain situations. In contrast to monocyte-derived macrophages which participate in an antibacterial process during acute inflammatory response, tissue-resident macrophages express different functional properties and play a central role in maintaining tissue architecture, function, and homeostasis [22–25], and their role in anastomotic healing is further discussed below.

The diversity and plasticity were recognized as hallmarks of macrophages, which contribute to their significant heterogeneity. In general, polarization of macrophages can be divided into two major extremes, that is, the classical activation which results in M1-type macrophages (M1) and the alternative activation which results in M2-type macrophages (M2). Those two types of macrophages perform diverse functional phenotypes in response to microenvironmental signals, like microbial products, damaged cells, and cytokines from activated lymphocytes. Specifically, ligands of Toll-like receptors (TLRs) and interferon-γ (IFN-γ) can induce macrophages to polarize into M1-type macrophages; on the contrary, interleukin-4 (IL-4) and interleukin-13 (IL-13) induce macrophages to polarize into M2-type macrophages [26–28]. However, such explanation may not fully illustrate all different activation scenarios. Murray et al. proposed that there should be some other subtypes between M1 and M2 [29], including the M2a subgroup induced by IL-4 and IL-13, the M2b subgroup activated by immune complexes (TLRs), and the M2c subgroup deactivated by glucocorticoids, transforming growth factor (TGF), or interleukin-10 (IL-10) [30, 31]. Moreover, it is also reported that there might be a supplementary subtype of M2 (M2d), which is elicited by TLR agonists and adenosine [32, 33]. It seems that the polarization of macrophages should be viewed as a continued spectrum, on which, two types of macrophages (M1 and M2) occupied the opposite ends. Another classification of polarization proposed by Mosser and Edwards suggested that macrophages are activated to form three populations in charge of host defense, wound healing, and immune regulation, respectively [34]. The authors classified macrophages on the basis of their fundamental functions rather than of the stimuli. Matching with the previously discussed conception of "M1-M2" paradigm, most of monocyte-derived macrophages are classically activated and express the M1 phenotype, which exerts host defense; reversely, tissue-resident macrophages are mainly activated in the alternative pathway which expresses M2-like characteristic and preserves tissue homeostasis and resolution of inflammation [21–23].

1.2. The Role of Polarized Macrophages in Physiological Anastomotic Healing. The wall of the alimentary tract contains four layers (i.e., mucosa, submucosa, muscularis propria, and serosa). For a classic end-to-end inverted bowel anastomosis, apposition of the serosa vanishes the gap between the two ends of the gastrointestinal tract, providing a barrier that insulates the sterile abdominal cavity from luminal contents and bacteria; moreover, this layer is important in providing a matrix for fibroblasts [35]. The submucosa consists of blood vessels, lymphatics, and nerve fibers; this layer is the source of fibroblasts that become active after gastrointestinal surgery and start to deposit collagen. The stapled or sutured collagen fibers in this layer provide most of the tensile strength of anastomosis [36]; hence, the submucosa is of great importance in anastomotic healing. The mucosal layer also plays a role in maintaining homeostasis to allow the healing process. A pool of macrophages in the gastrointestinal mucosa is the largest pool of tissue macrophages in the body, and a long-lasting macrophage absence or dysfunction impairs anastomotic healing [37, 38].

Tissue repair and healing after injury have been studied for centuries but remain understood to a limited object, that is, skin. Different from that, healing of the gastrointestinal tract is anatomically obscured from inspection, only allowing the surgeon to judge the success of the operation only on the patient's parameters of general well-being [36]. There are some differences between the skin and anastomotic healing including anatomy (e.g., no equivalent anatomic component of the serosa in cutaneous tissues) and collagen and collagenase activity [39]. However, classical response to injury occurs in all organs and tissues. The physiological course of anastomotic healing can also be divided into three overlapping but distinct stages, which include inflammation, new tissue formation, and remodeling (Figure 1) [40, 41].

1.2.1. Inflammation. In addition to infection of diverse microbial factors, injuries or traumas such as surgical strike can also lead to a non-pathogen-associated inflammation, which can be further divided into the early inflammatory response and the late one [42]. In the early inflammatory phase, neutrophils are recruited from circulating blood to local wounding tissue (the anastomotic area) at first. Those recruited polymorphonuclear cells remove the local foreign particles or bacteria and then undergo apoptosis or necrosis. After that, monocytes are recruited and differentiate into macrophages which are highly phagocytic. They phagocytose impaired neutrophils and other tissue debris to protect from further tissue damage. During this phase, in response to pathogen-associated modifying patterns (PAMPs) in a contaminative circumstance or damage-associated modifying patterns (DAMPs) in a sterile circumstance, macrophages are classically activated and express the M1 phenotype [43–45]. M1 macrophages release high concentrations of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), and interleukin-12 (IL-12); protease; and reactive oxygen species (ROS) [34], all of which are thought to be important for

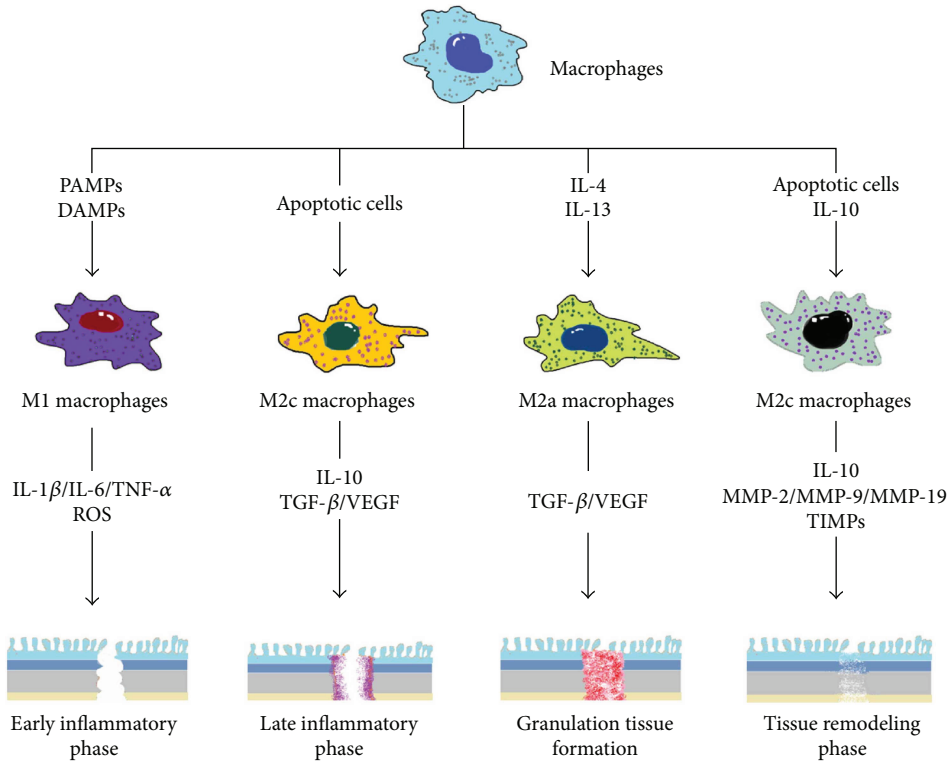

Figure 1: Polarization of macrophages in normal healing of anastomosis. Inactivated macrophages can be stimulated by various stimuli (e.g., PAMP, DAMP/IL-4, and IL-13/apoptotic cell) and polarize into M1- or M2- (M2a, M2c) type macrophages during different phases of normal anastomotic healing. Differentiated macrophages express a variety of cytokines (e.g., IL-1β, IL-6, IL-10, and TNF-α), growth factors (e.g., VEGF), and enzymes (MMPs). These biochemical substances acting upon tissues contribute to tissue repair and remodeling. PAMP: pathogen-associated modifying patterns; DAMP: damage-associated modifying patterns; IL: interleukin; TNF-α: tumor necrosis factor-α; VEGF: vascular endothelial growth factor; MMPs: matrix metalloproteinases.

microbial killing and proinflammatory response [13]. M1 macrophages can also produce collagenase, a high-activity enzyme that causes collagen degradation that results in low anastomotic strength early after the formation of an anastomosis [46]. In the late inflammation phase, with excessive phagocytosis of apoptotic neutrophils, engagement of β2 integrins on macrophages by apoptotic neutrophils activates macrophages to express anti-inflammatory mediator transforming growth factor (TGF) [47]. In contrast, the production of proinflammatory cytokines like TNF-α and IL-1β was inhibited [48, 49]. Thus, the phenotype of macrophages switches from proinflammatory M1-like to anti-inflammatory M2-like. These macrophages produce cytokines such as IL-10 and lay the foundation for new tissue formation by secreting other growth factors such as vascular endothelial growth factor (VEGF) [50, 51]. Because macrophages stimulated with IL-10, TGF, or glucocorticoids in vitro polarize into the M2c subtype that shares similarities with anti-inflammatory macrophages [30, 52–58], it suggests that anti-inflammatory macrophages belong to M2c-type macrophages and are able to amplify their anti-inflammatory response by secreting IL-10 and TGF in a feedforward loop. In addition, anti-inflammatory and regenerative capacities of anti-inflammatory macrophages were shown to be entirely IL-10-dependent in sterile environments, for example, in surgical wound [59].

1.2.2. New Tissue Formation. In this phase, macrophages resident in tissue or recruited from peripheral blood, known as profibrotic macrophages, generate various growth factors such as TGF, platelet-derived growth factor (PDGF), fibroblast growth factor-2, or insulin-like growth factor-1 [60]. Among them, TGF is a profibrotic cytokine that exerts on fibroblasts and activates them to differentiate into myofibroblasts in wound tissue. Myofibroblasts produce a mass of extracellular matrix (ECM) components including collagen and fibronectin to fill up the tissue defect. For the gastrointestinal tract, collagen can also be produced by smooth muscle cells [61]. Collagen subtypes in the gastrointestinal tract are collagens I, III, and V, compared to solely collagen I and III in the skin [62]. By efficient contractile forces from myofibroblasts, fractured wound tissue can be bound together and rebuild their integrity [63]. Meanwhile, profibrotic macrophages and activated fibroblasts release proangiogenic factors like VEGF, which elicit endothelial progenitor cells crawling towards wound tissue, to promote new vessel formation (angiogenesis). Invasion of the capillary increases the blood supply to local tissues and facilitates anastomotic healing. Furthermore, studies of the healing colonic mucosa of rabbits after experimental excision showed that an abundance of mesenchymal cells in the healing intestinal muscle layers accompanies capillary invasion; these cells can differentiate into smooth muscle cells and histiocytes, which are thought

to be responsible for the reestablishment of smooth muscle tissue [64, 65]. Profibrotic macrophages, myofibroblasts, and neovessels all together constitute granulation tissue, the most important fundamental compartment in the normal course of wound healing [40, 41]. These profibrotic macrophages are functionally classified as M2a-like macrophages because they can be induced in vitro by IL-4 and IL-13 [23, 30]. However, it is not clear whether anti-inflammatory and profibrotic macrophages can be clearly distinguished in vivo, and it appears that macrophage plasticity creates a mixture or continuous variant shifts during wound healing [50].

1.2.3. Remodeling. Remodeling of anastomosis is a dynamic process of maturation within healed tissue that is based on a balance between ECM deposition and breakdown and tissue remodeling [66, 67]. A part of tissue-resident macrophages termed as fibrolytic macrophages is critical for maintaining this dynamic balance. They produce matrix metalloproteinases like matrix metalloproteinase-2 (MMP2), matrix metalloproteinase-9 (MMP9), matrix metalloproteinase-12 (MMP12), and matrix metalloproteinase-19 (MMP19) [42, 68], to degrade matrix macromolecules, that is, collagen, one of the most important components of ECM. The submucosa is a strength layer of the gastrointestinal tract and made predominantly of collagen, and remodeling of this layer predominates the strength of the anastomosis. Depending on MMPs secreted by fibrolytic macrophages, initially deposited collagen fibers are rearranged and cross-linked, remodeled from type III collagen to type I collagen; the latter one is much stronger. Besides, fibrolytic macrophages also regulate the degradation by synthesizing the tissue inhibitor of metalloproteinases (TIMPs), which can inhibit the activities of MMPs. Furthermore, fibrolytic macrophages are responsible for the induction of fibroblast apoptosis, subsequent removal of apoptotic cells, and suppression of further inflammation via IL-10 release [60]. Fibrolytic macrophages are proposed to be classified as M2c-like macrophages which can be elicited in vitro by apoptotic cells and IL-10 [69, 70].

Thus, macrophages participate in whole physiological courses of anastomotic healing. Among the three main phases of tissue repair, macrophages express different phenotypes during different stages. There are at least four kinds of macrophages in a condition of normal tissue repair: (1) proinflammatory macrophages, (2) anti-inflammatory macrophages, (3) profibrotic macrophages, and (4) fibrolytic macrophages. If we sort out those four kinds of macrophages according to "M1-M2" paradigm, proinflammatory and profibrotic macrophages may, respectively, correspond to M1-type and M2a-type macrophages. Meanwhile, both anti-inflammatory and fibrolytic macrophages probably belong to M2c-type macrophages [42, 59].

1.3. Roles of Macrophages in Anastomotic Leakage. As we previously discussed in our review, occurrence of AL mainly contains three factors: communication, infection, and healing disturbances. Communication means defect of the alimentary tract in the anastomotic region that connects the gastrointestinal lumina and abdominal cavity. Infection indicates anastomotic site bacterial infection. Healing disturbances include all substances that disturb a normal healing process such as hypoxia or inflammation. These three factors actively interact with each other: one factor takes place, and a responsive chain that consists of all factors will be initiated, eventually leading to AL. For example, infection provokes inflammatory response at the anastomotic site, which impairs collagen deposition [71, 72], then interferes with the normal healing process, and leads to a communication between the intra- and extraluminal gastrointestinal walls. On the contrary, communication allows the bowel content (including bacteria) to dislocate into the abdominal cavity, causes intra-abdominal infection, and afterwards delays anastomosis healing. Clinically, communication in some extent is regarded as a macroscopic clinical outcome, while infection and healing disturbances are durative biological processes. For AL, macrophages are mainly involved in the latter two mechanisms, which is also observed in other poorly healed wounds [73–77].

Anastomotic infection may be caused by anastomotic dehiscence (intestinal contents leak to the sterile abdominal cavity) or pre-/intraoperative contamination. Regardless of the cause of infection, in the contaminative infective environment, macrophages polarize into the M1 type as mentioned above. However, instead of supporting resistance to intracellular bacteria and controlling the acute phase of infection, an excessive or prolonged M1 program is deleterious for patients, as demonstrated in acute infections with *Escherichia coli* [78]. *E. coli* as a resident flora of the gut can induce a typical M1 profile through the recognition of lipopolysaccharides (LPS) by TLR4 [79, 80]. Classical activated M1-type macrophages upregulate the expression of inducible nitric oxide synthase (iNOS), which is responsible for the generation of nitric oxide (NO). NO was first identified to mediate arterial vasodilatation [81–83] and then was found to have a role in host defense against pathogens [84, 85]. Moreover, a prominent role has been described for NO in collagen deposition, fibrosis, and scar formation [71, 72, 86, 87]. High levels of wound NO, as in infection or inflammation, severely impair wound collagen synthesis [88]. Decreased deposition of collagen seriously weakens the anastomotic strength, which may lead to the failure of anastomotic healing. Therefore, improper M1 polarization of macrophages in bacterial infection of the abdominal cavity contributes to the occurrence of AL.

The role of macrophages in leakage with healing disturbances is more complicated. During a normal condition, tissue repair initiates from clearance of tissue debris and dead cells, efficiently phagocytosing those "tissue rubbish" by macrophages, and is critical for timely resolution of inflammation and successful healing. Nevertheless, for those patients complicated with diabetes mellitus, advancing in years, or undergone chemotherapy, the ability of macrophages to phagocytose is severely influenced, which directly leads to an accumulation of apoptotic or necrotic cells at the anastomosis site. This accumulation of dead cells prolongs the inflammatory phase, disturbs the healing process, and compromises the resolution of inflammation [73, 74].

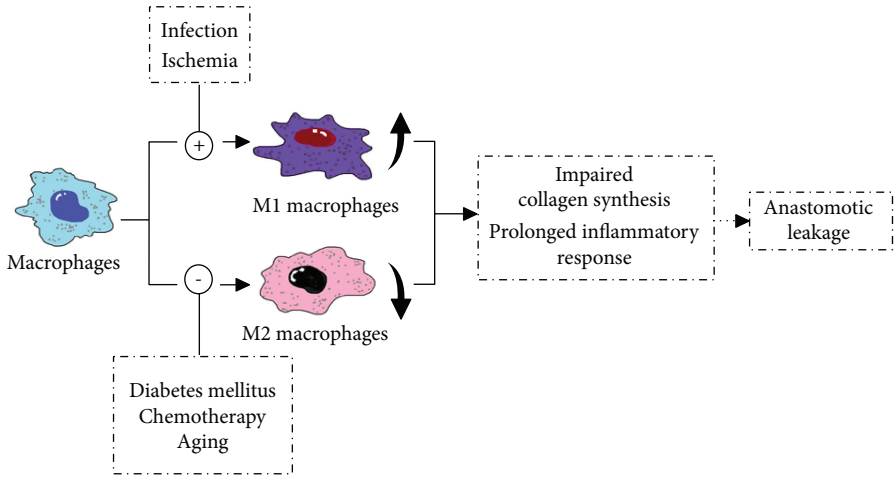

Figure 2: Imbalance of polarized macrophages contributes to the occurrence of anastomotic leakage. In some pathological conditions (infection, ischemia, diabetes mellitus, etc.), macrophages are abnormally activated into M1-type macrophages or inhibited to express the M2-type phenotype, which leads to long-time inflammatory response in the anastomotic site and influences collagen deposition and tissue repair; all of those are thought to be associated with anastomotic leakage.

Other disturbances such as ischemia or anastomotic hypoxia severely compromise the anastomotic healing [89, 90]. At a cellular level, exposing macrophages to an anoxic environment leads to the expression of proinflammatory cytokines like IL-1β and TNF-α and cytotoxic mediators like NO [91–93], which indicates that hypoxia can promote macrophages to polarize into the M1 phenotype. Excessively activated M1 macrophages sustain proinflammatory response and obstruct subsequent steps of the repair process that influences proper healing and remodeling of anastomosis [94–96], and the relevant mechanism is described above.

Based on the available evidence, it seems that classical activated macrophages which express the M1 phenotype are responsible for the pathological process of defective anastomotic healing, whereas alternative activated macrophages which express the M2 phenotype play a critical role in inflammation resolution and successful tissue repair (Figure 2). Although M1-type macrophages participate in the early phase of normal wound healing, the programmed transformation of their polarized orientation from M1 to M2 lays the foundation of transient inflammatory response and the following tissue regeneration.

2. Conclusion

Macrophages are the most versatile immune cells and possess significant plasticity and heterogeneity. Macrophages can polarize into two main extremes and express corresponding phenotypes (M1 and M2). As polarization is the premise for macrophages to exert their diverse biological functions, different polarized macrophages play different roles in the physiological process of anastomotic healing and pathogenesis of AL. Reacquainting AL in the perspective of macrophages contributes to the exploration of new diagnostic tools and therapeutic targets. For example, in different recovery phases after anastomosis construction, the spectrum of cytokines and inflammatory mediators such as IL-1β, IL-6, IL-10, IL-12, TNF-α, ROS, and NO, which are secreted by macrophages, may appear an alteration. Moreover, the level of these substances could indirectly reflect the situation of an anastomosis. An abnormal fluctuation of these substances probably indicates disorder and defection of anastomosis healing, which can be regarded as premonition of AL. Because M1-type macrophages show a stimulating effect on AL and M2 macrophages are essential for anastomosis healing, regulation of M1/M2 polarization may find its therapeutic roles in the treatment of AL in the future.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Authors' Contributions

Jinyao Shi and Zhouqiao Wu are the first authors.

Acknowledgments

This paper is supported by the Beijing Municipal Science and Technology Commission (nos. D141100000414004 and Z151100004015070) and Beijing Municipal Commission of Health and Family Planning (no. 2014-1-2151).

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Hindawi
Journal of Immunology Research
Volume 2018, Article ID 8695157, 14 pages
https://doi.org/10.1155/2018/8695157

Review Article

Receptors That Inhibit Macrophage Activation: Mechanisms and Signals of Regulation and Tolerance

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Received 26 July 2017; Revised 7 November 2017; Accepted 20 November 2017; Published 11 February 2018

Academic Editor: Kebin Hu

A variety of receptors perform the function of attenuating or inhibiting activation of cells in which they are expressed. Examples of these kinds of receptors include TIM-3 and PD-1, among others that have been widely studied in cells of lymphoid origin and, though to a lesser degree, in other cell lines. Today, several studies describe the function of these molecules as part of the diverse mechanisms of immune tolerance that exist in the immune system. This review analyzes the function of some of these proteins in monocytes and macrophages and as well as their participation as inhibitory molecules or elements of immunological tolerance that also act in innate defense mechanisms. We chose the receptors TIM-3, PD-1, CD32b, and CD200R because these molecules have distinct functional characteristics that provide examples of the different regulating mechanisms in monocytes and macrophages.

1. Introduction

1.1. Macrophages. Macrophages are phagocytic cells, which are localized through the whole human body. Monocytes give rise to these terminally differentiated cells. Monocytes and macrophages belong to the functional immune system known as mononuclear phagocyte system which includes dendritic cells, circulating monocytes, and their progenitors in bone marrow.

Macrophages have several functions to maintain immune homeostasis such as host protection, tissue repair, phagocytosis, clearance, and secretion of diverse factors, which contribute to innate and adaptive defenses against infection and counteract inflammatory processes, while distinct secreted signals restore tissue homeostasis and promote subsequent repair [1, 2]. To perform protective functions and repair damaged tissue, monocytes and macrophages express a wide range of surface, vacuolar and cytosolic receptors for recognition, and uptake of host-derived (damage signals) and foreign particles; many of these receptors facilitate phagocytosis, endocytosis, sense viral, bacterial, and parasitic molecules [3].

During organogenesis, macrophages expressed by the embryonic yolk sac and fetal liver remain as a resident cell, self-maintaining population, which turn over locally under steady-state conditions and perform a variety of clearance and organ-specific trophic functions [4]. After birth, bone marrow-derived blood monocytes replenish resident macrophage populations with high turnover rate, such occurs in the gut; larger numbers are recruited following injury, infection, and sterile inflammation and give rise to infiltrating and activated tissue macrophages [5].

Depending on the anatomical localization and its organ requirements, macrophages consist of variably mixed populations of resident macrophages and blood-derived monocytes. As a result of their complex origin, distribution, and physiologic responses to endogenous and exogenous stimuli, these cells will express a marked phenotypic heterogeneity (reviewed by Gordon and Plüddemann) [6].

Macrophages and monocytes are characterized by a multifunctional heterogeneity. For example, macrophages can be polarized in two main subtypes, "classical" and "alternative" activated macrophages. Classical macrophages depend on

the presence of proinflammatory cytokines such as INF-γ and TNF-α secreted by activated TH1 $CD4^+$ lymphocytes and NK cells and secreted bacterial components such as LPS. Classical macrophages are also known as M1 which has enhanced antimicrobial, inflammatory, and antigen-presenting properties [7]. Alternative macrophages are generated in presence of IL-4 and IL-13 cytokines secreted by activated TH2 $CD4^+$ lymphocytes. These macrophages play an important role limiting inflammatory responses, perform antiparasitic functions, and favor wound healing [8]. Moreover, alternative macrophages now can be classified in a new proposed scheme M2 like M2a, M2b, and M2c subtypes with specific functions and markers (reviewed by Martinez and Gordon) [8].

Activation through the surface, vacuolar, and cytosolic receptors results in signals to control or regulate functions in their neighbors and distant target cells. Their phagocytic capacity is variable and may even be undetectable but provides a well-developed machinery to internalize, degrade, and store cargo such as poorly degraded foreign particles [9].

Also, macrophages expressing regulatory surface molecules can attenuate or inhibit cell activation, which could be considered a tolerance or compensation mechanism as a result of an exacerbated immune response, so they have to preserve tissue homeostasis to avoid an inflammatory process which can compromise the homeostasis.

1.2. Inhibitory Receptors. Cells of the immune system are activated by endogenous or exogenous antigenic stimuli. Endogenous stimuli are often causal agents of autoimmune diseases but can also come from transformed cells (cancer cells). Exogenous stimuli include a broad range of environmental compounds, as well as other molecules that may come from pathogens like bacteria, fungi, parasites, and viruses that have entered into the organism where they can, potentially, establish infections [10]. These stimuli activate diverse cellular mechanisms whose purpose is to prevent or eliminate infections that are frequently accompanied by inflammatory processes. It is now well-accepted that the activation of the immune system is a process highly regulated through the expression and functioning of inhibitory receptors that work to prevent an exacerbated inflammatory response that can cause tissue damage or even autoimmune diseases.

Surface receptors are proteins that extend through the cell membrane. Their function is to transduce signals into the cell in response to some external stimuli. Receptors have intracellular domains that associate with cytoplasmic proteins such as adaptors, chaperone proteins, or enzymes which are required to perform adequately signaling. These receptors contain diverse conserved domains such as ITAM, ITIM, and ITSM. ITAM is perhaps the one that has been described in greatest detail. ITAM domains are found in several receptors, including T cell receptors (TCR), B cell receptors (BCR), and Fc region receptors (FcR). Studies of the structure of ITAM domains have identified a conserved sequence characterized by the presence of two tyrosine residues (Y), each one followed by two variable residues and one residue of leucine in the Y + 3 position, which is considered as a conserved domain that can trigger activation signals through conformational changes and phosphorylation of tyrosine residues, as well as the direct or indirect association with enzymatic elements present in the cytoplasm [11, 12]. Receptors with ITAM domains are associated with cell activation in response to stimuli or the ligands of those receptors. However, another group of receptors exists with the function of moderating cell activation. These are the inhibitory receptors.

The concept of the inhibitory receptor was introduced early in the 1990s [13, 14]. These receptors have been defined as molecules that negatively regulate the immune response to pathogenic microorganisms [15] and contain ITIM domains in the intracellular tail that, when phosphorylated, recruit enzyme phosphatases such as SHP1 and SHP2, which interfere with the activation pathways promoted by other activator receptors [16]. ITIM domains are similar to ITAM domains in that both have a specific arrangement of tyrosine/leucine [17]. This tyrosine/leucine arrangement in inhibitory receptors is located inside of a sequence of 13 amino acid residues that usually have a hydrophobic residue at position −2 [18].

Recent publications have demonstrated that inhibitory receptors may, or may not, have ITIM domains in the cytoplasmic tail [19]. Other mechanisms have also been described that allow these receptors to regulate activation of cells in the immune system. They include recognition and binding to ligands that perform the function of costimulatory molecules. In this way, inhibitory receptors block the cell from receiving the signals necessary for cell activation in response to specific antigens. In addition to recruiting enzyme phosphatases and competing for ligands, they can also be considered as a mechanism that regulates the status of cell activation/inhibition [20]. In general, inhibitory receptors can interfere in diverse stages of cell activation mediated by antigenic stimuli, inhibit the expression of genes involved in cell activation, and possibly induce the expression of other genes that inhibit the function characteristic of cell activation to produce deleterious changes at the level of cell metabolism, proliferation, and survival [21].

Therefore, we can define inhibitory receptors as molecules of the cell surface that interfere in various ways with intracellular signaling pathways to negatively regulate cell activation and cell function in response to tumors, infections, allografts, and even allergens and many other antigens. Numerous studies have described these receptors based on the diverse mechanisms of tolerance and anergy present in T lymphocytes and NK cells [22–26].

2. Receptors That Inhibit Macrophage-Monocyte Functions

2.1. PD-1 Belongs to the Superfamily of Immunoglobulins, and Its Function Is Associated with Cell Death and Regulating the Activation of Distinct Cell Types. PD-1 (CD-279) is an inhibitory protein made up of 288 amino acids from the superfamily of the immunoglobulins. Initial studies were associated with assays on cell death in the 1990s [27]. Since then, we have learned that PD-1 is a protein whose expression is induced during the apoptosis process or after

administration of apoptotic stimuli. Once synthetized, it is found in the cell membrane. The structure of PD-1 contains ITIM domains and an immunoreceptor tyrosine-based switch motif (ITSM) (Figure 1) [27]. Because PD-1 is a homologous protein to CD28, two ligands belonging to the B7 family were quickly identified: PD-L1 and PD-L2 [28, 29]. The function of PD-1 has been studied widely in T lymphocytes and other lymphoid cells, and it is now well documented that expression of this protein is associated with a dysfunctional state characterized by anergy in the presence of antigenic stimuli, a low rate of proliferation, and reduced cytokine production by PD-1+ cells. This phenotype, in which T lymphocytes express PD-1, is known as the phenotype of "exhausted" lymphocytes, and it has been identified in patients with chronic viral infections and oncological diseases [30, 31]. Blocking with monoclonal antibodies aimed at PD-1 or its ligands, PD-L1, and PD-L2 has been proposed as a therapeutic strategy that could revert the exhausted state of the lymphocytes [32–34].

2.1.1. PD-1 Regulates Cell Activation and the Production of Soluble Inflammatory Mediators. One of the mechanisms regulated by PD-1 in macrophages is IL-12 production, as was demonstrated in a group of patients with chronic HCV infection. That study showed that PD-1 expression increased in monocytes in peripheral blood and that this increase was inversely proportional to the production of IL-12 by those cells when compared to healthy subjects or those whose infections had been completely cured [35]. *In vitro* assays revealed that this reduction in IL-12 production is not secondary to the loss of recognition of the virus by the macrophages but, rather, to alterations in the intracellular signaling pathways that include a decrease in the phosphorylation of the JAK/STAT pathway [35]. Treatment with IFN-α and ribavirin reduced PD-1 expression, reversed the changes in STAT-1 phosphorylation, and increased production of IL-12 by macrophages in patients infected with HCV [35]. Research using ex vivo experimental models has demonstrated that blocking PD-1/PD-L1 with monoclonal antibodies in samples from patients infected with HCV restores IL-12 production in response to LPS [35, 36]. This mechanism is shared by TIM-3, another inhibitory receptor, since this molecule also negatively regulates TLR-mediated cell activation and IL-12 production [37]. Although these molecules do not belong to the same family, several studies have documented that blocking them is an efficient treatment that makes it possible to restore the activation of cells that are incapable of responding to antigenic stimuli. This was observed first in T lymphocytes [30, 31, 38] and, later, in other cells, including monocytes and macrophages [35, 36]. Despite the fact that the PD-1 molecule was discovered in the early 1990s [27], little is known about its functions in myeloid cells. In another hand, recent studies in the oncologic field have shown that PD-1 is expressed by TAMs in mouse models of cancer and primary human cancer. Gordon et al. demonstrate that PD-1 expression increases over time and with disease stages in humans [39]. Furthermore, PD-1 expression negatively correlates with the phagocytic function to eliminate tumor cells. In this way, these results support the therapy with anti-PD-1 or PD-L1 for distinct kind of tumors [40].

While various studies describe that macrophages and other immune cells express PD-L1 and 2 ligands and, through these molecules, can induce the death of PD-1+ cells [41, 42], the function of PD-1+ macrophages must be investigated deeper in another kind of pathologies such as viral, bacterial, and autoimmune diseases.

2.2. TIM-3 Regulates Diverse Functions in Macrophages

2.2.1. Introduction to TIM-3 Signaling and Functions in Macrophages. The TIM-3 protein was initially known as a membrane-specific marker for Th1 and Tc1 lymphocytes [43], but its expression was soon identified in other cell lines. Today, we know that TIM-3 is expressed in monocytes, macrophages, dendritic cells, NK cells, and even diverse cells in different tumor types [31, 44–47]. The extracellular region of TIM-3 consists of a mucin domain and an immunoglobulin domain to which the known ligands of TIM-3 bond, that is, galectin-9 (Gal-9) and phosphatidylserine (Ps) [48, 49]. Glycosylated sites are present in both domains [50] (Figure 1).

Scientific evidence suggests that the interaction of TIM-3 with Gal-9 functions as a negative regulation pathway in cell activation. The absence of this pathway has been associated with the development of autoimmune diseases [40–42]. The way in which signals are transduced into the interior of the cells is via the phosphorylation of tyrosine residues found in the intracellular tail of TIM-3. Currently, we know that there is evidence of the participation of diverse proteins and enzymes in TIM-3 signaling. A model based on the HEK 293 T cell line has shown that TIM-3-mediated signaling begins when the intracellular portion of TIM-3 is phosphorylated by the enzyme interleukin inducible T cell kinase—ITK—from the TEC family of kinases [51]. Phosphorylation by ITK kinase occurs in the tyrosine found at position 265 (Y265) only in the presence of Gal-9 [51]. Another study, this one using the Jurkat, D10, and 293 T cell lines, demonstrated that the Fyn and Lck kinases, which belong to the Src family of kinases, can perform phosphorylation of TIM-3, and further observed that Fyn does so more efficiently [51]. Finally, Lee et al. demonstrated that Bat-3 protein (human leukocyte antigen B-associated transcript 3) is associated with the intracellular portion of TIM-3 in T lymphocytes and that it also recruits a kinase belonging to the Src family (Lck). To date, there are no studies of which kinase participates in TIM-3 phosphorylation when this protein is expressed in monocytes and macrophages. Determining which proteins are involved in the myeloid signaling of TIM-3 could provide scientific knowledge about its function and how it can regulate the different processes identified in this section. Although studies about how TIM-3 function in monocytes and macrophages are scarce, we do know that some mechanisms can be regulated by TIM-3 in these cells in various pathologies, including infectious, autoimmune, and oncological diseases.

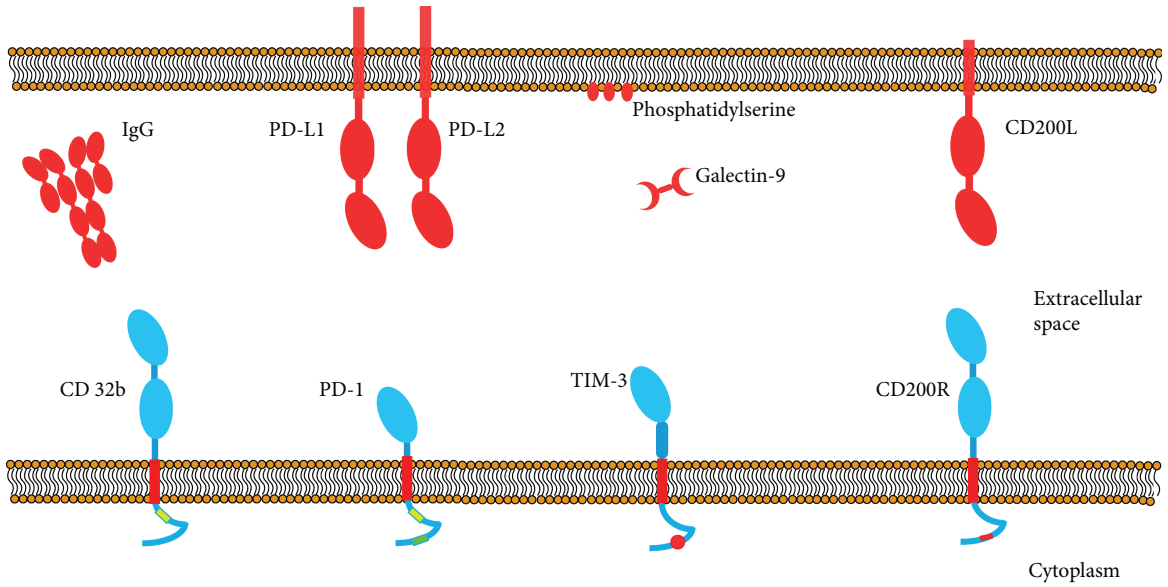

Figure 1: Schematic representation of the structures of inhibitory receptors. All receptors belong to the superfamily of immunoglobulins. They have a single transmembrane portion and an intracellular tail, through which they associate with proteins or effector enzymes. The immunoglobulin domains are represented as blue ovals for receptors and red ovals for ligands.

Besides being a regulator of activation in macrophages, TIM-3 participates in a process through which damaged cells and apoptotic bodies are removed and eliminated from pluricellular organisms. This mechanism is called efferocytosis [52], a term that refers to the mechanism that works to remove and eliminate cells that have culminated their life cycle or have been damaged by some other biological or physical processes. Some members of the TIM family, including TIM-1, TIM-3, and TIM-4, possess a binding site to phosphatidylserine in the Ig domain, a phospholipid that translocates to the external face of the plasmatic membrane of apoptotic cells and constitutes the principal *eat me* signal, which leads to the capture and elimination of those cells [53]. Crystallography studies have allowed us to determine that TIM-3 binds to phosphatidylserine (PS) through the IgV domain [54, 55]. Since PS is the principle signal for the phagocytosis of apoptotic bodies or cells, blocking recognition of this phospholipid with TIM-3 can induce immunological abnormalities, such as generating autoantibodies, since the detritus of apoptotic cells is not eliminated efficiently [55, 56].

2.2.2. TIM-3 Regulates Cell Activation Via TLR. Research has demonstrated that in addition to regulating Ps-dependent mechanisms, TIM-3 can also regulate macrophage activation and, later, cytokine production when it interacts with Gal-9. Gal-9 is a lectin that recognizes carbohydrates contained in the IgV domain of TIM-3. Based on this interaction, studies have identified that TIM-3 negatively regulates activation of diverse cell types through a mechanism that has been widely studied in T cells [30, 31, 57, 58]. In macrophages, however, little progress has been made in analyzing the process of inhibiting TIM-3-mediated activation. For example, we know that the association of TIM-3 with Gal-9 expressed in other cells (trans), or the macrophage itself (cis), has a distinct effect as a regulator of TLR-mediated activation. The *trans*-association of TIM-3 and Gal-9 negatively regulates TLR-mediated signaling reducing IL-12 production, increasing IL-23 production, and reducing phosphorylation of STAT-1, while also augmenting activation of STAT-3. Meanwhile, the association in Cis fosters corrects TLR signaling, and there is also evidence that the expression of Gal-9 increases through this mechanism [48]. This signaling pathway, which impacts the intracellular proteins STAT-1 and STAT-3, also causes alterations in cytokine production, especially IL-12, since it is solidly documented that IL-12 production is induced only after phosphorylation and translocation to the nucleus of the STAT1 factor. While other nuclear factors such as NF-κB and AP-1 exist and are activated through TLR4, phosphorylation of STAT1 has also been shown to occur, such that the interaction of this nuclear factor with TRAF6 could be involved [59, 60] (Figure 2). Additionally, research has identified that upon silencing TIM-3 expression with siRNA, IL-12 and IL-10 production increases in macrophages derived from the THP-1 line [37, 61].

Together, these data indicate that the expression and function of the TIM-3 receptor negatively regulate IL-12 production and, though to a lesser degree, other cytokines such as IL-10 and IL-6. They further suggest the dynamic character of inhibitory mechanisms and the fine balance between activation and inhibition signals. Therefore, regulation of activation is not restricted to lymphocytes and mechanisms of adaptive immunity in general but is also exerted on innate cells and must be the cause of several pathological disorders that would not have been contemplated in past years.

Although we have not identified all the elements that participate in TLR4-dependent activation, the interaction

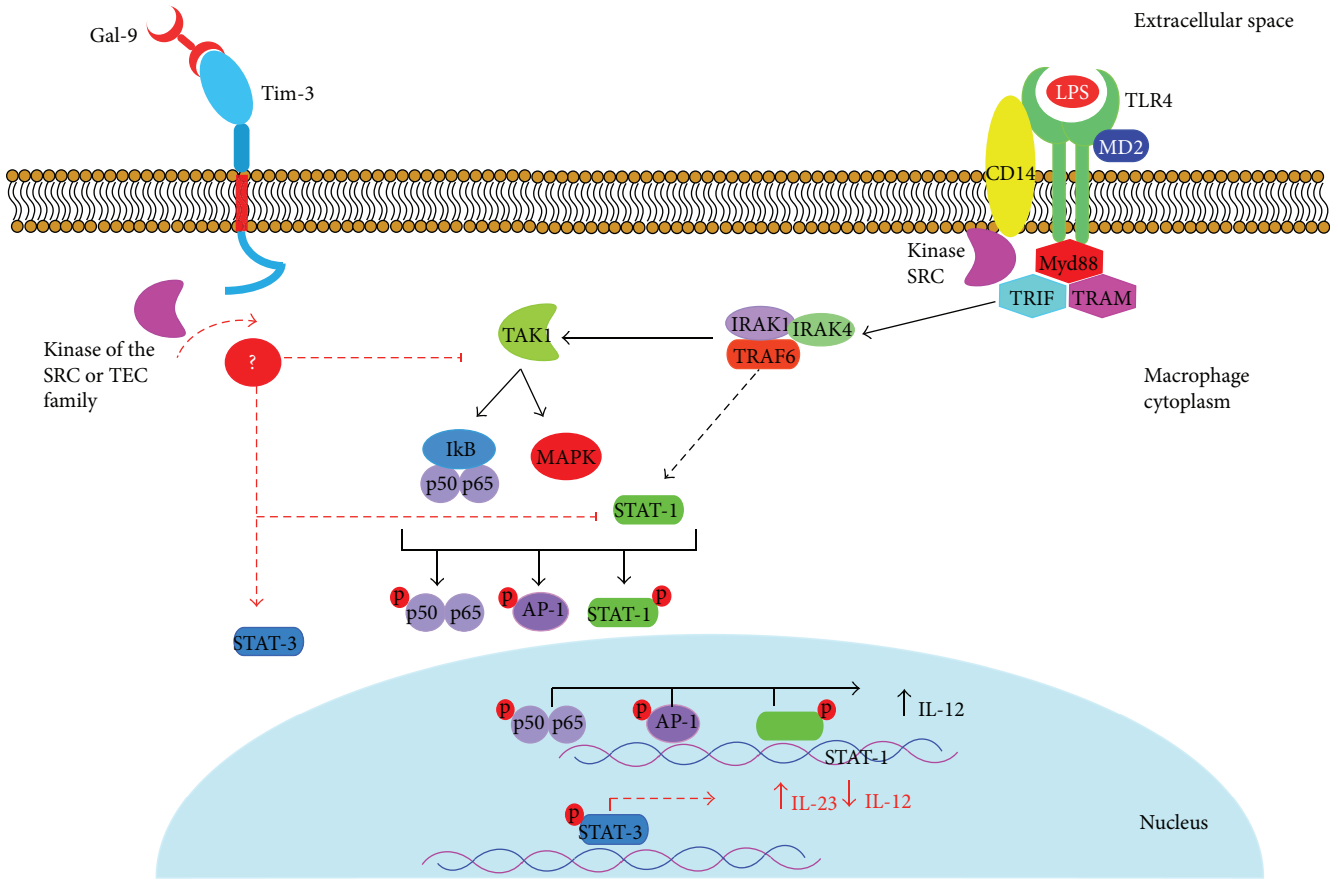

Figure 2: Inhibition mechanism of IL-12 production through interaction of TIM-3 and Gal-9. Graphic representation of the TLR4-signaling pathway (black arrows) when activated by LPS (to simplify, not all proteins involved are shown), which induces phosphorylation of diverse nuclear factors, such as NF-κB (p50-p65), AP-1, and STAT1. These nuclear factors induce production of proinflammatory molecules like IL-12. When TIM-3 interacts with Gal-9, phosphorylation of the intracellular portion of TIM-3 is induced and activates the regulatory pathway mediated by this protein. While we do not yet know the precise inhibition mechanism in macrophages, studies have identified a decrease in STAT1 phosphorylation and an increase in STAT3 phosphorylation (red arrows). The result of this change is reduced IL-12 production and increased IL-23 production.

of TIM-3 with kinases of the Src family must be considered. While several common adapters for different TLR participate in TLR4 activation—such as TRAM, TRIF, and MyD88/MAL—no direct interaction has been found between these elements and any member of the Src family, though interaction between the Src kinase and CD14 has been suggested [62], the latter being an accessory molecule in TLR4 functioning. Also, it has been shown that recognition of LPS by TLR4 is followed by induction of the activation of other members of this family of kinases, including SFK, c-Src, Yes, and Fyn [63]. The fact that the Src family can regulate TLR4-dependent activation as well as TIM-3-mediated inhibition makes analysis more complex and impedes identifying clear signaling pathways for these two processes.

In summary, TIM-3 can simultaneously foster and regulate several important functions in macrophages, including the internalization of apoptotic bodies and activation in response to stimuli captured by TLRs and mechanisms after activation. However, despite the accumulated scientific evidence on the structure and function of TIM-3, it is still not clear how it is that one protein with such distinct functions can act, or exert its effects, on one single cell. In addition to these questions, it seems that the phenomenon of activation/inhibition is being described more often each year. While some reports have documented or proposed possible mechanisms through which TIM-3 fosters the activation of macrophages and lymphocytes, the majority of studies support this protein's role as a negative regulator. This discrepancy may arise from our incomplete understanding of this protein's function and signaling pathway. Given that TIM-3 does not have a conventional ITIM domain, its function does not depend on an association with phosphatases. Hence, the mechanism through which TIM-3 negatively regulates activation pathways in macrophages and T cells must correspond, or perhaps be similar, to that of other inhibitor receptors, like CD200R, which do not require an association with phosphatases to inhibit cell activation. Discovering the precise mechanism that allows TIM-3 to regulate cell activation will propitiate a much better understanding of

autoimmune pathologies in whose development the loss of tolerance to autoantigens plays a crucial role. To different degrees, understanding the fine balance between activation and inhibition signals will allow us to develop new therapeutic strategies in diverse areas of immunology.

2.3. Fc-Gamma Receptor (CD32b), a Negative Regulator of Macrophage Activation. The family of immunoglobulins includes a heterogeneous group of receptors that can bind to crystallizable regions of immunoglobulins (FcR). The IgG receptors (IgGR) are the ones most widely expressed and may be present in the membrane of most immune cells. According to the functions they perform and the structural motifs they present, these receptors can be classified as activators—FcγRI, FcγRIIa, and FcγRIII—or inhibitors: FcγRIIb. FcR activators can trigger diverse events in cells that gain expression as increased intracellular Ca^{2+}. For these events to occur, the FcγRI, FcγRIIa, and FcγRIII receptors must be associated with accessory chains that possess an ITAM motif and are found in the intracellular tail of receptors, as well as in accessory chains, such as the common gamma chain. Research demonstrates that, like other receptors that have no extensive intracellular tail, FcγRI and FcγRIII receptors lose sequences that allow them to associate with proteins or enzymes of the cytosol and so become incapable of mediating signaling on their own once they bond to their ligand. Studies have also shown that the receptors for the Fc region with no extensive intracellular tail can associate with the common gamma chain [64] and, in this way, acquire the capacity to send signals to the interior of the cell. The FcγRIIa and FcγRIIb receptors, in contrast, have an intracellular tail with domains that allow them to initiate signaling directly once binding to their ligand occurs, with no need to form an association with accessory molecules. The intracellular tails of these two receptors show substantial changes, and alternative splicing generates more changes in the terminal amino and carboxyl regions of FcγRIIb [65]. The main differences between the intracellular portions of these molecules are their respective tyrosine-based activation and inhibition motifs.

In the case of the FcγRIIb inhibitory receptor, the motif is an off the ITIM type [66] (Figure 1). Although initially the capacity of the FcγRIIb receptor to modulate activation in a model of B cells was identified [17], we now know that this receptor represents a regulation point for activation that is found in many immune system cells. The mechanism by which the FcγRIIb receptor performs these functions involves enzymes that eliminate phosphate groups from ITAM domains [67]. The phosphatases usually present in the cytoplasm are SHP (Src homology 2-containing tyrosine phosphatase) and SHIP (Src homology 2-containing inositol phosphatase), which are recruited and associated with the ITIM domain in the FcγRIIb receptor through the Sh2 domain (Src homology 2) found in these phosphatases [18] (Figure 3). While the FCγRIIb receptor can bond to the enzymes SHP and SHIP, the function of the association with SHIP has been studied more widely in macrophages. The phosphatase SHIP can inhibit signals of cell activation by dephosphorylating phosphatidylinositol triphosphate (PIP3) [68]. In this way, it prevents PIP3-mediated signaling events, such as the translocation of kinases of the Tec family, like Akt and Btk, which are required to activate phospholipase C (PLC) [69, 70].

2.4. CD200R, a Receptor That Inhibits Activation of Macrophages and Propitiates the Survival of Intracellular Pathogens

2.4.1. Structure and Activation Mechanism. CD200R is a glycoprotein belonging to the superfamily of the immunoglobulins that are expressed on the surface of myeloid cells, principally in the subpopulation of regulator macrophages with the M2a phenotypical profile, which can be used as a specific marker [71, 72]. The extracellular portion of CD200R consists of two domains of immunoglobulin type (IgV and IgC2) [73]. This receptor contains a single transmembrane portion, which means that it can only cross the cell membrane through this region [73] (Figure 1). Unlike other inhibitory receptors, CD200R does not contain an ITIM domain in its intracytoplasmic tail; instead, studies have identified three tyrosine residues that are important for the function of CD200R (Y291, Y294, and Y302 in humans and Y286, Y289, and Y297 in mice). It has been shown that the interaction of CD200R with its ligand, CD200, in macrophages induces phosphorylation of tyrosine residues present in the intracellular portion of CD200R, a phosphorylation mediated by kinases of the Src family. The phosphorylation of these residues results in inducing recruitment of the adaptor protein Dok2 through its binding domain to the phosphorylated tyrosine (PTB), which then initiates the cascade of inhibitory signaling [74]. Once Dok2 has bound to the intracellular portion of CD200R, it can recruit other proteins, such as the activator protein of Ras-GTPase (RasGAP) [74]. In human macrophages, studies have shown that the recruitment and ensuing activation of RasGAP are essential for inhibiting the signaling pathway of the Ras-ERK and PI3K kinases [74] which, in turn, are essential for diverse, vital processes, including cell growth, differentiation, proliferation, and metabolism by activation of other transcriptional factor such as STAT-1 which also is involved in macrophage activation by IFN-γ [75]. This mechanism that regulates or inhibits activation in myeloid cells is clearly distinct from that of the receptors that contain ITIM motifs, which recruit phosphatases and are the principle effectors of the inhibition mediated by this type of receptor [76] (Figure 4).

Several studies have demonstrated that the interaction of CD200 with CD200R negatively regulates the activation of myeloid cells. This regulation may be caused by posttranslational modifications, such as tyrosine phosphorylation in the CD200R cytoplasmic tail, as well as increased expression of the receptor itself and its ligand CD200 in endothelial, epithelial and lymphoid cells, fibroblasts, and astrocytes [77, 78]. Also, it has been demonstrated that the expression of CD200 is induced by several nuclear factors such as NF-κB(p65), STAT1a, and IRF-1 when they bind to their corresponding *cis*-elements which are found in the CD200 promoter region. Furthermore, one of these factors

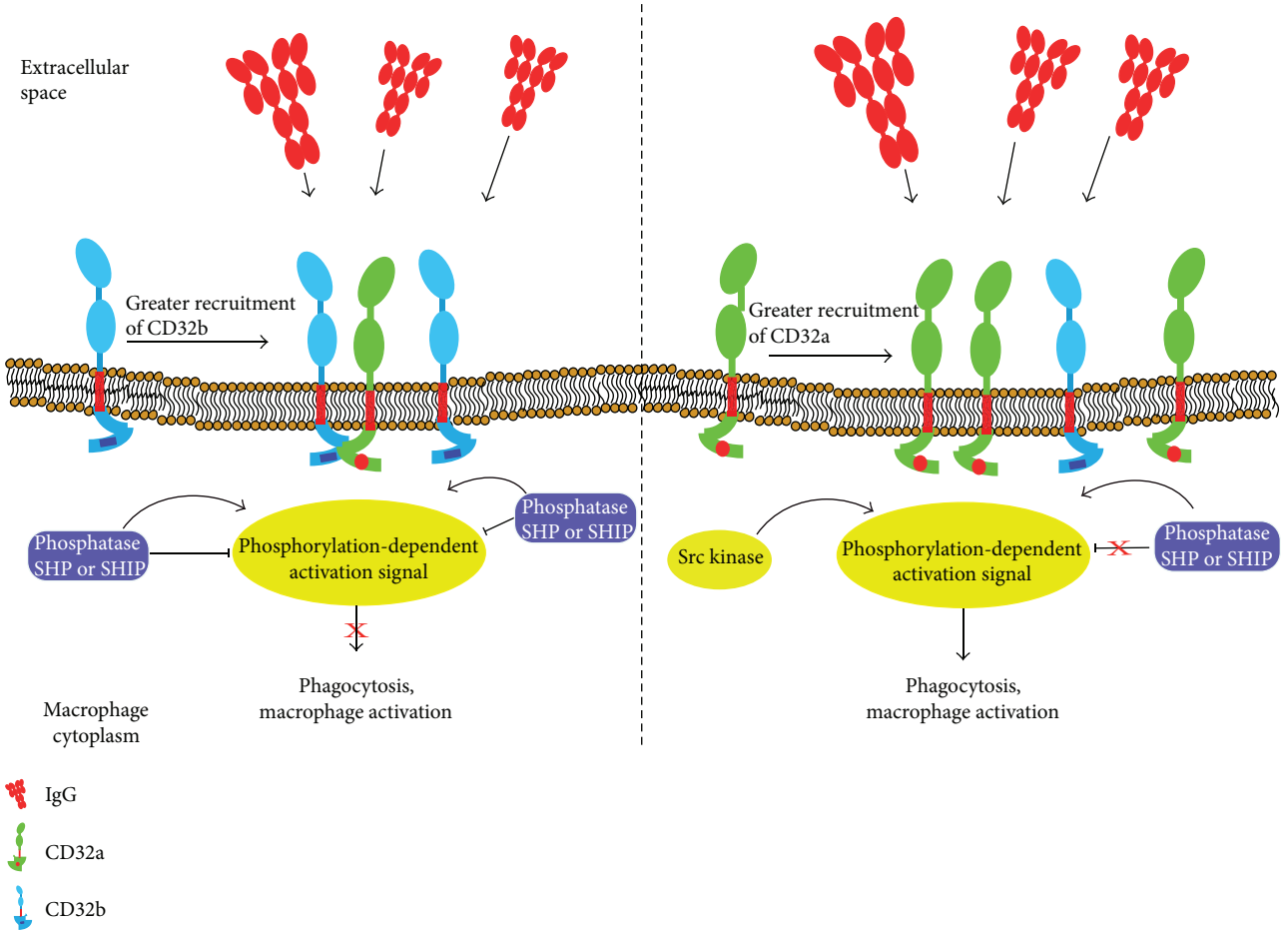

Figure 3: Inhibition mechanism of the FcγR receptor (CD32). (a) When CD32b recognizes the Fc fraction of IgG, the phosphatase SHP or SHIP is recruited into the ITIM domains of the intracellular portion of the receptor. Those enzymes inhibit phosphorylation of the ITAM domains or eliminate phosphorylation of the ITAM domains contained in CD32a. This mechanism inhibits the activation of macrophages and the phagocytosis of opsonized pathogens and other elements that are susceptible to recognition by IgG-class antibodies. (b) When the association of CD32a is greater than the frequency of CD32b receptors, phosphatase recruitment is not fostered and, therefore, no inhibition of the activation signals mediated by phosphorylation of the ITAM domains in the intracellular portion of CD32b is produced. As a result, the corresponding internalization mechanisms are activated.

is c-Rel, the NF-κB transcription factor, which is required for TLR-induced upregulation of CD200, probably induced by pathogens [77, 79]. Recently, it was determined that CD200R1 expression is regulated by C/EBPb and that overexpression of this transcription factor due to stimulation of microglia cells by LPS significantly reduces the expression of this receptor at the protein and mRNA level, but in the case of C/EBPb KO cells, this decrease was not seen [80]. The authors of that study suggested that the mechanism which inhibited CD200R expression was histone deacetylation since C/EBPb interacts with the histone deacetylase HDAC1 which, in turn, can bind directly to the CD200R promoter to inhibit transcription of the gene [80].

It has been reported that CD200R modulates the activation of cells in the microglia under conditions of acute, chronic inflammation by interacting with its ligand CD200 [81]. At the same time, CD200R expression is modulated by IL-4 [81]. An *in vivo* study with mice demonstrated that upon decreasing CD200R expression, activation of the cells in the microglia increases. CD200R expression decreased in IL-4KO mice, while stimulation with this cytokine increased expression of the receptor. These results are manifested in the macrophages of WT and IL-4 KO mice stimulated with LPS, as the latter showed higher production of the proinflammatory cytokines IL-1, IL-6, and TNF-α, and of the expression of CD40, while CD200R expression was null [82].

2.4.2. CD200 and Its Participation in Pathogenic Infections

(1) Parasites. In infections caused by intracellular parasites, cells increase their expression of CD200R and its ligand, but this has deleterious effects on the inflammatory response. For example, in wild-type mice infected with *Toxoplasma gondii*, research has documented the overexpression of CD200R in cells in the microglia and of CD200 in endothelial cells. This increase in CD200R expression has been associated with reduced cell activation and the production of molecules that are important in the immune response, such as TNF-α, iNOS, and MHC-II [83]. In contrast, in CD200 KO mice, it has been documented

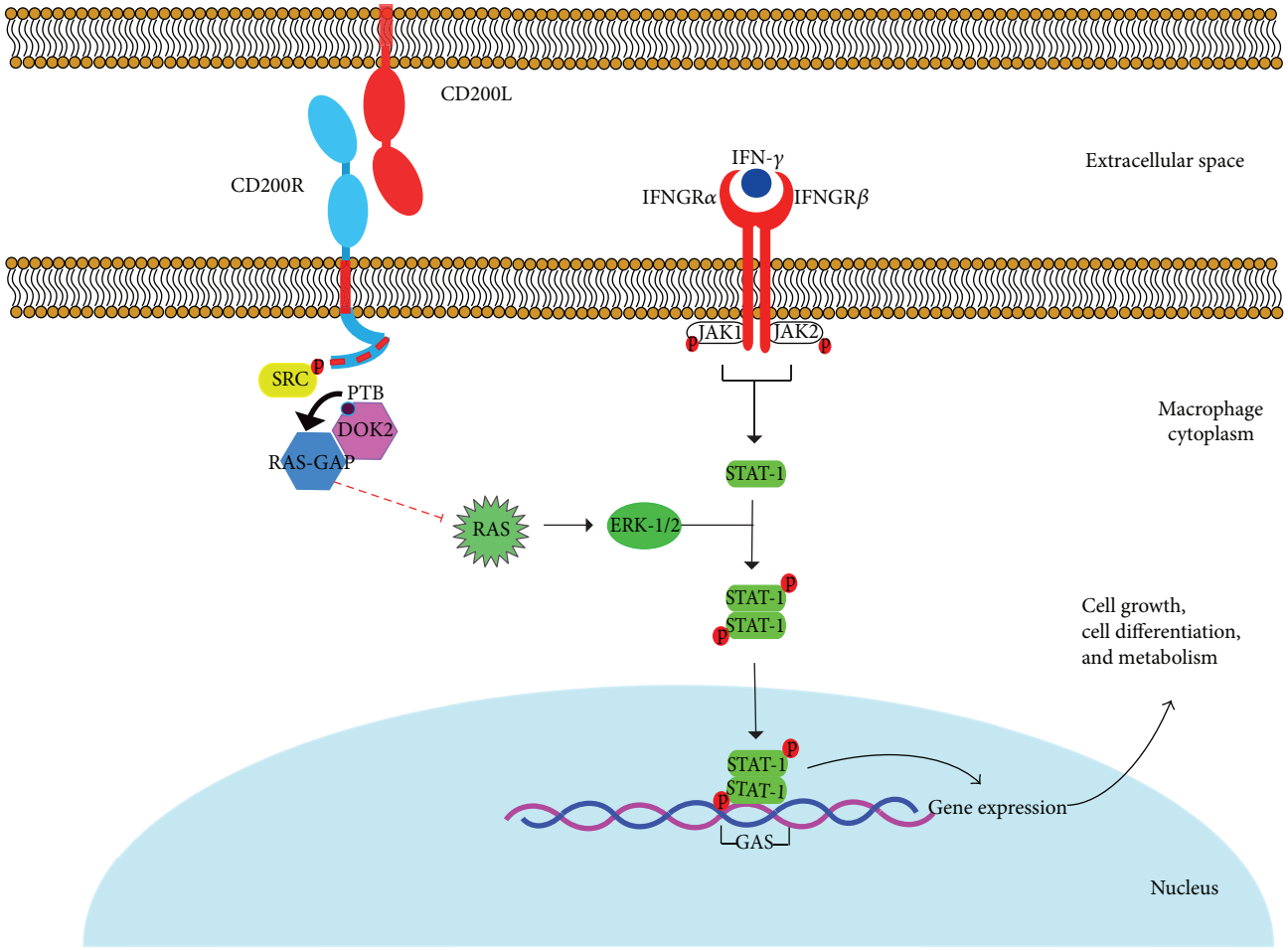

Figure 4: CD200R-induced signaling pathway. Interaction of CD200R with its ligand induces phosphorylation of the tyrosine residues (red squares) present in the intracellular portion of the receptor mediated by kinases of the Src family. This mechanism propitiates recruitment of Dok2 through its binding domain to tyrosine (PTB). Dok2 binds to the phosphorylated tyrosines, and it recruits the activator protein of Ras-GTPase (RasGAP), which inhibits Ras and downstream ERK activation. ERK is a MAP kinase which is involved in macrophages activation by IFN-γ. JAK/STAT-1 signaling pathway is required for cell activation, and STAT-1 is phosphorylated by JAK proteins and ERK-1/2 in turn to allow the gene expression for cell differentiation, growth and metabolism, so then, if CD200R binds to its ligand CD200 it allows the inhibition of cell activation [74, 95].

that the cells of the microglia increase their proliferation and activation rates, as well as the expression of MHC-II, TNF-α, and iNOS during chronic *Toxoplasma gondii* infection [78]. There is also evidence of a reduction in the parasitic load and mortality compared to WT mice that might be explained by the fact that the CD200 KO mice exhibit an increased inflammatory phenotype in response to ligands of TLRs, as shown by the increase in the production of TNF-α and IL-6, and the activation of the NF-κB pathway [78].

A mouse model of parasitic infection of bone marrow macrophages with *Leishmania amazonensis* (*L. amazonensis*) has also made it possible to evaluate the role of CD200/CD200R. It has been identified that infections by this parasite induce increased expression of CD200 at the protein and mRNA level compared to uninfected macrophages [84]. It is not clear yet, but this increase in CD200 expression favors survival of the pathogen, perhaps because CD200 affects normal Th1 lymphocyte development which is essential for immune response against Leishmania; in fact, IFN-γ-activated macrophages produce NO to kill intracellular Leishmania major, but if CD200 is increased, it can avoid this immune response which can lead to the development of systemic Leishmaniasis [84]. Infected macrophages treated with iNOS inhibitors also foster replication of the pathogen and increase CD200 expression, suggesting, at least concerning *L. amazonensis* infections, that the infected macrophages can inhibit activation of neighboring macrophages by expressing both CD200 and CD200R.

In addition to the aforementioned mechanisms in which CD200/CD200R can regulate cell activation and the response to intracellular pathogens, another mechanism has been described through which infected macrophages can regulate the activation of other cells without any direct interaction. This is the release of exosomes and small vesicles (30–150 nm) that contain membrane proteins and provide signals to naive macrophages. This type of regulation was described recently; [85] hence, it is likely that the exosomes contain the CD200 protein and that, once secreted, the CD200 on their

surface could bond to its receptor in neighboring macrophages with no need for cell-cell interaction.

(2) Bacteria. Aside from parasites, another pathogen that induces CD200 expression in macrophages in bone marrow is *Neisseria meningitidis*, through recognition of LPS by TLR4. A study in 2010 demonstrated that CD200 : CD200R interaction in WT mice negatively regulates the immune response against *N. meningitidis*. It has been documented that CD200 and CD200R expression is modified differentially when administered to macrophages of mice TLR agonists and for the inflammasome activators NOD2 and NALP3. That work showed that induction of CD200 expression is dependent on the c-Rel transcription factor and is negatively regulated when there is activation of the pathways activated through TLR and NOD2 [86]. This response turned out to be dependent on MyD88, but independent of the scavenger receptor A (SRA), which is considered one of the principle receptors that permits the interaction of macrophages with *Neisseria meningitidis* [87]. Additionally, agonists of pattern-recognizing receptors, such as NALP3, also induced CD200 expression, with c-Rel a member of transcription factor NF-κB—being essential in this signaling pathway. In contrast, an increase in the mortality of CD200 KO mice was observed in response to experimental meningococcal septicemia, due to high levels of proinflammatory cytokines and activated leucocytes [86]. Infection by *Chlamydia trachomatis* also increased CD200R expression in macrophages of the endometrium and CD200 expression in epithelial cells from the genital tract by regulating inflammation and suppressing collateral damage in the tissue during chronic infection, thus enabling persistence [88]. These mechanisms are consistent with the absence of changes in the vaginal epithelium, which complicates clinical studies of infected patients. It has also been reported that infection with *Chlamydia* increases the percentage of macrophages in the endometrium that coexpress CD200R and CD206 (mannose receptor), both of which are markers of alternately activated macrophages (M2) [88].

(3) Virus. Models of virus infections have also documented the importance of the CD200/CD200R pathway and its role as a regulator of the immune response. For example, it has been shown that CD200 KO mice infected with the influenza A virus manifest greater weight loss and higher mortality rates than native mice. These effects may cause the increase in NO levels in the lung and of the cytokines IL-6, TNF-α, IFN-γ, and MIP-1α measured in bronchoalveolar lavage [89]. In contrast, administering the anti-CD200R agonist (CD200-Fc) partially reverses the phenotype of CD200 KO mice by reducing weight loss and the number of cells compared to KO mice not treated with this agonist [90]. In this case, the function of CD200/CD200R interaction is to protect the host from "the cytokine storm" produced in response to the infection, which is the principal cause of the deleterious effects seen in CD200 KO mice.

In a 2011 study, Goulding et al. used an *in vivo* mouse model to show that the absence of the CD200R receptor in macrophages initially infected with the influenza virus and later with *Streptococcus pneumoniae* reduced the bacterial load and prevented mortality in WT mice [91]. That study suggested that the absence of CD200R is beneficial for the host because the exacerbated inflammation that occurred in the WT mice contributed to disease pathogenesis and increased the viral load [91]. In this sense, the inhibition or attenuation of cell activation by blocking the CD200R receptor would provide an advantage for treating secondary infections by counteracting the exacerbated inflammatory responses that might occur in such infections.

In another approach, Soberman et al. used a mouse model of encephalitis by the herpes simple virus (HSV-1) infection to demonstrate that increased morbidity and mortality that had been seen previously were related with the release of cytokines and chemokines that occurred once virus was recognized by TLR2. In contrast, in CD200 KO mice, observations showed a reduced inflammatory response against HSV-1, since the production of cytokines, such as IFN-γ, IL-6, and CCL5 (RANTES) decreased by 80% compared to WT mice [92]. However, the WT and CD200R KO mice did not differ in terms of IL-1β production, and in both types, the study detected adequate activation of inflammasome after stimulation with LPS and ATP [92]. This suggests that the pathway mediated by the CD200R receptor in mouse macrophages does not interfere with the activation of inflammasome and does not affect the production and release of IL-1β. Hence, we can consider that the CD200R receptor can regulate the activation mediated by membrane receptors for PAMPs, such as TLR4, but does not affect receptors of the NOD family. An additional way in which virus utilize the CD200/CD200R signaling pathway to counteract the host's inflammatory response is by incorporating into its genome proteins that are orthologues of the CD200 in the target cell. One of the most characteristic genes that codifies these orthologue proteins is the K14 gene of herpes 8 virus (HHV8), better known as the Kaposi sarcoma-associated herpes virus, which codifies a viral orthologue of CD200 (vOX2), whose expression on the surface of infected cells occurs during the lytic phase. Although vOX2 shares 36–40% of the identity of CD200 in humans, it bonds with similar affinity to its ligand and negatively regulates the release of TNF-α, G-CSF, and MCP-1 from macrophages activated with IFN-γ and LPS [93]. In addition to this protein, there is another viral orthologue of CD200: the R15 protein of the adenovirus that infects rhesus monkeys (RRV) and is a gamma herpes virus similar to HHV8. This protein is expressed on the surface of infected cells and is released into the supernatant of cell cultures. R15 reduces mRNA expression and the release of TNF-α from THP-1 macrophages activated with PMA, as well as the primary monocytes and macrophages of rhesus monkeys. These levels of inhibition were similar to those caused by CD200 in humans [94]. Therefore, these proteins of viral origin provide new therapeutic targets for which we can design and synthesize compounds to be directed against viral products of this kind to eliminate, or counteract, infection or viral propagation.

It is clear that diverse pathogens utilize, for their benefit, the signaling pathway realized by CD200/CD200R interaction to limit the inflammatory response and so survive inside the host cell. However, few studies have focused on

identifying the molecules that are associated with this signaling. Thus, understanding how infectious agents use this pathway to regulate the host's defenses may lead to the development of new clinical tools and therapeutic strategies. Depending on the context of the infection or pathology, a blocking antibody, for example, directed against CD200R to block its interaction with CD200, could be effective in counteracting infections by pathogens whose survival is favored by, or associated with, overexpression of CD200R, complemented by conventional antimicrobial treatment. In contrast, using agonists to activate the CD200/CD200R signaling pathway, or adjacent cells, may also contribute to inducing the effector immune response.

3. Conclusions

The nature of the innate mechanisms of the immune response has become increasingly complex. Immunologists used to speak of how these mechanisms, made up of cells and receptors with some soluble elements, functioned through nonspecific recognition, in contrast to adaptive immunity mechanisms. Today, we know that all proteins that participate in pathogen recognizing perform this task, in a pathogen-associated molecular patterns recognition dependent way. This recognition of patterns indicates that nonspecific recognition by the innate immune response does not exist but we consider it as nonspecific because the variety of antigens recognized is limited when compared to adaptive mechanisms. Something similar occurs with the regulation of activation. While we do not yet know exactly how many copies of an antigen must enter an organism to trigger activation of specific lymphocytes, we do know that the activation signals generated by the presence of these antigens must cross a threshold—or limit—in order to emit activation signals that are sufficiently intense to trigger a successful activation process. Also, the intensity of the activation signal must exeed the signal given by the inhibitory receptors present in the cells of immune system that, as mentioned earlier, participate in diverse signaling mechanisms and pathways whose purpose is to prevent an exacerbated, or even unnecessary, activation process.

This text has focused on four receptors that are known to have opposite functions to the receptors that mediate cell activation. TIM-3, PD-1, CD-32b, and CD200R form a group of receptors that use diverse mechanisms to inhibit cell activation, such as association with phosphatases or steric impedance in the plasmatic membrane. However, there are other receptors like SIRP-α/CD47, which have similar role and function recruiting phosphatases as CD32b. There is reliable evidence that the balance between activation and inhibition signals also occurs in cells with innate functions, such as monocytes and macrophages, suggesting that the function and activation of these cells are a highly regulated process in which it has often been demonstrated that the loss of regulation can generate such deleterious processes as exacerbated inflammation or the loss of tolerance to autoantigens. Several authors refer to these regulating mechanisms as response elements that are capable of generating immunological tolerance, although others consider that we are dealing with mechanisms that inhibit activation which, when functioning at a high level, cause a dysfunctional state in immune cells. In reality, we should consider that no such frontier exists between these two descriptions but that we are trying to define the extremes of a concept that consists of a broad range of levels of regulation, the lowest of which is inhibition. The level associated with exacerbated responses and the opposite level of regulation would be the one that, as an overinduced mechanism, causes lack of cell response or cell senescence upon recognizing an antigen. Given all the possible immunological scenarios that can emerge from the presence or absence of inhibitory receptors and their functions, it is extremely important to continue studying these proteins in both lymphoid and myeloid cells. Obtaining a thorough understanding of the function of these receptors may provide new ways of dealing, clinically, with diverse pathologies and of increasing the effectiveness of current treatments.

Abbreviations

Balb/c: Strain of laboratory-raised albino mice
BAT-3: Lymphocyte-associated transcript 3
BCR: B cell receptors
C/EBPb: Bonding beta protein to potentiator/CCAAT
CD: Differentiation group
ERK: Kinases regulated by extracellular signaling
Fc: Crystallizable region of immunoglobulin
FcR: Receptor of the crystallizable region of immunoglobulin
Gal-9: Galectin-9
GAS: Activation site of IFN-γ
G-CSF: Stimulating factor of granulocytic colonies
HBA: Hepatitis B virus
HCV: Hepatitis C virus
HDAC1: Deacetylase 1
HHV8: Human herpes 8 virus
HSV-1: Herpes simple 1 virus
IFN: Interferon gamma
IgC: Constant region of immunoglobulin
IgG: Immunoglobulin G
IgGR: Immunoglobulin G receptor
IgV: Variable region of immunoglobulin
IL: Interleukin
iNOS: Nitrous oxide inducible synthetase
ITAM: Tyrosine-associated activation motifs
ITIM: Tyrosine-associated inhibition motifs
ITK: Tyrosine kinase inhibitor
ITSM: Tyrosine-associated change motifs
JAK: Janus kinase
KO: Knockout
Lck: Leucocyte-specific tyrosine kinase protein
LPS: Lipopolysaccharide
M2a: Regulator macrophages
MAL: MyD88-type adapter
MHC: Principle histocompatibility compound
MIP-1α: Alpha 1 macrophage inducible protein
mRNA: Messenger ribonucleic acid
MyD88: Response gene 88 to myeloid differentiation
NF-κB: Nuclear factor kappa B

NK: Killer cell
NLRP3: NOD-associated protein
NO: Nitrous oxide
NOD2: Nucleotide 2 oligomerization domain
PD-1: Cell death protein
PDL1: PD-1 ligand
PI3K: Phosphatidyl inositol kinase
PIP3: Phosphatidyl inositol triphosphate
PLC: Phospholipase C
PMA: Myristyl phosphate acetate
Ps: Phosphatidylserine
PTB: Bonding domain to phosphorylated tyrosine
RasGAP: Activator protein of Ras-GTPase
RRV: Virus of the rhesus monkey, family *adenoviridae*
SHIP: Phosphatase 1 of inositol containing the SH2 domain
SHP: Tyrosine phosphatase containing the residue of homology 2 of Src proteins
siRNA: Interference ribonucleic acid
SRA: Scavenger receptor A
STAT: Signal transducer and activator of transcription 3
Tc1: Cytotoxic T lymphocytes
TCR: T cell receptor
Th1: T lymphocyte cooperator 1
THP-1: Human monocyte cell line from acute monocytic leukemia
TIM-3: Protein of family 3 of T lymphocytes containing mucin- and immunoglobulin-type domains
TIR: Homologous region to IL-1R/Toll
TLR: Toll-type receptor
TNF-α: Alpha tumor necrosis factor
TRAM: TRIF-related adaptor molecule
TRIFF: Adaptor with TIR domain, beta interferon inducer
vOX2: Viral orthologue of CD200
WT: Wild-type.

Conflicts of Interest

The authors declare that they have no conflict of interest.

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Hindawi
Journal of Immunology Research
Volume 2018, Article ID 5201759, 9 pages
https://doi.org/10.1155/2018/5201759

Review Article

The Role of Phospholipase C Signaling in Macrophage-Mediated Inflammatory Response

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Received 24 June 2017; Revised 6 October 2017; Accepted 5 November 2017; Published 8 February 2018

Academic Editor: Zissis Chroneos

Macrophages are crucial members of the mononuclear phagocyte system essential to protect the host from invading pathogens and are central to the inflammatory response with their ability to acquire specialized phenotypes of inflammatory (M1) and anti-inflammatory (M2) and to produce a pool of inflammatory mediators. Equipped with a broad range of receptors, such as Toll-like receptor 4 (TLR4), CD14, and Fc gamma receptors (FcγRs), macrophages can efficiently recognize and phagocytize invading pathogens and secrete cytokines by triggering various secondary signaling pathways. Phospholipase C (PLC) is a family of enzymes that hydrolyze phospholipids, the most significant of which is phosphatidylinositol 4,5-bisphosphate [PI(4,5)P2]. Cleavage at the internal phosphate ester generates two second messengers, inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG), both of which mediate in diverse cellular functions including the inflammatory response. Recent studies have shown that some PLC isoforms are involved in multiple stages in TLR4-, CD14-, and FcγRs-mediated activation of nuclear factor kappa B (NF-κB), mitogen-activated protein kinase (MAPK), and interferon regulatory factors (IRFs), all of which are associated with the regulation of the inflammatory response. Therefore, secondary signaling by PLC is implicated in the pathogenesis of numerous inflammatory diseases. This review provides an overview of our current knowledge on how PLC signaling regulates the macrophage-mediated inflammatory response.

1. Introduction

Inflammation is part of the complex biological response of body tissues to harmful stimuli, such as pathogens, damaged cells, or to molecular "irritants," and is a protective response involving both cellular and molecular mediators [1, 2]. Initially, both pro and anti-inflammatory signals with opposing effects are tightly regulated in a balanced status [3]. However, a disruption of this balance can result in an excessive inflammatory response resulting in cellular and tissue damage [4–6]. From extensive study, it has long been recognized that macrophages play a critical role in the initiation, maintenance, and resolution of inflammation.

Together with dendritic cells (DCs) and monocytes, macrophages are major components of the mononuclear phagocyte system. Macrophages participate in all phases of the immune and inflammatory responses [7]. Unstimulated macrophages are typically quiescent; however, stimulation of these cells by local micromilieu signals, however, results in their acquiring a polarized phenotype [8] either proinflammatory M1 macrophages or anti-inflammatory M2 macrophages. M1 macrophages, generally induced by LPS and IFNγ, generate high levels of proinflammatory cytokines [e.g., interleukin 1β (IL-1β), interleukin 6 (IL-6), interleukin 12 (IL-12), and tumor necrosis factor (TNF-α)] and oxidative metabolites [e.g., nitric oxide (NO) and ROS]; M2 macrophages stimulated by a variety of stimuli (e.g., IL-4/IL-13 and glucocorticoids) are important in the resolution of inflammation [9, 10]. Macrophages express a repertoire of pattern recognition receptors (PRRs) such as Toll-like

receptors (TLRs), CD14, nucleotide-binding oligomerization domain-like (Nod-like) receptors, and RIG-I-like receptors [11–15]. This sensor array enables them to recognize a diverse range of ligands and to initiate quickly appropriate responses, such as phagocytosis, and immunomodulation through production of various cytokines [3, 14, 16]. Macrophages have elaborate strategies for the regulation of the inflammatory response.

Stimuli, such as lipopolysaccharide (LPS) and cytokines, activate macrophages by ligation of corresponding receptors, such as Toll-like receptors (TLRs) [14]. Upon activation, a variety of intracellular signals are triggered to promote the production of proinflammation cytokines [e.g., IL-1β, IL-6, and TNF-α], chemokine [e.g., macrophage inflammatory factor (MIP-1α) and IL-8], and toxic molecules (e.g., NO and ROS) [17, 18]. The "cytokine storm" characterized by the hyperinduction of proinflammatory cytokines and chemokines is a pathogenic mechanism resulting in some pathogens causing tissue injury and multiorgan dysfunction [19–21]. For example, the lethal lung inflammation due to infection by influenza virus (e.g., 1918 H1N1 and H5N1) and porcine reproductive and respiratory syndrome virus (PRRSV) is mainly caused by cytokine storms induced by these viral infections [20, 22–24]. Macrophages are the major source of proinflammatory mediators [25–27] and are therefore implicated in the pathogenesis of numerous inflammatory diseases.

Members of the phospholipase C (PLC) family are thus involved in intracellular and intercellular signal transduction. Accumulated evidence has demonstrated that the PLC signaling inhibitor U73122 attenuates both acute and chronic inflammation mediated by macrophages both in vivo and in vitro [28–30], linking PLC signaling to macrophage-mediated inflammation. The involvement of PLCβ, γ, and δ in macrophage-mediated inflammation has been extensively studied, and herein the corresponding mechanisms are summarized and discussed.

2. The Spectrum of Expression of PLC Isoenzymes in Macrophages

PLC family enzymes are activated by numerous factors such as neurotransmitters, growth factors, histamine, and hormones, as reviewed by Nakamura and Fukami [31]. PI(4,5)P2 is the preferred substrate of PLC. Hydrolysis of PI(4,5)P2 leads to the generation of IP3 into the cytoplasm and DAG in the membrane. IP3 triggers the release of Ca^{2+} from intracellular stores, and DAG mediates the activation of protein kinase C (PKC). The activation of PKC and calcium signaling in turn activate downstream signaling [31, 32]. Concomitantly, PI(4,5)P2 also directly regulates a variety of cellular functions, including phagocytosis [33].

Protein kinase C (PKC) is a family of protein serine/threonine kinases that are involved in the phosphorylation of serine and threonine amino acid residues on other proteins, or other members of this family [34]. The PKC isoforms are divided into 3 subfamilies based on their activation requirements: classical PKCs (calcium dependent) (PKCα, βI, βII, and γ), novel PKCs (calcium independent) (PKCδ, ε, η, and θ), and atypical PKCs (PKC-ζ and λ/ι) [35, 36]. According to the literature, eight PKC isoforms (PKCα, βI, βII, δ, ε, η, ζ, and λ) are expressed in macrophages [37]. Though macrophages do not express detectable PKCθ, its expression is upregulated in response to LPS/IFNγ stimulation [38], suggesting that PKCθ expression in macrophages is inducible by certain inflammatory stimuli. It has been known that PKC inhibitors reduce LPS-stimulated cytokine secretion by macrophages, linking PKC activation to TLR4 signaling. It has been further evidenced that PKCα, δ, ε, and ζ are directly involved in multiple steps in TLR4 pathways, as well as in the downstream activation of inflammation pertinent signaling, such as MAPK and NF-κB [36, 39, 40]. PKCθ and PKCε also activate NF-κB-dependent pathways in muscle cells to promote expression of proinflammatory cytokines and chemokine [41]. PKCε regulates NF-κB-mediated NO production by macrophages in response to LPS stimulation [42]. Classical PKCs are critical components that control IRF-3-dependent gene expression downstream of TLR3 and TLR4 [43]. The role of PKC isoforms in TLR-dependent signaling transduction has been summarized in Figure 1. In view of the diversity of the PKC family and that PKC signaling is regulated by PLC enzymes, this further emphasizes the importance of PLC signaling in macrophage-mediated inflammation.

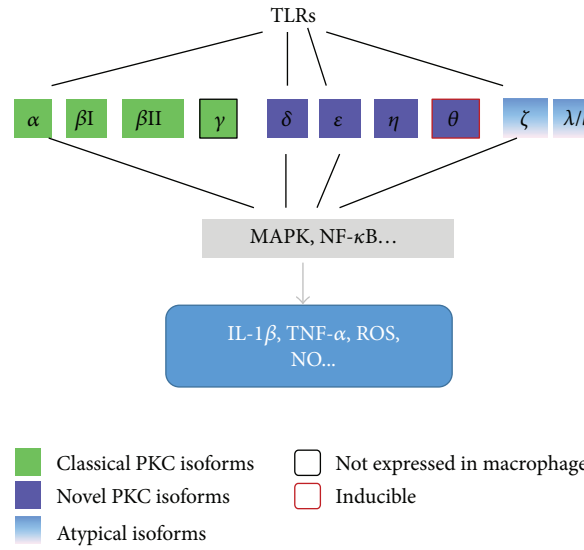

Figure 1: The expression of PKC isoforms in macrophages and their role in TLR-mediated inflammatory response. Among them eight, PKC isoforms (PKCα, βI, βII, δ, ε, η, ζ, and λ) are expressed in macrophages. PKCα, δ, ε, and ζ are directly related to TLR-induced inflammatory response. PKCθ expression in macrophages cannot be detected, but its expression can be induced by LPS/IFNγ stimulation.

Currently, there are a total of 6 classes of PLC isoenzymes discovered in mammals including the PLCβ, γ, δ, ε, η, and ζ. Each class of PLC is composed of many isotypes with distinct functions, domains, and regulatory mechanisms [44]. Based on the structure, they are further subdivided into 13 isoforms including PLCβ1–4, γ1-2, δ1, δ3-4, ε, ζ, and η1–2 [31]. The structures of these PLC isoforms show conserved domains

such as the X and Y domains that are responsible for catalytic activity, as well as regulatory specific domains including the PH domain, the C2 domain, and EF hand motifs involved in various biological functions of PLC isoenzymes [44, 45]. PLC isoforms are distinct in their activation mode, expression levels, cellular localization, and tissue distribution linking to a specific function for each isoform.

The spectrum of the expression of PLC isoforms in macrophages is phenotype-specific. It has been reported that in the case of human macrophages (derived from peripheral blood mononuclear cells), PLCβ1–4, γ1-2, δ1, and η1-2 are expressed in unstimulated macrophages, PLCβ1–3, γ1-2, δ1 and 3, and η1-2 are expressed in M1 macrophages, and PLCβ1–3, γ1-2, δ3, and η1-2 are expressed in M2 macrophages. In addition, these PLC isoforms showed different subcellular localization in differently polarized macrophages [46]. The distinct expression spectrum and subcellular localization of these PLC isoforms reflect the diverse roles that they play in the regulation of the inflammatory response.

3. The Role of PLCβ in Macrophage-Mediated Inflammatory Response

Macrophages express all the four PLCβ isoforms orchestrating the Ca^{2+} signaling [47, 48], for example, the clostridium difficile ToxB-stimulated Ca^{2+} signaling in macrophages is enhanced via PLCβ-4 signaling, but depressed by the PLCβ-3 signaling [49]. Ca^{2+} and Erk1/2 signaling play important roles in the regulation of inflammatory response. PLCβ is involved in the activation of Erk1/2 signaling in macrophages. It has been demonstrated that the glyceryl ester of prostaglandins activates Erk1/2 signaling in a dose-dependent manner through a pathway that requires PLCβ signaling [50].

Cell adhesion is required for monocyte differentiation into macrophages. In human cytomegalovirus- (HCMV-) infected monocytic THP-1 cells, the viral protein US28 promotes adhesion to the endothelial cells via the activation of PLCβ/PKC signaling cascade. Therefore, it is possible that PLCβ signaling may promote the differentiation of monocytes to macrophages via cell adhesion [51]. U73122 is a pan inhibitor for PLC isoforms. We have demonstrated that U73122 inhibits PMA-induced human promonocytic U937 cell adhesion, as well as the differentiation into macrophages [29]. These two independent studies indicated that PLC signaling regulates cell adhesion and the differentiation of monocytes to macrophages.

It has been reported that LPS suppresses PLCβ-2 and β-1 expression in macrophages in an MyD88-dependent manner, and the suppressed PLCβ-2 plays an important role in switching M1 macrophages into an M2-like state [52, 53], suggesting that PLCβ-2 signaling is closely involved in macrophage polarization.

PLCβ signaling broadly regulates the expression of proinflammatory cytokines or chemokines in diverse cell cultures. The binding of HIV-1 envelope glycoprotein gp120 to CCR5 leads to PLCβ-1 nuclear localization which promotes the release of chemokine CCL2 by macrophages [54], suggesting that activation of PLCβ-1 signaling stimulates the expression of CCL2 in macrophages. PLCβ-3 regulates IL-8 expression in bronchial epithelial cells via TLR-mediated activation of calcium signaling and NF-κB pathway [55]. However, whether PLCβ-3 regulates cytokine expression in macrophages has not been reported.

In summary, in macrophages, PLCβ-1 signaling regulates the expression of CCL2, and PLCβ-2 signaling regulates cell polarization, while PLCβ-3 and PLCβ-4 signaling regulates Ca^{2+} signaling with opposite effect.

4. The Involvement of PLCγ in Macrophage-Mediated Inflammatory Response

There are two main isoforms of PLCγ expressed in humans, PLCγ-1 and PLCγ-2, which regulate the development and functions of various hematopoietic cells [56, 57], for example, PLCγ1 regulates T cell activation and development through interaction with T cell receptor (TCR), and PLCγ-2 regulates development and maturation of B cells via interaction with pre-B cell receptor (BCR), reviewed by Nakamura and Fukami [31]. PLCγ-1 and PLCγ-2 are activated downstream of receptor (RTK) and nonreceptor tyrosine kinases, with tyrosine phosphorylation of PLCγ as the major mechanism. However, there is a novel mechanism towards the activation of PLCγ-2, which depends not on protein tyrosine phosphorylation, but on Rac GTPases [57–59]. Ubiquitously expressed PLCγ-1 is mainly activated by growth factors, including platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), and fibroblast growth factor (FGF) [60]. PLCγ-1 binds to the tyrosine-phosphorylated receptors of EGF via its SH2 domain and downstream proteins via the SH3 domain [61]. We have recently identified that the exposure of macrophages to the proinflammatory cytokines TNF-α and IL-1β, as well as to influenza virus H1N1, leads to activation of PLCγ-1 in macrophages, which expands the spectrum of upstream stimulators for PLCγ-1 signaling [30]. Influenza virus H1N1 infection activates PLCγ-1 signaling through EGR receptor (EGFR) in alveolar epithelial cell line (A549 cells) [62]. But whether EGFR or the other RTKs act as an upstream activator for PLC signaling in macrophages is largely unknown. PLCγ-2, being predominantly expressed in hematopoietic cells, is activated by immune cell (T cell, B cell, and Fc) receptors associated with multiprotein complexes [60]. So PLCγ-1 and PLCγ-2 may be differentially activated to perform diverse functions.

Upon stimulation by LPS, TLR4 signaling induces proinflammatory cytokine production. Generally, TLRs regulate TLR-specific gene expression through the recruitment of distinct combinations of TLR/IL1R (TIR) domain-containing adaptor proteins, such as myeloid differentiation primary response gene 88 (MyD88), Toll/IL-1 receptor domain-containing adaptor protein (TIRAP), TIR domain-containing adaptor inducing IFN-β (TRIF), TRIF-related adaptor molecule (TRAM), and sterile α- and armadillo motif-containing protein (SARM) to form a signalosome, which activates downstream signals [63]. TLR4 is unique among these TLRs in its ability to utilize all of the TIR domain-containing adaptors and mediate activation of

both MyD88-dependent and MyD88-independent (TRAM–TRIF-dependent) pathways [64–66], which are required to stimulate proinflammatory cytokine production in macrophages. In MyD88-dependent pathway, both MyD88 and TIRAP are required to activate NF-κB and MAPK cascades and proinflammatory cytokine production [67, 68]. The MyD88-independent signaling events are controlled by TRIF and TRAM and induce IRF3-dependent type I interferon production [65, 69]. So in TLR4-mediated signaling, distinct adaptors are recruited to form diverse complexes which activate various downstream inflammatory signaling.

The involvement of PLCγ signaling in TLR4-mediated inflammation has been well identified. Currently, it is clear that PI(4,5)P2 plays an important role in TLR4 signaling. Mechanistically, TIRAP localizes to the plasma membrane by binding to PI(4,5)P2; there it recruits TLR4 and MyD88 to PI(4,5)P2-rich sites on the plasma membrane to form the TLR4 signalosome [69]. The distinct cellular localization of TLR4 complex leads to optional activation of MyD88-dependent or MyD88-independent signaling. Once TLR4 complex resides at the plasma membrane, the MyD88-dependent NF-κB signaling is activated. Subsequently, the TLR-4 is delivered to the endosome compartment where MyD88-independent IRF3 signaling is activated [70]. The critical role that PI(4,5)P2 plays in TLR4 signaling is in linking TLR4 to PLCγ which controls the metabolism of PI(4,5)P2 [71]. Mechanisms for the regulation of LPS-induced TLR4 endocytosis and IRF3 activation by PLCγ-2 have been established: IP3, the cleavage product of PI(4,5)P2 by PLCγ-2, binding to IP3 receptors (IP3Rs) in the endoplasmic reticulum results in the release of Ca^{2+}. The increased cytosolic Ca^{2+} is required for translocation of TLR4 from the plasma membrane to endosomes, where TRIF-dependent IRF3 activation takes place. In contrast, LPS-induced activation of NF-κB pathway did not require PLCγ2-IP3-Ca^{2+} cascade [71]. Thus, signaling that affects TLR4 endocytosis could regulate TRIF-dependent signaling from endosome.

The LPS-binding protein CD14, together with TLR4 and MD-2, forms a multireceptor complex on the cell membrane [72]. CD14 controls the LPS-induced endocytosis of TLR4. LPS-induced clustering of CD14 triggers PI(4,5)P2 generation in macrophages [73], which may result in the activation of PLCγ2-IP3-Ca^{2+} cascade. The increase in cytosolic Ca^{2+}, released from intracellular calcium stores, promotes the translocation of TLR4 from the plasma membrane to endosomes and so results in the activation of downstream inflammatory signaling. In addition, the CD14-dependent endocytosis pathway is regulated by several cytosolic regulators. Among them, the tyrosine kinase Syk and its downstream effector PLCγ-2 have been identified. The stimulation of Syk/PLCγ-2 signaling by CD14 triggers an influx of Ca^{2+} from the extracellular environment, which promotes internalization of TLR4 [72, 74]. So the endocytosis of TLR4 in response to CD14 clustering is partially regulated by the increased concentration of cytosolic Ca^{2+} originating either from intracellular calcium stores or the extracellular environment, which emphasizes the important role of Ca^{2+} in TLR4-mediated inflammation. In addition, these results support the idea that PLCγ-2 regulates the inflammatory response by controlling the cytosolic level of Ca^{2+}. Apart from Ca^{2+}, PKC signaling is also involved in TLR4 signaling in macrophages. It has been reported that the infection of both *P. aeruginosa* and *K. pneumoniae* activates TLR4/PLCγ cascades which in turn activates the PKCα/Jun N-terminal protein kinase (JNK)/NF-κB axis and eventually induces the production of proinflammatory cytokines [75].

The generation of intracellular ROS in macrophages plays an important role in inflammation pertinent signaling transduction. The minimally oxidized LDL (mmLDL) stimulates ROS generation in macrophages through activation of NADPH oxidase 2 (Nox2), which is a suggested pathogenic mechanism for the development of atherosclerosis. It has been evidenced that mmLDL induces generation of ROS in macrophages through sequential activation of TLR4/Syk/PLCγ-1/PKCα/Nox2 cascade and thereby stimulates expression of proinflammatory cytokines IL-1β, IL-6, and RANTES [76, 77]. These studies indicate that PLCγ-1 regulates inflammatory response by the activation of PKCα, which is different from the role of PLCγ-2-dependent regulation of cytosolic Ca^{2+}. Interestingly, we have recently shown that influenza virus H1N1 infection activates PLCγ-1 signaling and triggers ROS expression in human macrophages dU937 cells, which can be blocked by the PLC inhibitor U73122 [30]. Taken together, these two independent results reveal that PLCγ signaling regulates the generation of an important messenger ROS.

Phagocytosis by macrophages is a process that involves engulfment and clearing of invading microbial pathogens, concomitantly stimulating an inflammatory response leading to upregulation of inflammatory genes, such as TNF-α, IL-1β, and IL-12. The mechanism for FcγR-mediated phagocytosis has been extensively investigated. The ingestion of IgG-opsonized targets is initiated by the engagement and clustering of FcγRs, which induce receptor tyrosine phosphorylation and subsequent activation of multiple downstream signaling pathways to promote the development of the phagocytic cup and the extension of pseudopods. The sequential process including cup formation, phagosome internalization, and phagolysosome formation is critical steps in the process of phagocytosis [78]. The translocation of PKCε to phagosome is a critical step to regulate the rate of FcγR-dependent phagocytosis [79]. Diverse mechanisms regarding as to how FcγR-dependent phagocytosis is regulated by PLCγ signaling have been revealed, for example, PLCγ-1 is consistently concentrated at phagosomes and provides DAG to facilitate PKCε localization to the phagosome [80]; Syk-dependent as well as Bruton's tyrosine kinase-(Btk-) and Tec-dependent activation of PLCγ-2 affects early and later stages of phagocytosis, respectively [78].

Peptidoglycan (PGN), the major cell wall component of Gram-positive bacteria, is able to stimulate proinflammatory cytokine production in macrophages. Normal human plasma from uninfected people contains low titer of anti-PGN IgG [81]. The anti-PGN IgG and FcγRs are the key mediators of systemic inflammation in Gram-positive bacteria-induced sepsis [81, 82]. The binding of

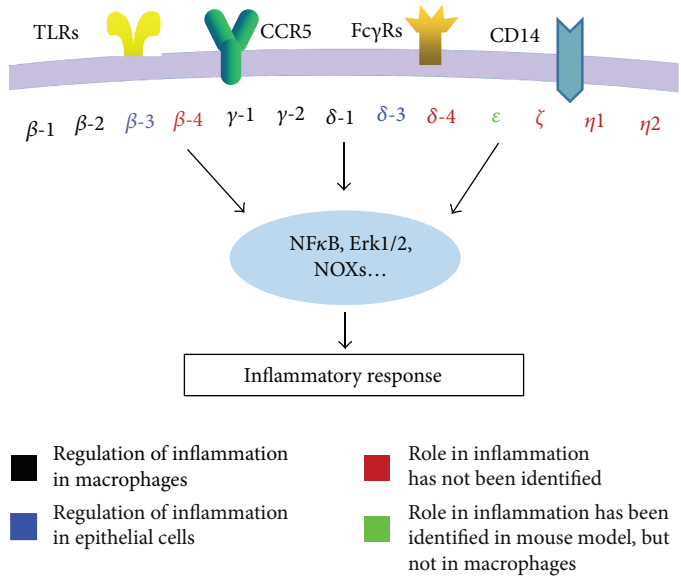

Figure 2: Schematic of macrophage-mediated inflammatory response through PLC signaling. PLCβ1-2, PLCγ1-2, and PLCδ shown in black indicated that these PLC isoforms are expressed in macrophages and are involved in macrophage-mediated inflammatory response. PLCβ3 and PLCδ3 shown in blue indicated that their involvement in inflammatory response has been identified in epithelial cell but not in macrophages. PLCβ4, PLCδ4, PLCζ, and PLCη1-2 shown in red indicated that whether they are involved in inflammatory response has not been identified. PLCε shown in green indicated that the involvement of inflammatory response has been identified with mouse model, in vivo. But whether it regulates inflammatory response in macrophages has not been identified.

PGN to anti-PGN IgG triggers FcγR-mediated phagocytosis, which consequently leads to an inflammatory response [81]. In this mechanism, the phagocytosis of PGN-IgG-FcγR complex in macrophages is triggered by Ca^{2+} release from intracellular Ca^{2+} stores controlled by PLCγ-2 signaling [82, 83], suggesting that the regulation of intracellular calcium signaling by PLCγ-2 is involved in IgG-FcγR-mediated phagocytosis and cytokine production.

5. PLCδ Controls Phagocytosis

The PLCδ1-PH domain negatively regulates FcγRII-mediated cell spreading and phagocytosis through destabilizing PI(4,5)P2 availability in macrophages [84]. In addition, it has been reported that LPS stimulation reduces PLCδ1 expression at both mRNA and protein levels, an effect which would allow upregulation of the TLR4-induced proinflammatory cytokine production and FcγR-mediated phagocytosis [85]. These studies suggest that PLCδ1 negatively regulates TLR4/FcγR-mediated inflammatory response in macrophages. The roles of the other PKCδ isoforms including PKCδ3 and PKCδ4 in macrophage-mediated inflammation are not yet defined.

6. The Involvement of PLCε in Inflammatory Response Has Been Characterized In Vivo, but Not in Macrophages

PLCε is involved in a variety of signaling pathways and controls different cellular functions. Its role in carcinogenesis has been documented. With a PLCε knockout mice model (PLCε−/−), PLCε has been identified as a novel tumor suppressor [86]. Also with this mouse model, it has been revealed that the airway inflammation induced by cigarette smoke *in vivo* was partially mediated by PLCε signaling [87]. The PLCε has also been convincingly demonstrated to regulate Ca^{2+} signaling in β cells and cardiomyocytes [88]. However, whether PLCε is expressed in macrophages, as well as it is having any role in the macrophage-mediated inflammatory response, has not been identified.

7. Conclusions and Perspectives

Evidence accumulating from multiple studies has indicated that the PLC enzymes which functionally rely on the hydrolysis of PI(4,5)P2 to produce IP3 and DAG with subsequent modulation of calcium and PKC signaling regulate macrophage-mediated inflammatory response. The macrophage inflammatory response, such as the expression of inflammation-related genes and endocytosis, is controlled by calcium and/or PKC signaling. The PKC family contains ten isoforms with individual regulatory mechanism (summarized in Figure 1). Intracellular Ca^{2+} levels regulate multiple signaling pathways. In addition, the PLC family contains at least 13 members with specific activity for each one. Diversity of PKC family and the versatile Ca^{2+} signaling networks confers PLC enzyme multiple functions in the regulation of inflammatory response. Therefore, PLC enzymes are promising targets for the development of novel anti-inflammatory drugs.

Macrophages express various receptors, such as TLRs, CD14, and FcγRs, which have been identified as important upstream activators of PLC signaling (summarized in Figure 2). These receptors, such as CD14 and TRL4, may independently or collaboratively regulate the same or distinct PLC isoforms. In addition, some PLC isoforms may have

opposite or synergistic effects on the same downstream signaling, for example, the concentration of intracellular Ca^{2+} is increased by PLCβ-4 signaling, but decreased by PLCβ-3. These studies indicate the complexity of the PLC-dependent signaling in the inflammatory response, and further research on PLC-dependent functions will contribute towards our understanding of the underlying mechanism of some inflammatory diseases.

Disclosure

Because of space limitations, the authors could not fully discuss all the important roles of PLC isozymes in other biological functions.

Conflicts of Interest

The authors declare that they have no competing interests.

Acknowledgments

The authors apologize to those researchers whose work was not cited. This work was supported by the Chinese National Science Foundation Grant (nos. 31472172 and 31772743), the National Key Research and Development Program of China (Grant no. 2016YFD0500704), the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD and TAPP), the USDA-NIFA Competitive Grants Program (13-01041 and 16-09370), and funds derived from Sitlington Endowment.

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Hindawi
Journal of Immunology Research
Volume 2018, Article ID 1436236, 8 pages
https://doi.org/10.1155/2018/1436236

Research Article

Distinct Profiles of CD163-Positive Macrophages in Idiopathic Interstitial Pneumonias

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Received 26 October 2017; Accepted 14 December 2017; Published 4 February 2018

Academic Editor: Zissis Chroneos

Background. The types of cells most significantly linked to individual subtypes of idiopathic interstitial pneumonias (IIPs) remain unclear. Few studies have examined $CD163^+$ macrophages in IIPs. *Objective*. We retrospectively aimed to immunohistochemically characterize the $CD163^+$ macrophages in IIPs. *Methods*. Paraffin-embedded lung tissue samples were obtained from 47 patients with IIPs, including idiopathic pulmonary fibrosis (IPF), idiopathic nonspecific interstitial pneumonia (NSIP), and cryptogenic organizing pneumonia (COP), and 12 normal controls were immunohistochemically analyzed, using primary antibodies against CD68 and CD163 as indicators of pan and M2 macrophages, respectively. *Results*. $CD68^+$ macrophage density was significantly increased in the 3 subtypes of IIPs relative to that in the control group, although no difference was detected within the different IIPs. $CD163^+$ macrophage density was significantly increased in NSIP and COP samples relative to that in IPF samples. The density ratio of $CD163^+$ macrophages to $CD68^+$ macrophages was significantly decreased in IPF/UIP samples relative to that in the others, while the densities in NSIP and COP were significantly higher than those in control cases. *Conclusion*. $CD163^+$ macrophages show distinct profiles among IIPs, and the standardized numerical density is decreased in IPF cases that have poor prognoses.

1. Introduction

Idiopathic interstitial pneumonias (IIPs) are a heterogeneous group of acute and chronic disorders with varying degrees of inflammation and fibrosis; the etiologies of which are unknown [1]. Idiopathic pulmonary fibrosis (IPF)/usual interstitial pneumonia (UIP), nonspecific interstitial pneumonia (NSIP), and cryptogenic organizing pneumonia (COP) have gained attention because of their relatively high incidences. The prognoses of IPF/UIP are poor relative to those of the latter two types, and different pathological features enable discrimination of these conditions [1]. IPF/UIP is histopathologically characterized by temporal heterogeneity in the degree of interstitial fibrosis in the alveolar septa, including in normal regions and severe fibrotic areas [2, 3]. The presence of intraluminal fibrotic lesions, known as fibroblastic foci, is associated with the prognosis of IPF/UIP [3]. NSIP is mainly characterized by a dense or loose interstitial fibrosis with a uniform appearance. COP primarily shows intraluminal fibrotic involvement with a patchy distribution [4, 5]. The background lung architecture is well preserved in COP and NSIP, while a honeycomb lung

represents a terminal status of tissue remodeling in IPF/UIP [2–5]. Importantly, the types of cells most significantly linked to differences in the pathogenesis and prognoses among the subtypes of IIPs remain unclear.

Macrophages constitute a heterogeneous population of cells of the innate immune system and display a variety of functions [6]. This functional diversity of macrophages develops from the response to local microenvironmental signals that allow them to adapt to this local environment, with the cells typically represented as M1 and M2 types of populations [7]. M1 macrophages are characterized by the ability to produce proinflammatory mediators, which is associated with phagocytosis, killing of microorganisms, and tissue injury [8–10]. In contrast, M2 macrophages have an anti-inflammatory effect, which is linked to the phagocytosis of apoptotic cells, tissue repair, and fibrosis [11–15]. Particularly, an increasing number of studies have revealed a significant association between fibrotic diseases and macrophages with positivity for CD163, an endocytic receptor for heme and ferroportin and M2 marker [16–20].

We hypothesized that subpopulations of macrophages are represented differently among the subtypes of IIPs, as macrophages exhibit plastic responses to different microenvironments. Different types of subpopulations indeed participate in individual processes corresponding to the development and repair of fibrosis models [21, 22]. However, limited information is available regarding the subpopulations of macrophages in IIPs. In the present study, we immunohistochemically characterized $CD68^+$ and $CD163^+$ macrophages in the three subtypes of IIPs: IPF/UIP, NSIP, and COP. CD68 is a single-chain glycoprotein of 110 kD expressed predominantly on the lysosomal membrane of myeloid cells and is thought to be a pan-macrophage marker [23]. Our findings revealed interesting characteristics for the counting of $CD163^+$ macrophages among IIPs.

2. Materials and Methods

2.1. Materials. A total of 87 patients who underwent video-assisted thoracoscopic surgery biopsy for the diagnosis of interstitial pneumonia at Iwate Medical University Hospital (Morioka, Japan) and Tohoku University Hospital (Sendai, Japan) from 2000 to 2010 were selected for the study. Of these, 40 patients were excluded from the study because of diagnoses of interstitial pneumonias other than IIPs, such as collagen vascular diseases or hypersensitivity pneumonitis. The remaining 47 patients were retrospectively diagnosed with idiopathic interstitial pneumonias based on multiple disciplinary discussions, as per international consensus criteria [24], and consisted of 23 with IPF/UIP, 17 with NSIP, and 7 with COP. No patient received treatment before surgical biopsy. After diagnosis, treatment was administered according to the guidelines in effect at the time of the diagnosis [24]. The mean follow-up of patients alive at the endpoint of analysis was 56.8 months. For the control lungs, normal regions distant from the cancer lesions in lung specimens obtained from 12 patients with preinvasive lung adenocarcinoma were used. These specimens were obtained from the archives of the Departments of Pathology at Iwate Medical University and Tohoku University Hospital, the Ethical Committees which approved the use of all samples in this study (IRB, H24-170, and 2014-1-446). The requirement for informed consent was waived because of the retrospective nature of this study.

2.2. Immunohistochemistry. As primary antibodies, mouse anti-human CD68 (clone PGM-1, DAKO, Glostrup, Denmark, dilution 1 : 50), mouse anti-human CD163 (clone 5C6, BMA Biomedicals, Augst, Switzerland, dilution 1 : 200), and anti-human CD163 (clone EDhu1, Serotec, Cambridge, UK, dilution 1 : 200) antibodies were used. As negative controls for each antibody, normal mouse IgG1 (Dako) and normal rabbit serum (Vector Labs, Burlingame, CA, USA) were used. The detailed protocol used for immunohistochemistry analysis has been previously described [25].

2.3. Morphometric Analysis. The numerical density of $CD68^+$ [N_A(CD68)] and $CD163^+$ [N_A(CD163)] mononuclear cells was counted at 200-fold magnification in 20 randomly sampled fields per slide [25]. Alveolar and interstitial macrophages were separately counted. Areas corresponding to 1–3 degrees based on Ashcroft's fibrotic score were estimated to compare the 3 conditions, as IPF samples exhibit temporarily heterogeneous lesions [26]. The numerical density of macrophages was standardized according to the interstitial number densities [N_A(int)], which were measured by point counting methods using a grid [25, 27]. In IPF samples, the alveolar and interstitial numerical density of $CD68^+$ [N_A(CD68)] and $CD163^+$ [N_A(CD163)] macrophages was estimated, which were divided into 2 fibrotic grades including mild and severe lesions. Densities were standardized by air space and interstitial area density as [A_A(Air)] and [A_A(int)], respectively [28]. Lung specimens were obtained from multiple lobes as far apart as possible. Morphometric analyses were performed in individual lobes, and average values were used as representative data of each patient. Morphometric examinations were performed independently by two pathologists (R.S., T.S.).

2.4. Pulmonary Function Tests. The forced vital capacity, forced expiratory volume in 1 s, and diffuse capacity of the lung for carbon monoxide were measured according to American Thoracic Society guidelines [29]. These values were also expressed as percentages of the predicted normal values calculated according to sex, weight, and age [30].

2.5. Statistical Analysis. Statistical significance was evaluated by one-way analysis of variance followed by Dunnett test or Fisher's exact test. Receiver operating characteristic (ROC) curves were plotted for standardized numerical density of $CD163^+$ macrophages and differential diagnosis between IPF and NSIP. A diagnostic test with an area under the curve (AUC) above 0.75 was regarded as contributive [31]. A p value less than 0.05 was considered to indicate statistical significance. Statistical analyses were performed using SPSS Statistics software (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Patient Characteristics. Patient characteristics are shown in Table 1. Patients with NSIP were younger than those with IPF/UIP ($p < 0.05$).

3.2. Morphological and Morphometric Analyses of CD68 Macrophages in IIPs. We immunohistochemically characterized $CD68^+$ and $CD163^+$ macrophages in the 4 groups, including normal control lungs, IPF/UIP, NSIP, and COP (Figures 1–3). $CD68^+$ macrophages were scattered in the control lungs (Figure 1(a)), while high numbers were observed in every type of IIP (Figures 1(b)–1(d)). Numerous $CD68^+$ macrophages were observed within airspace neighboring mild and severe fibrotic lesions (Supplementary Figure E1A and B), but were undetectable within fibroblastic foci of IPF/UIP (Supplementary Figure E1C). The numerical density of $CD68^+$ macrophages was significantly increased in the 3 types of IIPs relative to that in the control, although no difference was observed among the 3 disease groups (Figure 3(a)). While categorizing fibrotic lesions in IPF/UIP into 2 severity grades, $CD68^+$ macrophages were detected in both lesions (Supplementary Figure E1C and D). However, the standardized density of $CD68^+$ alveolar macrophages $[N_A(CD68)/A_A(air)]$ showed significantly higher levels in severe lesions relative to those in mild lesions, although no difference was detected in interstitial density $[N_A(CD68)/A_A(int)]$ ($p < 0.0001$) (Supplementary Figure E2A and B).

3.3. Morphological and Morphometric Analyses of $CD163^+$ Macrophages in IIPs. $CD163^+$ macrophages showed a scattered distribution in normal control samples (Figure 2(a)). In the airspace neighboring the mild lesions of IPF/UIP, numerous macrophages showed weak or no expression of CD163, although a few $CD163^+$ macrophages were observed (Figure 2(b), Supplementary Figure E1E). In interstitial lesions of IPF/UIP, very few $CD163^+$ macrophages were detected (Supplementary Figure E1G and H). In contrast, these cells were abundant in NSIP and COP (Figures 2(c) and 2(d)). The numerical density of $CD163^+$ macrophages was significantly increased in NSIP and COP relative to those in the control group and IPF/UIP (Figure 3(b)). Although $CD163^+$ mononuclear cells locally formed cluster aggregation in the airspace neighboring severe fibrotic lesions, there was no difference in the standardized numerical density of $CD163^+$ alveolar macrophages between the airspaces adjacent to mild and severe fibrotic lesions (Supplementary Figure E1E and F and Figure E2C). There was no difference in the numerical densities of $CD163^+$ interstitial macrophages between the two severity grades of lesions of IPF/UIP (Supplementary Figure E2D). In the present study, although the data are represented as the results obtained with anti-CD163 antibody (clone EDhu1), both antibodies of clone 5C6 and EDhu1 showed similar results.

3.4. Density Ratio of $CD163^+$ Macrophages to $CD68^+$ Macrophages. The density ratio of $CD163^+$ macrophages to $CD68^+$ macrophages was significantly decreased in mild lesions of IPF/UIP relative to that in the others, although the densities in NSIP and COP were significantly higher than those in control cases (Figure 3(c)). The significant difference in the ratio was also observed in alveolar and interstitial macrophages (Supplementary Figure E3).

3.5. Differences between Nonsmokers and Smokers. We also explored the influence of smoking on $CD68^+$ and $CD163^+$ macrophage densities in normal control, IPF/UIP, and NSIP cases. We did not determine the effects of smoking in patients with COP because the number of patients was too less. There was no difference in $CD68^+$ macrophage densities between nonsmokers and smokers in every condition (Supplementary Figure E4). However, $CD163^+$ macrophage density was significantly lower in smokers with NSIP, and the ratio of $CD163^+$ macrophages to $CD68^+$ macrophages showed a decreasing trend in smokers with IPF/UIP (Supplementary Figure E4F and H).

3.6. Diagnostic Value of $CD163^+$ Macrophage Densities in Differentiation between IPF/UIP and NSIP. We explored the diagnostic value of the numerical density of $CD163^+$ macrophages in the differentiation between IPF/UIP and NSIP, using ROC analysis. The total numerical density of $CD163^+$ macrophages showed an ROC-AUC value of 0.898 (95% confidence interval, CI, 0.783–1.000) for the differentiation (Figure 4). A cut-off level of 12.0 in total numerical density of $CD163^+$ macrophages yielded a sensitivity of 90.5% (95% CI = 78.2–96.2%) and specificity of 88.2% (95% CI = 73.1–95.3%). Moreover, we evaluated the relation between the response to the treatment and numerical density of $CD163^+$ macrophages. No statistically significant relation was detected in any group; the coefficient of correlation was determined to be 0.49 in patients with NSIP and COP who received corticosteroids for treatment.

4. Discussion

In the present study, we found that the numerical density of $CD68^+$ macrophages was higher in the 3 types of IIPs relative to that in the normal control lungs, while $CD163^+$ macrophages density was higher in NSIP and COP than in IPF/UIP. The density ratio of $CD163^+$ macrophages to $CD68^+$ macrophages was significantly lower in IPF/UIP relative to those in the other 3 groups, while the ratios in COP and NSIP were significantly higher relative to that in the normal control lungs.

Very limited information is available regarding the characterization of $CD163^+$ macrophages in IIPs. Wojtan et al. estimated the proportion of $CD163^+$ macrophages in bronchoalveolar lavage fluids by immunocytochemistry [32]. The proportion of $CD163^+$ macrophages did not differ between IPF/UIP and NSIP, which is inconsistent with our results. However, as they did not use pan-macrophage markers, the proportions represented in their study are unclear. In addition, it is difficult to draw conclusions regarding the association between IIPs and $CD163^+$ macrophages in their study, as they used a very small sample size of 6 patients with IPF/UIP and 8 with NSIP.

There are two mechanistic possibilities explaining how the higher ratio of $CD163^+$ macrophages to $CD68^+$

Table 1: Characteristics of healthy volunteers and patients with IIPs.

	Healthy control	UIP	NSIP	COP	
n	12	23	17	7	*p* value
Age	64.3 ± 3.1	65.3 ± 1.6	54.3 ± 2.7	64.0 ± 3.9	0.015
Female (%)	6 (50.0)	10 (43.5)	8 (46.6)	2 (28.6)	n.s
Smoke (%)	6 (50.0)	12 (52.2)	5 (29.4)	4 (57.2)	n.s
Pulmonary function tests					
FVC (L)	3.03 ± 0.24	2.48 ± 0.19	2.55 ± 0.30	2.19 ± 0.23	n.s
FVC (%)	112.7 ± 3.0	83.3 ± 4.2	78.4 ± 7.2	68.3 ± 5.9	n.s
FEV/FVC (%)	77.8 ± 2.1	87.6 ± 2.0	85.0 ± 1.41	81.4 ± 1.9	n.s
DLco (%)	114.2 ± 7.6	73.7 ± 5.1	67.8 ± 6.9	63.4 ± 4.8	n.s
Lung specimens obtained					
Upper lobe (%)	6 (50.0)	5 (21.7)	1 (5.8)	1 (14.3)	n.s
Lower lobe (%)	6 (50.0)	7 (30.4)	3 (17.6)	3 (42.9)	n.s
Both lobes (%)	0 (0)	11 (47.8)	13 (76.5)	3 (42.9)	<0.001
Treatments					
Corticosteroid (%)	0 (0)	4 (17.4)	13 (74.5)	5 (71.4)	<0.001
Immunosuppresants (%)	0 (0)	2 (8.7)	5 (29.4)	0 (0)	n.s
Pirfenidone (%)	0 (0)	2 (8.7)	1 (5.9)	0 (0)	n.s
None (%)	12 (100.0)	16 (69.6)	3 (17.6)	2 (28.6)	<0.001

Data are shown as mean ± SD. Brackets represent percentage. IPF/UIP: idiopathic pulmonary fibrosis/usual interstitial pneumonia; NSIP: nonspecific interstitial pneumonia; COP: cryptogenic organizing pneumonia; FVC: forced vital capacity; $FEV_{1.0}$: forced expiratory volume in 1 second; DLco: diffusing capacity of the lungs for carbon monoxide; n.s: no statistical significance.

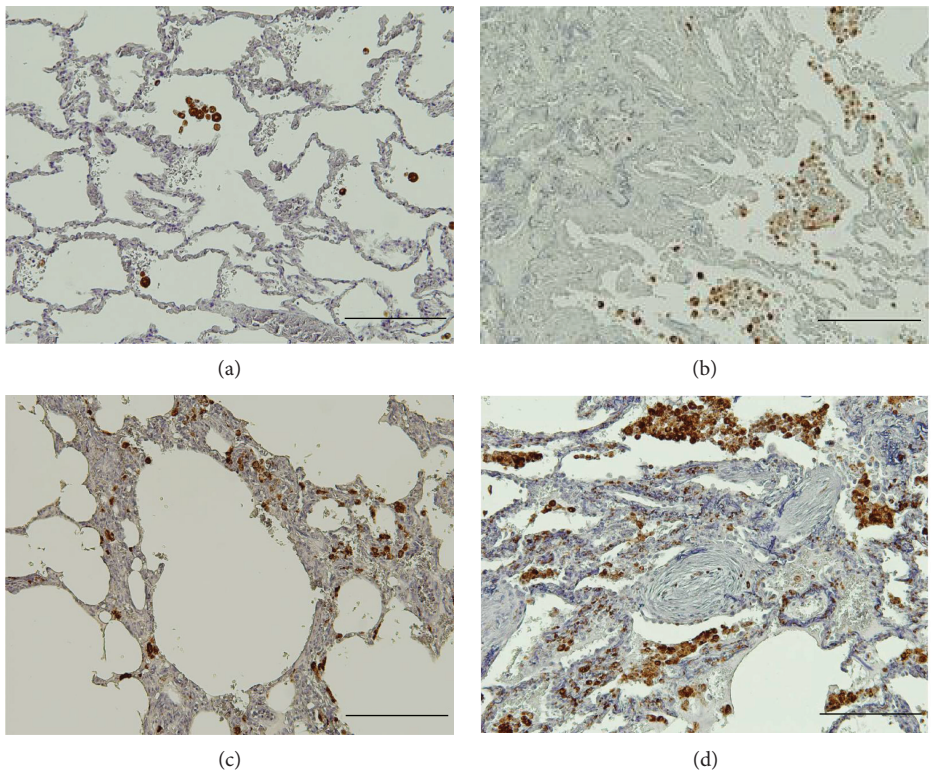

Figure 1: Immunohistochemical analysis of CD68 expression in IIPs. (a) $CD68^+$ macrophages (*Brown*) were scattered in the alveolar space of normal control lungs. Numerous $CD68^+$ macrophages were observed in the alveolar space in IIPs, including IFF/UIP (b), NSIP (c), and COP (d). In the interstitium, $CD68^+$ macrophages were observed in NSIP and COP, but barely detectable within the intraluminal fibrosis in COP. Resorcin-fuchsin and hematoxylin were used as counterstains. Scale bar, 200 μm.

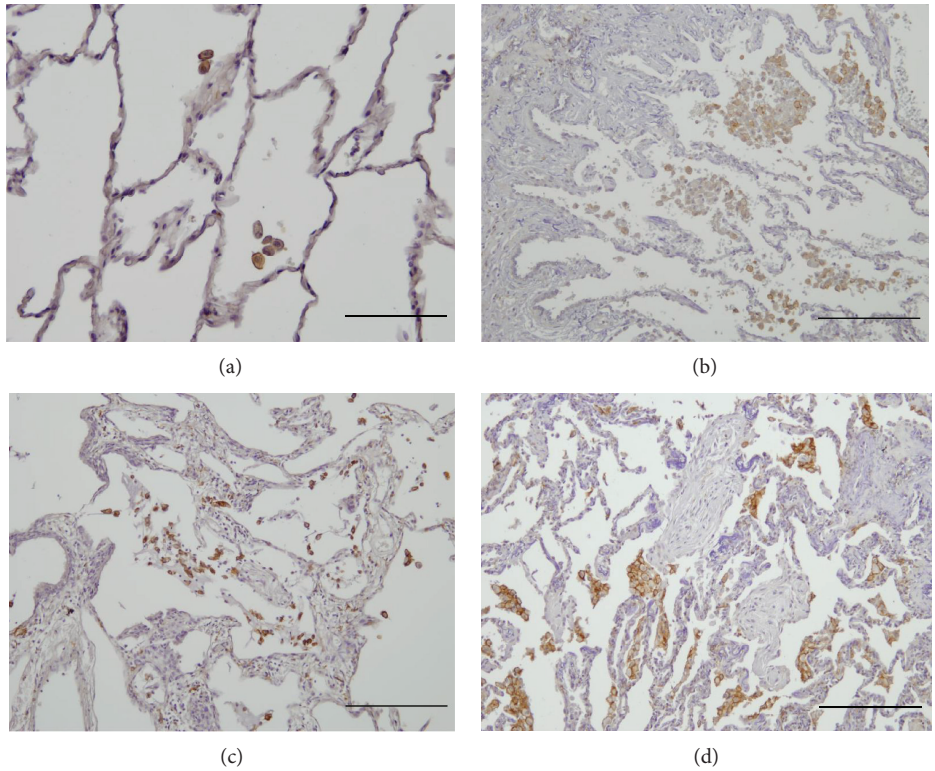

Figure 2: Immunohistochemical analysis of the expression of CD163 in IIPs. $CD163^+$ macrophages (*Brown*) are observed to be scattered in normal control lungs (a). In IPF/UIP, numerous alveolar macrophages show weak or no expression of CD163 (b). Numerous $CD163^+$ macrophages are observed predominantly in alveolar space of NSIP (c) and COP (d) and in interstitium too. $CD163^+$ macrophages were rarely detected within fibroblastic foci in IPF/UIP and the intraluminal fibrosis in COP. Resorcin-fuchsin and hematoxylin were used as counterstains. Scale bar: (a) 100 μm and (b–d) 200 μm.

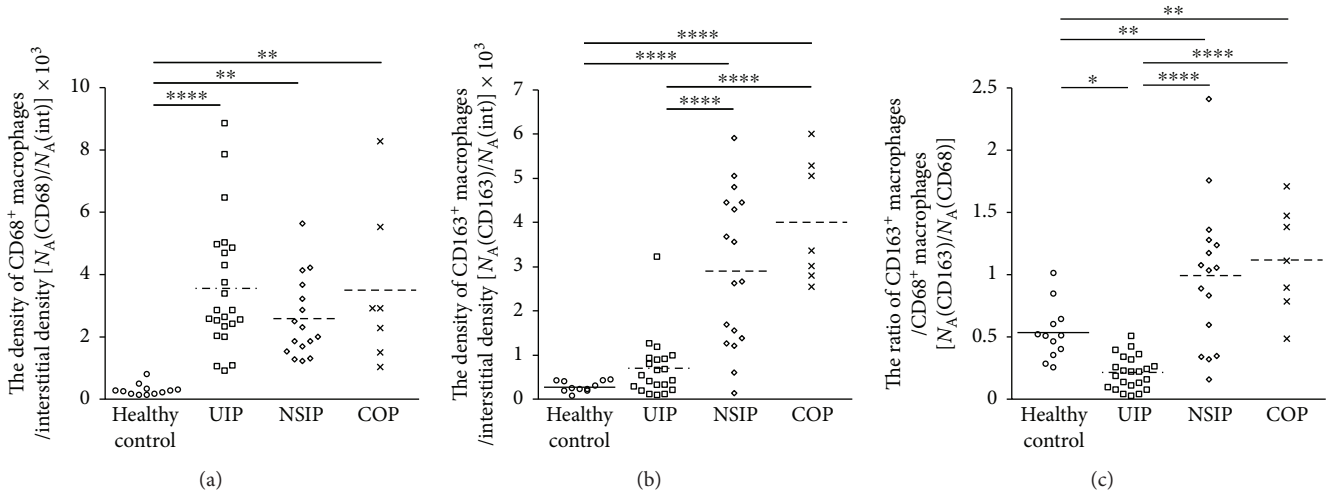

Figure 3: Comparison of $CD68^+$ and $CD163^+$ macrophage densities among the four groups. The numerical densities of $CD68^+$ macrophages standardized by interstitial density $[N_A(CD68)/N_A(int)]$ were significantly increased in IPF/UIP, NSIP, and COP relative to those in normal control lungs (a). The numerical densities of CD163 macrophages $[N_A(CD163)/N_A(int)]$ were significantly increased in NSIP and COP relative to those in normal control lungs and IPF/UIP (b). The ratio of $CD163^+$ macrophages to $CD68^+$ macrophages was significantly increased in IPF/UIP and normal control lungs relative to those in the other 2 groups (c) $[N_A(CD163)/N_A(CD68)]$. The values of the numerical densities described in the figures represent actual values multiplied by 10^3. $^*p < 0.05$, $^{**}p < 0.01$, and $^{****}p < 0.0001$.

macrophages is related to interstitial pneumonia, although we could not determine the pathogenic roles of the macrophages in IIPs in the present study. The first possibility is that $CD163^+$ macrophages have a protective role against tissue injury associated with IIPs. Ye et al. reported decreased expression of heme oxygenase-1 in alveolar macrophages in

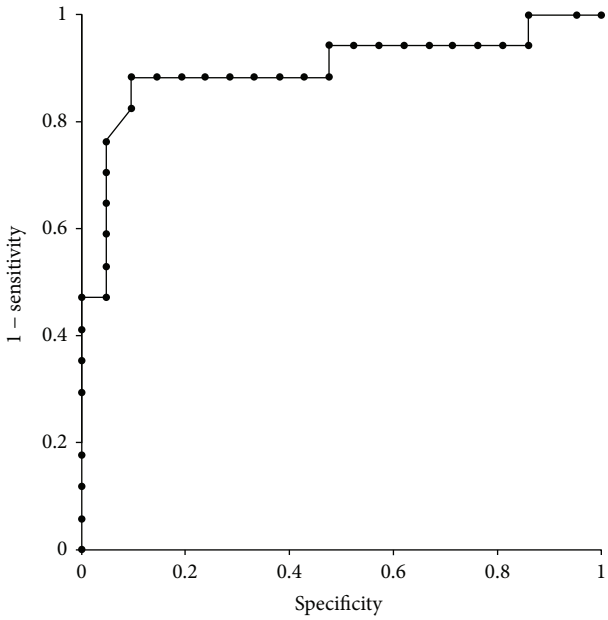

Figure 4: The results of receiver operating characteristic (ROC) curve analysis. The value of ROC-area under the curve shows 0.898 (95% confidence interval, CI, 0.783–1.000) for the diagnostic differentiation between IPF/UIP and NSIP. A cut-off level below 12.0 in total numerical density of $CD163^+$ macrophages yielded a sensitivity of 90.5% (95% CI = 78.2–96.2%) and specificity of 88.2% (95% CI = 73.1–95.3%) for the diagnosis of IPF/UIP.

idiopathic pulmonary fibrosis patients [33]. CD163 is a scavenger receptor for the heme-haptoglobin complex, which reduces the toxicity of heme-oxygenase. Our findings and those of previous studies suggest a protective role of $CD163^+$ macrophages against IIPs. In contrast, the second possibility is that CD163 macrophages accelerate fibrosis in non-IPF/UIP, which has a relatively better prognosis, such as NSIP and COP. Christmann et al. reported that mRNA expression of CD163 was upregulated in the lung specimens obtained from patients with systemic sclerosis-associated interstitial lung diseases (SSc-ILD), which mainly consisted of NSIP, and that CD163 gene expression levels were correlated with the progression of fibrosis based on HRCT [34]. Mathai et al. reported that mRNA expression of CD163 was upregulated in monocytes in the peripheral blood of patients with SSc-ILD relative to that in healthy controls [35].

Interestingly, our results showed that $CD163^+$ macrophages were associated with IPF/UIP to a lesser extent, although increasing evidence suggests a positive association between $CD163^+$ macrophages and fibrogenic conditions. It is very important to consider the possibility that the development of fibrotic lesions in IPF/UIP does not depend on acceleration by $CD163^+$ macrophages, and it remains to be elucidated which types of cells most significantly regulate the prolonged activity of myofibroblasts in IPF/UIP.

It has been reported that smoking influences macrophage polarization [36]. In the present study, we examined the influence of smoking on $CD68^+$ and $CD163^+$ macrophage densities in normal control lungs, IPF/UIP, and NSIP. $CD163^+$ macrophage density was decreased in NSIP patients who smoked, and the ratio of $CD163^+$ macrophages to $CD68^+$ macrophages showed a decreasing trend in IPF/UIP patients who smoked. The comparative data of CD163 macrophage density among the 3 groups were unlikely to be biased by smoking because the ratios of patients with smoking were equivalent in the 3 conditions.

In the clinical setting, NSIP is diagnostically differentiated from IPF/UIP, and histopathologic analysis is routinely required for diagnosis. However, it is not always easy to differentiate between the two conditions, and multiple disciplinary discussions are often required to determine the diagnosis [37, 38]. No information is available regarding a diagnostic marker for the differentiation of IIP subtypes. In the present study, the high value of the ROC-AUC suggests the potential of $CD163^+$ macrophage density as a useful differential marker.

The present study has some limitations. The first limitation is its retrospective nature. It is unlikely that there was selection bias in patients with IIPs because we consecutively enrolled patients at both institutes. Second, we could not estimate the numerical densities of $CD68^+$ and $CD163^+$ macrophages in multiple lobes in all patients with IIPs. However, no difference in the numerical densities of $CD68^+$ and $CD163^+$ macrophages was observed between the upper and lower lobes in each group (data not shown). Third, the study population was relatively small. Further studies on a larger cohort of patients are needed to validate the diagnostic value of $CD163^+$ macrophage density in IIPs.

We clearly demonstrated the distinct profiles of $CD163^+$ macrophage counts among the subtypes of IIPs. The lower ratio of $CD163^+/CD68^+$ macrophages was related to IPF/UIP, and $CD163^+$ macrophages may be diagnostically useful markers for differentiating IIPs. Our results provide insight into the pathogenic and clinical perspectives of IIPs and may facilitate further investigations of the heterogeneity of macrophages in IIPs.

Conflicts of Interest

The authors do not have any conflicts of interest.

Authors' Contributions

Masahiro Yamashita designed and performed the experiments, analyzed the data, and wrote the manuscript. Ryoko Saito and Tamotsu Sugai performed the morphometric analyses. Hironobu Sasamo and Tamotsu Sugai provided the research materials. Shinji Yasuhira, Yuh Fukuda, and Kohei Yamauchi provided crucial ideas.

Acknowledgments

Masahiro Yamashita acknowledges partial support from JSPS KAKENHI Grant no. JP16K08940. The authors would like to thank Mr. Noriyuki Yamada and Ms. Miyuki Niisato for excellent help.

Supplementary Materials

Supplementary 1. Figure E1: immunohistochemical analyses of CD68 and CD163 expression in mild and severe fibrotic lesions of IPF/UIP. Many $CD68^+$ macrophages with strong expression (brown) were observed in the airspaces neighboring mild (A) and severe (B) interstitial fibrotic lesions. $CD68^+$ macrophages were scattered within mild (C) and severe (D) interstitial fibrotic lesions. $CD68^+$ macrophages were not detected within fibroblastic foci (arrows in C). In contrast, numerous macrophages showed weak or no expression of CD163 in the alveolar space near mild fibrotic lesions, although few $CD163^+$ macrophages were detected (E). In the alveolar space near severe fibrotic lesions of IPF/UIP, $CD163^+$ macrophages showed cluster aggregation (F). $CD163^+$ macrophages with weak expression (brown) were occasionally observed in mild (G) and severe (H) interstitial fibrotic lesions. Resorcin-fuchsin and hematoxylin were used as counterstains. Scale bar, 100 μm.

Supplementary 2. Figure E2: comparison of $CD68^+$ and $CD163^+$ macrophage densities between mild and severe fibrotic grades of lesions in IPF/UIP. (A) A comparison of the numerical densities of alveolar $CD68^+$ macrophages between airspaces near mild and severe fibrotic lesions in IPF/UIP. The numerical density of $CD68^+$ alveolar macrophages standardized by airspace area density $[N_A(\text{CD68})/A_A(\text{air})]$ in severe fibrotic lesions showed a significant increase relative to that in mild lesions. In contrast, no difference was detected in the numerical densities of $CD68^+$ interstitial macrophages standardized by interstitial numerical density $[N_A(\text{CD68})/N_A(\text{int})]$ (B). There was no difference in the numerical densities of $CD163^+$ alveolar macrophages $[N_A(\text{CD163})/A_A(\text{air})]$ and interstitial macrophages $[N_A(\text{CD163})/N_A(\text{int})]$ between mild and severe lesions (C and D). The values of the numerical densities described in the figure represent actual values multiplied by 103. $^{****}p<0.0001$.

Supplementary 3. Figure E3: comparison of alveolar and interstitial densities of $CD68^+$ and $CD163^+$ macrophages among the 4 groups. The results of alveolar macrophages are similar to those of total macrophages in the case of $CD68^+$ $[N_A(\text{CD68})/N_A(\text{int})]$ (A), $CD163^+$ macrophages $[N_A(\text{CD163})/N_A(\text{int})]$ (B), and ratio of $CD68^+$ macrophages to $CD163^+$ macrophages $[N_A(\text{CD68})/N_A(\text{CD163})]$ (C). The interstitial density of $CD68^+$ macrophages $[N_A(\text{CD68})/N_A(\text{int})]$ is similar to those of total macrophages, although $CD68^+$ interstitial macrophages were not detected in 9 of 12 control cases (D). The results of $CD163^+$ interstitial macrophages $[N_A(\text{CD163})/N_A(\text{int})]$ are similar to those of alveolar and total macrophages (E). The interstitial ratio of $CD68^+$ macrophages to $CD163^+$ macrophages $[N_A(\text{CD163})/N_A(\text{CD68})]$ showed a significant increase in IPF/UIP relative to that in the others (F). The values of numerical densities described in the figure represent actual values multiplied by 103. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$, and $^{****}p<0.0001$.

Supplementary 4. Figure E4: comparison of numerical densities of $CD68^+$ and $CD163^+$ macrophages between nonsmokers and smokers. There was no difference in $CD68^+$ and $CD163^+$ macrophage densities and the ratio of $CD163^+$ macrophages to $CD68^+$ macrophages between nonsmokers and smokers in the normal control lungs (A–C). The ratio of $CD163^+$ macrophages to $CD68^+$ macrophages showed a decreasing trend in smoker patients with IPF/UIP relative to that in nonsmokers (F). A significant decrease was detected in smoker patients with NSIP relative to that in nonsmokers (H). The values of numerical densities described in the figure represent actual values multiplied by 103. $^{*}p<0.05$.

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Hindawi
Journal of Immunology Research
Volume 2018, Article ID 8917804, 25 pages
https://doi.org/10.1155/2018/8917804

Review Article

Macrophage Polarization in Chronic Inflammatory Diseases: Killers or Builders?

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Received 23 July 2017; Revised 1 November 2017; Accepted 15 November 2017; Published 14 January 2018

Academic Editor: Kebin Hu

Macrophages are key cellular components of the innate immunity, acting as the main player in the first-line defence against the pathogens and modulating homeostatic and inflammatory responses. Plasticity is a major feature of macrophages resulting in extreme heterogeneity both in normal and in pathological conditions. Macrophages are not homogenous, and they are generally categorized into two broad but distinct subsets as either classically activated (M1) or alternatively activated (M2). However, macrophages represent a continuum of highly plastic effector cells, resembling a spectrum of diverse phenotype states. Induction of specific macrophage functions is closely related to the surrounding environment that acts as a relevant orchestrator of macrophage functions. This phenomenon, termed polarization, results from cell/cell, cell/molecule interaction, governing macrophage functionality within the hosting tissues. Here, we summarized relevant cellular and molecular mechanisms driving macrophage polarization in "distant" pathological conditions, such as cancer, type 2 diabetes, atherosclerosis, and periodontitis that share macrophage-driven inflammation as a key feature, playing their dual role as killers (M1-like) and/or builders (M2-like). We also dissect the physio/pathological consequences related to macrophage polarization within selected chronic inflammatory diseases, placing polarized macrophages as a relevant hallmark, putative biomarkers, and possible target for prevention/therapy.

1. Introduction

Macrophages belong to the mononuclear phagocyte system (MPS), a family of professional phagocytes that includes monocyte and dendritic cells (DCs). Over the past few decades, classification of the cells within the MPS system has generated considerable controversy given the different, often confusing, nomenclature to identify macrophages in different physio/pathological conditions as a consequence of their plasticity, resulting in very different phenotype/functions.

The first open debate arises already in the definition of macrophage cell of origin. The classic scenario of the MPS stated that monocytes recruited from the periphery, under the influence of specific tissue-local growth factors, developed into macrophages. According to this scenario, macrophages derive from hematopoietic progenitors of bone marrow that differentiate under the influence of specific growth factors within the hosting tissues [1]. These cells primarily enter the blood as monocytes and further infiltrate tissues as macrophages, where they adapt to the local microenvironment to play out specific functions, such Kupffer cells in the liver, microglial cells in the brain [2], and mesangial cells in the kidney [3].

This view has been completely reconsidered over the last decade, and the ontogeny of macrophages has been totally rewritten, based on genetic approaches of cell fate mapping. New evidence demonstrated that macrophages can originate from embryonic precursor cells that colonized developing tissues before birth (foetal tissue macrophages) and that tissue-resident macrophages have self-maintaining abilities

in the adulthood. Murine models allow the definition of three main sources for tissue-resident macrophages: (i) the yolk sac in the embryo as a source for progenitor cells by primitive hematopoiesis; (ii) the foetal liver, where the hematopoiesis takes places, shifting form the yolk sac, and (iii) the bone marrow that becomes the elicit hematopoietic centre in late embryos and adult organisms [4–6]. Another intriguing scenario, concerning the origin and persistence of macrophages, has been proposed by Gomez et al. [7]. The model proposed that resident macrophages, developing in the embryo independently of the hematopoietic stem cell (HSC) compartment [2, 8–11], still persist in adults and can coexist with the so termed "passenger" leucocytes that include monocytes and DCs, which originated from bone marrow HSCs and myeloid progenitors [1, 12, 13].

The abundance of macrophages within tissues is finely controlled through the axis colony-stimulating factor-1 or macrophage-colony-stimulating factor (CSF-1 or M-CSF), IL-34, and colony-stimulating factor-1 receptor (CSF-1R) [14].

It has been reported that recruited macrophages differ from the resident tissues in terms of transcriptional profiling. Even if the term "macrophage activation" has been commonly used to describe macrophage activity in response to diverse stimuli, several studies pointed out that the results of cell activation deeply depend on the macrophage location and on the stimulus that triggers their activation.

In vitro and *in vivo* studies have shown that the phenotypic heterogeneity of macrophages correlates with peculiar functions specific to their local microenvironment [15] and this plasticity enables the appropriate response to pathogen or injury challenge.

Macrophage activation can be obtained in response to a plethora of diverse stimuli, including microbial products, damaged cells, activated lymphocytes, and inflammatory cells, and can result in the acquisition of distinct functional subsets undergoing different phenotypic polarizations.

Macrophage plasticity and heterogeneity give rise to a still opened debate, concerning the nomenclature to identify cell subsets/subtypes undergoing in such different phenotypic, functional (cytokine release), metabolic, regulatory (versus other arms of innate and adaptive immunity) rearrangements.

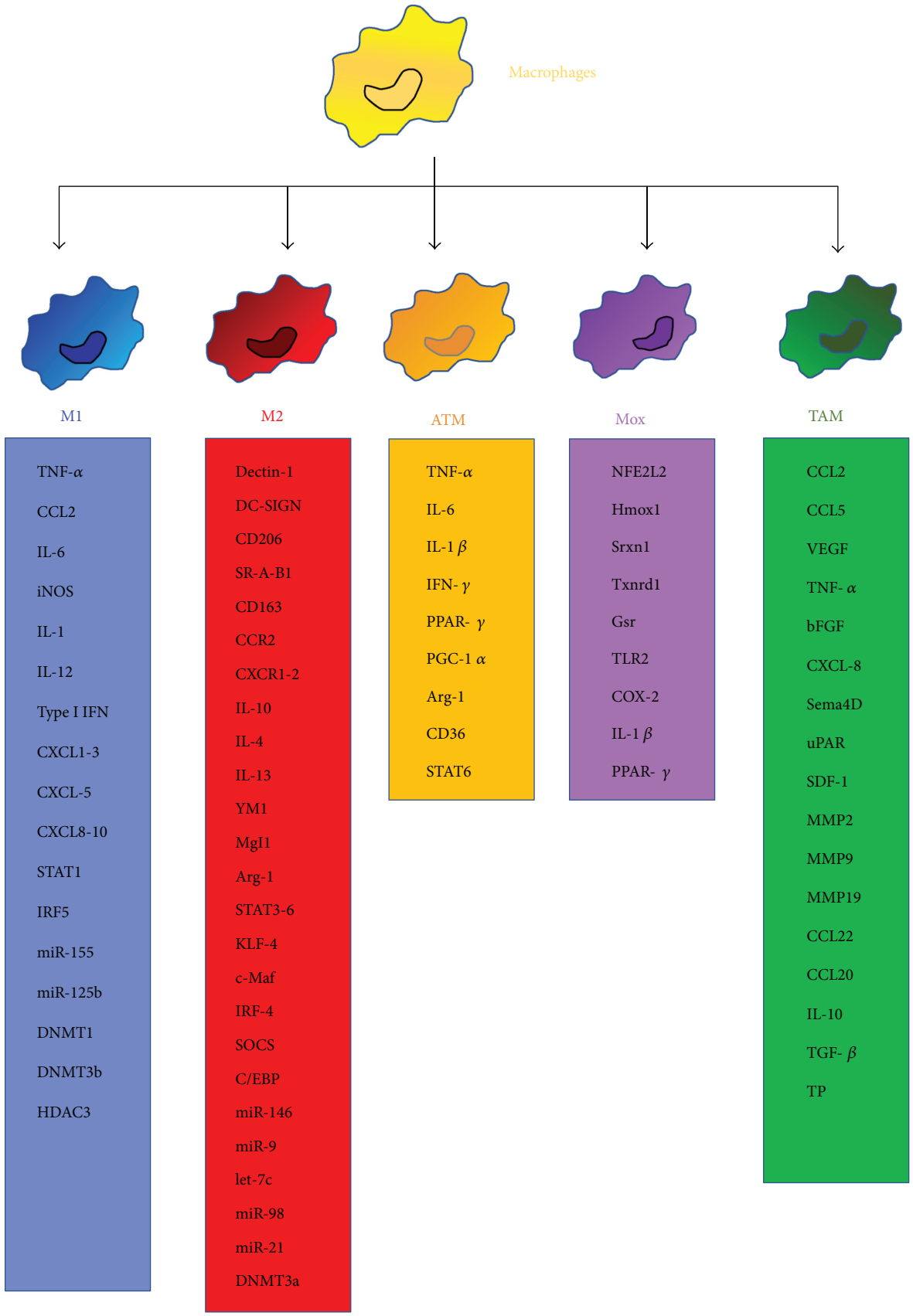
On the basis of the type-1/type-2 helper- T(h-) cell polarization concept [16, 17], phenotypically polarized macrophages have been defined according to two primary activation states, termed classically activated M1 and alternatively activated M2 (Figure 1(a)). M1 and M2 nomenclature has been long and lastly employed to define the "supposed" main subsets of macrophages, which originates in 2000 by Mills et al. [18]. Basically, M1 and M2 responses exemplify the opposing activities of killing (proinflammatory, "killer M1") and repairing (anti-inflammatory, "builder M2") [19].

However, macrophage polarization in many physiologic and pathologic conditions represents a continuum, involving high plasticity and heterogeneity of these effector cells, and resemble mainly to a spectrum of distinct polarization states that do not fit to the oversimplified M1/M2 classification. Hence, in line with a consensus recommendation, we decide to use "M1" to indicate only IFN-γ and LPS-driven macrophage phenotypes and "M2" to refer to macrophage phenotypes triggered only by IL 4 or IL 13. Furthermore, we use "M1-like" to illustrate diverse signal-induced polarization states that leads to cell cytotoxic function (killer) and antitumour activities and "M2-like" in relation to distinct phenotypes that share the functional capacity of repair, inducing new vessels and remodelling (builder) in parallel with tumour promotion and immunosuppressive ability toward T-cell responses [20] (Figure 1(b)).

In a normal tissue, the ratio of M1-like/M2-like macrophages is highly regulated and increases during the inflammation process [21]. Gene expression profile analysis showed that M1 macrophages can release high levels of proinflammatory cytokines, including tumour necrosis factor-α (TNF-α), CCL2 also known as monocyte chemoattractant protein-1 (MCP-1), IL-6, inducible nitric oxide synthase (iNOS), IL-1, IL-12, type I IFNs, CXCL1–3, CXCL5, and CXCL8–10 [22]. On the contrary, M2 macrophages have been demonstrated to express high levels of dectin-1, DC-SIGN (CD209), mannose receptor (CD206), scavenger receptor A, scavenger receptor B-1, CD163, CCR2, CXCR1, and CXCR2 [23] and to produce a large amount of IL-10, YM1, macrophage and granulocyte inducer-form 1 (MgI1), and arginase-1, highlighting their relevance during tissue remodelling and repair [24].

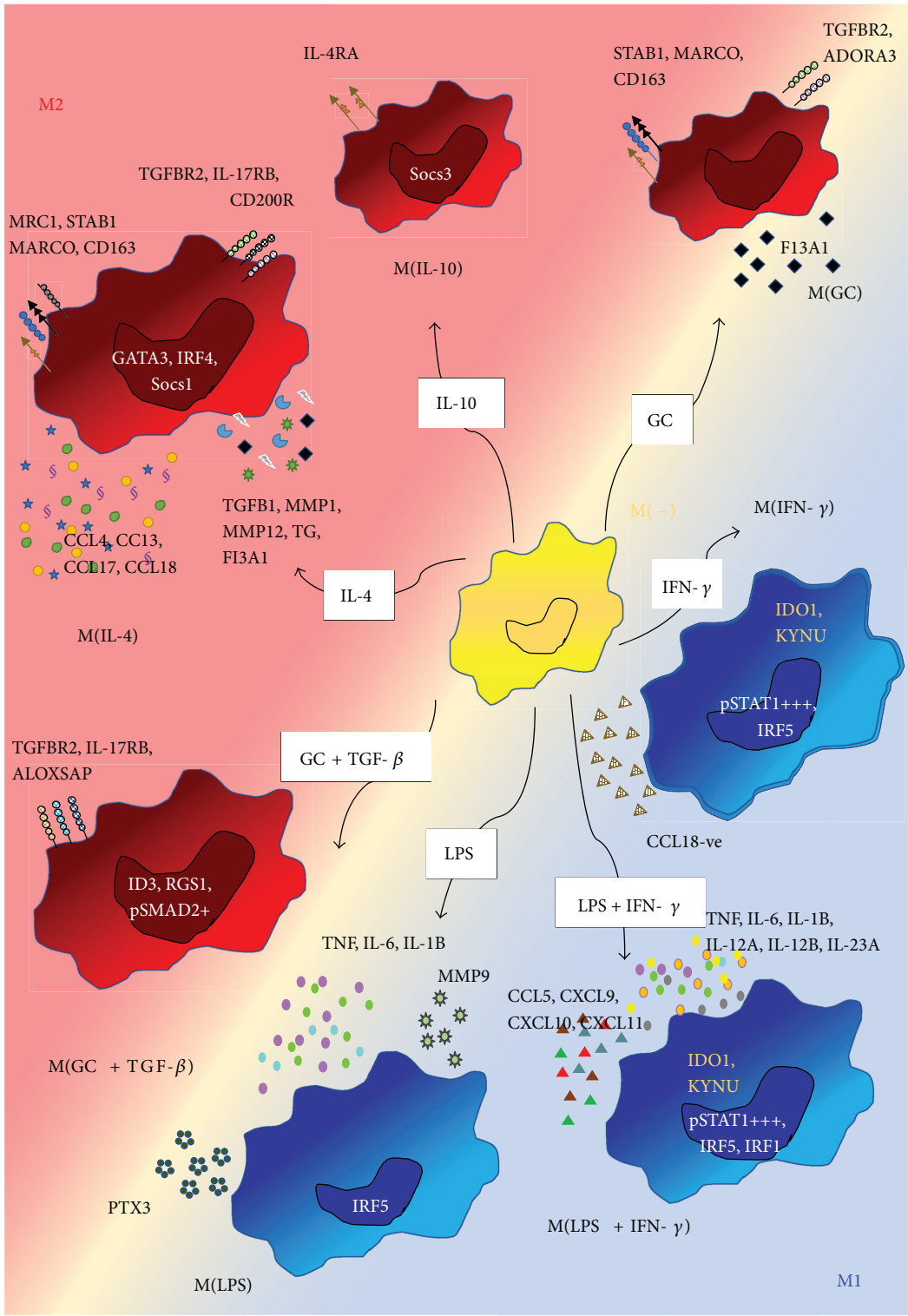
Macrophage polarization and functions are tightly regulated through the activation of several interconnected pathways. Among all, the balance between activation of STAT1 and STAT3/STAT6 has been demonstrated to play a crucial role; indeed, the predominance of STAT1 activation promotes M1 macrophage polarization, resulting in cytotoxic and proinflammatory functions. In contrast, STAT3 and STAT6 activation by IL-4/IL-13 and IL-10 signaling increases M2 macrophage polarization, associated with active tolerance and tissue repairing [22]. Moreover, the downstream effector of STAT6 and KLF-4 promotes M2 macrophage functions by suppressing the NF-κB/HIF-1α-dependent transcription. IL-10 promotes M2 polarization inducing p50 NF-κB homodimer, c-Maf, and STAT3 activities. In addition, IL-4 induces c-Myc that activates the IRF4 axis that inhibits IRF5-mediated M1 polarization, resulting in the M2 promotion [22]. Bouhlel et al. also demonstrated the relevance of PPAR-γ (peroxisome proliferator-activated receptor gamma) in skewing human monocytes toward an anti-inflammatory M2 phenotype. Indeed, the authors showed that PPAR-γ is highly upregulated in M2 macrophages and PPAR-γ agonists have been demonstrated to induce directly M2-like differentiation of monocytes *in vivo* and *in vitro* [25].

In the past decade, a novel class of small noncoding RNAs, termed microRNAs (miRs), has emerged as important regulators in biological processes. Accumulating evidence suggest a relevant role for several miRs in the polarization process (Figure 1(a)). In particular, miR-155 and miR-223 are involved in modulating macrophage activation state by targeting SOCS1, C/EBP (a hallmark of M2 macrophages), and Pknox1 [26]. Overexpression or silencing of miR-155 has been demonstrated to drive macrophages to M1 or M2 phenotype, respectively, confirming that miR-155



(a)

Figure 1: Continued.



(b)

Figure 1: Past and new concept in macrophage polarization. (a) Schematic overview of the different stimuli that can induce the diverse macrophage polarization state. M1: classically activated phenotype; M2: alternatively activated macrophages; ATM: adipose tissue-derived macrophages; Mox: atherosclerosis-associated macrophages; TAMs: tumour-associated macrophages. (b) The polarization landscape of macrophages. According to the different stimulation conditions, macrophages can acquire peculiar M1 or M2 phenotype, governed by the different surface antigen expressions, including scavenger receptors, chemokine, matrix-associated protein and cytokine release, and different patterns of transcription factors and metabolic pathway activated. The driver stimuli include IL-4, IL-10, glucocorticoids (GC) with TGF-β, glucocorticoids alone, LPS, LPS and IFN-γ, and IFN-γ alone.

plays a central role in regulating Akt-dependent M1/M2 polarization of macrophages. It has been also shown that miR-155 downregulates the expression of IL-13Rα1, suppressing the polarization toward M2 phenotype [27, 28]. Some studies have observed that let-7c was expressed at a higher level in M2 macrophages than in M1 macrophages. Accordingly, the upregulation of let-7c in macrophages diminished M1 phenotype and promotes M2 polarization targeting C/EBP-d [29, 30]. miR-146, miR-125b, miR-155, and miR-9 can inhibit TLR4/IL-1R signaling by regulating IRAK-1, TRAF6, IKKe, p50 NF-κB, and TNF-α [29]. Further, miR-17, miR-20a, and miR-106a reduce the expression level of the signal regulatory protein (SIRPa), an important macrophage differentiation-related marker. miR-98 and miR-21 downregulate the expression of inflammatory genes in monocytes and macrophages via controlling IL-10 level [31].

Emerging data have demonstrated that epigenetic mechanisms, including chromatin remodelling, DNA methylation (DNAm), histone modifications, and regulation of target gene expression, are also involved in the orchestration of macrophage polarization in response to local environmental signals [22, 32, 33]. M1 and M2 macrophages have been shown to express different levels of DNA methyltransferase (DNMT) 1, 3a, and b that are associated with gene silencing [34]. DNMT1 drives the M1 polarization in atherosclerosis by directly targeting the promoter of PPAR-γ in macrophages [35]. The DNMT3b binding of the promoter of PPAR-γ contributes to the M1 phenotype in adipose tissue during inflammatory process [33].

Lund et al. demonstrated that atherogenic lipoproteins can promote global DNA hypermethylation in monocyte [36]. Thus, DNMT inhibition or knockdown could decrease the M1 polarization, providing novel strategies for atherosclerosis prevention and therapy. Accordingly, the treatment with 5-aza-2-deoxycytidine (decitabine), a recognized inhibitor of DNMTs, results in an increased M2 polarization induced by the inhibition of the PPAR-γ promoter, which in turn prevents obesity-induced inflammation, atherosclerosis, and insulin resistance [37, 38]. DNMT3a and DNMT3al expression levels have been shown to be increased significantly in M2 compared to M1 macrophages, and this is associated with AMPK signaling [33]. On the contrary, DNMT3b was significantly lower in M2 compared with M1 adipose macrophages [39]. Histone H3 and H4 acetylations were found to be toughly associated with the maturation of human monocytes [40]. M1 polarization induced by IFN-γ increases histone H4 acetylation at the TNF-α promoter throughout the ERK and p38 mitogen-activated protein kinase (MAPK) signaling pathways [41]. STAT3 and MAPK activation and the simultaneous acetylation of histones H3 and H4 on the SOCS-3 promoter suppress the inflammatory responses in microglial cells and promote M2 polarization [42]. Histone deacetylase 3- (HDAC3-) deficient macrophages showed a decreased expression of IFN-β and Cox-1 showing an M2-like phenotype and thereby ameliorate many inflammatory diseases, such as pulmonary inflammation [43–45].

Such heterogeneity in macrophage phenotypes and functions generated the still open questions of whether they act as killers or builders. During inflammation, macrophages drive in the autoregulatory loop characterizing this process, as they release a wide range of biologically active molecules which participated in both detrimental (killers) and beneficial (builders) in inflammation [46–48]. Therefore, inflammation stands as the typical environmental setting where macrophages show their "Janus" behaviour [46–48]. During the first events occurring during inflammation, macrophages are endowed to kill/remove pathogens and damaged cells, while at the end of the inflammatory process, termed resolution of inflammation, macrophages act as builders that promote damaged tissue regeneration and return to homeostasis [49–51]. Since inflammation represents a shared hallmark from diverse chronic diseases and direct involvement in insurgence and progression of these conditions, here, we discuss whether macrophages can act as killers or builders within the inflammatory landscape of selected and apparently "distant" pathologic conditions.

2. Macrophages in Cancer: Killers or Builders?

Macrophages represent the most abundant tumour infiltrating inflammatory cells [52, 53]. Reflecting their extreme plasticity within healthy tissues, macrophages infiltrating tumours can acquire distinct phenotype and functions resulting in the attenuation of antitumour activity and induction of tumour-supporting functions and have been defined as tumour-associated macrophages (TAMs) with M2-like features (Figure 2). However, in the initial phases of carcinogenesis, macrophages can act as protective killer cells, cooperating with T lymphocytes in the control of early proliferating cancer cells in the immunoediting process [54]. Instead, in developing tumours, compelling evidence indicate that subverted macrophages or TAMs exert a major role in driving tumour progression by different mechanisms and pathways, depending on the types of tumour, tissues, and inflammatory mediators. The builder option of macrophages in the tumour microenvironment (TME) can lie to conditions in which a chronic nonresolving inflammation is established, a feature that has been defined a hallmark of cancer [55] and that points out TAMs as key inflammatory mediators able to link chronic inflammation with cancer development and progression [56, 57].

Among soluble factors that mediate their displacement, there are CCL2, CCL5, CSF-1, VEGF, and complement elements, which are often produced by the cancer cells and stromal cells in the TME. Moreover, some TAMs can derive from differentiation of monocytic myeloid-derived suppressor cells (M-MDSCs) via upregulation of CD45 tyrosine phosphatase activity in response to tumour hypoxia and following downregulation of STAT3 [58].

Tumour promoting or builder activities exerted by TAMs have been demonstrated by several studies. Elevated TAM infiltration has been correlated with worse clinical outcome in most malignant tumours, such as breast, cervical, ovarian, prostate, and thyroid cancers; Hodgkin's lymphoma; hepatocellular carcinoma; lung carcinoma; and cutaneous melanoma [56, 59–65]. In contrast to these findings, some reports have instead highlighted that tumour infiltrating macrophages correlated to increased survival in

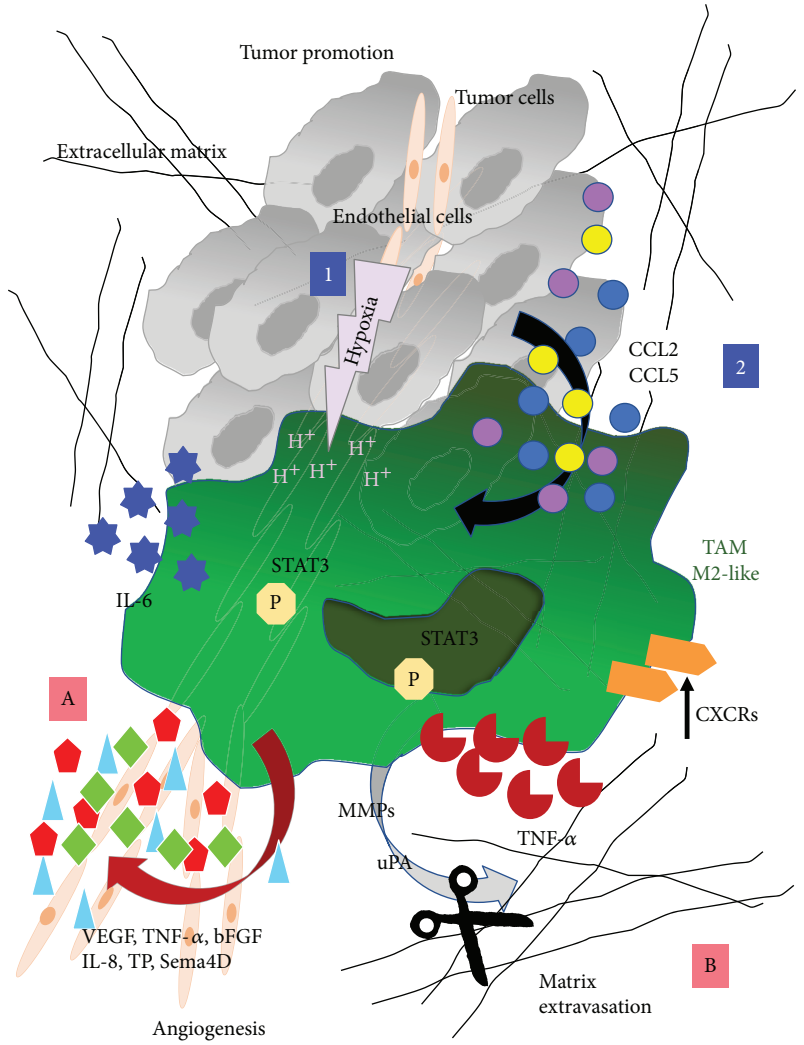


Figure 2: Macrophage polarization in tumour progression. Macrophage recruitment in tumours and their polarization are regulated by several factors. Among all, hypoxia can induce the differentiation of monocytic myeloid-derived suppressor cells (M-MDSCs) via upregulation of CD45 tyrosine phosphatase activity (1). Further, soluble factors, such as CCL2 and CCL5 that are produced by the cancer cells and stroma cells, can increase macrophage infiltrate (2). In the TME, infiltrating associated to tumours (TAM/M2-like macrophages) can orchestrate tumour progression by several mechanisms including the release of cytokine, chemokines, and tissue remodelling proteins. Hypoxia increases the expression of CXCRs in TAMs and promotes tumour angiogenesis by enhancing the production of VEGF, TNF-α, bFGF, IL-8, TP, and Sema4D that can induce endothelial cell proliferation, sprouting and migration, tube formation, and maturation of new vessel, followed by its stabilization by attaching mural cells (A). TAMs can regulate the extracellular matrix degradation by producing different types of enzymes and proteases, such as matrix metalloproteinases (MMPs), in particular MMP2, MMP9, plasmin, urokinase plasminogen activator (uPA) and cathepsins acting on connective tissue surrounding the tumour, and allow tumour cells to detach from the mass of origin and to disseminate, leading to the formation of distant metastases (B).

colorectal, prostatic, and lung cancer patients [66–70]. The main builder features of TAM include the ability to support tumour angiogenesis as well as lymphangiogenesis, to increase the breakdown of extracellular matrix, to promote tumour cell invasion and migration, and to suppress the anti-tumour immune responses [56, 62, 71, 72]. These functions are shared with M2-like macrophages that, in a physiological context, are induced during vascular and matrix remodelling, necessary for damage resolution [73–77].

TAM infiltrate is also associated with the onset of resistance to different chemotherapeutic agents through the activation of diverse pathways. In breast cancers, TAMs can induce IL-10/STAT3/Bcl-2 signaling, leading to an inhibition of apoptosis upon paclitaxel treatment [78]. In advanced lung adenocarcinomas, TAMs are also reported to decrease the responsiveness to target therapy based on the epidermal growth factor receptor tyrosine kinase inhibitors [79].

M2-like TAMs support tumour growth directly by producing cytokines able to stimulate the proliferation of tumour cells or indirectly, by fostering endothelial cell (EC) proliferation and angiogenesis (Figure 2). It has been reported that the growth of subcutaneous Lewis lung tumour is impaired in the CSF-1-deficient and macrophage-deficient mice [80]. Furthermore, the treatment of tumour-bearing

mice with recombinant CSF-1 reestablished the tumour growth, indicating a role for macrophages in tumour growth. TAMs can produce IL-6, whose release impacts on cell proliferation by a STAT3-dependent mechanism. Inhibition of STAT3 signaling blocks the antiapoptotic activity of IL-6 in human liver cancer cells [81]. TAMs are lower producers of TNF-α, resulting in enhanced tumour growth. Hypoxia significantly impacts on the TAM tumour cell interaction that induces the expression of CXCR4 and its ligand, CXCL12 (SDF-1), further supporting tumour cell dissemination and angiogenesis [82]. The number of TAMs within a tumour has been positively correlated with its metastatic potential, suggesting a role for TAMs in the distant dispersion of tumour cells [52, 83, 84]. By producing different types of enzymes and proteases, such as matrix metalloproteinases (MMPs), in particular MMP2 and MMP9, plasmin, urokinase plasminogen activator (uPA), and cathepsins [85–87] (Figure 2), TAMs can regulate the degradation of the extracellular matrix (ECM) and dictate tumour invasion and the metastatic process [19]. These factors act by relaxing the connective tissue surrounding the tumour, allowing tumour cells to detach from the mass of origin and to disseminate, leading to the formation of distant metastases.

TAMs sustain tumour angiogenesis by producing VEGFA (VEGF), the master growth factor involved in the angiogenic process. Besides VEGF, TAMs release a panel of proangiogenic factors which include TNF-α, basic fibroblast growth factor (bFGF), CXCL8/IL-8, thymidine phosphorylase (TP), adrenomedullin (ADM), and semaphorin 4D (Sema4D) [88–91] (Figure 2). These factors released by TAMs act by inducing endothelial cell proliferation, sprouting and migration of ECs into the tumour, tube formation, and maturation of new vessel, followed by its stabilization by attaching mural cells [92].

It has been recently reported that the expression of Sema3A from tumour cells is able to promote TAM accumulation inside the tumour, particularly in the avascular areas and required neuropilin-1 (NRP-1)-signaling cascade [93]. Macrophages are not only critical regulators of angiogenesis, but also crucial participants in lymphangiogenesis via VEGFC and VEGFD release, both in inflammatory settings and in tumour progression [94]. Thus, TAM-derived factors can link tumour angiogenesis and lymphangiogenesis [95–97].

Among TAMs, a relevant proangiogenic monocyte/macrophage subset, characterized by some distinctive features, has been further identified. These macrophages can express the angiopoietin receptor Tie2, termed TEMs (Tie2-expressing macrophages), and are closely associated with the vasculature [98, 99]. These cells have been implicated in the interference and in the resistance of action of antiangiogenic therapeutics, in particular vascular disrupting agents, and experimental data support the notion that inhibition of TEMs can foster antiangiogenic treatments with higher inhibition of angiogenesis and tumour spreading [100, 101].

Apart from their extreme plasticity, TAMs also sustain an immunosuppressive milieu aiding tumours to escape from immune surveillance [102]. TAM contribution to tumour progression acts also through synergistic interaction with other arms of the innate and adaptive immunity [46–48, 103] within the immunosuppressive TME. TAMs can interact with MDSCs, neutrophils, and DCs [104, 105]. TAMs also orchestrate the recruitment of T regulatory cells, by secreting CCL20 [106, 107] and CCL22 [108], and their activation through a bidirectional interaction by the release of IL-10 and TGF-β [107, 109–111].

Moreover, TAMs represent an important factor for the establishment of the premetastatic niche [112–116].

Different therapeutic strategies have been developed to target TAM physiology with encouraging preclinical and clinical results, either by blocking their tumour recruitment and functions or by redirecting their features to antitumour effector activities [57, 81, 117–121]. In several preclinical experimental models, including prostate, breast, and lung cancer and melanoma, the specific inhibition by antibodies of CCL2 has proven its promising effects, and when they are delivered in combination with chemotherapy shown enhancement of the effectiveness of treatment [122, 123]. However, though in a mouse model of breast cancer, it has been reported that a rebound effect following inhibition of CCL2 pathway resulted in the recruitment of monocytes/macrophages into the tumour and enhancement of lung metastasis [124]; different antibodies targeting CCL2 have been entered phase I and II clinical trials. Regarding the CCL5-CCR5 axis blocking strategies, a CCR5 antagonist has been approved as a treatment for patients with liver metastases of advanced refractory colorectal cancers and preliminary results indicated that this approach can lead to clinical responses [125]. Another interesting TAM-specific therapeutic treatment involves interferences with the CSF-1-CSF-1R axis, and in particular the receptor tyrosine kinase CSF-1R. Several compound and antibody inhibitors have been developed and evaluated in preclinical models and in patients with different types of cancer [120]. Important clinical regressions were obtained from patients with diffuse-type tenosynovial giant-cell tumour, which experienced CSF-1R tumour overexpression [120]. Interestingly, in a mouse glioblastoma multiforme model, CSF-1R blockade did not affect the TAM numbers but instead the M2-like TAM polarization, which is associated with the block of glioma progression and improvement of survival [119]. Also, bisphosphonates, usually used to treat osteoporosis and to prevent bone metastases-related complications, can be used to target macrophages in the tumour context, although their cytotoxic effects have been illustrated initially toward osteoclasts [126]. Combination chemotherapy or hormonal therapy with bisphosphonates in different types of tumour has shown clinical synergistic effects, in particular in postmenopausal women with breast cancer [127]. Another encouraging therapeutic strategy is related to agonistic anti-CD40 antibody and gemcitabine in pancreatic ductal adenocarcinoma patients. This approach revealed clinical responses and importantly demonstrated that in treated mice the CD40 agonist approach is responsible for reeducation of M2-like TAM toward an M1-like phenotype and of effective antitumour responses [128, 129]. Finally, a recently identified compound that found application in soft tissue sarcomas and ovarian cancer patients is trabectedin, which induces

selective TRAIL-dependent apoptosis of monocytes, macrophages, and M-MDSCs in the blood, spleens, and tumours with reduction of TAM numbers and angiogenesis [130, 131].

3. Macrophages in Type 2 Diabetes: Killers or Builders?

Type 2 diabetes (T2D) is a metabolic disorder, and its incidence has increased significantly in recent years. T2D is characterized by a peripheral resistance to the action of insulin and a failure of beta cells to compensate, leading to hyperglycaemia. It is now widely accepted that obesity increases the risk of T2D by inducing a chronic low-grade inflammation [132] and progression in local adipose tissue.

Accumulating evidence supports a role for tissue macrophages in a broad spectrum of inflammatory conditions [133], including obesity-associated metabolic diseases, such as insulin resistance and T2D [68, 134].

Macrophages together with other immune cells account almost 10% of the normal adipose tissue and play a key role in maintaining homeostasis. However, diet-induced obesity compromises homeostasis, resulting in an increased infiltration of macrophages representing up to 50% of the cells in adipose tissue [135, 136].

Several studies have established the crucial role of macrophage polarization in the development of T2D. The M1/M2-like polarization of tissue-destructive (killers) versus tissue-reparative (builders) macrophages is of great interest in clinical strategies because of their role in β-cell proliferation [137]. Recent evidence demonstrate that the high plasticity and phenotypic diversity of macrophages promote the cross-talk between β-cells, non-β endocrine cells, endothelial cells, mesenchymal cells, and other circulation-derived blood cells [138–140]. Builder-M2-like macrophages regulate β-cell proliferation through the release of a variety of trophic factors such as TGF-β1, which directly induce upregulation of SMAD7 in β-cells. SMAD7 in turn promotes β-cell proliferation by increasing CyclinD1 and CyclinD2 and by inducing nuclear exclusion of p27 [141] (Figure 3). In addition, M2-like macrophages also secrete Wnt ligands, thus activating the Wnt signaling pathway, and β-catenin, supporting β-cell replication [138] (Figure 3). Conversely, only a few studies investigating the polarization state of macrophages in pancreatic microenvironment have been described in literature [16–19], where an overall increase of macrophages/islets has been detected by immunohistochemistry. Eguchi et al. [142, 143] showed that Ly6c$^+$ M1 macrophage was expanded in the diabetic mouse islet. Ly6c$^+$-killer-M1 macrophage has been shown to secrete IL-1β, resulting in potent inhibition of insulin secretion, followed by islet destruction (Figure 3). The use of IL-1R antagonists and anti-IL-1β-neutralizing antibodies was able to abolish these effects on pancreatic islets [21–24].

Several studies in T2D have shown that M1-like macrophages resulted in increased inflammation, obesity, and insulin resistance, while M2-like macrophages are associated with a reduction in both obesity and insulin resistance [144]. M2-like macrophages are reported to not only suppress inflammatory cytokine IL-10 [145] but also provide a niche for preadipocytes to keep the number and quality of them, thus maintaining insulin sensitivity [146].

These data clearly suggest that macrophages play a nonredundant role in the pathogenesis of T2D [147]. An important aspect of diabetes prevention is a better understanding of the underlying mechanisms behind obesity-induced visceral adipose tissue inflammation, crucial for the development of T2D.

Obesity is associated with the accumulation of proinflammatory cells in visceral adipose tissue, which is an important underlying cause of insulin resistance and progression to T2D [148–150]. Establishing the initiating events leading to the switch from an anti-inflammatory M2-like state to M1-like phenotype remains elusive.

Recent studies show that obesity-induced adipocyte hypertrophy results in upregulated surface expression of stress markers. Adipose stress is detected by local sentinels, such as NK cells and CD8$^+$ T cells, which produce IFN-γ, driving M1-like adipose tissue macrophage (ATM) polarization [148–150]. Adipocyte hypertrophy has been reported to create hypoxic area and activates hypoxia-inducible factor-1, which induces inflammatory cytokines and suppresses preadipocyte-related angiogenesis and causes insulin resistance [151].

Normal adipose tissue macrophages phenotypically resemble the alternatively activated M2-like phenotype, expressing the mannose receptor, the CD206 surface antigen, and releasing Arg-1 and IL-10. In contrast, diet-induced obesity leads to a shift toward an M1 classically activated macrophage, characterized by the F4/80, CD11b, and CD11c expression [152] (Figure 3).

Low-grade inflammation in this setting is mediated by the polarization of recruited and resident macrophages to the M1-like phenotype in tissues, such as liver and adipose tissues [153, 154]. In contrast, M2 macrophage activation appears to protect against obesity-associated inflammation and insulin resistance [155, 156]. Several cytokines and chemokines, such as CCL2, interleukin IL-6 and IL-1β, macrophage migration inhibitory factor (MIF), and TNF-α, can be released by both adipocytes and macrophages [157, 158]. Macrophages within adipose tissue are recruited from the bone marrow and are characterized by a wide panel of factors that track with the degree of obesity [136, 159, 160]. Indeed, the paracrine as far as the endocrine activity was exerted by the proinflammatory cytokines, including TNF-α, IL-6, and IL-1β released by ATMs can induce decreased insulin sensitivity through the activation of Jun N-terminal kinase (JNK), inhibitor of IKκB (IKK-β), and other serine kinases in insulin target cells [161, 162].

The unbalance in the ratio between M1-like and M2-like adipose macrophages has been considered to be directly related to the development of insulin resistance [21, 149]. Insulin resistance resulted from a transition in macrophage polarization from the M2-like activation state, induced by STAT6 activation and PPAR, to a classic M1-like activation state, further driven by NF-κB, AP1, and other related factors [163–165].

The network of molecular mediators that regulate M2 polarization in response to hypermetabolism is not fully

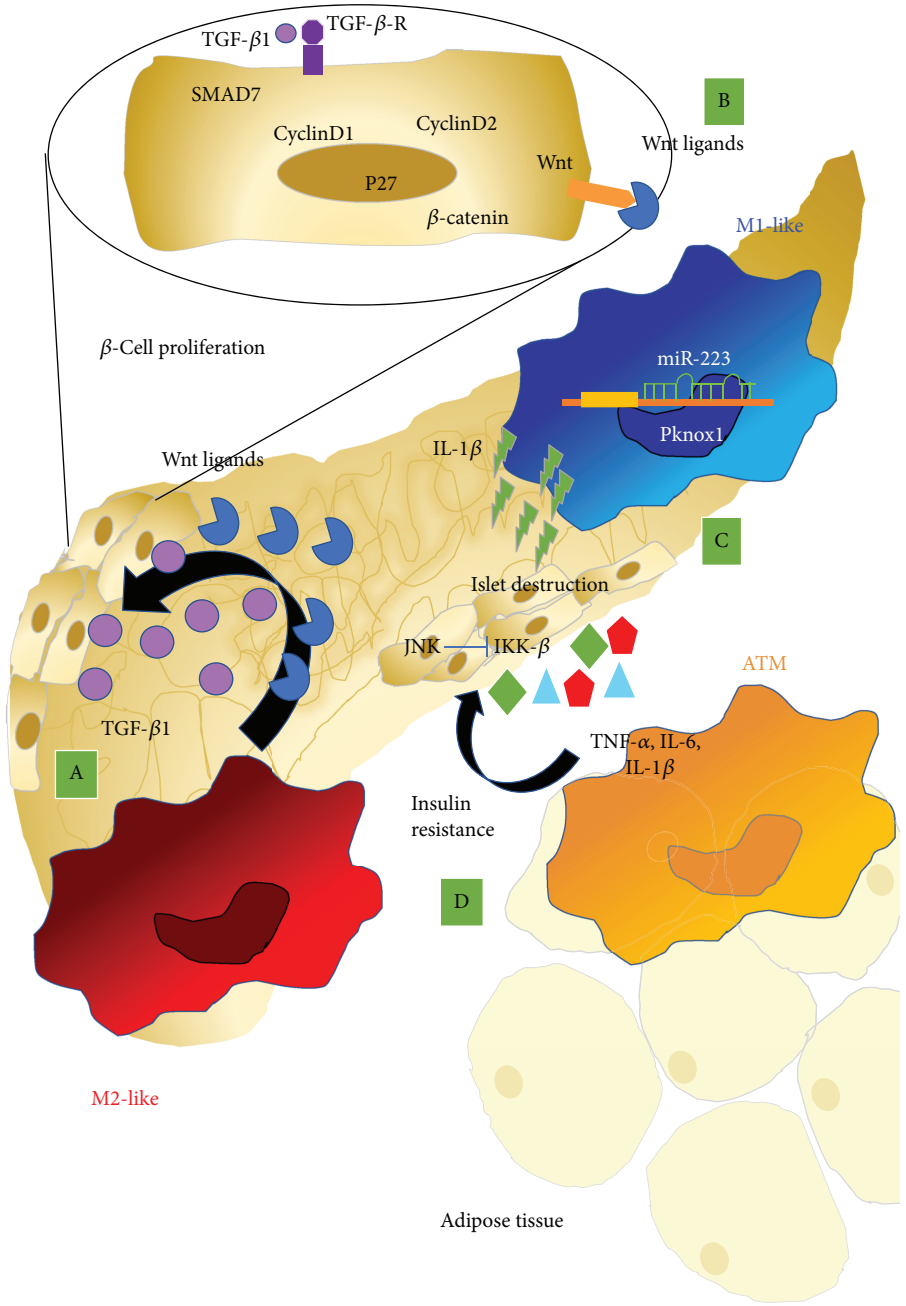


Figure 3: Macrophage polarization in type 2 diabetes. Macrophage within pancreatic tissues can be switched toward different functionalities according to the environment stimuli. M2-like macrophage supports B-cell proliferation by several trophic factors like TGF-β1 which directly induce upregulation of SMAD7 and increases of cyclinD1, cyclinD2, and p27 (A). Moreover, M2-like macrophages release Wnt ligands, thus activating the Wnt signaling pathway, and β-catenin, supporting β-cell replication (B). M1-like macrophage in pancreatic tissues can secrete IL-1b, inhibiting insulin secretion, followed by islet destruction (C). Adipose-derived macrophages (ATM) can release proinflammatory cytokines, including TNF-α, IL-6, and IL-1β that decrease insulin sensitivity through the activation of Jun N-terminal kinase (JNK), inhibitor of IKκB kinase (IKK-β), and other serine kinases in insulin target cells (D).

understood, but peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) and PPAR-γ target genes, such as arginase-1 and CD36, are implicated in this process. PPAR-γ has been proven to be essential for macrophage M2 polarization with the function of anti-inflammation and associated with metabolic dysfunction [145, 156, 166]. PPAR-γ was found to be a miR-130b target gene in regulating macrophage polarization insulin tolerance via repression of PPAR-γ [167]. Several studies have shown that PPAR-γ interacts with NF-κB, in the modulation of macrophage polarization. PPAR-γ blocked the proinflammatory pathway of NF-κB and inhibited the expression of relative factors, such as TNF-α [168].

Further, it was shown that IL-6 acts as a Th2-builder cytokine in obesity by stimulating M2-like polarization and local ATM proliferation, presumably due to upregulation of

the IL-4 receptor α [169]. Recently, it has been reported that adenosine monophosphate kinase (AMPK) β1 plays an important role in protecting macrophages from inflammation under high lipid exposure resulting in a modulation of obesity-induced insulin resistance (Figure 3). Genetic deletion of the AMPK β1 subunit in mice reduced macrophage AMPK activity, acetyl-CoA carboxylase phosphorylation, and mitochondrial content, resulting in reduced rates of fatty acid oxidation [170].

Inhibition of proinflammatory cytokines and chemokines, such as TNF-α, IL-1β, IL-6, and CCL2, may reduce adipose tissue inflammation and insulin resistance [147, 171, 172]. For instance, several studies have demonstrated that treatment with neutralizing IL-1β antibody or blockage of IL-1β signaling improved glycaemic control in diet-induced obese mice and insulin sensitivity in patients with T2D [173–176]. Other findings suggest that the CCL2-CCR2 signaling pathway disruption reduces adipose tissue macrophage content ameliorating insulin resistance and improves insulin sensitivity [160, 177]. CCL2 knockout mice receiving intact monocytes or mice receiving CCR2-deficient monocytes were both protected from the accumulation of macrophages in adipose tissue and the liver. [178] So far, targeting the CCL2-CCR2 signaling pathway may provide the basis for the development of novel therapies against T2D. *In vivo* studies have shown that circulating levels of free fatty acid (FFA) promote the generation of M1 macrophages via TLR4 signaling in adipocytes and macrophages in the setting of obesity [179–181]. In this context, adipose tissue inflammation is aggravated by the secretion of TNF-α, which in turn increases lipolysis leading to further production of FFAs establishing a vicious circle. Resistin is another potential target to combat insulin resistance or T2D. In fact, resistin induction which in turn stimulates secretion of several proinflammatory cytokines by increased infiltration of macrophages causes inflammation-induced insulin resistance [182–184].

Several phase II and III clinical trials have been initiated to inhibit key immunological processes of adipose tissue inflammation in T2D patients, such as NF-κB signaling, IL-1β function, or arachidonic acid metabolism, with promising results [148].

A shift in the polarization of adipose tissue macrophages from an M2-like state to an M-like proinflammatory state resulting in insulin resistance favours inflammation and insulin resistance [145]. Thus, targeting of inflammatory M1/M2-like polarization process of obese patients appears to be a promising future strategy for prophylaxis against diabetes development. For instance, adipose tissue macrophages from CCR2 knockout mice are polarized to the M2-like macrophages, even after obesity and CCR2 knockout mice were found to be protected from diet-induced insulin resistance [145, 160]. Furthermore, it has been shown that inhibition of IL-10 secreted by M2-like macrophages enhances the impairment of insulin signaling confirming its protective role in T2D [185].

Insulin-sensitizing thiazolidinediones (TZDs), clinically used for T2D patients [186], target the PPAR-γ that plays a key role in the maturation of M2-like macrophage and insulin sensitivity. PPAR-γ deletion prevents polarization of the monocyte/macrophage to the M2-like phenotype, and PPAR-γ-deficient mice exhibit glucose intolerance and insulin resistance [187]. Therefore, existing and future drug mechanisms may be involved in modulating the phenotypical and functional features of macrophages. For instance, metformin is a drug widely used to treat T2D, to decrease insulin resistance; it has been proposed that the benefit may result, at least in part, from modulating macrophage differentiation and polarization [188, 189]. How metformin can modulate the differentiation of Ly6C monocytes into M2-like macrophages remains the subject of ongoing interesting studies. In addition to glucose-lowering drugs, T2D patients are typically treated with low-dose aspirin (acetylsalicylic acid) that has off-target anti-inflammatory properties. Aspirin exerts its anti-inflammatory effects via inhibition of cyclooxygenase and a subsequent decrease in the proinflammatory prostaglandins [190]. Recently, it has been demonstrated that aspirin-triggered resolvin D1 into a degradable biomaterial after injury was able to significantly increase the accumulation of anti-inflammatory monocytes and M2-like macrophages while limiting the infiltration of neutrophils and increase proregenerative immune subpopulations [191].

Incretin-based treatments and the cannabinoid 1 receptor (CB1) blocker rimonabant have anti-inflammatory effects and may protect the pancreatic islets from IL-1β-driven. However, this anorectic antiobesity and glucose-lowering drug had also psychiatric side effects [164, 192, 193].

Several studies highlight the role of miRs as key regulators of cell fate determination and significant contributors to the pathogenesis of complex diseases, such as inflammatory responses and T2D [194]. It was found that miR-223 inhibits Pknox1, suppressing proinflammatory activation of macrophages, and protects against diet-induced adipose tissue inflammatory response and systemic insulin resistance [195]; miR-130b was found to be a novel regulator of macrophage polarization via repression of PPAR-γ and a promising target for T2D therapy [167]; miR-27a was also proposed as a target of intervention for inflammation and insulin resistance in obesity [196].

In summary, M1/M2-like macrophage polarization and switching hold the key to the regulation of insulin sensitivity and T2D. Macrophage polarization toward the alternative M2-like phenotype may play a preventive role and also be a novel and useful strategy for the treatment of insulin resistance and T2D.

Novel macrophage-targeted strategies that are both tissue-specific and disease-specific hold a promise for the future management of the chronic inflammatory disorders that were covered in this review.

4. Macrophages in Atherosclerosis: Killers or Builders?

Atherosclerosis is a chronic inflammatory disease driven by an imbalance in lipid metabolism and a maladaptive immune response [197]. This disease is characterized by the accumulation of lipids in large- and medium-sized arteries forming plaque deposits that block the flow of the blood. Several

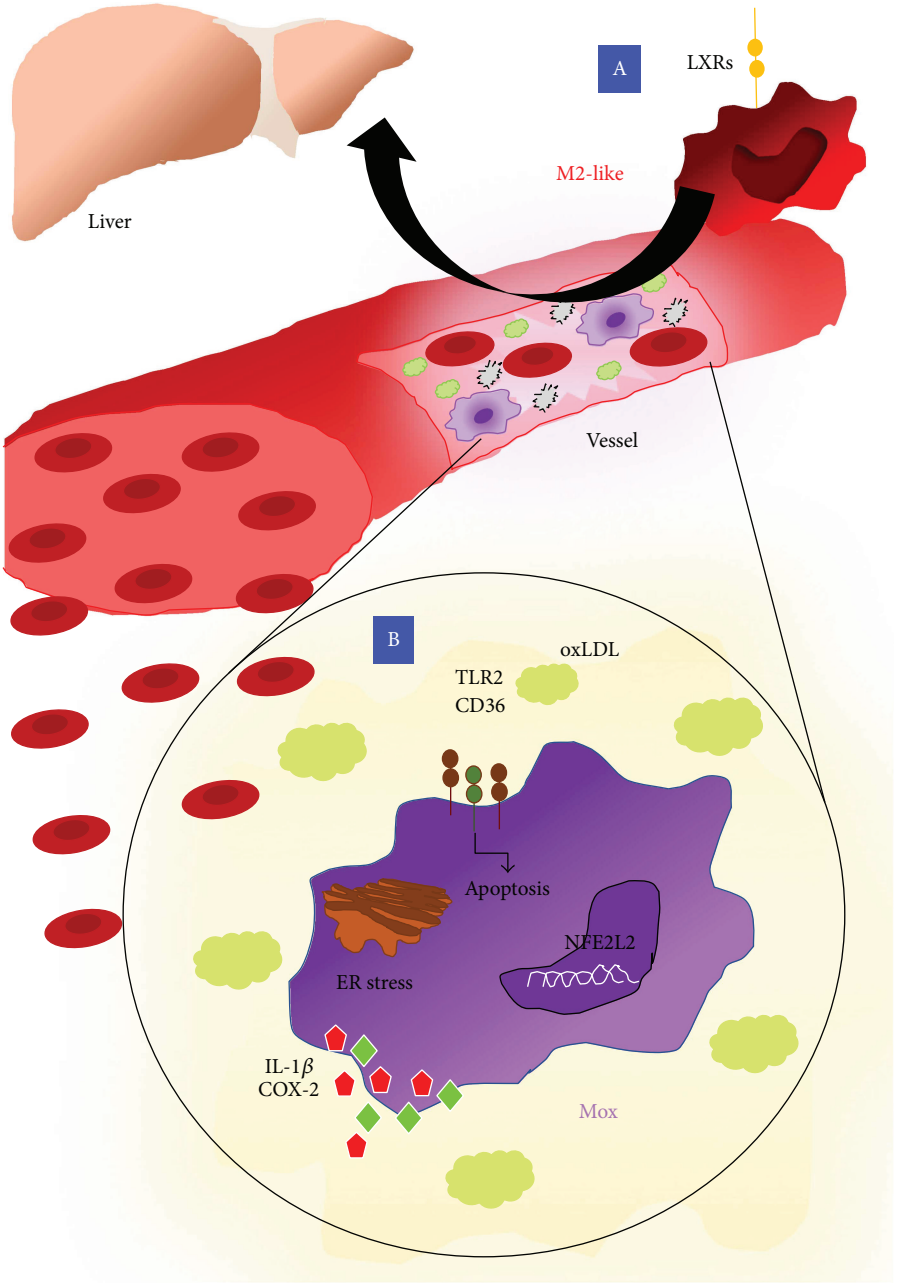


Figure 4: Macrophage polarization in atherosclerosis. Macrophages are crucial players involved in the atherosclerosis development due to their ability to regulate cholesterol efflux. In this context, the upregulation of LXRs in M2 macrophages has been found to exert a protective role. Indeed, LRXs reduce peripheral tissue excess cholesterol that is returned to the liver by releasing HDL in the plasma (A). Apart from M1 and M2 polarization, a third macrophage state has been described in the atherosclerosis context that is termed Mox. Macrophages exposed to oxidized phospholipids display reduced phagocytic and chemotactic abilities compared with M1- and M2-like macrophages and are characterized by the expression of the transcription factor NFE2L2 as far as Hmox1, Srxn1, Txnrd1, and Gsr genes. Mox macrophages also activate TLR2dependent mechanisms in response to oxidized lipids leading to an increase of IL1β and COX-2 (B).

factors have been correlated with the development of atherosclerotic diseases, among which the elevated low-density lipoprotein (LDL) cholesterol, hypertension, obesity, and both T2D and T1D. The accumulation of LDL promotes the recruitment of monocytes that lead to the formation of the atherosclerotic plaques [198]. Further, the exposure to CSF-1 and the uptake of oxidized LDL (ox-LDL) induce monocyte differentiation into macrophage and results in foam cell formation with the proliferation of smooth muscle cells [199]. The scavenger receptors lead the ox-LDL recognition, and the intracellular cholesterol is metabolized and transported to exogenous acceptors, such as high-density lipoprotein, through efflux proteins, such as ATP-binding cassette transporters [200] (Figure 4).

Macrophage apoptosis has been observed in patients with defects in the Acyl-CoA:cholesterol acyltransferase (ACAT),

the enzyme that re-esterificates free cholesterol in cholesteryl fatty acid esters [198]. Seimon et al. showed that oxidized phospholipids, oxidized LDL, saturated fatty acids (SFAs), and lipoprotein(a) can induce apoptosis in ER-stressed macrophages through a CD36- and TLR2-dependent mechanism [201] (Figure 4).

Several *in vivo* studies have demonstrated macrophage heterogeneity within the atherosclerotic plaque in response to the exposition of lipids and their oxidized derivatives [202]. Indeed, within atherosclerotic microenvironment, macrophages adapt their phenotype activating specific transcriptional programs. Cholesterol crystals that accumulate during the early stages of the atherosclerotic process might be involved in the activation of macrophages [202]. Cholesterol crystals can promote the caspase1-activating NLRP3 inflammasome, which results in the cleavage and secretion of IL-1 and may act as a M1-polarizing stimulus [203]. The proinflammatory M1-like phenotype can also be promoted by a mechanism that involves inhibition of the transcription Kruppel-like factor 2 [204, 205] or the activation of the TLR4-mediated pathway that in turn leads to the activation of NFκB [206]. Conversely, the anti-inflammatory M2-like phenotype is induced by 9-oxononanoyl cholesterol, a major cholesteryl ester oxidation product that can enhance TGFβ secretion [207]. Moreover, sphingolipid metabolites, such sphingosine1phosphate (S1P), promote the switching phenotype of mouse macrophages from M1- to M2-like state, by activating S1P1 receptor [208].

Recently, a third macrophage phenotype has been described in the atherosclerosis context that has been termed Mox (Figure 4) and represents macrophages exposed to oxidized phospholipids [209–211]. In advanced atherosclerotic lesions of mice, Mox macrophages comprise approximately 30% of the total number of macrophages [212]. Mox phenotype can be triggered by the activation of transcription factor NFE2L2 [212, 213]. Mox macrophages display reduced phagocytic and chemotactic abilities compared with M1- and M2-like macrophages. In mice, Mox macrophages typically express NFE2L2-mediated redox regulatory genes, including *Hmox1*, *Srxn1*, *Txnrd1*, and *Gsr* [212]. Nevertheless, in response to oxidized phospholipids, Mox macrophages activate TLR2-dependent mechanisms that lead to an increase of IL-1β and COX-2 expression [214].

Circulating monocytes in murine models have been classified into two major subsets, described as Ly6C^{hi} and Ly6C^{low} monocytes. In apolipoprotein E-deficient (ApoE$^{-/-}$), mice the increase of Ly6C^{hi} subset (corresponding to human M1-like subset) has been observed within atherosclerotic plaques [215].

Several studies have also correlated macrophage polarization with the clinical course of atherosclerosis. Among all, de Gaetano et al. [216] observed a marked difference in a macrophage subset between symptomatic and asymptomatic plaques. Indeed, M1 macrophages were found to be abundant in the developed lipid core of the symptomatic plaque and were rarely found in the intimal regions of the plaque, while M2-like macrophage number was higher in asymptomatic atherosclerotic plaques, suggesting a potential protective role of M2-like macrophages. Moreover, in mouse models, it has been demonstrated that in the regressing plaque a decrease in the number of macrophages occurs and, in some, a switch of their phenotypic characteristics has been observed, with an enrichment in M2-like phenotype, suggesting that this is a common signature of regressing plaques [217].

Despite several current standard therapies for atherosclerosis that may influence general immune responses, including angiotensin-converting enzyme (ACE) inhibitors, β-blockers, aspirin, and corticosteroids, these drugs lack specific macrophage targeting and may only be recognized as mild modifiers of macrophage activity [218]. Several common pharmacological agents have already been proposed to modulate macrophage activity for the prevention as well as the treatment of inflammatory-related diseases, including atherosclerosis. PPAR-γ is a crucial factor involved in the regulation of macrophage lipid metabolism and inflammatory responses and, as already discussed above, is upregulated in M2-like macrophages [25]. PPAR-γ activators might have therapeutic potential, and studies conducted by Bai et al. [219] suggest that mediator 1 (MED1) is required for the PPAR-γ-induced M2 phenotype switch and showed that MED1 in macrophages has an antiatherosclerotic activity via PPAR-γ-regulated transactivation, suggesting MED1 as a promising target for atherosclerosis therapy.

Natural ligands such prostaglandins and some pharmacological agents including anti-TZD that have been demonstrated to activate PPAR-γ have also been shown to decrease atherosclerosis progression. Choi et al. demonstrated that 5-(4-hydroxy-2,3,5-trimethylbenzylidene) thiazolidine-2,4-dione (HMB-TZD) reduced leukotriene B4 (LTB4) production and cytokine production by RAW264.7 macrophages and attenuates atherosclerosis possibly by reducing monocyte recruitment to the lesion [220]. In *in vivo* studies, selective inactivation of macrophage PPAR-γ impairs M2-like activation exacerbating diet-induced obesity [154], suggesting that PPAR-γ inducer might have a therapeutic potential. Likewise, liver X receptors (LXRs) have been found to be upregulated in M2-like macrophages and exert atheroprotective effects by modulating cholesterol metabolism and M1 macrophage-induced inflammatory genes, including iNOS, COX-2, and IL-6 [221] (Figure 4). Tangirala et al. have observed that in experimental models of atherosclerosis, LXR agonists induced a reduction of preexisting plaque size and this was associated with LXR macrophage activity. Indeed, macrophage-specific loss of LXRs resulted in a statistically significant increase in lesion size [222]. Moreover, the immunomodulatory drug fingolimod (FTY720) that has been described as a S1P1 receptor modulator has been shown to increase the proportion of M2-like macrophages in atherosclerotic lesions and reduce lesion progression in mice [223]. Statins, effective cholesterol-lowering agents, have also been reported to dampen immune responses through inhibition of macrophage inflammatory activity by increasing efferocytosis *in vitro* in a 3-hydroxyl-3-methylglutaryl coenzyme A (HMG-CoA) reductase-dependent manner, decreasing membrane localization of RhoA and preventing impaired efferocytosis by lysophosphatidic acid, a potent inducer of RhoA [224].

Stimulation of the macrophage autophagy-lysosomal system by the natural sugar trehalose has been reported to reduce the formation of the atherosclerotic plaque by limiting macrophage apoptosis and necrosis in the plaque cores [225].

Finally, some *Lactobacillus* has been observed to regulate M1/M2-like macrophage ratio by suppressing ox-LDL phagocytosis, thus blocking foam cell formation [226]. These data supported the employment of prebiotic or probiotic in atherosclerosis.

5. Macrophages in Periodontitis: Killers or Builders?

Gingivitis and periodontitis are two common diseases affecting the oral tissues and the health of the supporting structures of a tooth that share inflammation as a common feature. While in gingivitis the inflammatory process is limited to the soft tissues, epithelium, and connective tissue, in periodontitis, the inflammation is extended to the supporting tissues, including the alveolar bone [227].

Chronic periodontitis (CPD) occurs in response to specific bacteria within the oral biofilm and involves the destruction of tooth-supporting tissues. Major features for CPD are accumulation of immune cells in gingival connective tissue, resorption of alveolar bone, and the degradation of periodontal connective tissues, which lead to increased tooth mobility and eventual tooth loss [228, 229].

Chronic periodontitis is strongly associated with the presence of Gram-negative anaerobic bacteria in subgingival plaque, in particular, *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola*. Although initiated by bacteria, the bone pathology in CPD is mediated almost entirely by the host response that is thought to be responsible for the local tissue destruction observed in periodontitis [230]. In addition, the response to oral pathogens has systemic consequences. For example, infection and chronic inflammatory conditions, such as periodontitis, may influence the atherogenic process [231, 232].

It has been reported that monocyte/macrophages act as relevant killers in periodontal diseases by contributing to tissue breakdown. Elevated numbers of macrophages/monocytes associated with greater collagen breakdown and higher level of MMPs have been observed in samples from periodontitis [233]. Studies have shown that IL-1 was expressed predominantly by macrophages in the tissue isolated from periodontal patients [234]. In addition, higher levels of Receptor activator of nuclear factor kappa-B ligand (RANKL) protein, associated with macrophages, have been observed in the periodontitis tissues [235].

Activated macrophages have been found in the gingival epithelium, *lamina propria*, and perivascular tissues and in the blood vessels in human CPD. As lesions are associated with chronic periodontitis progress, increasing numbers of macrophages infiltrate into the gingival tissues [236]. Therefore, the gingival tissue and crevicular fluid of patients with chronic periodontitis have been reported to contain significantly increased amounts of CCL3, also known as macrophage inflammatory protein- (MIP-) 1α and CXCL-8/IL-8, as compared to healthy subjects [237, 238].

Porphyromonas gingivalis (*Pg*) is a key periodontal pathogen that promotes dysbiosis between host-and plaque-associated bacteria, thus resulting in both periodontal disease onset and progression [239, 240]. LPS from *Pg* activates macrophages through both TLR2 and TRL4 [241], and specifically, TLR2 activation by *Pg* LPS triggers the downstream stimulation of NF-κB, leading to the production of proinflammatory cytokines [242–244] (Figure 5).

Macrophages are frequently used as the *in vitro* model cells to define immune cell function in CPD studies. Transfer of TLR2 expressing macrophages to TLR2-deficient mice restored host sensitivity to *Pg* oral challenge [245] (Figure 5).

Pg LPS, in the presence of IL-1 and TNF-α, has been shown to induce cultured human fibroblasts and epithelial cells to release PGE2, a factor associated with periodontal bone resorption that promotes the proinflammatory M1-like macrophage polarization [229, 246–250] (Figure 5). IL-1 and TNF-α not only enhance inflammation but also promote bone resorption, a major concern in periodontitis [251–253]. Oral infection with *Pg* in BALB/c and C57BL/6 mice resulted in the influx of M1 macrophages into the submandibular lymph node (SMLN) and gingival tissue, together with an increase in alveolar bone resorption, as compared with untreated mice in a murine model of periodontitis [254, 255]. Selective SMLN macrophage *in vivo* depletion, using liposomes containing the proapoptotic agent clodronate, resulted in decreased *Pg*-induced alveolar bone *in vivo* resorption.

Pg infection enhances the secretion of the cytokines IL-1β, IL-6, IL-12, TNF-α, CSF-3 (G-CSF), and CSF-2 (GM-CSF), in addition to the chemokines eotaxin and CCL2–4 from macrophages, reflecting a M1 proinflammatory response (Figure 5). These cytokines and chemokines are known to act as proinflammatory mediators, to induce monocytes to migrate from the bloodstream into the gingival tissue, and to act synergistically to further stimulate proinflammatory cytokine production [246, 248, 249, 256]. IL-10, which is mainly produced by macrophages, was detected among the wide array of cytokines released during *Pg* infection [257]. IL-10 strongly supports M2-like macrophage and polarized functions including increased production of arginase-1, higher collagen deposition, and induction of fibrosis in gingival tissue, all common clinical features of chronic periodontitis [258–260].

In a recent study, Lam et al. observed that *Pg* can persist in naïve and M2-like, but not M1-like, macrophages for 24 hours. Phagocytosis of *Pg* also induced high levels of TNF-α, IL-12, and iNOS in M1 macrophages, but not in naïve macrophages (MØ) or M2 macrophages [254].

T. forsythia expresses a well-characterized TLR2 ligand, the BspA protein, and N- and O-glycan-linked glycoproteins that comprise its surface- (S-) layer, covering the outer membrane [261]. This S-layer has been shown to be important in delaying the cytokine responses of monocyte and macrophage cells *in vitro* [262, 263]. BspA and other ligands of *T. forsythia* induce TLR2 signaling favoring the development of Th2-type inflammatory responses detrimental to the alveolar bone that has been shown to be limited in TLR2$^{-/-}$ mice [242].

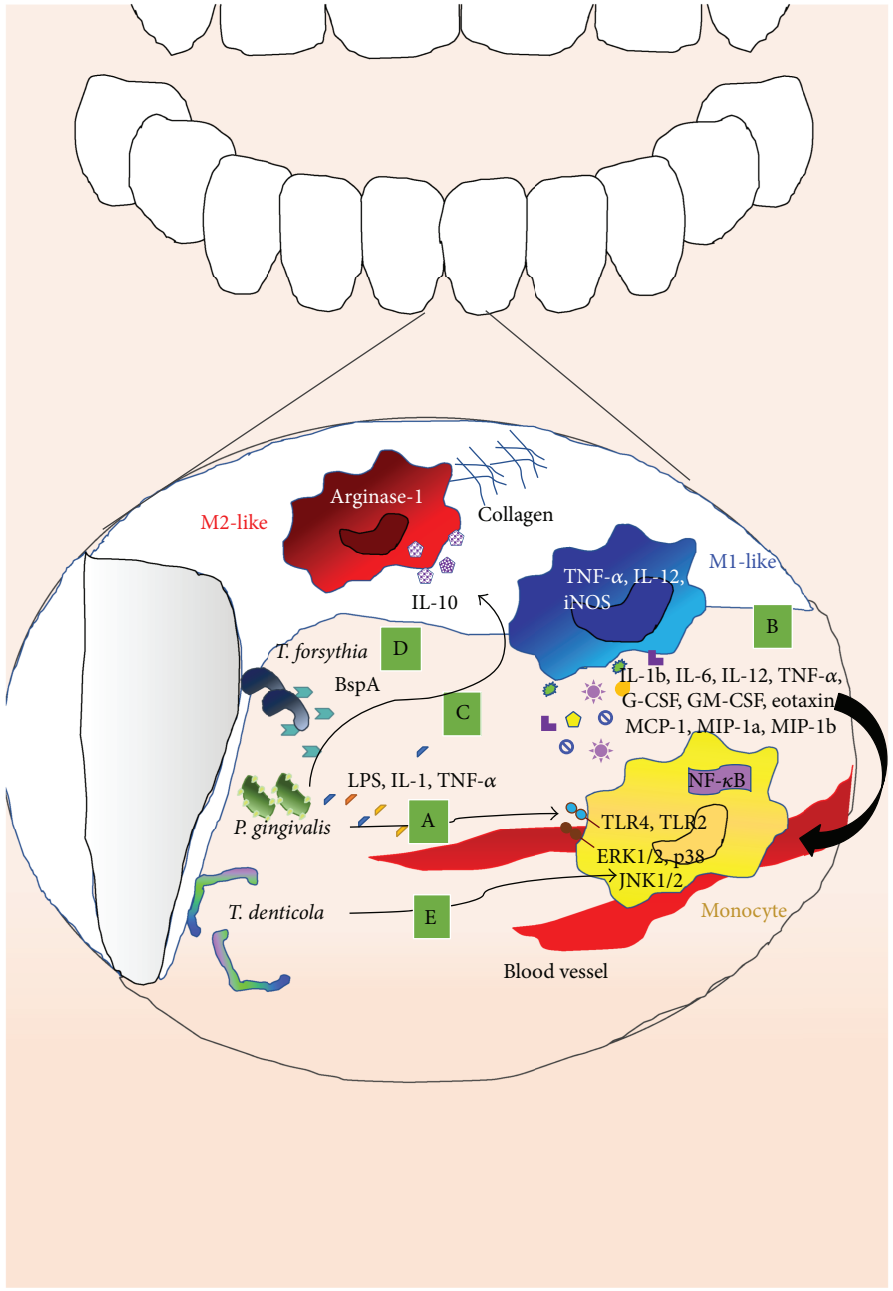


Figure 5: Macrophage polarization in periodontitis. Macrophages that have been found in the gingival epithelium can be activated by several microorganisms able to induce macrophage polarization toward M1- or M2-like phenotype. *P. gingivalis* releases LPS, IL-1, and TNF-α that promote the proinflammatory M1 macrophage polarization (A). Moreover, Pg infection enhances the secretion of IL-1β, IL-6, IL-12, TNF-α, G-CSF, GM-CSF, and the chemokines eotaxin, MCP1, MIP-1α, and MIP-1β from macrophages, reflecting a M1-like proinflammatory response (B). In spite of this, it has also been reported that *Pg* infection can also be associated with the increase of IL10, supporting M2 macrophage and increasing arginase-1 production and collagen deposition, leading to periodontitis (C). *T. forsythia* releases BspA and other ligands that induce TLR2 signaling favouring the development of Th2-type inflammatory responses (D). *T. denticola* induces TLR2 signaling that stimulates the prolonged activation of both ERK1/2 p38 and JNK1/2 in monocytes (E).

T. forsythia whole cells induced significantly greater amounts of IL-6 and IL-10 in wild-type (BALB/c) bone marrow-derived dendritic cells (BM-DCs) and macrophages, markers related to an M2-like polarization, as compared with TLR2$^{-/-}$ cells. The macrophage-inducible C-type lectin receptor (Mincle), a FcγR-coupled pathogen recognition receptor (PRR) [263, 264], has been reported to contribute to macrophage polarization [265]. THP-1 macrophages infected with the purified S-layer on whole wild-type *T. forsythia* elicit a M2-like polarization (IL-10, TNF-α) that is limited in Mincle knockdown macrophages or where infection is performed with the S-layer TfΔtfsAB-mutated form [265] (Figure 5).

Treponema denticola is among the most frequently isolated oral spirochetal species in patients with periodontitis

[266, 267]. Major surface protein complex (MSPc), which is expressed on the envelope of this treponema, plays a key role in the interaction between *T. denticola* and gingival cells and the related cytopathic effects [268]. *Treponema denticola* within the periodontium of the host has been reported to be associated with localized inflammation. MSPc has been showed to stimulate the release of the proinflammatory cytokines NO, TNF-α, and IL-1β from murine macrophages, both in LPS-responsive and LPS-nonresponsive murine macrophages [269]. Furthermore, IL-1β, IL-6, and TNF-α secretion by *T. denticola*-activated macrophages has been shown to exhibit potent bone reabsorption effects due to their proosteoclastic properties [270].

T. denticola-mediated macrophage response is mainly mediated by TLR2 and via MAP kinases [271]. One of the most highly conserved signaling cascades activated in both the innate and the adaptive immune systems involves a family of MAPKs including ERK1/2, p38, and JNK1/2 [272].

T. denticola stimulates the prolonged activation of both ERK1/2 and p38 in monocytes, and pharmacological inhibition of these pathways plays major roles in regulating both pro- and anti-inflammatory cytokine productions by *T. denticola*-stimulated monocytes [271] (Figure 5).

A study from Miyajima et al. reported a correlation between periodontitis-activated monocytes/macrophages and aortic inflammation in an *in vivo* ligature-induced experimental model of periodontitis. Gene expression profiling in circulating monocytes in this experimental model showed that periodontitis induced a M1-like specific signature with high levels of TNF-α and IL-6 as compared to controls, indicating that a M1-like phenotype of macrophages is induced by periodontitis [273]. This in turn supports the hypothesis that periodontitis-induced M1-like macrophages are the inflammatory orchestrators driving specific proinflammatory messages to the systemic vasculature [273]. The work from Miyajima et al. also showed that periodontitis-induced M1 macrophages can increase macrophage adhesion to aortic endothelial cells through the NF-κB/VCAM-1 axis [273]. These results clearly suggest that local-tissue alterations of macrophages during periodontitis can impact on circulating monocyte polarization and are associated to vascular alterations involved in apparently distant pathologies that shares inflammatory cell polarization as common features.

6. Conclusion

It is now widely accepted that inflammation represents a host hallmark of diverse chronic diseases, ranging from cancer, diabetes, and metabolic, cardiovascular, and neurological/neurodegenerative disorders. In the same way, inflammation has been recognized as a relevant condition for insurgence, maintenance, and progression of such disorders. Cell plasticity is a key and shared feature of inflammatory cells within the host organism that can potentially acquire killer (M1-like) or builder (M2-like) properties, based on the surrounding environment. Macrophages are the clearest example of immune cells that can be switched from killers to builders and vice versa, and this has been observed in all the inflammatory-based/associated disorders. Here we discussed the cellular and molecular mechanisms involved in macrophage switching to killers or builders in differently and apparently distant disorders, pointing out the attention on how the macrophages/microenvironment reciprocal interaction shape their polarization and distinct functional states.

Further, we discussed some approaches aimed at resolving this process, by interfering with aberrant macrophage killer/builder reciprocal switch. With this knowledge, it is clear that the identification of novel preventive and intervention strategies, along with effective compounds able in targeting/limiting/reverting proinflammatory macrophage polarization, are urgently needed and may represent a relevant tool to shape macrophage function action directly on them or on the hosting/surrounding environment.

Abbreviations

ACAT: Acyl-CoA:cholesterol acyltransferase
ADM: Adrenomedullin
AKT: Protein kinase B
AMPK: Adenosine monophosphate kinase
ATMs: Adipose tissue macrophages
Bcl2: B-cell lymphoma 2
bFGF: Basic fibroblast growth factor
BspA: Bark storage protein A
c-Maf: Avian musculoaponeurotic fibrosarcoma oncogene homolog
c-Myc: Avian myelocytomatosis viral oncogene homolog
C/EBP: CCAAT-enhancer-binding proteins
CCR: Chemokine receptor
CD: Cluster of differentiation
COX: Cyclooxygenase
CPD: Chronic periodontitis
CSF-1: Colony-stimulating factor 1
CSF-1R: Colony-stimulating factor 1 receptor
CXCL: C-X-C chemokine ligand
CXCR: C-X-C chemokine receptor
DC-SIGN: Dendritic cell-specific ICAM-grabbing nonintegrin
DC: Dendritic cells
DNAm: DNA methylation
DNMT: DNA methyltransferase
ECM: Extracellular matrix
ER: Endoplasmic reticulum
ERK: Extracellular signal-regulated kinase
G-CSF: Granulocyte colony-stimulating factor
GM-CSF: Granulocyte macrophage colony-stimulating factor
GSR: Glutathione-disulfide reductase
HDAC: Histone deacetylase
HIF: Hypoxia-inducible factor
HMB-TZD: 5-(4-Hydroxy-2,3,5-trimethylbenzylidene) thiazolidine-2,4-dione
HMG-CoA: 3-Hydroxyl-3-methylglutaryl coenzyme A
Hmox1: Heme oxygenase 1
IFN: Interferon
IKK: IκB kinase

IKKβ: Inhibitor of IKκB kinase
IL: Interleukin
iNOS: Inducible nitric oxide synthase
IRAK: Interleukin receptor-associated kinase
IRF: Interferon regulatory factor
JNK: Jun N-terminal kinase
KLF4: Kruppel-like factor 4
LDL: Low-density lipoprotein
let-7: Lethal-7
LPS: Lipopolysaccharide
LTB4: Leukotriene B4
LXRs: Liver X receptors
M-CSF: Macrophage colony-stimulating factor
M: Macrophage
MAPK: Mitogen-activated protein kinase
MCP: Monocyte chemoattractant protein
MCP1: Monocyte chemoattractant protein-1
MDSCs: Myeloid-derived suppressor cells
MED1: Mediator 1
MgI1: Macrophage and granulocyte inducer-form 1
MIF: Macrophage migration inhibitory factor
Mincle: Macrophage-inducible C-type lectin receptor
MIP: Macrophage inflammatory protein
miRNA/miR: Micro-RNA
MMPs: Metalloproteases
MØ: Naïve macrophages
Mox: Macrophages exposed to oxidized phospholipids
MPS: Mononuclear phagocyte system
MSPc: Major surface protein complex
NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B-cells
NFE2L2: Nuclear factor- (erythroid-derived 2) like 2
NLRP3: NLR family pyrin domain containing 3
NO: Nitric oxide
NRP1: Neuropilin-1
ox-LDL: Oxidized LDL
PD: Periodontitis
Pg: *Porphyromonas gingivalis*
PGC-1α: Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PGE2: Prostaglandin E2
Pknox1: PBX/knotted 1 homeobox 1
PPAR: Peroxisome proliferator-activated receptor
PRR: Pathogen recognition receptor
RANTES: Regulated on activation, normal T cell expressed and secreted
S1P: Sphingosine1phosphate
SDF-1: Stromal cell-derived factor-1
Sema4D: Semaphorin 4D
SFAs: Saturated fatty acids
SIRPa: Signal-regulatory protein
SMAD: Small mother against decapentaplegic
SMLN: Submandibular lymph node
SOCS1: Suppressor of cytokine signaling 1
Srxn1: Sulfiredoxin-1
STAT: Signal transducers and activators of transcription
T2D: Type 2 diabetes
TAM: Tumour-associated macrophage
TEMs: Tie2-expressing monocytes
TGF: Transforming growth factor
Th: T helper
TLR: Toll-like receptor
TNF: Tumour necrosis factor
TP: Thymidine phosphorylase
TRAF: TNF receptor-associated factor
Txnrd1: Thioredoxin reductase 1
TZD: Thiazolidinediones
uPA: Urokinase-type plasminogen activator
VCAM: Vascular cell adhesion molecule
VEGF: Vascular endothelial growth factor
YM1: Chitinase 3-like 3.

Disclosure

Antonino Bruno was a FIRC (Fondazione Italiana per la Ricerca sul Cancro) fellow and a fellow of Fondazione Umberto Veronesi (FUV).

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' Contributions

Luca Parisi and Elisabetta Gini share equal contribution as first authors. Giampietro Farronato, Antonino Bruno, and Lorenzo Mortara share equal contribution as last authors.

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Hindawi
Journal of Immunology Research
Volume 2017, Article ID 8160589, 7 pages
https://doi.org/10.1155/2017/8160589

Review Article

Macrophage Polarization in Cerebral Aneurysm: Perspectives and Potential Targets

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Received 27 July 2017; Revised 20 October 2017; Accepted 13 November 2017; Published 27 December 2017

Academic Editor: Hao Liu

Cerebral aneurysms (CAs) have become a health burden not only because their rupture is life threatening, but for a series of devastating complications left in survivors. It is well accepted that sustained chronic inflammation plays a crucial role in the pathology of cerebral aneurysms. In particular, macrophages have been identified as critical effector cells orchestrating inflammation in CAs. In recent years, dysregulated M1/M2 polarization has been proposed to participate in the progression of CAs. Although the pathological mechanisms of M1/M2 imbalance in CAs remain largely unknown, recent advances have been made in the understanding of the molecular basis and other immune cells involving in this sophisticated network. We provide a concise overview of the mechanisms associated with macrophage plasticity and the emerging molecular targets.

1. Introduction

Cerebral aneurysms (CAs) are a major cause of subarachnoid hemorrhage (SAH) [1]. Up to 50% of SAH patients die within the first 30 days after aneurysm rupture, and 30–50% survivors suffer from moderate-to-severe disabilities [2]. Clarification of mechanisms underlying the pathogenesis of CA is fundamental for developing effective therapies. In recent years, it is well recognized that inflammation plays an etiological role in the formation and rupture of CAs [3, 4], though several other factors mainly hemodynamic, genetic, environmental, and hormonal have been identified [5–8]. In particular, macrophages have been confirmed as critical effector cells in the progression of CAs [9]. In animal models, both macrophage depletion and inhibition of monocyte chemotactic protein-1 (MCP-1), a key chemoattractant of macrophages, are associated with a reduced incidence of CAs [10]. Macrophages are not homogeneous, and they are generally categorized into two subsets known as classically activated macrophages (M1-like) and alternatively activated macrophages (M2-like), respectively [11]. In general, M1 cells exhibit a proinflammatory effect while M2 cells facilitate resolution of inflammation and promote tissue repair. In response to various environmental cues (e.g., microbial products, damaged cells, and activated lymphocytes), macrophages can acquire distinct functional phenotypes via undergoing different phenotypic polarization, which are finely regulated processes [12, 13]. Their imbalances have been thought to be associated with various diseases [14]. Hasan et al. found that M1 and M2 cells were present in equal proportions in unruptured aneurysms; however, a marked predominance of M1 over M2 cells was documented in ruptured aneurysms [15]. Therapies targeting macrophage activation or preventing the M1/M2 imbalance may potentially halt aneurysm formation and rupture. In this review, we will focus on the factors that influence macrophage polarization in CAs. We will also discuss potential targets for CA therapies.

2. Molecular Mechanisms of Macrophage Polarization in Cerebral Aneurysm

Extensive research efforts have been made in defining the molecular networks underlying macrophage polarization. As shown in Figure 1, IRF/STAT (interferon-regulatory factor/signal transducer and activator of transcription) signaling is a central pathway in modulating macrophage M1-M2 polarization. A detailed description of these processes is

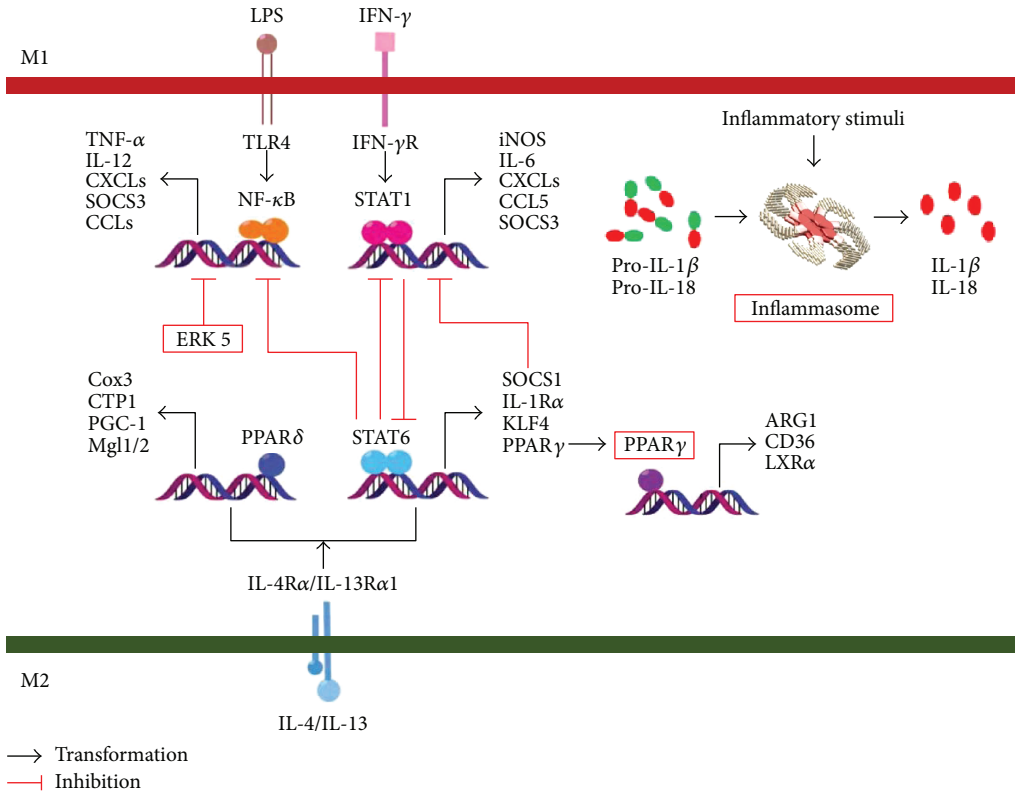

Figure 1: Mechanisms of macrophage polarization. Activation of IRF/STAT signaling pathways by IFN and TLR signaling skews macrophage function toward the M1 phenotype (via STAT1), while activation of IRF/STAT (via STAT6) signaling pathways by IL-4 and IL-13 skews macrophage function toward the M2 phenotype. PPARγ and ERK 5 participate in the promotion of M2 macrophage in cerebral aneurysms. NLRP3 inflammasome may contribute to M1 polarization.

provided in the excellent recent reviews on this subject [12, 16]. Here, we focus on the molecular mechanisms of macrophage polarization in cerebral aneurysm.

Toll-like receptor signaling, particularly the activation of TLR4 (Toll-like receptor 4), drives macrophages to a preferential M1 phenotype in cerebral aneurysms [17, 18]. The signaling pathway through the Myd88 (myeloid differentiation primary response gene-88) adaptor results in the activation of IKKβ (inhibitor kappa B kinase β). In addition, the activation of IKKβ leads to the phosphorylation and degradation of IκB (inhibitor kappa B), which permits the translocation of free NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) to the nucleus. As a key transcription factor related to macrophage M1 polarization, NF-κB activates the expression of a large number of inflammatory genes, resulting in tissue damage [19]. On the other hand, M2 phenotype is promoted by several transcription factors. For example, a recent study has shown that the activation of PPARγ (peroxisome proliferator-activated receptor γ) by pioglitazone promoted M2 activation to protect mice from CAs [20]. Besides, it has been reported that ERK5 (extracellular signal-regulated kinase 5) activation reduced the M1/M2 ratio by inhibiting the NF-κB pathway in CAs [21]. Although these promising results expand our knowledge of macrophage polarization in CAs, the molecular mechanisms that govern the phenotype switch of macrophages remain largely unknown. Recently, NLRP3 (nucleotide-binding domain and leucine-rich-repeat-containing protein 3) inflammasome has been detected in T cells and macrophages in the tissue of human CAs [22]; however, it remains unclear how NLRP3 inflammasome further regulates macrophage polarization. In addition, the role of miRNA (microRNA) in the development of cerebral aneurysm has been of particular interest. Several miRNAs (e.g., miRNA-133, miRNA-140-3p, and miRNA-145-5p) involved in the differentiation of macrophages have been identified in CAs, but their targets need further investigation [23].

3. Classically Activated Macrophages in Cerebral Aneurysm

Cerebral aneurysms are characterized by disruption of the internal elastic lamina (IEL), phenotypic modulation of smooth muscle cells (SMCs), apoptosis of mural cells, and extracellular matrix (ECM) degradation, which are considered as the hallmarks of CA [24]. Mechanistic links between chronic inflammatory response and these features have been provided by repeated animal studies [25, 26]. Cerebral aneurysm development is characterized by increasing polarization towards the M1 macrophage phenotype. Nowicki and coworkers have reported that the M1 to M2 macrophage phenotype ratio increased during the 2-week period as aneurysms developed in mice [27]. Inflammatory cytokines derived from M1 cells initiate the pathological changes of

aneurysmal walls, especially mediated by tumor necrosis factor α (TNF-α) [28, 29] and interleukin-1β (IL-1β) [30]. For example, TNF-α, an essential cytokine in the pathogenesis of CAs, initiates SMC phenotypic modulation that is an alteration from contractile to a proinflammatory and matrix remodeling phenotype [31]. In addition, IL-1β inhibits ECM biosynthesis in SMCs, thereby exacerbating degeneration of CA walls [32]. Sustained inflammatory response may eventually trigger apoptosis of mural cells, which ultimately leads to aneurysm rupture. On the other hand, the irritated SMCs further propagate the inflammatory cascades by secreting cytokines [31], which drive macrophages to M1 polarization. The interaction between M1 macrophages and SMCs may exacerbate the progression of CA through a positive feedback loop.

4. Inducing Alternative Activation of Macrophages Relieves Inflammation in CAs

Sustained chronic inflammation may result from dysregulated macrophage polarization. Macrophages can be driven to M2 phenotype not only by canonical M2 stimuli (e.g., IL-4, IL-13, and IL-10) but also by several transcription factors, including PPARγ and Kruppel-like factor 4 (KLF-4) [13]. PPARγ was identified as a critical factor in modulating macrophage M2 polarization induced by IL-4 or IL-13 [33, 34]. Recent study indicated that a PPARγ agonist, pioglitazone, exhibited a protective effect on preventing CA rupture in mice [35]. Moreover, Shimada et al. reported that decreased infiltration of M1 macrophage into the CAs and the macrophage M1/M2 ratio were documented following pioglitazone treatment. Interestingly, the beneficial effect of pioglitazone treatment was abolished in macrophage-specific PPARγ knockout mice. The authors concluded that activation of PPARγ in macrophages may act against CA rupture through reducing macrophage-related cytokines, including IL-1, IL-6, and MCP-1 [20]. Their study sheds light on noninvasive treatment of CAs by inducing inflammation regression, such as promoting M2 shift. However, the underlying mechanisms governing these processes remain to be elucidated.

5. Regulation of Macrophage Plasticity by Other Immune Cells

Besides macrophages, the representation of several other immune cell populations, such as neutrophils, natural killer (NK) cells, mast cells, and lymphocytes, is altered in CA walls [36]. As the initial responder to cellular stress, macrophages can contribute to the further recruitment and activation of adaptive immune cells. These immune populations elicit their effects on the potentiation or repression of inflammation by altering the activation state of macrophages, suggesting a highly complex regulation of the inflammatory processes in vascular wall (Figure 2). Over the last decade, orchestration of inflammation by these immune cells in CAs has been extensively investigated. For example, it has been reported that neutrophil blockade using anti-CXCL1 (C-X-C motif ligand 1) antibody attenuated polarization towards the M1 phenotype during the 2 weeks postaneurysm induction in mice, suggesting that CXCL1-dependent neutrophil inflammation may have an important role in macrophage polarization to M1 phenotype in the development of CAs [27].

Although best known for the contribution of mast cells in microbial defense and allergy, previous study has found that mast cells were invariably present in CA walls and were more pronounced in ruptured than in unruptured human CAs [15]. Reduced infiltration and activation of mast cells effectively attenuate destruction in aneurysmal walls, suggesting their roles in CA development [37]. Degranulation of mast cells led to increased expression of MMP-2 (matrix metalloproteinase-2) and MMP-9 and induced nitric oxide synthase, which result in damage to the vascular wall [38]. Moreover, they release cytokines, including TNF-α, IL-1β, and MCP-1, which potently activate M1 macrophages. By using mast cell degranulation inhibitors, decreased macrophage infiltration was evident in a rat model [37]. However, the biological mechanisms underlying interaction between M1 macrophages and mast cells remain unclear. Further studies are needed to determine the potential role of mast cells in macrophage polarization and the pathology of CAs.

Studies of specimens of human CAs have shown that both T and B lymphocytes robustly infiltrate the vessel wall, especially around the site of CA rupture; presumably, they are involved in the progression of CAs [36]. Nonetheless, the role of lymphocytes in the pathogenesis of CAs is controversial. Sawyer et al. found that CA formation and rupture in lymphocyte-deficient mice were significantly less prevalent than that in wild-type group, though they were equally subjected to a robust CA induction protocol [39]. Conversely, a recent study indicated that deficiency of T cells in rats failed to affect CA progression, degenerative changes of arterial walls, and macrophage infiltration in lesions [40]. As T lymphocytes can differentiate into distinct subsets following the local stimuli within the CA walls, it is tempting to speculate that a certain subset of T cells may contribute to the pathogenesis of CAs. In clinical, patients with CAs exhibited a $CD4^+$ T cell skewing in their peripheral blood, with more Th17 (T helper cell 17) and fewer Th2 cells. In line with these findings, IL-17 level was elevated while IL-10 was decreased. Although the representation of Th1 and Treg cells (regulatory T cells) in CA patients was not distinguished from that of healthy controls, altered cytokine profiles were detected. In patients suffered from CAs, the Th1 cytokines (IFN-γ, TNF-α) were increased whereas the production of IL-10 was declined significantly [41]. The imbalance of $CD4^+$ T cell was likely to facilitate inflammation in CAs. Their findings do not fully describe the range of functions that activated macrophages exert, but specialized T cells (Th1, Th2, Th17, and Treg cells) presumably participate in macrophage polarized activation [42, 43]. Considering their crucial roles in adaptive immune response, the effect of specific T subsets on macrophage polarization remains to be revealed.

6. Current Antiinflammatory Therapeutic Strategies and Future Directions

Since chronic inflammation is a key etiologic factor in CA formation and rupture, therapeutic attempts to interfere with

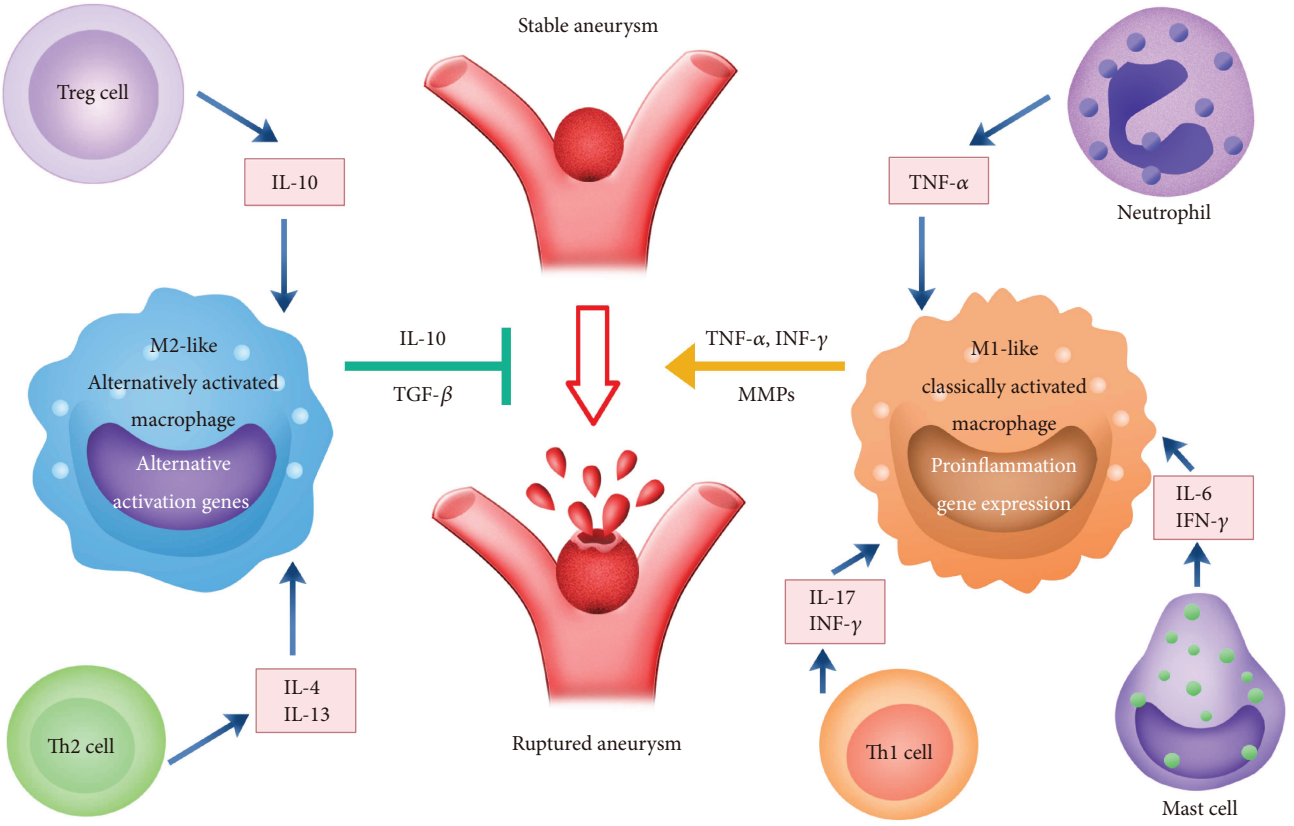

FIGURE 2: Summary of mediators and immune cells involved in M1/M2 polarization. The proinflammatory cytokines released by neutrophil, Th1 cells, and mast cells contribute to the maintenance of classically activated macrophage. Polarized M1 cells increase inflammation gene expression, promoting the progression of cerebral aneurysm to rupture. Conversely, alternatively activated macrophages may halt aneurysm rupture by facilitating inflammation regression. MMP indicates matrix metalloproteinase.

inflammatory response have potential importance. Several clinical agents have been investigated with varied success, perhaps the most promising one being aspirin [44]. Both direct macrophage imaging and histological examination have confirmed that aspirin ameliorates the inflammation of CA walls in human [45, 46]. Growing evidences indicate that administration of aspirin is associated with the reduced risk of CA rupture in humans [47, 48]. A detailed discussion of this subject can be found in recent reviews [49, 50].

Given the critical role of macrophages in the etiology of cerebral aneurysm to rupture, macrophage-mediated therapies, by directly effecting on macrophages or indirectly targeting other immune cells that regulate M1/M2 polarization, are likely to represent novel strategies for CA treatment [16]. As mentioned above, PPARγ was identified as a key factor inducing alternative M2 phenotype. In human atherosclerotic lesion, PPARγ activation primes monocyte toward an alternative M2 phenotype [51]. In parallel, reduced infiltration of M1 macrophage and the M1/M2 ratio are observed following pioglitazone in mouse model, raising the possibility that inflammatory cell PPARγ is emerging as a potential target for preventing CA rupture. Recently, NLRP3 inflammasome, a multiprotein complex initiating the maturation of pro-IL-1β and pro-IL-18, is detected in T cells and macrophages within the wall of human CAs [22]. Activation of NLRP3 inflammasome results in IL-1β and IL-18 production, which potently induce M1 polarization. It has been confirmed that Nlrp3-knockout mice show decreased M1 but increased M2 gene expression in adipose tissue macrophages [52]. These studies implicate that genetic elimination of the components of NLRP3 inflammasome may dampen the inflammatory response mediated by M1 macrophage. In contrast to recruitment of monocytes to arterial walls, the process of macrophage emigration from CAs may be impaired. In murine models of atherosclerosis, Netrin-1 was found to block macrophage movement by inhibiting actin reorganization, making cells refractory to emigration from plaques [53]. The mechanisms preventing macrophage egress from CAs warrant further exploration. Finally, with our refined recognition of the complex interactions between macrophages and other immune cells in CA wall, we are likely to enter a new era in which immune modulation can be proposed as a therapeutic strategy against cerebral aneurysm.

7. Concluding Remarks

In recent years, progress has been made in our understanding of dysregulated macrophage polarization in CAs; however, detailed processes remain fragmentary. It is likely that in the next few years, ongoing work in this field will continue. Future studies to delineate the mechanisms involving

macrophage plasticity in the environment of aneurysmal walls will enable new strategies for attacking CAs.

Abbreviations

CA: Cerebral aneurysm
SAH: Subarachnoid hemorrhage
MCP-1: Monocyte chemotactic protein-1
IRF: Interferon-regulatory factor
STAT: Signal transducer and activator of transcription
TLR4: Toll-like receptor 4
Myd88: Myeloid differentiation primary response gene-88
IκB: Inhibitor kappa B
IKKβ: Inhibitor kappa B kinase beta
NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells
PPAR: Peroxisome proliferator-activated receptor
ERK: Extracellular signal-regulated kinase
NLRP3: Nucleotide-binding domain and leucine-rich-repeat-containing protein 3
miRNA: MicroRNA
IEL: Internal elastic lamina
SMC: Smooth muscle cell
ECM: Extracellular matrix
TNF-α: Tumor necrosis factor α
IL-1β: Interleukin-1β
KLF-4: Kruppel-like factor 4
NK: Natural killer
CXCL1: C-X-C motif ligand 1
MMP: Matrix metalloproteinase
Th17: T helper cell 17
Treg: Regulatory T cell
TGF-β: Transforming growth factor-β
IFN-γ: Interferon-γ
SOCS3: Suppressor of cytokine signaling 3
iNOS: Inducible nitric oxide synthase.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this review.

Acknowledgments

This study was supported by the Science Foundation for Youth Scholars of Wuhan University (no. 2042016kf0128 to Lingmin Shao) and the National Natural Science Foundation of China (no. 81571147 to Xiaoxing Xiong). The authors thank Xueting Wu for her artistic drawing.

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Hindawi
Journal of Immunology Research
Volume 2017, Article ID 5482768, 10 pages
https://doi.org/10.1155/2017/5482768

Research Article

Modulation of Tumor-Associated Macrophages (TAM) Phenotype by Platelet-Activating Factor (PAF) Receptor

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Received 10 October 2017; Accepted 7 November 2017; Published 27 December 2017

Academic Editor: Zissis Chroneos

Platelet-activating factor (PAF) plays an important role in the pathogenesis of several types of tumors. The biological effects of PAF are mediated by the PAF receptor (PAFR), which can be expressed by tumor cells and host cells that infiltrate the tumor microenvironment. In the present study, we investigated the role of PAFR expressed by leukocytes that infiltrate two types of tumors, one that expresses PAFR (TC-1 carcinoma) and another that does not express the receptor (B16F10 melanoma) implanted in mice that express the receptor or not (PAFR KO). It was found that both tumors grew significantly less in PAFR KO than in *wild-type* (WT) mice. Analysis of the leukocyte infiltration shown in PAFR KO increased the frequency of neutrophils ($Gr1^+$) and of $CD8^+$ lymphocytes in B16F10 tumors and of $CD4^+$ lymphocytes in TC-1 tumors. PAFR KO also had a higher frequency of M1-like ($CD11c^+$) and lower M2-like ($CD206^+$) macrophages infiltrated in both tumors. This was confirmed in macrophages isolated from the tumors that showed higher iNOS, lower arginase activity, and lower IL10 expression in PAFR KO tumors than WT mice. These data suggest that in the tumor microenvironment, endogenous PAF-like activity molecules bind PAFR in macrophages which acquire an M2-like profile and this promotes tumor growth.

1. Introduction

Platelet-activating factor (PAF, 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine) is an inflammatory lipid mediator produced through the activation of A_2 phospholipase in response to different stimuli [1]. PAF is secreted by many different cell types, and the biological effects of this molecule are mediated by the activation of PAF receptor (PAFR), a G protein-coupled receptor expressed in monocytes/macrophages, polymorphonuclear leukocytes, platelets, endothelial cells, and other cell types as well as tumor cells [2–5].

Emerging evidence indicates that PAFR plays an important role in tumor growth [6–8]. Systemic treatment with PAFR antagonists resulted in the inhibition of tumor growth in murine melanoma, B16F10, and the human melanoma cell line, SK-MEL-37, engrafted in nude mice [9]. Transgenic mice overexpressing PAFR spontaneously developed melanocytic tumors [10]. In the tumor microenvironment, PAFR ligands can promote tumor growth, either by suppressing antitumor immune responses or by inducing tumor cell proliferation angiogenesis and production of growth factors [11, 12].

TAMs (tumor-associated macrophages) have been the subject of study for many research groups through the last few years. These are plastic cells that respond to the environment displaying a large phenotypic heterogeneity but that have been classified into two distinct extreme populations: classically activated macrophages (M1), which are characterized by high production of nitric oxide (NO) and reactive oxygen intermediates (ROI) and CD11c/IL-12 expression, and the alternatively activated macrophages (M2), identified by the expression of CD206 (mannose receptor) and IL-10, with high arginase activity and low NO production. In murine and human tumors, TAM generally exhibits an

alternatively activated phenotype which is associated with the promotion of tumor growth, extracellular matrix remodeling, angiogenesis, and the suppression of adaptive immunity [13, 14].

Some tumor cells also express PAFR; upon activation of the receptor, intracellular programs are switched in tumor cells that promote their survival and proliferation [11, 15, 16]. We have recently shown that TC-1 carcinomas express PAFR and the addition of PAF increased tumor cell proliferation *in vitro*. Moreover, the addition of PAF to human carcinoma cells transfected with PAFR (KBP) increased cell proliferation, whereas in KBM cells, devoid of the receptor, PAF had no effect [17]. Human cancer cells derived from uterine adenocarcinoma (HEC-1A) have been shown to secrete PAF, and treatment with PAF receptor antagonists inhibited their proliferation [18]. There is also evidence that leukemia cell lines and cells derived from esophageal cancer express PAFR since the addition of PAF was able to stimulate transcription of the cyclooxygenase-2 enzyme, the activity of which was associated with tumor growth [19].

The tumor-promoting effect of PAF-like activity molecules generated in the tumor microenvironment can be dependent either on their effect on host cells or on tumor cells. The experiments that showed a reduction of tumor growth after *in vivo* treatment with PAFR antagonists do not allow to discriminate whether they blocked the receptor in host or tumor cells. Experiments by Sahu et al. [20] favor the first hypothesis. The authors showed that melanoma cells treated *in vitro* with PAF before implantation potentiated tumor growth in wild-type but not in PAFR KO mice.

In an attempt to understand the relative contribution of PAFR in the tumor microenvironment, we used two different tumor cell lines, B16F10 and TC-1 to inoculate wild-type mice (WT) or genetically deficient PAFR mice (PAFR KO). These tumor cells have different embryonic origins, generate subcutaneous tumors in 100% of the inoculated mice, and are very well characterized in the literature. Using these experimental models, we investigated tumor growth, tumor leukocyte infiltrate, and the TAM phenotype.

2. Methods

2.1. Cell Lines and Animals. The B16F10 melanoma cell lineage was purchased from the American Type Culture Collection (ATCC CRL6475™, Manassas, VA, USA) and was maintained in DMEM (Dulbecco's Modified Eagle's Medium, GIBCO, Waltham, MA, USA) supplemented with 10% fetal calf serum (GIBCO), penicillin (100 units/mL), and streptomycin (100 μg/mL). The TC-1 cell line was kindly donated by Dr. Wu (John Hopkins, Baltimore), this cell line is a murine carcinoma derived from lung epithelium, transduced with HPV16 E6/E7 and c-Ha-ras oncogenes [21]. TC-1 cell line was maintained in 10% FCS in RPMI supplemented with 400 μg/mL neomycin. Cells have been regularly tested for mycoplasma and were free of this contamination. All cell cultures were incubated at 37°C under a humidified atmosphere of air containing 5% CO_2.

C57BL/6 wild-type mice (WT, PAFR expressing; age 6–8 week) and age-matched PAFR-deficient (PAFR KO) mice on a C57BL/6 background, generated as described [22], were a kind gift of Professor Takao Shimizu (Department of Biochemistry, University of Tokyo). All mice were housed in the Department of Immunology's Animal Facility at the University of São Paulo. The animals were maintained in specific pathogen-free conditions, with 12 h light/dark cycles and water and chow ad libitum. All experimental procedures were performed following the guidelines adopted by the Brazilian College of Animal Experimentation (COCEA) and were approved by the Ethical Committee for Animal Research of the Institute of Biomedical Sciences of the University of São Paulo (protocol number 130/2015).

2.2. Mouse Tumor Model. Tumor cells lines (B16F10 or TC-1) were injected subcutaneously in the dorsal flank of C57BL/6 WT and PAFR KO mice as single cell suspensions (5×10^5 in 100 μL) in PBS^{++} (phosphate-buffered saline supplemented with 1 mM $CaCl_2$, 0.5 mM $MgCl_2$). Tumor formation and size were measured with a caliper until the 15th day. Mice were observed and measured with intervals of 2 or 3 days from the day when they were injected. Tumor volume was calculated using the equation: $V = D^* d^2/2$, where V is the tumor volume, D is the largest measured diameter, and d is the smallest measured diameter of the tumor.

2.3. Cell Suspension Preparations. All cell preparations were made using ice-cold 1x Hanks' solution with 15 mM HEPES, pH 7.4, 0.5 U/mL DNase I (Worthington Biochemical, Lakewood, NJ, USA) and 5% FBS. Tumors were harvested after mouse euthanasia. The tumor cell suspensions were obtained by the digestion of finely minced tissue with 1 mg/mL collagenase I and IV (Worthington Biochemical Corp., Lakewood, NJ) in the buffer described above in a ThermoMixer (Eppendorf, Germany) at 37°C for 45 min. Spleen-nucleated cell suspensions were obtained by tissue dissociation through a 70 μm metal mesh and red cell lysis with ACK (ammonium-chloride-potassium) Lysis Buffer (Invitrogen, Invitrogen-Life Technologies, Carlsbad, CA, USA). Peritoneal macrophages were harvested after mouse euthanasia, by washing the peritoneal cavity with 5 mL ice-cold PBS. Cell viability, accessed by trypan blue staining, in the final suspensions was between 90% and 95%.

2.4. Flow Cytometry Analysis. Single cell suspensions were stained with different fluorochrome-conjugated antibodies (indicated in each figure). The antibodies used in this work were anti-CD4 (clone GK1.5), anti-CD8 (clone 53-6.7), antiGr1 (clone RB6-8C5), anti-CD11b (clone M1/70), anti-CD45 (clone 30-F11), and anti-F4/80 (clone BM8) purchased from BD Biosciences (San Diego, CA). Flow cytometry was performed in a FACSCanto II (BD Biosciences, San Jose, CA, USA), where 30,000–50,000 events were acquired. During data acquisition, debris and doublets were excluded. Data obtained were analyzed with the FlowJo software version 5.0 (TreeStar, Ashland, OR, USA).

2.5. Cell Sorting. $CD45^+$ cells and leukocytes were sorted from total tumor suspensions by positive selection after

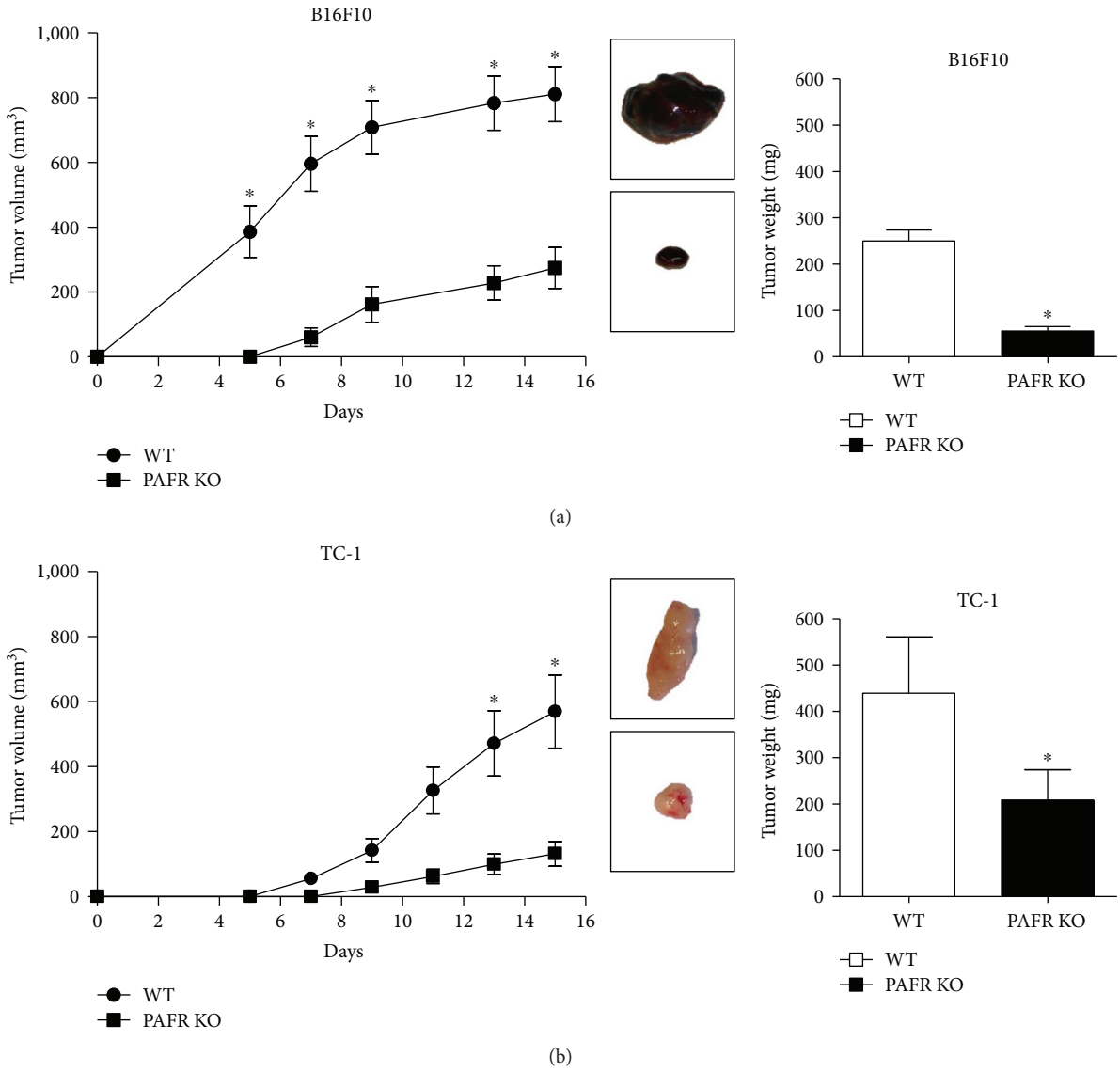

Figure 1: PAFR is important for B16F10 melanoma and TC-1 tumor growth. B16F10 melanoma cells and TC-1 tumor cells were injected (5×10^5 cells) into the dorsal flank of C57BL/6 WT or PAFR KO mice. The left panel shows the tumor growth kinetics of B16F10 melanoma (a) and TC-1 (b). Representative macroscopic images of the tumors at 15 days postinoculation are shown to the right of the tumor growth kinetics; each tumor depicted in the images correspond to the tumors displayed in the adjacent curve. $*$ indicates $p < 0.05$ (Mann–Whitney U test). In the right panels, we show the average weight of the tumors at 15 days postinoculation. Data were obtained from 3 independent experiments with 4 animals per experimental group. $*$ indicates $p < 0.05$ (t-test).

incubation with biotin-conjugated anti-CD45 magnetic beads (Miltenyi Biotec, Germany) and loading in columns exposed to a magnetic field (MACS LS$^+$ Separation Columns, Miltenyi Biotec). In general, we obtained 80–95% pure cells with at least 90% viability.

2.6. Quantification of Nitric Oxide, Arginase Activity, and IL-10. CD45$^+$-sorted cells were seeded in 6-well culture plate (10^6 cells/mL) in 10% RPMI treated with 10 ng/mL LPS (Sigma-Aldrich, St Louis, MO, USA) at 37°C for 72 h. Supernatants were then harvested for NO production and assessed by nitrite production in culture using the Griess reaction [23]. Arginase activity assay in total cell lysates was done as previously described [24] Aliquots of cell lysates were used for protein quantification by the Bradford assay (Bio-Rad; ref. 32). Murine IL-10 production was determined by ELISA (BD Biosciences, San Diego, CA, USA) according to the manufacturer's specifications.

2.7. Statistical Analyses. Tumor growth kinetics experiments were tested using the nonparametric Mann–Whitney U test. Data from all other experiments were tested by t-test, using the Prism 5.0 statistical program (GraphPad Software, San Diego, CA, USA). In all cases, $p < 0.05$ was considered significant. The number of animals or samples used in each experiment is indicated in the figure legends. Each experiment was repeated at least three times. Mostly, our data are represented as the average value of parameters

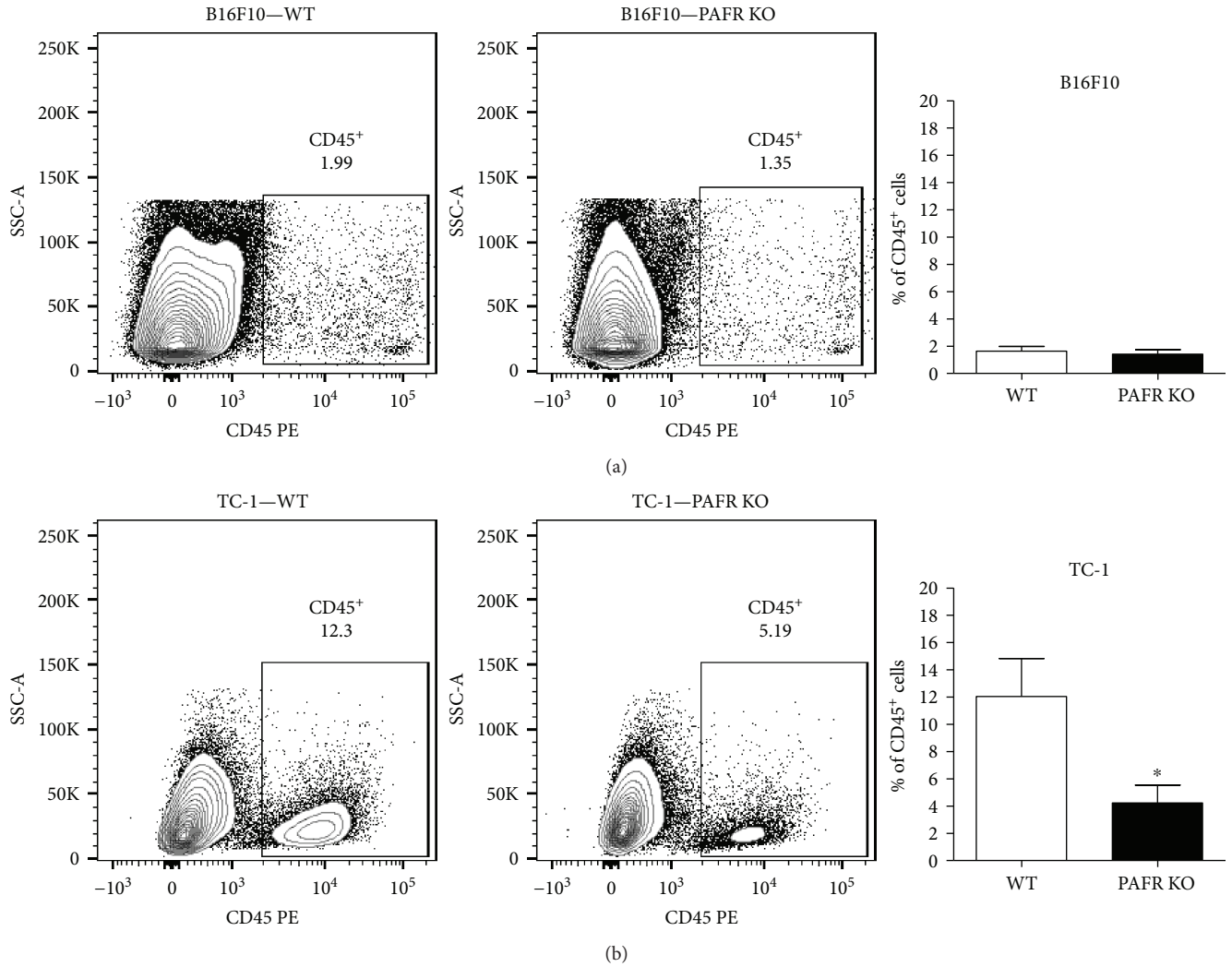

Figure 2: Inflammatory infiltrate in B16F10 melanoma and TC-1 tumors. Single cell suspensions of TC-1 (a) or B16F10 (b) tumors from WT and PAFR KO animals injected 15 days earlier were analyzed by flow cytometry. Cells were labeled with anti-CD45 antibody and 50,000 events were acquired per sample, using a FACSCanto cytometer. In the left panel: dot plots depicting SSC-A X CD45 expression (panleukocyte marker) of tumors from WT and PAFR KO animals. These plots were obtained by previously gating out debris and doublets. The gates indicate the $CD45^+$ populations. In the right panels, we show the average percentage of $CD45^+$ cells in each experimental group. Data were obtained from 3 independent experiments with 4 animals per experimental group. $*$ indicates $p < 0.05$.

obtained per experiment ± standard deviation (s.d.) unless otherwise indicated.

2.8. Data Availability. The datasets generated during and analyzed during the current study are available from the corresponding author on reasonable request.

3. Results

3.1. PAFR and Tumor Growth. We injected B16F10 melanoma and TC-1 carcinoma cells subcutaneously in WT and PAFR KO mice and followed tumor growth for 15 days. TC-1 cells express PAFR [17] whereas B16F10 do not [20]. After 6 days of cell inoculation, it was possible to detect palpable tumors in both mouse strains. However, in PAFR KO animals, the tumors were significantly smaller than in the WT. These results were consistently observed throughout the experiments for both, melanoma (Figure 1(a)) and for TC-1 carcinomas (Figure 1(b)). At the end of the experiment (day 15), tumor weight was also significantly smaller in PAFR KO mice. It is noteworthy that melanoma tumors had higher volume/weight rate (3.2) compared to TC-1 (1.2), which was compatible with the observation that melanoma was more edematous. Thus, the presence of PAFR in host cells seems to be relevant for tumor growth.

3.2. PAFR and Tumor Inflammatory Infiltrate. Our previous data suggest that PAFR signaling in the host plays a role in tumor growth. The tumor microenvironment is not only constituted by neoplastic cells but also several cell types recruited from the bloodstream, constituting the tumor inflammatory infiltrate. The inflammatory infiltrate can provide signals that inhibit or favor tumor growth [25]. Moreover, several of the cell types present in the tumor

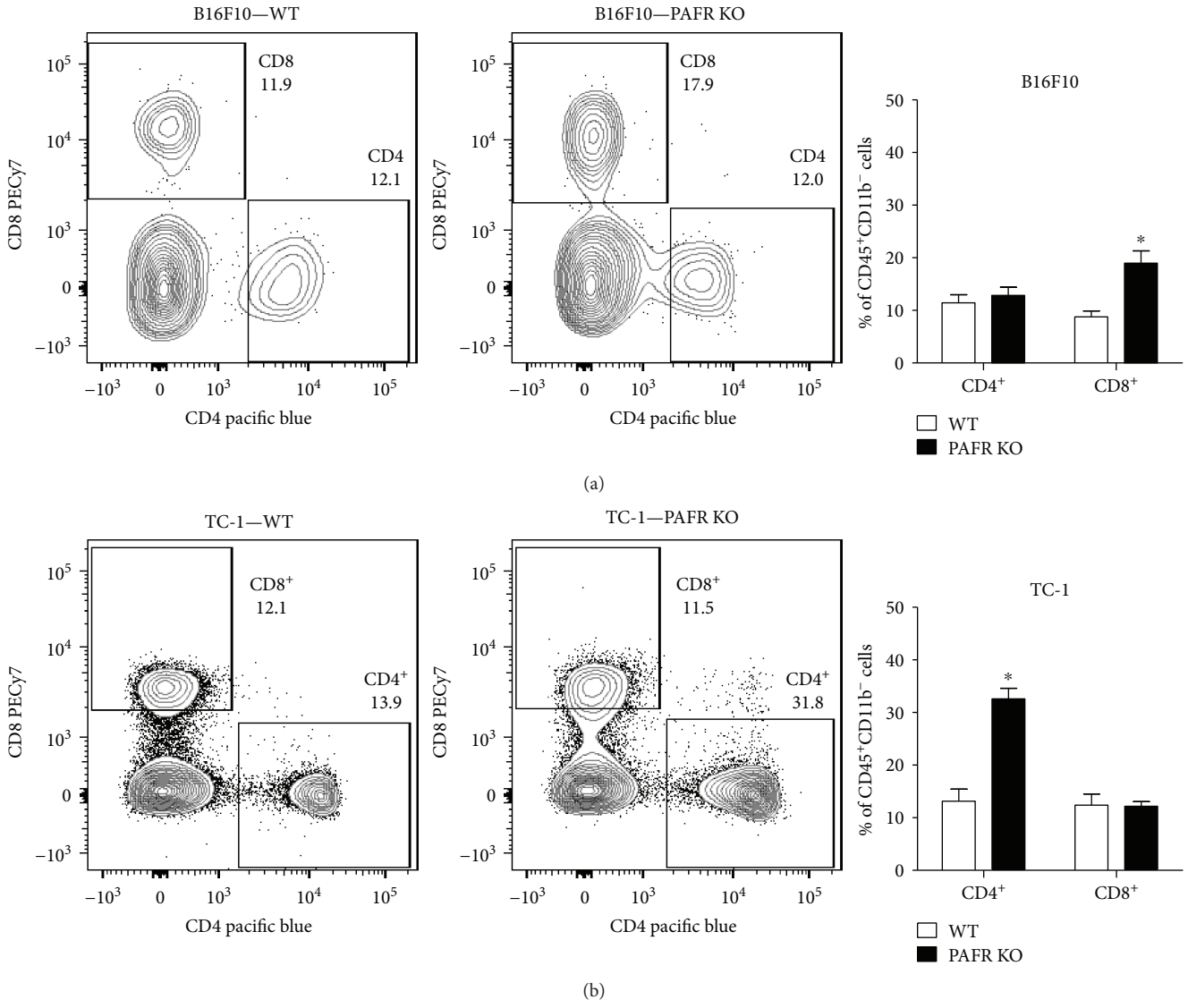

Figure 3: Characterization of lymphoid tumor infiltrate. Total single cell suspensions of B16F10 (a) or TC-1 (b) tumor from WT and PAFR KO animals were analyzed by flow cytometry (50,000 events). The left panels show a representative analysis of the lymphocyte ($CD4^+$ and $CD8^+$) populations. The CD4 and CD8 populations were analyzed within the $CD45^+CD11b^-$ gate, after gating out debris and doublets. The panels to the right show the quantification of these experiments, represented as the average percentage of cells expressing CD4 or CD8, within the $CD45^+CD11b^-$ population. Data were obtained from 3 independent experiments with 4 animals per experimental group. $*$ indicates $p < 0.05$.

microenvironment express PAFR and can be modulated by its ligands. Therefore, we decided to investigate whether PAFR could control the tumor inflammatory infiltrate in our experimental models.

We harvested and digested tumors from wild-type and PAFR KO mice 15 days after tumor cells inoculation and analyzed the inflammatory infiltrate by flow cytometry. In melanoma tumors, the tumor inflammatory infiltrate ($CD45^+$ cells) corresponded to $1.6 \pm 0.3\%$ of the total cells and there was no difference in the percentage of $CD45^+$ cells between the two groups of animals (WT versus PAFR KO; Figure 2(a)). In TC-1 tumors, however, the inflammatory infiltrate was almost 10 times higher than in B16F10 melanoma and a significant reduction in the number of infiltrated cells was observed in PAFR KO (Figure 2(b)).

Next, we evaluated the frequency of lymphocyte populations in the tumors. We observed a twofold increase in the frequency of $CD8^+$ T cells, within the $CD45^+$ population in PAFR KO animals injected with melanoma when compared to WT animals (Figure 3(a)). In contrast, in TC-1 tumors, it was the $CD4^+$ cell population that was increased in PAFR KO (Figure 3(b)).

Regarding the myeloid populations (macrophages and neutrophils) that were recruited to the tumors, we found that B16F10 melanoma recruited more macrophages ($F4/80^+$ cells) than TC-1 tumors; macrophages corresponded to $42 \pm 3\%$ of the $CD45^+CD11b^+$ population whereas in TC-1 tumors, macrophages corresponded to $28.7 \pm 2.6\%$ of this population (Figure 4). Interestingly, in both tumor models, the frequency of neutrophils ($CD45^+CD11b^+Gr1^+$) was

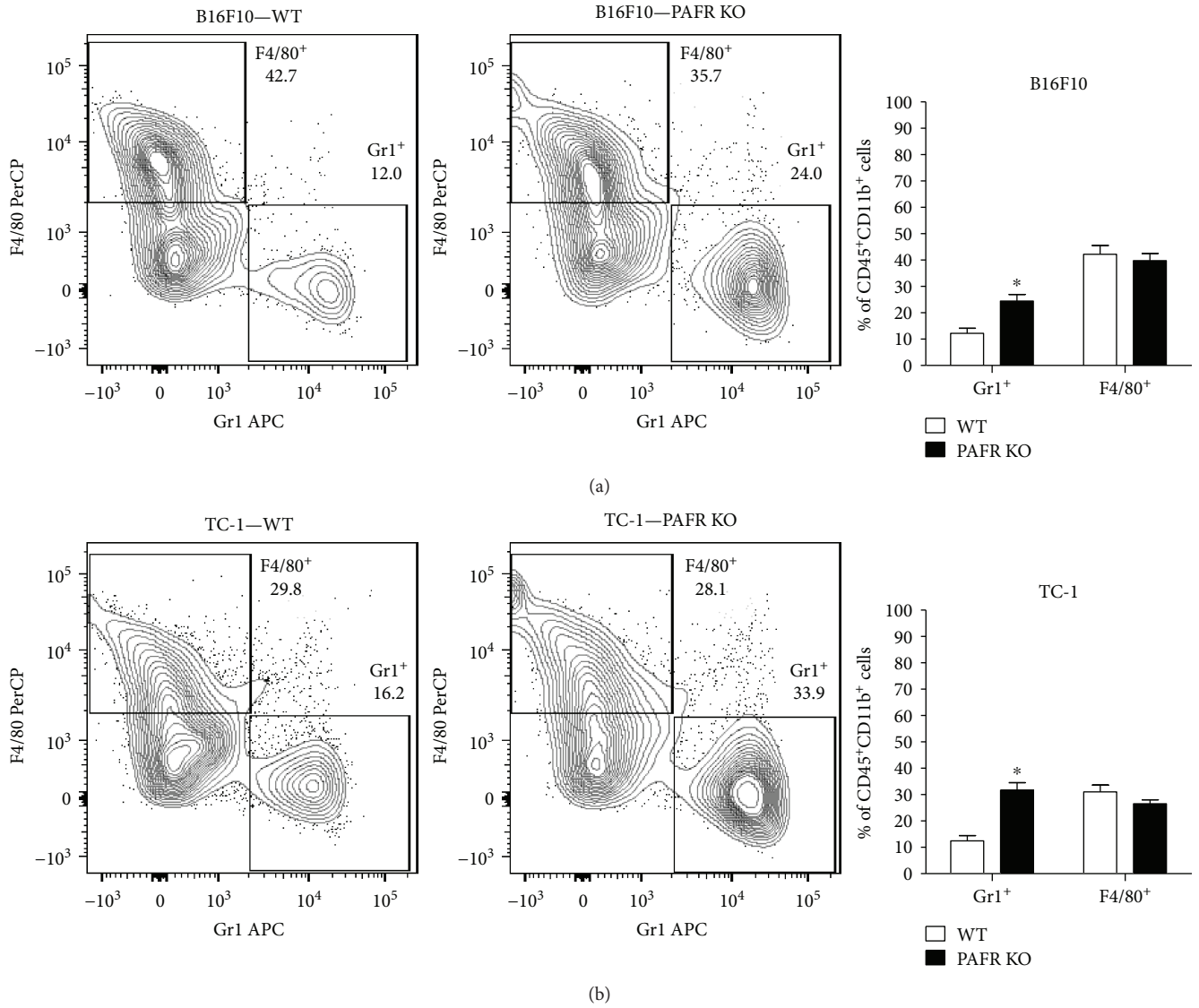

Figure 4: Characterization of myeloid tumor infiltrate. Total single cell suspensions of B16F10 (a) or TC-1 (b) tumors from WT and PAFR KO animals were labeled with antibodies against the indicated markers plus anti-CD45 and anti-CD11b and analyzed in a FACSCanto, where 50,000 events were acquired. In the left panels, we show a representative analysis of the myeloid populations from 3 independent experiments with 4 animals per group. The cells were analyzed within the $CD45^+CD11b^+$ gate, after exclusion of debris and doublets. In the right panel, we show the mean percentage of the frequency of $Gr1^+$ and $F4/80^+$ cells within the $CD45^+CD11b^+$ population. $^*p < 0.05$.

significantly higher in PAFR KO mice than in WT mice (Figures 4(a) and 4(b)). Together, these results indicate that the absence of PAFR in the host cell determines the recruitment of different leukocyte populations into the tumor stroma.

3.3. PAFR and Tumor-Associated Macrophages (TAM) Phenotype. We have previously shown that the activation of PAFR reprogram mice and human macrophages towards an anti-inflammatory phenotype [26]. We therefore decided to investigate the phenotype of TAM in our experimental models. Although these cells are highly heterogeneous, they are classified into two extreme subtypes: the classically activated M1, which has a proinflammatory profile and expresses CD11c as a phenotypic marker, and the alternatively activated macrophages M2, which exhibit an anti-inflammatory profile and express CD206 [27]. Thus, we determined the frequency of CD11c and CD206 cells within the $CD45^+CD11b^+F4/80^+$ macrophage population.

Figure 5 shows that PAFR KO mice had a significantly higher frequency of TAM expressing the CD11c (M1-like) and a lower frequency of cells expressing CD206 (M2-like) molecule in both melanoma (Figure 5(a)) and TC-1 (Figure 5(b)) tumors when compared to the WT groups of each strain.

This was confirmed in macrophages ($CD45^+$ cells) isolated from the tumors and stimulated with 10 ng LPS for 72 hours in culture. We observed that macrophages from PAFR KO animals produced significantly higher concentration of nitrite (Figure 6(a)), indicative of iNOS activity, and

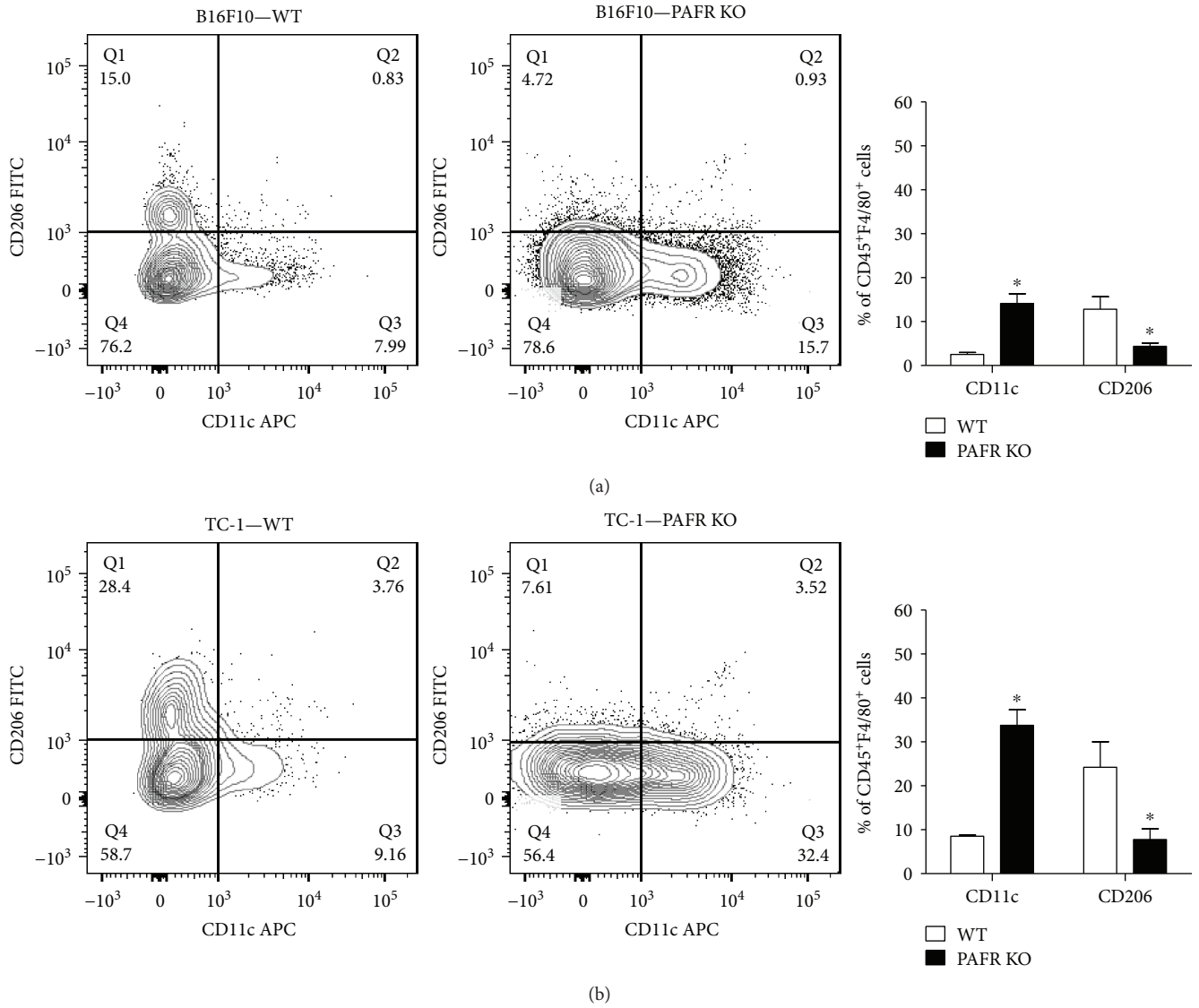

Figure 5: PAFR-modulated TAM phenotype. In a (B16F10) and b (TC-1), we show a representative analysis of the macrophage populations ($CD45^+CD11b^+$ $F4/80^+$) expressing the M1-like marker, CD11c, or the M2-like marker, CD206, from WT and PAFR KO tumors. $*$ indicates $p < 0.05$ for WT compared with PAFR KO tumors. Each experimental group contained 4 animals.

had lower arginase activity (Figure 6(b)) than cells from WT animals. There was also lower concentration of IL-10 in the supernatant of cultures of leukocytes from tumors from PAFR KO mice (Figure 6(c)). Thus, in the WT mice, the TAMs are predominantly M2 whereas in PAFR KO mice, the TAMs are predominantly M1. It can be suggested that during tumor growth, the generation of PAFR ligands by activating the receptor reprograms the macrophages towards the M2 phenotype which favors tumor growth. In the absence of PAFR, the activated M1 macrophages are able to control tumor growth.

4. Discussion

In the present study, we showed that the growth of B16F10 melanoma and TC-1 carcinoma is reduced in mice lacking the PAF receptor when we compared to WT mice. Our results indicate that during the growth of melanoma B16F10 and TC-1 carcinoma PAF receptor ligands are produced in the tumor microenvironment and control the recruitment and phenotype of inflammatory cells to the tumor, promoting the accumulation of M2 macrophages and stimulating tumor growth. Our observations are made even more robust in light of the different PAFR status of B16F10 and TC-1 cell lines. TC-1 cells express PAFR [17], whereas B16F10 do not [20], which indicates that PAFR signaling in the tumor cells was not important in our experimental context.

Evidence from a previous work showed that PAFR has an important role in tumor growth based on studies employing selective antagonists of PAFR. Blockade of PAFR with the antagonist, WEB2170, reduced the growth of Ehrlich ascites tumor (EAT) [28] and melanoma B16F10 growth in C57BL/6 mice [29]. PAF receptor

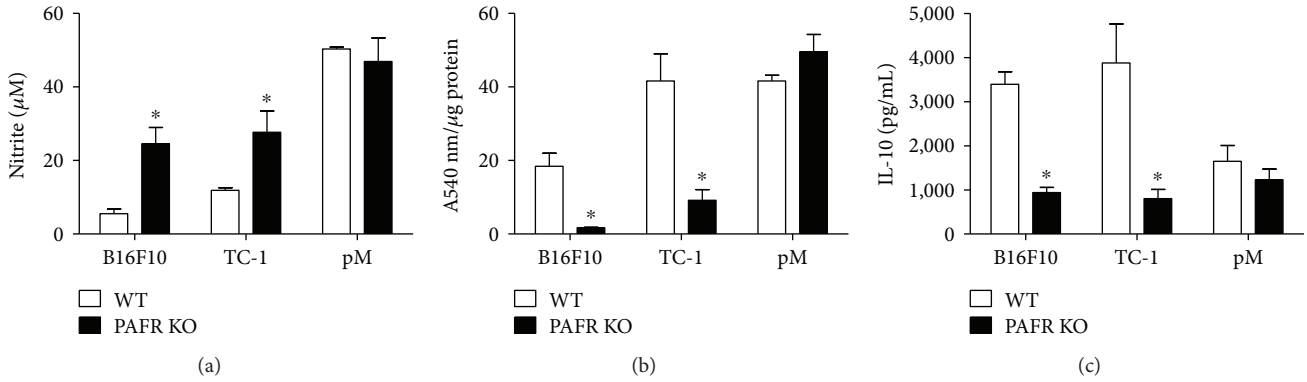

Figure 6: TAMs from PAFR KO tumors show the M1-like phenotype. $CD45^+$ cells were purified from tumor suspensions from animals by positive selection using CD45-coated magnetic beads (Miltenyi Biotec) and stimulated with 10 ng/mL of LPS. After 72 hours of incubation at 37°C in 10% SFB RPMI, the culture supernatants were harvested for nitric oxide (nitrite) production detection by Griess reaction (a) and IL-10 detection by ELISA (c). The cellular extracts were used for determination of arginase activity (b) normalized by the total protein concentration measured by BCA kit. $*$ indicates $p < 0.05$ for WT compared with PAFR KO tumors. Each experimental group contained 4 animals. pM are peritoneal macrophages treated in exactly the same way as tumor infiltrating cells, used as control for NO detection and arginase activity assays.

antagonist ginkgolide B inhibits tumorigenesis and angiogenesis in colitis-associated cancer [30]. Several tumor cells express PAFR and its activation is involved in tumor cell survival and proliferation. In the present work, we showed that the protumoral effect of PAF ligands was due to the modulation of TAM phenotype rather than on tumor cell proliferation.

Interestingly, while we observed that deficiency in PAFR expression caused a reduction in total leukocyte recruitment ($CD45^+$ population), this effect was directed to specific populations, as within the $CD45^+$ infiltrate, neutrophils and T cells still had higher frequency in tumors from PAFR-deficient mice than in tumors from WT mice, suggesting that PAFR mediated the recruitment of monocytes/macrophages. Our results also indicate that during tumor growth, chemokines that promote leukocytes migration to the tumor are produced in response of PAFR activation inside the tumor microenvironment. Previous work has shown that in PAFR KO animals with Ehrlich tumor ascites, elevated levels of CXCL2 and CCL2 chemokines controlled the recruitment of myeloid cells to the tumor [31].

In our study, the increased frequency of neutrophils and intratumoral $CD4^+/CD8^+$ lymphocytes correlated with the inhibition of tumor growth, suggesting that PAFR may be involved in the recruitment of these cells into the tumor stroma. Although we did not investigate the phenotype of these lymphocytes specifically, our data suggest that in PAFR KO mice, these cells might have an antitumor function. Indeed, when macrophages were depleted from TC-1 tumors, not only was an increase in the T cell tumor infiltration found but also the presence of antitumor-specific CD8 cells in the infiltrate [32].

PAF appears to have a pivotal role in macrophage function. Macrophages undergo functional and phenotypic changes in response to signals from the tumor microenvironment. The role played by macrophages in the biology of neoplasias is complex, because macrophages may assume anti- or protumor phenotype [27, 33–35]. M1 phenotype or "classically activated" macrophages have antitumor activity and macrophages of the M2 phenotype are considered protumor [36–38]. In our study, we found no significant differences in the frequency of macrophages that infiltrate melanoma and TC-1 tumors when comparing PAFR KO and WT animals. However, when we analyzed the activation profile of these cells, we observed that in the PAFR KO animals there was a significant shift in the frequency of macrophages from M1 to M2 phenotype. Previous work from our group showed that during EAT growth, macrophages in the ascites presented morphology of nonactivated macrophages and after treatment *in vivo* with PAFR antagonists (BN52021 or SRI63441), the macrophages acquired an activated morphology, and this was accompanied by a significant reduction in EAT growth [28, 39]. The clearance of apoptotic cells by macrophages requires the scavenger receptor CD36 and PAFR and induces macrophage reprogramming towards the M2 phenotype [26]. De Oliveira et al. [29] showed that PAFR antagonist decreased the phagocytosis of apoptotic cells by macrophages and inhibit the production of anti-inflammatory cytokines and mediators. These results suggest that during tumor growth, the clearance of apoptotic cells by TAM as well as the generation of PAF or PAF-like activity molecules in the tumor microenvironment modulates the macrophages into the M2 suppressor phenotype.

Solid tumors can display systemic effects on leukocyte populations, modulating the immune response even before cells reach the tumor microenvironment or promoting the proliferation of protumoral cells, such as myeloid-derived suppressor cells [40]. This is an important aspect to be considered during tumor growth since molecules produced in the tumor microenvironment can circulate and signal to lymphoid organs, increasing hematopoiesis, leading to accumulation, mainly of myeloid cells, in secondary lymphoid organs [41, 42]. We have previously shown that myeloid cells accumulate in the spleen of TC-1 tumor-bearing mice [32].

Here, we also observed that mice bearing tumors have larger spleens and significantly higher cellularity than those without tumors. Interestingly, this tumor-associated splenomegaly did not occur in PAFR KO mice (Supplementary Figure S1). Whether this was a direct effect on cell proliferation and survival in lymphoid organs or a result of the diminished tumor growth, with concomitant reduction in the secretion of molecules that could stimulate leukocytosis, is yet to be investigated. Either way, it seems that the activation of PAFR-dependent pathways can interfere with the balance of leukocyte populations in the spleen and thus potentially modulate the adaptive immune responses to the tumor.

Together with the data presented in this manuscript, we can assume that part of this mechanism may relate to the signaling through PAFR. Interestingly, STAT3 upregulation is a hallmark of many cancer models, not only in cancer cells but also in the inflammatory infiltrate [43]. For instance, cervical carcinoma cells can induce the tolerogenic phenotype in macrophages through the secretion of IL-6 and PGE2 [44]. PAF/PAFR can activate the IL-6/STAT3 axis contributing to the epithelial-mesenchymal transition in nonsmall lung cancer cells [45]. Therefore, the idea that PAFR signaling may have direct and indirect effects in promoting cancer progression and growth seems consistent.

Our results clearly show that PAFR ligands modulate inflammatory cells in the tumor microenvironment, mainly macrophages, promoting protumoral effects, through the induction of the M2 macrophage phenotype.

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' Contributions

Ildefonso Alves da Silva Junior, Sonia Jancar, and Ana Paula Lepique participated in the conception and design of the study. Ildefonso Alves da Silva Junior, Simone Cardozo Stone, and Renata Marques Rossetti performed acquisition of data and analysis and interpretation of data. Ildefonso Alves da Silva Junior and Ana Paula Lepique wrote the manuscript.

Acknowledgments

The authors would like to sincerely thank Marlise BA Montes and Sandra Alexandra da Silva for the expert technical support.

Supplementary Materials

Figure S1: PAFR controls spleen cellularity in tumor bearing animals. Number of nucleated single cells in suspensions from spleens of WT (white bars) or PAFR KO (black bars) mice bearing B16F10 or TC-1 and animals without tumors (naïve) were determined using a hemocytometer. Results are expressed as the average of cell numbers of spleens of 4 mice per experimental group ($n = 3$). $*$ indicates $p < 0.05$ for WT compared with PAFR KO tumors. *(Supplementary Materials)*

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Hindawi
Journal of Immunology Research
Volume 2017, Article ID 5150678, 12 pages
https://doi.org/10.1155/2017/5150678

Review Article

The Role of Microglia and Macrophages in CNS Homeostasis, Autoimmunity, and Cancer

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Received 29 July 2017; Revised 20 October 2017; Accepted 27 November 2017; Published 19 December 2017

Academic Editor: Hao Liu

Macrophages are major cell types of the immune system, and they comprise both tissue-resident populations and circulating monocyte-derived subsets. Here, we discuss microglia, the resident macrophage within the central nervous system (CNS), and CNS-infiltrating macrophages. Under steady state, microglia play important roles in the regulation of CNS homeostasis through the removal of damaged or unnecessary neurons and synapses. In the face of inflammatory or pathological insults, microglia and CNS-infiltrating macrophages not only constitute the first line of defense against pathogens by regulating components of innate immunity, but they also regulate the adaptive arms of immune responses. Dysregulation of these responses contributes to many CNS disorders. In this overview, we summarize the current knowledge regarding the highly diverse and complex function of microglia and macrophages during CNS autoimmunity—multiple sclerosis and cancer—malignant glioma. We emphasize how the crosstalk between natural killer (NK) cells or glioma cells or glioma stem cells and CNS macrophages impacts on the pathological processes. Given the essential role of CNS microglia and macrophages in the regulation of all types of CNS disorders, agents targeting these subsets are currently applied in preclinical and clinical trials. We believe that a better understanding of the biology of these macrophage subsets offers new exciting paths for therapeutic intervention.

1. Introduction

The central nervous system (CNS) has been long recognized as an immune-privileged site [1]. But over the last several years, evidence has accrued suggesting that the CNS contains resident immune cells that actively participate in immune surveillance and shape the CNS development and neuronal function under steady states. These resident cells include various types of macrophages, including the most abundant and best studied population, microglia [2]. In the face of pathological insults, CNS microglia and macrophages, including CNS-infiltrating macrophages derived from circulating monocytes, constitute the first line of defense against pathogens by regulating components of both innate and adaptive immune responses. Dysregulation of these responses underlies the pathogenesis of many CNS disorders. Here, we summarize the current understanding of CNS microglia and macrophages, including their development, homeostasis, and functions in physiological and pathological status (autoimmune disease and tumor), the interaction of CNS microglia and macrophages with other immune components (innate and adaptive immune cells), and the therapeutic potential of CNS microglia and macrophages as drug targets.

2. The Development, Homeostasis, and Function of CNS Microglia and Macrophages

Macrophages are myeloid cells that survey their immediate and local environment by ingesting and degrading dead cells, debris, and potentially hazardous agents, such as pathogens [3, 4]. As part of the mononuclear phagocyte system, macrophages are present in almost all tissues and have a crucial role in maintaining tissue homeostasis during development and in adulthood. Tissue-resident macrophages are nonmigratory cells that comprise many subsets, including microglia

(brain), osteoclasts (bone), alveolar macrophages (lung), histiocytes (interstitial connective tissue), and Kupffer cells (liver). There are also various mononuclear phagocyte subpopulations in the circulation that can differentiate into macrophages once they migrate into tissues, called monocyte-derived macrophages [5, 6]. Although the phenotypes and names of these macrophage populations vary on the basis of their anatomical location, they all acquire similar functional capability when stimulated appropriately [7]. Here, we summarize the current view of the developmental requirement and functional specialization of CNS microglia and macrophages.

2.1. The Development and Homeostasis of CNS Microglia and Macrophages. Most tissue-resident macrophages are prenatally established and then maintained through adulthood [8]. Embryonic yolk sac and fetal liver-derived macrophage precursors are the origin of all tissue-resident macrophages, although the contributions of these two progenitors vary among different tissues [8]. Primitive macrophages in the yolk sac appear around embryonic day 7 (E7) and disseminate throughout embryonic tissues following the establishment of blood circulation around E9.5. Fetal liver monocytes infiltrate peripheral tissues, except the CNS, and give rise to tissue-resident macrophages. While macrophages from both origins usually coexist, the fetal liver-derived cells can progressively outcompete yolk sac-derived tissue macrophages. Thus, the generation and maintenance of tissue-resident macrophages are independent from ongoing hematopoiesis, despite the fact that these cells can be complemented by adult monocyte-derived macrophages [9]. For example, during adulthood, bone marrow-derived circulating Ly6C^{hi} monocytes can give rise to relatively short-lived, non-self-renewing tissue-resident macrophages in organs, such as the intestine, heart, and remodeling mammary glands [5, 6]. Despite the similarities of microglia with various other tissue-resident macrophages, two remarkable properties of microglia are their restricted prenatal origin and their capacity for self-renewal and longevity. After birth, myeloid progenitors from the circulation cannot significantly contribute to the pool of adult microglia, and the increase in microglial cell number results from the expansion of resident microglia [10, 11]. While the numbers of microglia increase during aging, their structure changes from a highly ramified shape to a morphology with less elaborate processes accompanied by an irregular tissue distribution pattern and slower responses to environmental signals [12, 13]. In contrast to microglia, circulating monocytes and other tissue macrophages are continually replaced by circulating myeloid cells after birth [14].

In the steady state, the CNS hosts several myeloid populations, including parenchymal microglia, perivascular cells, meningeal macrophages, and choroid plexus macrophages [15]. CNS macrophages have been characterized and classified mainly according to their localization, morphology and surface-marker expression, and *in vitro* responses. Despite the fact that all of these macrophage populations share numerous myeloid- and macrophage-specific markers, such as ionized calcium binding adaptor molecule 1 (Iba1), F4/80 (mouse) (or EMR1 (human)), and CX3CR1, microglia have their unique signatures. Transcriptome analyses comparing microglia, myeloid, and other immune cells have identified 239 genes and 8 microRNAs that are highly expressed and unique to microglia. These molecular signatures include *Sall1*, *Tgfbr1*, *P2ry12*, *Fcrls*, and *Gpr34* genes that are dependent on the transforming growth factor-β (TGFβ) signaling—an essential pathway required for the development of microglia [16]. Moreover, the same analyses have identified the purinergic receptor P2y12 (P2ry12) as a specific marker for microglia [16]. In addition to the varying markers among different macrophage subsets, CNS-associated myeloid populations also have distinct ontogenesis. Current view supports that microglia originate exclusively from yolk sac-derived hematopoietic progenitors, whereas the other CNS resident macrophage subsets arise later during embryonic development [10, 11, 17]. This view is supported by a series of elegant genetic fate-mapping and parabiosis studies. By injection of tamoxifen into pregnant mice between E7 and E8.5, when embryonic hematopoiesis is limited to the yolk sac, to induce Cre recombinase activity from the runt-related transcription factor 1 (*Runx1*) locus [10] or from the colony-stimulating factor 1 receptor (*Csf1r*) locus, these fate-mapping experiments have demonstrated that the majority of adult microglia are derived from the yolk sac [11]. A similar pattern of microglial cell development also occurs in humans [18]. Parabiosis experiments have also recently shown that the other CNS macrophage subsets, except choroid plexus macrophages, arise from hematopoietic precursors later during embryonic development and become stable populations [19]. Due to the blood-brain barrier, circulating leukocytes (e.g., monocytes, T, B, and natural killer (NK) cells) normally stay within the blood vessels and do not enter the healthy brain, unless the blood-brain barrier is disrupted during CNS diseases, including inflammation, autoimmunity, and cancer. The CNS-infiltrating monocytes give rise to disease-related macrophages and execute distinct functions that differ from resident microglia [20], which we will discuss in Sections 3 and 4.

The development of microglia is controlled by many molecular elements including transcription factors, growth factors, chemokines, microRNAs, and others [21]. One of the important factors that control the microglia population are the signals emanating from the binding of colony-stimulating factor 1 (CSF1) and interleukin 34 (IL-34) to the microglial CSF1 receptor (CSF1R). Mice deficient in the CSF1R or IL-34 or the CSF1R adaptor protein DNAX activation protein of 12 kDa (DAP12) contain substantially reduced numbers of tissue macrophages, including microglia [22, 23]. The transcription factor interferon regulatory factor (IRF)-8 is also essential for the development of microglia, as IRF8-deficient mice show a significantly reduced microglia density in adults [17]. Once the CNS is fully developed, the population size of microglia is maintained via a balance between mitosis and apoptosis [24]. In contrast, the generation of other CNS macrophages relies on the transcription factor PU.1, but not MYB, BATF3, and NR4A1 [19]. A more complete understanding of molecular circuits that regulate the development and homeostasis of CNS microglia and

macrophages may lead to improved strategies for better modulating the size of these cellular populations.

2.2. Physiological Functions of CNS Microglia and Macrophages. Generic effector functions of macrophages include activities associated with their highly developed lysosomal compartment that bears critical protease and bactericidal activity [25]. Microglia and macrophages are phagocytic cells that constitutively express several families of receptors that facilitate the removal of aged, necrotic tissues, and toxic molecules from the circulation and their surroundings [5, 8]. These receptors include scavenger receptors (e.g., CD36, SR1, and macrophage receptor with collagenous structure (MARCO)), low-density lipoprotein (LDL) receptor family members (e.g., LDLR, ApoER2, and VLDL), and three receptor tyrosine kinases (Tyro3, Axl, and Mertk) [5, 26]. Mertk and Axl are expressed in resting and activated macrophages, respectively [5]. Engagement of Tyro3, Axl, and Mertk by binding to soluble proteins, growth arrest-specific 6 (GAS6) and protein-S, results in opsonization of apoptotic cells [5, 21]. Macrophages also capture and endocytose immune complexes and complement-opsonized protein complexes through Fc receptors and complement receptors [5, 8, 21, 25]. In addition, macrophages often express chemokine receptors (e.g., CX3CR1 and CXCR4) and integrins (e.g., CD11b and CD11c), which control the migration and positioning of microglia and macrophages within the CNS and enhance their capacity to phagocytose and eliminate bound target cells [21].

Microglia interact with neurons and constitute important components that support the development of the healthy brain [27]. Disruption of these interactions can have a severe negative impact on the functioning of the CNS. Here, we summarize several vital microglia-mediated homeostatic functions that help establish and maintain the overall health of the nervous system, including regulation of neuronal survival and death as well as synaptogenesis. During embryonic development, microglia and perivascular macrophages are uniquely positioned through the pial surface and migrate along the abluminal surface of penetrating vessels to influence the early sprouting, migration, anastomosis, and refinement of the growing CNS vasculature [10]. Microglia also produce various neurotrophic factors that promote the differentiation and survival of neurons. For example, insulin-like growth factor 1 (IGF-1) is released by surrounding microglia to promote the survival of layer V cortical neurons during postnatal development [28]. In adulthood, IGF-1 induces multipotent rat hippocampus-derived neural progenitor cells to differentiate into oligodendrocytes [29]. IGF-1 can also protect immature oligodendrocytes from glutamate-mediated apoptosis [30]. In addition to IGF-1, microglia also secrete other trophic factors, such as basic fibroblast growth factors (FGF), hepatocyte growth factors (HGF), platelet-derived growth factors (PDGF), epidermal growth factor (EGF), nerve growth factor (NGF), and brain-derived neurotrophic factor (BDNF). All of these factors play significant roles in neuronal development, maintenance, and function throughout life [31]. Microglia not only support neuronal survival, but also function as a scavenger to eliminate immature faulty neurons resulting from defective differentiation and/or migration [32]. Microglia induce such neuronal death through the release of soluble factors, such as NGF and reactive oxygen species (ROS) [33, 34].

In addition, microglia play a crucial role in shaping and maintaining the neuronal synaptic network, which occurs constantly throughout life [35]. This type of microglia-mediated remodeling of synapses, called synaptic pruning, is a process in that damaged or unnecessary synapses are eliminated in order for the developing neurons to establish the mature CNS circuit and maintain synaptic homeostasis [35, 36]. The synaptic pruning occurs when an "eat me signal" is created by the engagement of microglial receptor CR3 by the complement protein C3 [37]. In addition to synaptic pruning, microglia also produce various trophic factors and synaptogenic signals to properly regulate synaptic function and plasticity [38]. As a result, reduced microglia in the brain may result in aberrantly increased synaptic activity and a delay in synaptic pruning, leading to cognitive impairments [36, 39]. Finally, the release of neurotransmitters and neuropeptides by neurons promotes neuron-glia communications that fine-tune the homeostatic regulation by microglia [40, 41]. Taken together, the establishment and maintenance of a healthy nervous system requires a tight control of microglia function.

2.3. Pathological Function of CNS Microglia and Macrophages. Microglia and macrophages normally function independently of activating stimuli. However, to meet with greater demand for the control of infection or tissue injury, the functional activity of microglia and macrophages can be increased by a variety of stimuli. The nature of these stimuli often determines the distinct morphology and movement of activated microglia to better cooperate their function, as reviewed by others [42–44]. Although this enhanced function allows microglia and macrophages to become more responsive to changes in their surroundings, it also bears the inherent risk of hyperactivation and the ensuing collateral tissue damage. To counterbalance the activatory program, microglia and macrophages are subjected to silencing programs that set tissue-specific thresholds for their activation and allow them to gradually respond to and gauge the quality and intensity of the stimulus [8]. The intensity and duration of this activation or inhibition are balanced through the activating or inhibitory receptors they express. For example, the immunoglobulin superfamily (Ig-SF) molecules deliver either activating or inhibitory signals through protein tyrosine kinase and protein tyrosine phosphatase pathways, respectively. The triggering receptor expressed on myeloid cells 2 (TREM2) is an activating receptor that binds to phospholipids [45], while binding TGFβ receptor (TGFβR), CD33, CD200R1, and signal regulatory protein α (SIRPα) to TGFβ, sialic acids, CD200, and CD47 delivers inhibitory signals, respectively [32]. However, less is understood about the roles of tumor necrosis factor (TNF) receptor (TNFR) family members and signaling lymphocytic activation molecule (SLAM) family members in the regulation of

macrophage activity [46]. Thus, the imbalance between the activating and inhibitory signals that regulate the activity of microglia and macrophages may pertain to the occurrence of tissue pathology, including both CNS autoimmunity and tumor.

Plasticity and diversity are hallmarks of cells in the macrophage lineage. In response to different stimuli, microglia and macrophages undergo either classical (M1) or alternative (M2) activation [47]. This type of polarized activation of macrophages is often controlled by intrinsic (e.g., epigenetic program) or extrinsic (e.g., inflammatory cytokines) regulatory factors [48]. The M1/M2 continuum has been applied to CNS infiltrating macrophage/monocytes in the context of inflammation or tumor. M1 activation is a proinflammatory and neurotoxic state typically induced by simultaneous triggering of toll-like receptors (TLRs) and interferon (IFN)-γ signaling pathways, which is generally associated with immunity to bacteria and intracellular pathogens. These M1 macrophages produce proinflammatory cytokines and chemokines, such as TNF-α, interleukin (IL)-6, IL-1β, IL-12, and C-C chemokine ligand 2 (CCL2) [47]. M1 macrophages also express the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which in turn generates superoxide and ROS, as well as inducible nitric oxidase that converts arginase into nitric oxide (NO) [49]. NO increases the toxic effect of glutamate, thereby potentiating N-methyl-d-aspartate (NMDA) receptor-mediated neurotoxicity [47, 49]. Another important inflammatory mediator produced by M1 macrophage is matrix metalloproteinase (MMP)-12 [47]. Lastly, M1 macrophages often express high amounts of MHC class I or II, costimulatory molecules, Fc receptors, and integrins, which also facilitate induction of inflammation and neurotoxicity [49].

M2 activation describes the anti-inflammatory and tissue remodeling activities of macrophages, which are usually observed in settings dominated by type 2 responses, such as helminth immunity, asthma, and allergy [48]. It can be induced by IL-4, IL-10, IL-13, ligation of Fc receptors by immunocomplexes, and detection of apoptotic cells. Moreover, activation of the transcription factors peroxisome proliferator-activated receptor gamma (PPARγ), liver X receptor (LXR), and retinoic acid receptor (RXR) by fatty acids, oxysterols, and 9-cis-retinoic acid can also trigger the M2 activation state [47]. M2 activation promotes the release of prosurvival factor progranulin [50, 51] and anti-inflammatory cytokines, such as IL-10 and TGFβ, and induces arginase 1, which promotes the conversion of arginine into polyamines [47, 49]. M2 macrophages secrete growth factors such as IGF-I, FGF, and CSF1, as well as neurotrophic factors such as NGF, BDNF, neurotrophin 4/5, and glial cell-derived neurotrophic factor (GDNF). In turn, these neurotrophic factors engage a family of receptor tyrosine kinases known as tropomyosin-receptor-kinase (Trk) receptors, which regulate synaptic strength and plasticity [27].

Although the M1 and M2 categories have been helpful for conceptualizing macrophage activities *in vitro*, it is increasingly accepted that the M1/M2 paradigm is inadequate to describe microglia and macrophage activation *in vivo*, as they rarely display a significant bias toward either the M1 or M2 phenotype. Indeed, a recent study based on single-cell transcriptome analysis has described a novel microglial cell type associated with neurodegenerative diseases, called disease-associated macrophage (DAM). The genetic programming of this microglial subset involves downregulation of microglial inhibitory-checkpoint pathways in a TREM2-independent manner and subsequent activation of the TREM2-dependent program [52]. This new microglial cell has the potential to restrict neurodegeneration. Another recent study has also identified a type of microglial cell from models of amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS), and Alzheimer's disease (AD) and from tissues surrounding neuritic β-amyloid (Aβ)-plaques in the brains of people with AD. This microglial cell carries a specific apolipoprotein E- (APOE-) dependent molecular signature that depends on TREM2-induced APOE signaling pathway, which switches the microglia from a homeostatic to a neurodegenerative phenotype after phagocytosing apoptotic neurons. Targeting the TREM2-APOE pathway has prevented neurodegeneration by restoring the homeostatic signature of microglia [53]. These findings suggest that microglia may have a disease-associated signature common to many CNS disorders, including neurodegenerative diseases, autoimmunity, and possibly cancer, which is worth further investigation.

3. CNS Microglia and Macrophages in Autoimmunity: Multiple Sclerosis

CNS microglia and macrophages play important roles in communication between the systemic immune system and the brain. These cells not only regulate the innate immune responses to mediate host defense against cellular or pathogenic components [32, 54], but also modulate the adaptive immune components functioning as antigen-presenting cells [55, 56], or accessory helper cells [32]. Here, we summarize the roles of CNS microglia and macrophages in the regulation of both aspects of immune responses and discuss the contribution of these dysregulated responses to the pathogenesis of CNS disorders, exemplified by multiple sclerosis here.

3.1. Microglia and Macrophages in Innate Immunity. As a component of innate immunity, macrophages are critical players in the first line of defenses against infection or tissue injury. This is largely attributed to the vast array of receptors expressed on macrophages. These receptors include pattern recognition receptors (PRRs) that detect pathogen-associated molecular patterns (PAMPs) or tissue damage-associated molecular patterns (DAMPs). PRRs include TLRs (e.g., TLR4 and TLR1/2) and their coreceptors, such as CD14, nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), receptors for nucleic acids, retinoic acid-inducible gene I (RIG-I)-like receptors, and C-type lectin receptors (CLRs) (e.g., CLEC7A) [57]. Microglia and macrophages also express the receptors for proinflammatory and anti-inflammatory cytokines, such as IFNα/β, IFNγ,

TNFα, IL-1β, IL-10, and TGFβ, to regulate the intensity of the inflammatory responses [57].

Although the repertoire of these receptors varies among different tissue macrophages and likely reflects local adaptation, these receptors all have important roles in the induction of innate immune responses. They enable microglia and macrophages to engulf and destroy foreign particles and dying cells to promote an M1-like phenotype [57]. Engagement of these receptors also connects to the adaptor myeloid differentiation primary response gene 88 (MyD88). This relays activating signals to regulate inflammasome formation and induce the production of cytokines (e.g., TNFα and IL-1β), which can further enhance the functional activity of M1 cells [58]. Microglia and macrophages with the M2 phenotype are intimately involved in CNS repair and regeneration, as demonstrated by the role of growth factors, cytokines, and chemokines released by these cells in response to the CNS injury [59, 60]. Microglia and macrophages also secrete MMPs that regulate the deposition of extracellular matrix components at the injured sites [60]. In addition, the protective role of M2 cells may reflect their expression of the IL-4 receptor. Engagement of this cytokine receptor by IL-4 prevents a proinflammatory skew of microglia and macrophages, which influences normal neuronal function and behavior [61, 62]. Finally, microglia and macrophages play a critical role in orchestrating the inflammatory response by provision of chemokines and cytokines, which recruit and activate neutrophils, monocytes, and lymphocytes, to intensify the inflammation [57].

3.2. Microglia and Macrophages in Adaptive Immunity. Besides functioning as sentinels, macrophages also function as antigen-presenting cells and participate in the activation of the adaptive arm of the immune response [5]. Upon immunological insults, other innate immune cells, such as NK cells, often provide the initial source of IFNγ that enables macrophages to develop a classical activated M1 phenotype [63]. These M1 macrophages then produce large amounts of TNFα, IL-12, and IL-23, which are important drivers of type 1 helper T (T_H1) cell and type 17 helper T (T_H17) cell responses [64]. T cell-derived IFNγ provides a positive amplification feedback loop that expands the M1 cells while increasing their microbicidal and tumoricidal activities [63]. M1 cells are generally believed to display antitumor properties by antagonizing the suppressive activities of tumor-associated macrophages (TAM), myeloid-derived suppressor cells (MDSC), and alternatively activated macrophages and regulatory macrophages, which in turn promote tumor growth, invasion, and metastasis by suppressing adaptive antitumor immune responses [65] (see Section 4). Because M1 macrophages secrete large amounts of TNFα and IL-1β that contribute to the differentiation of T_H17 cells, they are also believed to be important drivers of chronic inflammatory and autoimmune diseases, including multiple sclerosis, rheumatoid arthritis, atherosclerosis, pulmonary fibrosis, and Crohn's disease [66–68] (see Section 3.3).

In contrast to M1 cells, M2 cells mainly display suppressive or immunoregulatory activity. They antagonize M1 responses, dampen inflammation, suppress antitumor immunity, and promote wound healing, tissue remodeling, and angiogenesis [5, 69]. Regulatory macrophages that secrete IL-10 have similar roles in adaptive immune responses, although they are particularly adept at suppressing antimicrobial immunity [63]. Regulatory macrophages also facilitate the maintenance of immune homeostasis in the gut by inducing the development of regulatory T cells (Treg) [70], whereas M2 cells mediate secondary immunity to gastrointestinal worms [71]. Although alternatively activated macrophages are induced by a variety of innate IL-4- and IL-13-producing cells, including basophils [72], T_H2 cells are thought to serve as the main inducers of M2 cells when the adaptive immune response is activated, as in many chronic inflammatory and fibrotic diseases [73, 74]. In the CNS, IL-4 produced by T cells in the meninges and cerebrospinal fluid prevents local inflammation, possibly benefiting cognition through regulation of M2 cells [75].

3.3. CNS Microglia and Macrophages in Multiple Sclerosis. As discussed in Section 2.3, M1 macrophages may contribute to many autoimmune diseases. Here, we focus on multiple sclerosis (MS) (Figure 1). MS is a CNS disease that affects over 2 million people and has no known cure. MS is considered as a chronic autoimmune inflammatory disease affecting brain, nerve, and spinal cord tissues, which causes demyelination of neurons, axonal damage, and neurodegeneration [76]. Myelin-specific T_H17 and T_H1 cells and B cells are believed to help initiate and/or promote the development of MS [76]. Experimental autoimmune encephalomyelitis (EAE) is the most commonly used animal model for MS and is induced by $CD4^+$ T cells specific for myelin-derived antigens, either generated after immunization or injected directly [77]. Studies using this EAE model have shown that microglia and macrophages contribute to aggravating the CNS pathology [78]. In mice with the deletion and/or inactivation of microglia, delayed EAE onset and reduced severity of clinical symptoms are observed along with decreased inflammation, confirming the crucial role of microglia in the pathogenesis of MS [78].

Microglia contribute to EAE disease initiation by presenting antigens to naive T cells and secreting cytokines, such as IL-6, IL-23, IL-1β, and TGFβ, that are required for the differentiation and activation of encephalitogenic T_H17 cells. It remains unclear if microglia and macrophages regulate the other T_H cells that modulate EAE and MS progression. It is known, however, that activation or inhibition of effector T cells by microglia is controlled by other neighboring immune cells. For example, a subset of microglia has the capacity to suppress effector T cell proliferation by inducing $FoxP3^+$ Treg, leading to attenuation of EAE disease progression [79]. Although it is believed that microglia have a neurotoxic role in MS and EAE, there is conflicting evidence that suggests microglia exert a neuroprotective function in MS and EAE [42]. Potential beneficial effects of microglia in EAE and MS are thought to occur in at least three major ways: (1) microglia clear myelin debris and apoptotic cells; (2) microglia release protective cytokines and mediators for remyelination; and (3) microglia trigger recruitment of oligodendrocyte precursors and stimulate neurogenesis [27, 32].

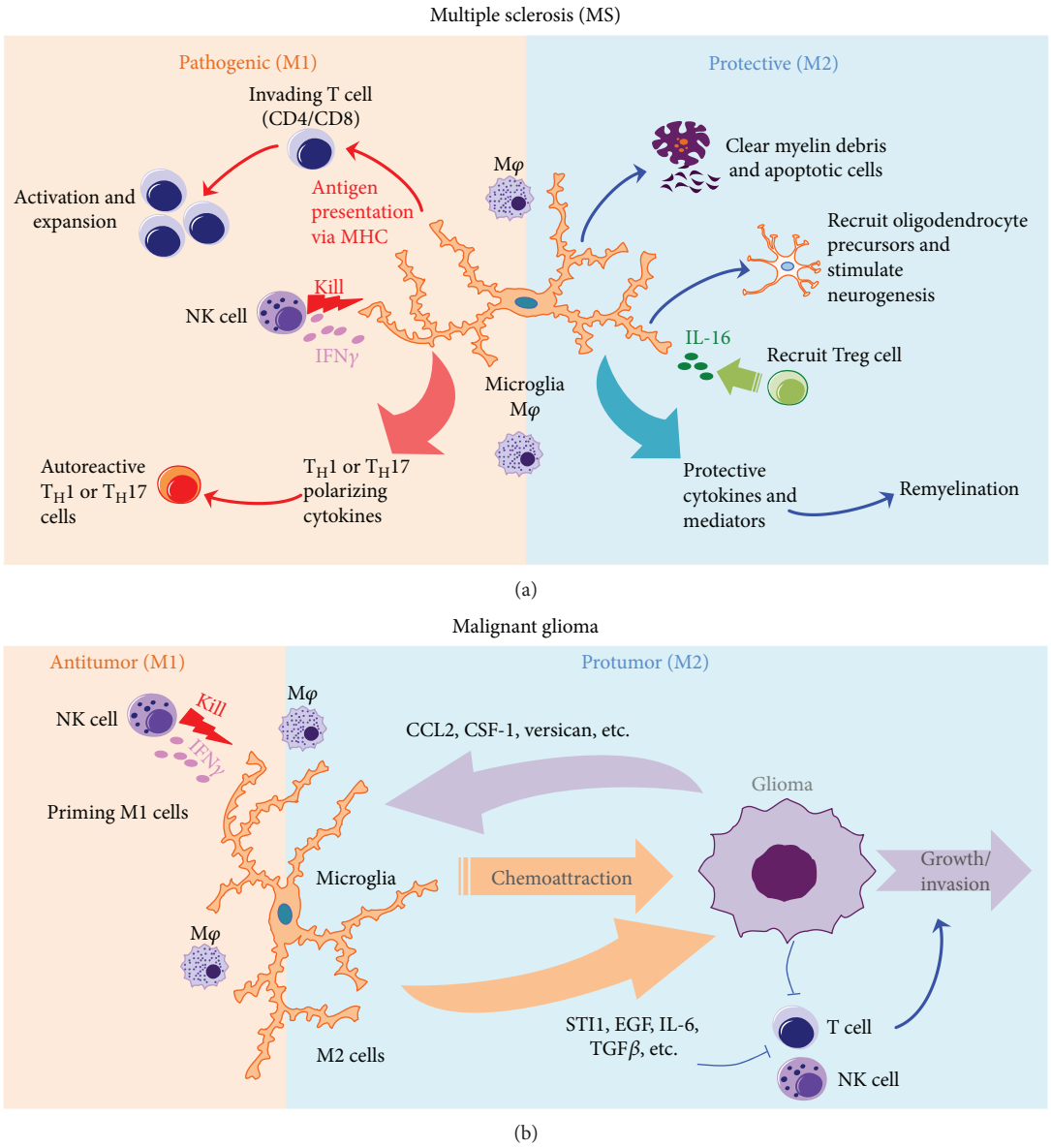

Figure 1: Immune regulation of MS and malignant gliomas by CNS microglia and macrophages. (a) CNS microglia and macrophages (M1) activate autoreactive T cells and program encephalitogenic T_H1 and T_H17 cells to induce and exacerbate MS, while NK cells reduce numbers of M1 cells, and M2 cells recruit Treg, contributing to disease amelioration. Microglia also provide protective roles by helping remyelination and neurogenesis. (b) NK cells may prime M1 macrophage and microglia or reduce numbers of M2 cells to promote antitumor response. M2 cells are regulated by factors derived from glioma and further produce suppressive factors to intensify the immunosuppressive environment within the glioma, contributing to the tumor growth and invasiveness.

The neurotoxic and neuroprotective functions of microglia may depend on the CNS disease stages and activation status of microglia, which awaits further investigation. Interestingly, a recent study using the parabiosis model combined with highly efficient permanent labeling of blood monocytes has elegantly revealed that circulating monocytes invade the inflamed CNS during EAE pathogenesis and have an essential role in promoting disease progression [80]. A precise understanding of these two pools of CNS macrophage subsets during CNS inflammation and autoimmunity may provide insights into better strategies for the treatment of these disorders.

4. CNS Microglia and Macrophages in Cancer: Malignant Glioma

As discussed in Section 2.3, M1 macrophages display antitumor activity while M2 cells are protumorigenic. Here, we discuss one of the most deadly brain cancers, malignant glioma (Figure 1). Gliomas, a type of brain tumor that grows from glial cells, include astrocytoma, oligodendroglioma, and glioblastoma. Gliomas are complex tumors composed of both neoplastic and nonneoplastic cells. The majority of nonneoplastic cells are TAMs, which account for 50% of the cellular fraction of gliomas. TAMs include infiltrated

monocyte-derived macrophages and brain-resident microglia. These cells constitute a supportive stroma for neoplastic cell expansion and invasion [81]. Therefore, understanding the cellular and molecular mechanisms for the regulation of microglia and macrophages may suggest novel strategies to target these cells for immunotherapy of gliomas.

The importance of microglia and macrophages in glioma is underscored by clinical observations. The number of infiltrated TAMs and microglia, identified by CD68 and Iba-1 antibodies, respectively, is positively correlated with tumor grade [82] and inversely correlated with the recurrence-free survival of patients [83]. While monocytes represent 10–15% of the cell population in normal nonneoplastic brain specimens, 15–30% of cells in low-grade gliomas are TAMs [84]. Moreover, the proportion of microglia can reach 35–50% within the gliomas, depending on the region in which the tumor arises and the degree of tumor invasiveness [85]. Microarray analyses have revealed approximately 1000 transcripts that are highly enriched in glioma-associated microglia and macrophages relative to control microglia. Interestingly, these genes show little overlap with reported gene signatures for M1 or M2 phenotypes [86].

Despite the positive correlation between the number of intratumoral TAM and microglia with glioma malignancy, it remains controversial and to be determined whether these cells display antitumor activity or protumorigenic properties. Understanding these mechanisms is important for directing future therapeutic strategies for glioma. Deletion of microglia and macrophages increases glioma tumor volume by 33%, suggesting that these cells may contribute to the antitumor response [87]. In contrast, pharmacological activation of microglia and macrophages results in increased glioma size, indicating that these cells may promote tumor growth and invasion [88]. Moreover, in the presence of microglia, the motility of the murine glioma cells is increased threefold *in vitro* [89]. Using transgenic mice expressing the herpes simplex virus thymidine kinase gene under the control of the *Cd11b* promoter, Galarneau et al. have shown that targeted reduction of $CD11b^+$ microglia and macrophages concomitantly results in attenuated glioma growth *in vivo* [44, 90]. Within the tumor microenvironment, the crosstalk between glioma cells and microglia/macrophages may determine the glioma aggressiveness and invasiveness. Microglia release several factors to promote glioma proliferation and/or migration. Microglia synthesize and release stress-inducible protein 1 (STI1), a cellular prion protein ligand that increases the proliferation and migration of glioblastomas *in vitro* and *in vivo* [91], as well as EGF, which stimulates glioblastoma cell invasion [92]. TGFβ, predominantly released from microglia, also increases the migration of glioma cells; moreover, blocking TGFβ signaling impairs glioma growth [93]. In addition, TGFβ2 induces the expression of MMP2 in glioma cells and suppresses the expression of tissue inhibitor of metalloproteinases (TIMP)-2, which degrades the extracellular matrix and subsequently promotes glioma invasion [94]. TAMs not only target glioma cells, but also indirectly affect tumor growth through angiogenesis. This likely occurs via expression of the receptor for advanced glycation end product (RAGE) and vascular endothelial growth factor (VEGF), an important proangiogenic factor [95].

On the other hand, factors produced from glioma cells facilitate the glioma-promoting activity of microglia. CSF1, constitutively released by the glioma cells, acts as a chemoattractant for microglia and also converts microglia into a protumorigenic phenotype [96]. CCL2 is another factor released from glioma cell lines and acts on the CCL2 receptor (CCR2) expressed on microglia [97]. CCL2 can trigger the release of IL-6 from microglia, promoting the glioma invasiveness [98]. Glioma-derived versican interacts with TLR2, inducing CNS microglia and macrophages to express membrane type 1-matrix metalloproteinase 1 (MT1-MMP) that activates MMP2 [99]. In its active form, MMP2 amplifies the glioma-brain macrophage interaction network and potentiates glioma growth and invasiveness [99]. Furthermore, the suppressive factors produced from both glioma and microglia or TAMs inhibit the antitumor activity of effector $CD4^+$ and $CD8^+$ T cells and NK cells, but promote the recruitment and suppressive activity of Treg and MDSC, which constitute the immunosuppressive microenvironment and enhance glioma growth [100].

Glioblastomas contain a subpopulation of cells with stem cell-like properties, called glioma stem cells (GSCs), which have the capacity for self-renewal, the potential for multilineage differentiation, and are capable of reconstituting the native tumor following implantation into naive hosts [101]. However, these GSCs reside in the perivascular niche and are highly resistant to radiation and chemotherapy [101]. There is a positive correlation between the density of GSCs and TAMs, indicating that GSCs may recruit TAMs more efficiently than their more differentiated neoplastic counterparts [102]. GSCs also release periostin, which acts as a chemoattractant for TAMs through interactions with TAM's integrin receptor $\alpha_v\beta_3$ [103]. TAMs also influence the properties of GSCs, in that TGFβ released from TAMs induces MMP-9 expression and increases GSC invasiveness [104]. In addition, naive microglia can reduce the sphere-forming ability of human stem cells and in turn, suppress glioma growth. In contrast, microglia or TAMs cultured from glioma patients lack this antitumorigenic potential [105]. It is likely that GSCs secrete factors, which inhibit the phagocytosis activity of TAMs and induce the secretion of cytokines to prevent antitumor responses [106].

Due to the importance of microglia and TAMs in glioma growth and invasiveness, these cells are currently considered as therapeutic targets. Interfering with CSF1 signaling by antibody-mediated blockade or use of CSF1R inhibitors is a potential approach to regulate glioma growth by targeting TAMs [96]. Periostin has also emerged as an interesting target for attenuating the tumor-supportive phenotype of TAMs by interrupting integrin $\alpha_v\beta_3$ signaling [103]. Interfering with this pathway via a blocking peptide impairs TAM recruitment. Finally, Minocycline, an antibiotic that interferes with the process of microglia activation and has the unknown effects on tumor growth, is currently being tested in a clinical trial of MS patients [107]. However, as discussed above, the dual antitumoral and protumoral activities of microglia and macrophages should be taken into account

when the therapeutic strategy for malignant glioma is configured. Additionally, therapeutic strategies should evaluate the crosstalk of microglia and macrophages with other immune cells, as reviewed below.

5. Regulation of CNS Disorders: Crosstalk between Macrophage and NK Cells

We have discussed the highly diverse and complex function of microglia and macrophages during CNS autoimmunity—multiple sclerosis and cancer—malignant glioma. Considering the important roles of innate immune components in host defenses against these two types of CNS disorders, here we emphasize the crosstalk between CNS microglia/macrophages and NK cells, one of the important components of innate immunity, which has not been reviewed elsewhere. We focus on the discussion of how this type of cellular interactions impact on the pathological processes of both CNS disorders.

Macrophages regulate the functional activity of various innate immune subsets, including neutrophils, innate lymphocyte cells, and NK cells. NK cells exhibit potent cytotoxicity and produce cytokines in response to inflammation and stressed conditions, contributing to many facets of immune surveillance and tolerance [108]. It is well-recognized that the macrophage-NK interaction is a major first-line defense against pathogens. However, the crosstalk between macrophages, particularly microglia, and NK cells in the regulation of tissue-specific immune responses remains largely unknown.

Macrophages can activate or inhibit NK cell activity through either direct cell-to-cell contact via a diverse receptor-ligand interaction or soluble mediators, such as cytokines [109]. Conversely, NK cells also regulate the population size and functional activity of macrophages [109]. The outcome of the macrophage-NK interaction depends on the tissue origin of macrophages [110]. Interestingly, macrophages derived from peripheral blood mononuclear cell (PBMC) do not display the similar regulatory property as tissue-resident macrophages. The intensity and duration of macrophage-NK crosstalk depend on the nature of stimuli. For example, high doses of lipopolysaccharide (LPS) induce the expression of various ligands of the activating receptor NKG2D in human macrophages, UL16-binding proteins (ULBP1, ULBP2, and ULBP3) and MHC class I-related chain A (MICA) [111]. Human NK cells that are in contact with LPS-activated macrophages express increased levels of NKG2D. Consequently, NK cells lyse these macrophages stimulated with high doses of LPS to prevent endotoxic shock [111]. In contrast, LPS-stimulated microglia are less susceptible to NK cell-mediated cytotoxicity compared to resting microglia, likely due to reduced NKG2D expression in NK cells upon interactions with LPS-stimulated microglia [111]. Subsequently, this may help microglia present antigens to infiltrating T cells and initiate the immune response in the brain [112]. Other receptor-ligand pairs, including 2B4-CD48, NKp46-NKp46 ligand, CD226-CD112/CD155, and NKp80-AICL, also induce similar crosstalk effects as the NKG2D-NKG2D ligand on macrophage-NK cells, but only NKp46 engagement has been implicated in the NK-mediated killing of microglia [112]. Besides increased NK cytotoxicity, activated macrophages may also induce the release of IFNγ by NK cells that further amplifies the ongoing immune responses. In addition to activating interactions between NK cells and macrophages, there is also inhibitory crosstalk. We and others have previously reported that Qa-1, the homologue of human HLA-E and a ligand for the NK cell inhibitory receptor NKG2A, is upregulated on the surface of activated macrophages. Despite the unaltered NKG2A expression, the NKG2A-Qa-1 interactions allow the macrophages to escape NK cell-mediated lysis [113–115]. Consequently, blockade of the interaction between NKG2A on NK cells and Qa-1 on microglia by an anti-NKG2A antibody unleashes NK cell activity, reduces microglia activation, and decreases T cell infiltration into the CNS, leading to amelioration of EAE [114]. Due to the enhanced NK cell activity via the anti-NKG2A-mediated blockade, this antibody has also been applied in the clinical trials of multiple cancers (e.g., NCT02331875 and NCT02557516). It will be worthy to test the therapeutic efficacy of anti-NKG2A as a new generation of checkpoint inhibitors in the treatment of malignant glioma. Besides direct contact between macrophages and NK cells, their crosstalk is also regulated by the cytokines they produce. Macrophages produce IL-12, IL-15, IL-18, and IL-23 to induce the production of IFNγ, TNFα, or granzyme B by NK cells [109], whereas TGFβ1 and IL-10 released by macrophages, especially TAMs, inhibit NK cell function [109]. The latter may contribute to the exhausted or dysfunctional phenotype of NK cells, as observed in many tumors, including malignant glioma [100], which promotes tumor growth and invasion. Given the dynamic interaction between microglia/macrophages and NK cells that regulates the CNS inflammation, autoimmunity, and tumor, a more complete understanding of their molecular interplay may guide the development of optimal interventions of these CNS disorders.

6. Conclusion

As a resident macrophage population, microglia are critical components in the establishment and maintenance of a healthy nervous system. They not only purge damaged or unnecessary neurons and synapses, but also act as the primary form of active immune defense against infectious and stress-derived agents. Microglia and the CNS-infiltrating monocyte-derived macrophages actively participate in the regulation of innate and adaptive immune responses under pathological insults. We have discussed two types of CNS disorders here, multiple sclerosis with excessive immune responses and glioma with extreme immunosuppression. Although the cellular components share similarities between these two types of diseases (Figure 1), the mechanistic actions of CNS microglia and macrophages and their interactions with other immune cells are fully context-dependent. Additional studies are needed to dissect the differential contribution of microglia versus CNS-infiltrating monocyte-derived macrophages to these disorders. The discovery of P2ry12 as a specific marker for microglia definitely facilitates a more

precise understanding of these macrophage populations. In the future, a better understanding of molecular circuits that regulate the homeostasis and function of these macrophage populations may also direct more effective therapeutic strategies that specifically target individual subsets for better therapy of CNS autoimmunity, cancer, and other neurodegenerative disorders.

Conflicts of Interest

The authors declare that they have no conflict of interests.

Acknowledgments

The authors thank Dr. Jonathan D. Leavenworth for the critical reading and discussion. This work was funded by the American Cancer Society Institutional Research Grant (no. IRG-60-001-53 to Jianmei W. Leavenworth) and the University of Alabama at Birmingham Start-up funds (to Jianmei W. Leavenworth).

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Hindawi
Journal of Immunology Research
Volume 2017, Article ID 9570129, 9 pages
https://doi.org/10.1155/2017/9570129

Review Article

TNF Tolerance in Monocytes and Macrophages: Characteristics and Molecular Mechanisms

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Received 27 July 2017; Accepted 25 September 2017; Published 8 November 2017

Academic Editor: Kebin Hu

Tumor necrosis factor (TNF) tolerance in monocytes and macrophages means that preexposure to TNF reduces the sensitivity in these cells to a subsequent restimulation with this cytokine. Differential effects arise following preincubation with both low and high doses of TNF resulting in absolute as well as induction tolerance affecting specific immunologically relevant gene sets. In this review article, we summarize the relevance of TNF tolerance *in vivo* and the molecular mechanisms underlying these forms of tolerance including the role of transcription factors and signaling systems. In addition, the characteristics of cross-tolerance between TNF and lipopolysaccharide (LPS) as well as pathophysiological aspects of TNF tolerance are discussed. We conclude that TNF tolerance may represent a protective mechanism involved in the termination of inflammation and preventing excessive or prolonged inflammation. Otherwise, tolerance may also be a trigger of immune paralysis thus contributing to severe inflammatory diseases such as sepsis. An improved understanding of TNF tolerance will presumably facilitate the implementation of diagnostic or therapeutic approaches to more precisely assess and treat inflammation-related diseases.

1. Introduction

TNF is a potent proinflammatory master cytokine modulating inflammatory processes, and its rapid induction is fundamental for the orchestration of the immune response [1]. The application of TNF over a certain time period can result in a reduced sensitivity of cells, organs, or organisms towards a subsequent stimulation with the same cytokine, a phenomenon known as tolerance [2, 3]. TNF tolerance can be induced by a pretreatment of monocytes and macrophages with both low and high doses of TNF and can occur as absolute tolerance or induction tolerance following restimulation [3, 4]. In addition to pure TNF tolerance, several forms of TNF/LPS cross-tolerance have been described [5-7]. In this review, we will summarize the *in vivo* relevance of TNF tolerance as assessed in different animal models. In addition, we will refer to general features and the known molecular mechanisms underlying different forms of tolerance including the role of transcription factors, signaling systems, and receptors. Finally, the pathophysiological impact of TNF tolerance on both the resolution of inflammation and immune paralysis as well as potential diagnostic and therapeutic aspects targeting tolerance-associated mechanisms or molecules will be discussed.

2. TNF Tolerance *In Vivo*

TNF tolerance was first described in rats and mice on the basis of reduced physiological responsiveness towards a subsequent stimulation with TNF following a repeated application of sublethal doses of this cytokine within a certain time period [2, 8]. Monocytes and macrophages were early supposed to be important cellular mediators of TNF tolerance [2], and later experiments confirmed that assumption [3, 9] although other cell types (e.g., hepatocytes, cardiomyocytes, or epithelial cells) also proved to be prone to tolerization [10, 11]. *In vivo*, TNF tolerance is characterized by organo- and cytoprotective effects, as represented by the protection of tolerized mice against subsequent injections of normally lethal TNF doses [12]. Appropriately treated mice, rats, and guinea pigs are protected from inflammation-related symptoms such as fever [13, 14], gastrointestinal toxicity

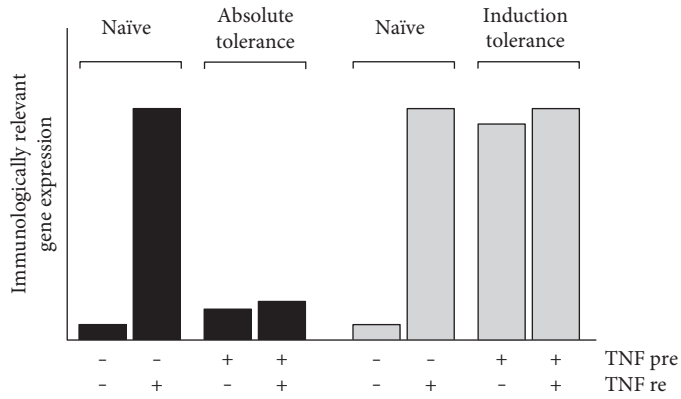

Figure 1: Two forms of TNF tolerance. TNF preincubation (pre) leads to reduced sensitivity towards further restimulation (re) with TNF (absolute tolerance) or causes elevated gene expression levels resistant to further upregulation following TNF restimulation (induction tolerance) in comparison to medium preincubated cells (naïve).

[15], liver injury [16], anorexia [17], hypertension, hypothermia, and lethality [18, 19]. Apoptosis, an important and common feature of inflammation-related diseases such as sepsis [20], is also suppressed in tolerant cells [16, 21]. In addition, disease-related alterations of physiological functions such as food intake normalized faster in TNF-tolerized mice than in control individuals [18, 22]. Since *in vitro* studies demonstrated in Hep G2 cells that TNF-associated cytotoxicity was more severe at fever-like temperatures, it has been speculated that TNF tolerance may be of importance for hepatic decompensation during febrile episodes [23]. In a sarcoma mouse model, it has been shown that the pretreatment with sublethal TNF doses may not only prevent the toxic effects of lethal doses of TNF but also reduce its antitumour effects, even when further increased doses were applied in tolerant mice [8]. An infection with adenoviruses can induce a tolerant-like state towards TNF, an effect which prevents LPS-induced mortality and liver injury/failure of the affected mice [24]. In addition, acquisition of TNF tolerance has been presumed in the case of malaria-infected mice, in which Plasmodium infection and released TNF did not result in perceivable symptoms of disease [25].

3. Molecular Characteristics

3.1. General Features of TNF Tolerance. TNF tolerance can be induced by a pretreatment of monocytes and macrophages (or other eligible cells) with both low and high doses of TNF [3, 4, 26]. Typically, low-dose preexposure/incubation was performed using up to 10 μg/kg (mostly human) TNF in animal experiments or up to 20 ng/ml in cell culture experiments [3, 27], while high doses included up to 100 μg/kg in animal studies [2]. It also has to be taken into account that human TNF can induce tolerance in animal experiments using higher doses due to a reduced cytotoxicity, for example, in comparison to murine TNF which proved to be lethal at significantly lower doses [19]. Since the biological activity of different TNF samples and batches could vary significantly, TNF is latterly applied for cell culture studies in amounts standardized to the biological activity, that is, ≤40 U/ml for low and >40 U/ml for high TNF doses [4]. Following restimulation which is always performed using higher TNF concentrations, tolerance can be observed as absolute tolerance or induction tolerance. In absolute tolerance, a low expression of immunologically relevant genes can be found following TNF preincubation which remains on this level even in the case of a subsequent restimulation when compared to the short-term stimulation of naïve cells (Figure 1). Induction tolerance is characterized by an increased expression of genes following long-term preincubation with TNF which is in general roughly comparable with the level observed in naïve cells after short-term stimulation. Following TNF restimulation, the expression of these genes is "frozen" on this level and cannot be further induced (Figure 1). Gene groups affected by absolute and/or induction tolerance are related to cellular functions such as inflammation, growth/differentiation, chemotaxis/migration, signaling/transcription, and metabolism [4]. For instance, genes such as TNF, interleukin (IL-) 1β, IL-6, IL-8, or tissue factor are affected by absolute tolerance, whereas IL-18, IL-28A, IL-32, or toll-like receptor (TLR) 2 are prone to induction tolerance. Although TNF is normally applied for 48–72 h during preincubation [4, 26], a time period of 18 to 24 h has been generally described to be minimally required for the induction of TNF tolerance [5, 12] and enables the maintenance of the tolerized state for several days [12]. However, under certain conditions, even a TNF pretreatment for 2 h appears to be sufficient to induce protection from TNF-dependent cell/organ damage [21]. The signaling network involved in development, consolidation, and regulation of monocytic/macrophagic TNF tolerance is only partially investigated, and only a limited number of cell culture studies characterizing the molecular basis of TNF tolerance exist yet. Currently, it is realized that TNF tolerance is based on a variety of distinct but connected and mutually depending molecular events covering several regulatory spectra of intracellular signal transduction. In the following, the known molecular mechanisms determining TNF tolerance are discussed.

3.2. Low-Dose-Induced Tolerance. Long-term preincubation with low doses of TNF predominantly leads to the

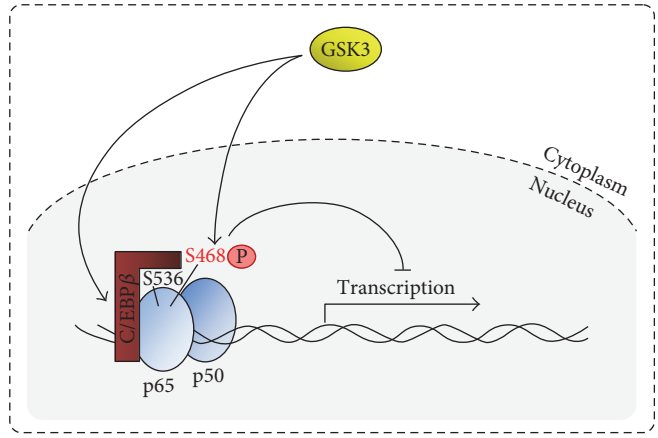

Figure 2: Transcriptional repression during low-dose-induced TNF tolerance. In low-dose TNF preincubated cells, p65 phosphorylation of the activating phosphorylation site Ser536 is blocked via direct protein-protein interaction with C/EBPβ. In addition, an increased phosphorylation of the inhibitory site p65-Ser468 can be observed. Both events may be influenced by GSK3 and negatively regulate the transcription of immunologically relevant genes in low-dose TNF-tolerant cells.

development of absolute tolerance [4]. The expression of most of the genes suppressed under these conditions is regulated by the transcription factors nuclear factor κB (NF-κB) and/or activator protein (AP-) 1 [4, 28] indicating that their limitation is an essential step in restricting the expression of these tolerance-sensitive genes. In low-dose-tolerized and restimulated monocytic cells, IκBα proteolysis, nuclear translocation of p65, and NF-κB DNA binding activity are only weakly affected [26]. However, following restimulation of low-dose-tolerized cells, activation of IL-8 promoter- and κB-dependent transcription is inhibited and p65 phosphorylation at the activating site Ser536 is markedly reduced in murine macrophage-like and monocytic THP-1 cells ("Tohoku Hospital Pediatrics-1", [29]) [4, 9] (Figure 2). The latter effect may be ascribed to an increased association of p65 with the transcription factor CCAAT/enhancer binding protein (C/EBP) β in low-dose-tolerized cells [9]. In this context, protein-protein interaction of p65 and C/EBPβ, which is an important regulator of proliferation and differentiation in myelomonocytic cells [30], results in a blockade of p65-Ser536 (and possibly other activating phosphorylation sites) [9, 31]. In addition, under these conditions (but also in high-dose-tolerized cells), p65 phosphorylation is intensified at Ser468 [4], a phosphorylation site negatively regulating p65 activity [32]. Interestingly, p65-Ser536 phosphorylation is resumed and low-dose TNF-induced tolerance is reversed by glycogen synthesis kinase (GSK) 3 inhibition using SB6763 [4], an effect also described within TNF-induced cross-tolerance towards LPS [6]. Furthermore, it has been reported that GSK3 phosphorylates C/EBPβ [33] as well as p65-Ser468 [32] which suggests a multistep influence of this kinase in TNF tolerance.

Moreover, both low- and high-dose TNF-preincubated THP-1 cells are characterized by attenuated phosphorylation of c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), and p38 [4, 5], that is, kinases targeting (amongst others) AP-1 subunits of the Jun and Fos protein families [34]. Total levels of these kinases, however, appear not to be significantly affected [4, 5]. As a consequence, concentrations of phosphorylated c-Jun were considerably lower in tolerized and restimulated monocytic cells than in stimulated naïve cells [4].

In murine models, it has been shown that a blockade of the glucocorticoid receptor (GR) using the antagonist RU-38486 can prevent the occurrence of low-dose-induced TNF tolerance *in vivo* suggesting that glucocorticoids and their receptor(s) contribute to the formation of tolerance [19]. This might be due to GR-dependent functions such as inhibition of proinflammatory signaling pathways, reduction of AP-1 DNA binding and NF-κB translocation, and induction of anti-inflammatory (e.g., IL-10) as well as down-regulation of proinflammatory cytokine/chemokine (e.g., IL-6, IL-8) expression [35]. For other transcription factors, a role within TNF tolerance has not been established yet. TNF-inducible factors such as activating transcription factor (ATF) or suppressor of cytokine signaling (SOCS-) 1, 2, and 3 have also been discussed with respect to an association with TNF tolerance [21]. However, ATF has not been analysed yet and for SOCS1-3, no influence on tolerance formation could be shown [21].

Taken together, the reported findings indicate that low-dose TNF-induced absolute tolerance is predominantly controlled via transcriptional mechanisms, that is, C/EBPβ-dependent suppression of p65 phosphorylation and potential GSK3 activity (Figure 2). In addition, reduced c-Jun/AP-1 activation and GR-dependent transcriptional repression may contribute to the development of low-dose-induced tolerance.

3.3. High-Dose-Induced Tolerance. Long-term preincubation with high TNF concentrations may result in the development of both absolute and induction tolerance [4]. Following restimulation, nuclear levels of p65 and NF-κB DNA binding activity are significantly reduced in high-dose-tolerized monocytic cells [4] in comparison to naïve and low-dose-tolerized cells [26]. In cells, long-term pretreated with high-dose TNF, IκBα turnover is increased and IκBα amounts are reduced to a lower level [4]. Following restimulation,

however, no further stimulus-induced proteolysis of IκBα can be observed. Consistently, it has also been observed that IκB kinase (IKK) phosphorylation is completely inhibited in high-dose pretreated and restimulated cells when compared to naïve cells [4, 36]. In TNF-overexpressing murine cardiomyocytes, additional activation of p50 homodimers has also been found in comparison to control cells and it was speculated that this effect might represent an adaptive response to reduce the detrimental inflammatory consequences of the permanent presence of TNF [11].

Due to the observation that A20 is a key molecule in the establishment of TNF/LPS cross-tolerance [6], its importance within TNF tolerance has also been assessed. A20 is a ubiquitinase/deubiquitinase known for its role as a repressor of NF-κB signaling [37, 38] and characterized by three major functionalities, that is, noncatalytic mechanisms mediating the repression of IKK activation, ubiquitin ligase activity leading to K48-labeling of proteins such as receptor interacting protein (RIP) to induce their proteasomal degradation, and protease activity towards K63 and M1 polyubiquitins [39]. A20 mRNA and protein amounts are markedly upregulated in THP-1 cells as well as primary human monocytes and macrophages pretreated with high TNF doses [4, 36], an effect equivalently occurring in cross-tolerance [6]. The siRNA-dependent knockdown of A20 leads to a strong upregulation of IKK phosphorylation and proinflammatory gene expression in monocytic cells in which TNF tolerance was induced by a high TNF dose [4, 36] indicating that A20 is a major regulator of high-dose-induced tolerance (Figure 3).

A20 interacts and cooperates with additional factors to form the A20 ubiquitin-editing complex, that is, the adaptor molecules Tax1-binding protein 1 (TAX1BP1) and RING-finger protein (RNF) 11 [40, 41] as well as the E3 ubiquitin protein-ligase Itchy homolog (Itch) [42] which have been described to support A20 during the regulation of ubiquitin-dependent TNF signaling [43]. Due to their essential contribution to A20 activity, these three proteins appear to be promising candidates in the initiation of TNF tolerance, but neither TAX1BP1 nor RNF11 or Itch was significantly regulated on the mRNA level in monocytic cells incubated with the high TNF dose for 48 h [4, 36]. Beyond initial mRNA expression analyses, however, an involvement of TAX1BP1, RNF11, and Itch in TNF tolerance has not been addressed yet and remains to be established. In contrast, mRNA and protein expression of two other regulators cooperating with A20 is increased in high-dose TNF pretreated monocytic cells [36]: A20 binding inhibitor of NF-κB (ABIN) 1, an A20 adjuvant protein [44], and cylindromatosis susceptibility gene (CYLD), a deubiquitinase possessing activities partly overlapping with A20 [39]. ABIN1 knockdown induced a modest elevation in IL-8 mRNA in TNF long-term-incubated cells which also could not be further elevated by TNF restimulation [36] indicating that ABIN1 cooperates with A20 in mediating TNF tolerance (Figure 3). CYLD knockdown results in an elevation of IL-8 mRNA in TNF long-term preincubated cells which was not further increased when the cells were restimulated with TNF. Effects of A20-siRNA application can be slightly further enhanced using a combination of A20- and CYLD-siRNA [36] suggesting that CYLD contributes to the A20-induced development of TNF tolerance and provides a certain amount of additive effects (Figure 3).

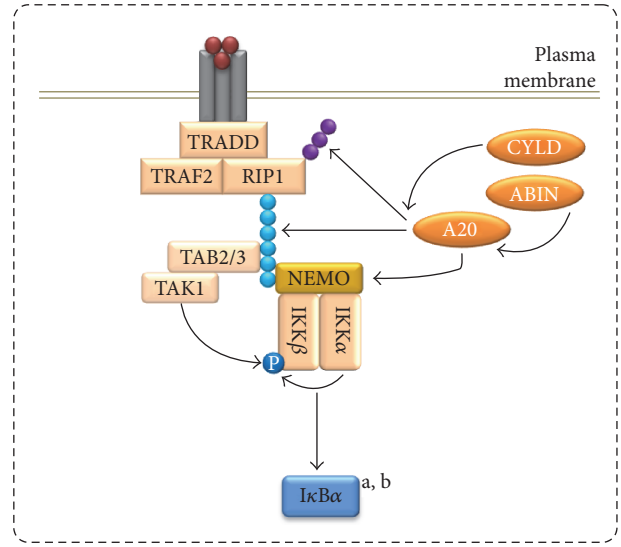

Figure 3: Inhibition of NF-κB-associated signaling during high-dose-induced TNF tolerance. The signaling complex at the TNFR1 consists of tumor necrosis factor receptor type 1-associated DEATH domain protein (TRADD), TNF receptor-associated factor 2 (TRAF2), and RIP1. Via RIP-associated K63-linked polyubiquitin chains (blue dots), other proteins are recruited, especially the IKK complex (consisting of NF-κB essential modulator (NEMO), IKKα, and IKKβ) and the TGF-β-activated kinase (TAK) 1/TAK 1 binding protein (TAB) 2/3 complex. In high-dose TNF preincubated cells, IKK phosphorylation is inhibited by A20 presumably via noncatalytic binding of NEMO, induction of RIP degradation by K48-polyubiquitination (purple dots), and/or hydrolyzation of K63 polyubiquitins on several signaling proteins. A20-mediated restriction of IKK activity is supported by ABIN1 and CYLD and results in modulated IκBα proteolysis: a, during pre-incubation, an increased IκBα turnover leads to lower IκBα levels; b, following restimulation, no further decrease of IκBα levels occurs.

It has also been demonstrated in high-dose-tolerized primary human monocytes that protein phosphorylation is affected by protein phosphatase (PP) 1, an enzyme [45] characterized under these conditions by increased mRNA expression of the catalytic subunit PP1CB and downregulation of the regulatory subunit PP1R14C. Repression of PP1 activity by PP1R14C overexpression or calyculin A treatment resulted in an abolition of high-dose TNF-induced absolute tolerance [4].

Together, these data suggest a model in which high-dose TNF-induced tolerance especially depends on the suppression of NF-κB-associated signaling by A20 which is supported by CYLD and ABIN1 (Figure 3). In addition, phosphatases may also be involved in the formation of TNF tolerance by reducing the global phosphorylation level in TNF preincubated cells.

3.4. Additional Mechanisms. In murine models, TNF tolerance can be induced by human TNF which acts as a selective inducer of murine TNFR1 but not murine TNFR2 [19]. Moreover, tolerance can be induced in TNFR2 knockout mice [16, 21] and the expression of glucocorticoids which may be involved in low-dose-induced TNF tolerance is activated by TNFR1-dependent signaling [19]. Thus, TNF tolerance appears to be mediated via TNFR1 [16, 19]. While total amounts of TNFR1 were not significantly affected during long-term treatment in monocytic THP-1 (high-dose TNF) or SW480 epithelial cells (low-dose TNF) [27, 36], TNF stimulation led to an internalization of the receptor within 2 h irrespective of the dose used [36]. Application of low-dose TNF during the preincubation phase, however, resulted in a (slow) recurrence of the initial level of TNFR1 within 48 h. In contrast, the use of high-dose TNF led to a permanent reduction of the receptor on the cell surface during the entire preincubation period, which could be reversed within 24 h when TNF was removed from the medium [36]. This suggests that receptor scarcity at the cell surface is another point restricting TNF-dependent signaling in tolerant cells, at least in primary human monocytes, since TNF pretreatment did not result in the downregulation of TNFR1 at the surface of tolerant murine hepatocytes [16]. However, the major functional role within the establishment of high-dose-induced TNF tolerance appears to be mediated by A20 as discussed above (see Section 3.3).

In mice and primary murine hepatocytes, low-dose TNF preexposure yields decreased amounts of hepatic ubiquitin-specific protease (USP) 2 [10]. Downregulation of USP2, either induced by TNF treatment or artificially using siRNA, resulted in the prevention of TNF-induced apoptosis, an effect occurring in combination with increased levels of cellular FLICE-like inhibitory protein (cFLIP), an antiapoptotic molecule [46], and downregulation of Itch [10], which acts as a cFLIP inhibitor in that context [47]. Vice versa, USP2 overexpression inhibited the establishment of TNF tolerance [10] indicating that USP2 represents a powerful inhibitor of tolerization, at least in the murine hepatic system.

4. Cross-Tolerance

The TNF-tolerant state may further include the refractoriness towards other stimuli. For instance, cells or animals tolerized with TNF are cross-tolerant towards gram-negative bacteria [7, 18], LPS [2, 6, 27], or other bacteria-derived agents such as lipophilic outer membrane vesicles [7]. Vice versa pretreatment with LPS [5, 48] or macrophage-activating lipopeptide 2 [49] induced the development of tolerance towards subsequent TNF application in THP-1 cells or mice and rats. Interestingly, tolerance may also be a result of certain infections, since adenoviral infection of mice has been described to yield a tolerance-like condition towards TNF treatment [24].

Due to the equivalent conditions they are creating especially in monocytes and macrophages, TNF tolerance and cross-tolerance appear to be based in part on overlapping or at least similarly operating molecular mechanisms. This assumption is already well substantiated in the literature revealing that both TNF- and LPS-induced tolerance/cross-tolerance are characterized by (1) decreased NF-κB activity in the nucleus under certain conditions [4, 50], (2) GR-dependent gene regulation [19, 51], (3) attenuated mitogen-activated protein kinase phosphorylation [4, 5], (4) regulatory influence of GSK3 activity [4, 6], (5) upregulation of A20 [6, 36], and (6) receptor downregulation at the cell surface [36, 51]. Together, these events finally lead to an inhibition of (primarily NF-κB-dependent) proinflammatory gene expression and the manifestation of a widely inert cellular state. However, since TNF tolerance and LPS/TNF cross-tolerance are established via different receptors (TNFR1 versus TLR4) [19, 50] and TNF tolerance is induced less rapidly by TNF than by LPS [22], specific mechanisms (depending on the respective inducer) influencing the unique features of both phenomena also have to exist.

Moreover, it has been described that the application of further agents can positively or negatively modulate the occurrence of TNF tolerance. For instance, the combined treatment of mice with TNF and leukemia inhibitory factor results in a significantly increased protective effect against a (generally lethal) LPS dose when compared to TNF application alone [52]. In contrast, the addition of human IL-1 completely prevented the development of TNF tolerance in mice normally induced by human TNF [19]. This indicates that an interaction or cross-reaction with other signaling pathways and regulatory events may modulate the events determining the occurrence of tolerance.

5. Pathophysiological Aspects of TNF Tolerance

To prevent deleterious consequences of inflammatory events such as excessive or chronic inflammation, a strictly controlled and fine-tuned termination of inflammatory processes is required [53-55]. As illustrated in this review, different forms of TNF tolerance can be observed following long-term (>24 h) preexposure to TNF [4, 5, 26]. The signaling quality occurring during the development of TNF tolerance significantly differs from the massive temporary activation of TNF-dependent signaling within the first 12 h of stimulation which has been characterized extensively [1, 56] and designated as phases I and II of TNF signaling [57]. Thus, TNF tolerance may play a role as a mechanism mediating refractoriness of monocytes and macrophages towards sustained proinflammatory cell activation in phase III (>12 h) of cytokine stimulation [36].

Due to its immunomodulatory effects, TNF tolerance may comprise several clinical implications in inflammatory and malignant diseases and represents a "Jekyll and Hyde"-like cellular process. On the one hand, tolerance may be a beneficial mechanism contributing to the resolution of inflammation and the protection from sepsis-associated hyperinflammation or prolonged chronic inflammatory diseases. In this context, loss of tolerance may favour the formation of chronic TNF-dependent inflammatory diseases in which NF-κB is activated such as rheumatoid arthritis [58], inflammatory bowel disease [59], or autoimmunity [60]. On the other hand, "too much tolerance" may be regarded as a deleterious event presumably involved in the development

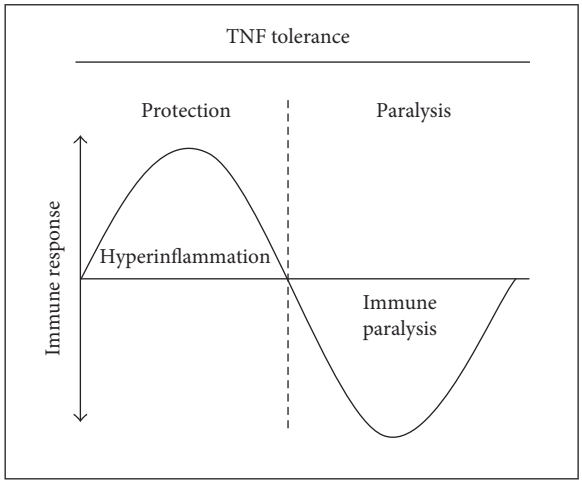

Figure 4: Two faces of TNF tolerance. To prevent hyperinflammation, refractoriness towards TNF may serve as a protective mechanism for cells and organisms to resolve inflammation and prevent acute or chronic inflammatory disease. On the other hand, excessive TNF tolerance may result in the paralysis of cellular immune functions, thus contributing to the state of immune paralysis characteristic for severe inflammatory diseases such as sepsis.

of immune paralysis and the shutdown of the immune system observed during certain phases of sepsis [61-63] (Figure 4). The balance between protection against excessive inflammation and immune paralysis may finally determine a patient's fate [62]. In malignant diseases, refractoriness towards TNF may also play a role [64]. For instance, it has been observed that tumour formation may occur as an adverse event of anti-TNF treatment in several inflammatory diseases [1].

6. Conclusions

As summarized in this review, TNF tolerance is generally characterized by the inability of affected cells, organs, or organisms to fully respond to a restimulation with TNF following a preceding long-term incubation with the same stimulus. Thus, TNF tolerance may play a role in severe acute or chronic inflammatory diseases in which TNF is present in large amounts. As such, TNF tolerance appears to be an interesting target within the efforts to improve the therapeutic modulation of exaggerated and/or prolonged dysregulated immune responses. However, despite a variety of experimental approaches and considerable progress in the characterization of its molecular regulation in recent years, the complex network of mechanisms determining occurrence and establishment of TNF tolerance is not completely elucidated yet. In addition, the distinct role of TNF tolerance in development and progression of inflammation-related diseases as well as its clinical relevance has to be precisely established [65].

A better characterization of the mechanisms determining TNF tolerance will improve the understanding of its clinical relevance and presumably facilitate the development of diagnostic approaches to assess different forms and states of tolerance. In addition, increased knowledge on TNF tolerance potentially offers the implementation of therapeutic approaches to treat inflammation-related diseases either by initiating or breaking the establishment of TNF tolerance.

Abbreviations

ABIN: A20 binding inhibitor of NF-κB
AP-1: Activator protein 1
ATF: Activating transcription factor
C/EBP: CCAAT/enhancer binding protein
cFLIP: Cellular FLICE-like inhibitory protein
CYLD: Cylindromatosis susceptibility gene
ERK: Extracellular signal-regulated kinase
GSK: Glycogen synthesis kinase
GR: Glucocorticoid receptor
IκB: Inhibitor of (NF-)κB
IKK: IκB kinase
IL: Interleukin
Itch: E3 ubiquitin protein-ligase Itchy homolog
JNK: Jun N-terminal kinase
LPS: Lipopolysaccharide
NF-κB: Nuclear factor κB
PP: Protein phosphatase
RIP: Receptor interacting protein
RNF11: RING-finger protein 11
SOCS: Suppressor of cytokine signaling
TAX1BP1: Tax1-binding protein 1
THP-1: Tohoku Hospital Pediatrics-1
TLR: Toll-like receptor
TNF: Tumor necrosis factor
TNFR: Tumor necrosis factor receptor
USP: Ubiquitin-specific protease.

Conflicts of Interest

The authors declare that they have no conflict of interests.

Acknowledgments

The authors are very thankful to Professor Dr. H.W. Löms Ziegler-Heitbrock, Professor Dr. Kyeong-Hee Lee, Dr. Nico Föger, Dr. Konstantin Neumann, and Mrs. Katharina Preuß for numerous critical discussions. The authors thank Mrs. Cornélia La Fougère-Brand for critical reading of the manuscript and apologize to all authors/working groups whose work has not been considered for this review due to space limitations or oversight. This work was supported by the German Research Foundation (Grant SFB 566/B17) and the Stiftung für Pathobiochemie und Molekulare Diagnostik der Deutschen Gesellschaft für Klinische Chemie und Laboratoriumsmedizin (DGKL).

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Hindawi
Journal of Immunology Research
Volume 2017, Article ID 3780572, 9 pages
https://doi.org/10.1155/2017/3780572

Review Article

Are the Therapeutic Effects of Huangqi (*Astragalus membranaceus*) on Diabetic Nephropathy Correlated with Its Regulation of Macrophage iNOS Activity?

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Received 6 July 2017; Revised 19 September 2017; Accepted 26 September 2017; Published 8 November 2017

Academic Editor: Hao Liu

Objective. To investigate the correlation between the clinical effects of Huangqi (*Astragalus membranaceus*) on different stages of diabetic nephropathy (DN) and the pharmacological effect of Huangqi on the activity of inducible nitric oxide synthase (iNOS) in macrophages in different states. *Methods*. The PubMed, China National Knowledge Infrastructure, and Wanfang databases were searched. Clinical data was sourced from papers on treatment of different stages of DN with Huangqi, and pharmacological data was from papers on the effects of Huangqi on the iNOS activity of macrophages in a resting or an activated state. *Results*. Meta-analysis of Huangqi injections on stages III and III-IV DN and randomized controlled trials on other stages showed that Huangqi had therapeutic effects on different stages of DN and on macrophages in different states: inducing normal macrophages in a resting state to generate nitric oxide (NO), tumor necrosis factor-α, and so forth upon iNOS activation; inhibiting NO generation by normal lipopolysaccharide- (LPS-) activated macrophages; and enhancing NO generation by LPS-induced macrophages from patients with renal failure. *Conclusions*. Huangqi can regulate iNOS activity of macrophages in different states *in vitro*. These biphasic or antagonistic effects may explain why Huangqi can be used to treat different stages of DN.

1. Introduction

Diabetic nephropathy (DN) is one of the most serious chronic microvascular complications of diabetes mellitus (DM) and is also the main cause of renal failure in end-stage chronic kidney disease (CKD). Mogensen et al. divide DN into five stages, according to the course and pathophysiological process of the disease (Table 1) [1]. Proteinuria is a hallmark of diabetic kidney disease and is also an independent risk factor for the progression of renal failure [2]. A pharmacological study showed that Huangqi (*Astragalus membranaceus*), a traditional Chinese medicine (TCM), attenuates proteinuria in a streptozotocin- (STZ-) induced model of diabetes [3].

Recent research showed that there are significant differences between urinary mRNA of podocyte-associated

Table 1: Mogensen's criteria for stages of DN [1].

Stage	Features
I	Hyperfunction, hypertrophy. Increased urinary albumin excretion (microalbuminuria).
II	Morphologic lesions without clinical signs of disease. Increased GFR. Poor diabetic control or exercise increase microalbuminuria.
III	Incipient DN. Persistent proteinuria (30–300) mg/24 h. Microalbuminuria slowly increasing over the years. Increased GFR.
IV	Overt DN. Persistent proteinuria (>0.5 g/24 h). Untreated hypertension leads to decreased GFR.
V	End-stage renal failure with uremia due to DN.

Note. DN: diabetic nephropathy; GFR: glomerular filtration rate.

molecules in relation with albuminuria stage [4], and the activation of macrophages causes podocyte damage in DN [5]. Macrophages in a resting state appear to have no obvious effect on renal injury, and macrophages in an activated state are very important to the disease progression. After inducible nitric oxide synthase (iNOS, nitric oxide synthase type II) is induced by lipopolysaccharide (LPS), high glucose, or the like, a large amount of nitric oxide (NO) is generated, a marker of macrophage activation [6].

There has been extensive research on Huangqi in the clinical treatment of DN, and Huangqi has been shown to exhibit particular therapeutic effects on different stages of DN [7–28]. Existing studies have shown that Huangqi has an inhibitory effect on the generation of NO by LPS-induced macrophages [29–42] and also that Huangqi itself can induce macrophages to generate NO [41–49]. This article attempts to analyze the correlation between these apparently antagonistic pharmacological effects of Huangqi on macrophages and the clinical effect of Huangqi in the treatment of different stages of DN, based on existing clinical data and the results of relevant pharmacological studies.

2. Materials and Methods

2.1. Literature Retrieval Strategy. The Chinese journal full-text database of China National Knowledge Infrastructure (CNKI), Wanfang database (Wanfang), and Medline were electronically searched, from the inception of the databases until August 2017. (*Astragalus membranaceus* OR Huangqi) AND (diabetic nephropathies OR renal failure) were selected as MeSH terms for clinical data. (*Astragalus membranaceus* OR Huangqi) AND macrophages AND nitric oxide synthase type II were selected as the MeSH terms for pharmacological studies.

2.2. Incorporation of Literature. Clinical data was incorporated from articles satisfying the following criteria: papers that demonstrate the therapeutic effects of Huangqi on different stages of DN, involving randomized controlled trial (RCT), semirandomized controlled trial (CCT), and meta-analyses of RCTs and CCTs. In such data, the DM should be diagnosed according to the diagnostic criteria of the WHO (1980, 1985, or 1999) or the American Diabetes Association (1997 or 2010), and the stages of DN should be diagnosed according to Mogensen's criteria for stage diagnosis [1].

Pharmacological studies were incorporated from articles satisfying the following criteria: *in vitro* pharmacological studies on the influence of Huangqi on the generation of NO, tumor necrosis factor-α (TNF-α), and so forth by macrophages upon iNOS expression. The macrophages include normal macrophages and immunocompromised macrophages, in either resting state or activated state.

3. Results

3.1. Preliminary Analysis of Clinical Studies on Treatment of DN with Huangqi. In this study, CNKI was initially used for a preliminary analysis of the clinical reports of treatment of different stages of DN with Huangqi. The retrieval results show that therapeutic effects of Huangqi on all stages of DN have been reported. 484 clinical research papers relate to treatment of different stages of DN with Huangqi, with the majority of these (243) referring to Huangqi injections.

3.2. Clinical Studies on Treatment of Different Stages of DN with Huangqi Injections. Based on the preliminary retrieval results of Section 3.1, Medline, Wanfang, and CNKI were searched. The papers involving treatment of DN with Huangqi injections were classified, collected, and summarized according to different stages: meta-analyses of therapeutic effects on different stages were first selected, and then relevant RCT or CCT studies were selected, so as to clarify the therapeutic effects of Huangqi injections on the five stages of DN. As shown in the flowchart in Figure 1, ultimately 22 references were chosen from a total of 356 references, including 5 meta-analyses and 17 RCTs [7–28].

There was only one RCT report about Huangqi injections against stages I-II of DN, with a relatively small sample size (28 in total, 13 in control group and 15 in Huangqi injection treatment group). The main improvement indicators reported on included urine albumin excretion rate (UAER), transforming growth factor β1 (TGF-β1), amongst others [7].

There are four meta-analyses that demonstrate the therapeutic effects of Huangqi injections on stage III DN. The studies incorporated into these four meta-analyses on stage III were conducted from 1998 to 2015, including 49 RCTs

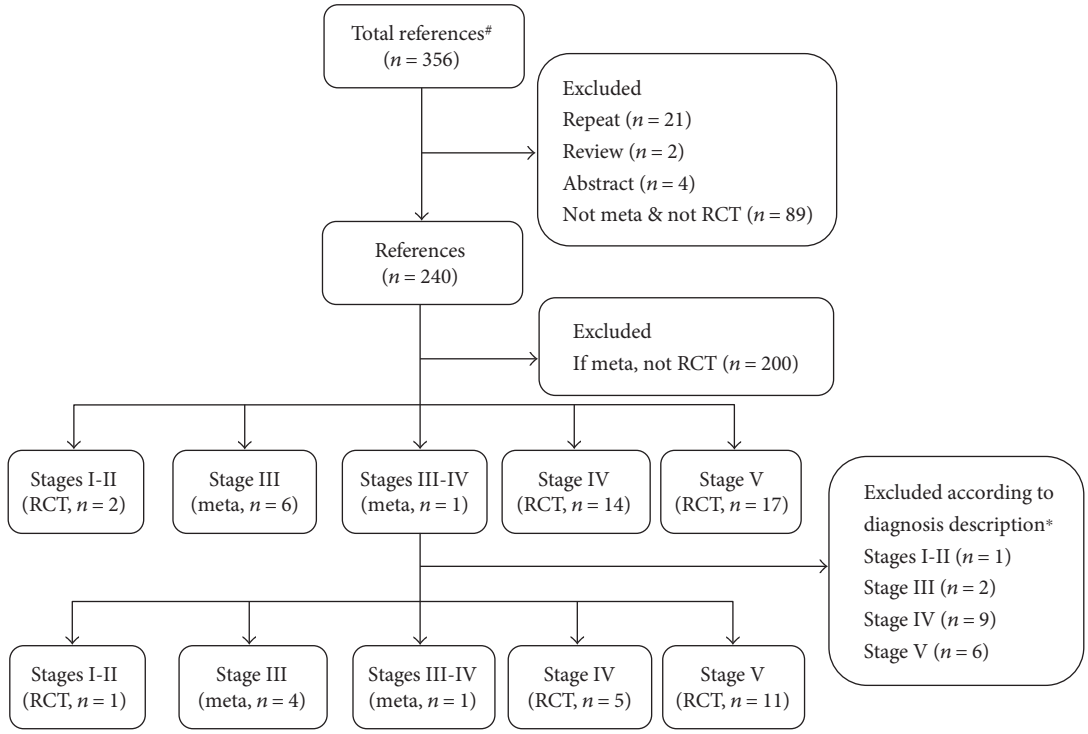

Figure 1: Flowchart on clinical trials. *Note.* #Total references included 243 from the Chinese journal full-text database of China National Knowledge Infrastructure and 113 from Wanfang database. *The diabetes mellitus should be diagnosed according to the diagnostic criteria of the WHO (1980, 1985, or 1999) or the American Diabetes Association (1997 or 2010), and the stages of diabetic nephropathy should be diagnosed according to Mogensen's criteria for stage diagnosis [1].

(29 RCTs were cited in more than one meta-analysis). The total cases were 3368 in which 1644 cases were in the control group and 1724 in the treatment group. UAER improved in these four meta-analyses. Both 24-hour urinary protein and serum creatinine (Scr) were reported in three analyses, and blood urea nitrogen (BUN) in two [8–11].

One meta-analysis related to stages III-IV was reported in 2011, including 21 RCTs and 4 CCTs [12]. A report about Huangqi injections at stage IV included five RCTs. The total cases were 455 (199 in the control group and 256 in the treatment group [13–17]). The improvement indicators included 24-hour urinary protein, Scr, BUN, and creatinine clearance rate (CCr).

An RCT report about stage V included 32 cases in the treatment group and 30 in the control group [18]. There are another 10 RCT papers related to renal failure research using Huangqi injections. We extracted 210 cases of renal failure due to DN (120 cases in the treatment group and 90 cases in the control group) from a total of 1372 cases [19–28]. All these were included in Table 2 stage V, and Scr, BUN, and CCr were improved [18–28].

3.3. Pharmacological Studies on Regulation of iNOS Activity of Macrophages in Different States by Huangqi. There are 21 articles about the influence of Huangqi on the generation of NO, TNF-α, and so forth by normal macrophages upon iNOS expression *in vitro* [29–49], including 9 in Chinese [29–32, 41–45], and 12 in English [33–40, 46–49], as shown in Table 3. The macrophages used in the studies were derived from either RAW264.7 macrophage cell line, peritoneal macrophages, or human mononuclear macrophage line U937.

Twelve articles reported that Huangqi could inhibit the generation of NO by LPS-activated macrophages [29–40]; eight studies were performed on Huangqi extracts, including crude extracts, active fractions, and compounds [31, 34–40], two studies on polysaccharides [29, 30], and another two on saponins and total flavonoids separately [32, 33]. Seven articles separately reported that Huangqi could induce normal macrophages in a resting state to generate NO, focusing on polysaccharides and saponins, with polysaccharides (6 studies) being the most studied [43–49]. Two articles reported that Huangqi could both induce the generation of NO by macrophages in a resting state and inhibit the generation of NO by macrophages in an activated state (polysaccharides and various combinations of polysaccharides with saponins) [41, 42].

Only one study concerned immunocompromised macrophages, derived from macrophages isolated from the dialysate of dialysis patients with renal failure. The results showed increased ability of macrophages to generate NO in the presence of LPS induction after Huangqi injection was administered to patients with renal failure for 9 days [50].

4. Discussion

In China, the estimated prevalence of DM in adults aged 18 and older is 11.6%, equating to about 114 million patients [51]. A study indicates that diabetes-related CKD has become

Table 2: Analyses of clinical studies on treatment of different stages of diabetic nephropathy with Huangqi *(Astragalus membranaceus)* injections.

Stage	Number of cases		Study design	Main improvement indicators	Publication year
	Control group	Treatment group			
I-II [7]	13	15	RCT	UAER, TGF-β1, HbA1c, C-IV	2004
III [8–11]	1644	1724	4 metas, including 49 RCTs	UAER [8–11], 24-hour urinary protein [8–10], Scr [8, 10, 11], BUN [8, 10], TG [8, 9], TC [8, 9], FBG [9, 10]	2013 (2004–2012)* 2013 (2005–2011)* 2014 (1998–2012)* 2017 (1998–2015)*
III-IV [12]	859	945	Meta, including 21 RCTs, 4 CCTs	24-hour urinary protein, Scr, BUN, CCr	2011 (1999–2006)*
IV [13–17]	199	256	5 RCTs	24-hour urinary protein [13–17], Scr [14, 16], BUN [14, 15], CCr [13, 17], TG [13, 16, 17], TC [13, 16, 17]	2000–2015
V [18–28]	120	152	11 RCTs	Scr [18, 20, 22, 23, 25–28], BUN [18, 22, 23, 25–28], CCr [22, 26, 27]	1997–2016

Note. *Publication year of cited paper of RCT or CCT. UAER: urine albumin excretion rate; TGF-β1: transforming growth factor β1; HbA1c: glycosylated hemoglobin; C-IV: type IV collagen; Scr: serum creatinine; BUN: blood urea nitrogen; TG: glycerin trilaurate; TC: total cholesterol; FBG: fasting blood glucose; CCr: creatinine clearance rate.

Table 3: Number of papers on Huangqi (*Astragalus membranaceus*) regulation of inducible nitric oxide synthase activity in different states of MΦ.

Sample type	On resting MΦ	On LPS-activated MΦ	On resting and LPS-activated MΦ	On LPS-activated immune compromised MΦ
Polysaccharides	6 [43, 45–49]	2 [29–30]	1 [41]	
Saponins	1 [44]	1 [33]		
Total flavonoids		1 [32]		
Extracts		8 [31, 34–40]		
Polysaccharides and saponins			1 [42]	
Injection				1 [50]

Note. On resting MΦ: effects of Huangqi on resting MΦ; On LPS-activated MΦ: effects of Huangqi on MΦ after LPS activation; On resting and LPS-activated MΦ: effects of Huangqi on resting MΦ and LPS-activated MΦ; On LPS-activated immune compromised MΦ: effects of Huangqi on immune compromised MΦ after LPS activation; MΦ: macrophages; LPS: lipopolysaccharide.

the main substantial effect on the observed spectrum of CKD [52]. Clinicians are facing the challenge of how to effectively control the occurrence and progression of DN.

The recommended therapeutic regimens for DN worldwide include controlling blood glucose and hypertension and reducing urinary albumin and blood lipids. In recent years, attempts to treat DN with TCM in combination with western medicine in China have achieved some efficacy. In a national basic research and development project, the efficacy and safety of conventional western medicine combined with TCM in the treatment of DN were evaluated by a multicenter, prospective cohort study, to investigate syndrome-based use of TCM for DN. The results showed that the rule of prescription of TCM was based on supplementing *qi* and nourishing *yin* and promoting blood circulation to remove blood stasis, for which Huangqi was used most frequently [53]. Based on this research, the results retrieved from CNKI showed that simple recipes, compound recipes, and other different formulations of Huangqi all have therapeutic effects on different stages of DN, and the majority of the papers concern Huangqi injections.

Huangqi injection was initially used clinically to treat hepatitis B in 1979 [54], and now is widely used in the treatment of leukopenia [55], viral myocarditis [56], and DN [7–28]. The quality standard for preparation of Huangqi injection, revised by the China Food and Drug Administration in 2002, specified that the amount of Astragaloside IV should be above 0.08 mg/mL [57]. Since Huangqi polysaccharides have many reported pharmacological effects, some of the existing studies investigate the molecular weights and distribution of polysaccharides in Huangqi injection, to provide further basis for the improvement of the quality standard for Huangqi injection [58]. A quantitative assay for simultaneously measuring saponins, such as Astragaloside IV, and flavonoids, such as calycosin and formononetin, contained in Huangqi injection is also being further improved [59, 60]. A pharmacokinetic study on Huangqi injection combined with other drugs such as gliquidone has

also provided further reference and basis for the clinical use of Huangqi injection [61]. An effective usage and management system has been established for Huangqi injection with respect to clinical dosage, course of treatment, monitoring of adverse reactions, and so forth through more than 30 years of formulation standardization and gradual improvement of the measurement of various effective ingredients.

The use of Huangqi injection for the treatment of DN was first reported in 1998 [62]. According to our research, Huangqi injection is mainly used on stages III and IV. After many years of multiple RCT observations, meta-analyses as a secondary evaluation can help us more accurately and objectively assess the therapeutic effects of Huangqi injection. Five meta-analyses related to stages III and III-IV were chosen in this paper (Figure 1).

Microalbuminuria occurs in stage III patients, and clinically stage III is normally called the "early stage" of DN. Persistent proteinuria, 30–300 mg/24 h, is a main feature in this stage [1]. If an effective intervention is delivered in this stage, the progression of DN is likely to be delayed or even reversed. This could explain why Huangqi injections are used far more in stage III than other stages. Research showed that Astragaloside IV improved proteinuria, UARE, and BUN in the rat STZ-induced model of diabetes [3]. In Table 2, in total, 70 RCTs and 4 CCTs in stages III and III-IV also showed improvement in indicators including 24-hour urinary protein, UARE, and BUN, and the therapeutic effect of Huangqi injection plus a conventional therapy was better than that of the control group receiving the conventional therapy alone [8–12].

The pathogenesis of DN involves many aspects, such as oxidative stress and immune inflammation. It is considered that macrophages play an important role in the development and progression of DN [5, 6]. In a mouse model of renal ischemia-reperfusion injury (IRI), macrophages were found to highly express iNOS in early IRI, which induced the apoptosis of renal tubular epithelial cells [63].

LPS is a potent inducer and activator of iNOS of macrophages to generate NO. The effect of Huangqi on the generation of NO by macrophages upon activation has been a focus of studies. The 14 articles [29–42] incorporated into this study separately reported that Huangqi extracts and the active ingredients thereof, including polysaccharides, saponins, and total flavonoids, had inhibitory effects on the generation of NO by normal macrophages induced by LPS. Three of these studies simultaneously reported that Huangqi can inhibit the LPS-induced generation of TNF-α [29, 30, 38], another specific indicator for activation of macrophage iNOS. The studies above showed that Huangqi inhibited the generation of NO by inhibiting LPS-induced iNOS activity.

Some animal experiments supported the view that the therapeutic effect of Huangqi injection on early DN might be achieved by inhibiting the activity of iNOS. Renal blood flow and glomerular filtration rate were significantly increased in diabetic mice after 4 weeks of STZ induction, with increased expression of iNOS in the renal cortex and medulla. The expression of iNOS was significantly decreased after administration of Huangqi injection [64].

A clinical study showed that after administration of Huangqi injection to patients with renal failure, the secretion of both NO and TNF-α from the macrophages isolated from their dialysate was enhanced upon LPS induction [50]. This study suggests that after Huangqi is administered to DN patients with end-stage renal failure, their immunocompromised macrophages in an LPS-activated state may present different functions from normal macrophages in an activated state.

DN is a progressive, chronic metabolic disease. There is still a question as to whether iNOS has a pathogenic effect or protective effect upon activation according to different periods of the disease and different states of the body. A study on different courses of DN in an animal model of STZ-induced DN indicated that iNOS might have different effects in different stages of the disease: in a model of very early stage DN induced by STZ for one week, glomerular hypertrophy and high filtration were associated with the high expression of insulin-like growth factor-1 (IGF-1) in the kidney. It was confirmed that the increase in IGF-1 was associated with an increase in NO, the source of which might be eNOS and iNOS [65].

A number of studies also demonstrated the protective effect of iNOS during the progression of DN. A study in a model of chronic DN for 40 weeks showed that iNOS-derived NO modulates glomerulosclerosis and tubulointerstitial fibrosis in chronic STZ nephropathy [66]. The drugs for treating DN such as pentoxifylline could exert therapeutic effects by increasing the expression of iNOS protein in the kidneys of a mouse model of STZ-induced DN [67].

Of the studies on Huangqi regulation of iNOS activity shown in Table 3, two studies compare the effects of Huangqi polysaccharides and polysaccharides plus saponins on normal macrophages in a resting state and macrophages activated by LPS induction. The results showed that Huangqi polysaccharides and saponins could not only inhibit the production of NO in LPS-induced macrophages but also induce macrophages to generate NO [41, 42]. Seven papers incorporated into this article reported that Huangqi could induce macrophages to generate NO and were focused on polysaccharides and saponins [43–49].

The results of a mechanistic study showed that Huangqi polysaccharides could significantly induce RAW264.7 cells to release NO and enhance iNOS activity. The nuclear factor-kappa B (NF-κB) cell signaling pathway was involved in the induction of NO generation and TNF-α secretion in macrophages by Huangqi polysaccharides, but NF-κB inhibitors did not completely inhibit the induction by Huangqi polysaccharides, suggesting that the NF-κB cell signaling pathway may not be the only pathway for this function [45]. Huangqi polysaccharides could induce the generation of NO and improve the phagocytosis of macrophages, which could be blocked by iNOS inhibitors [46]. The studies above suggest that Huangqi can increase the generation of NO by inducing the expression of iNOS in macrophages, thereby exerting pharmacological effects.

It is known that NO exerts biphasic and often antagonistic effects in many processes, depending on factors such as the local tissue concentrations and cell types and the

intensity and duration of the inflammatory phase where iNOS is initially produced [68]. Therefore, our first hypothesis is that the pathogenic or protective effect of iNOS generated upon induction may be related to different pathological stages of the same disease and different states of the same patient. The role of iNOS in the initiation, progression, and renal failure of DN still needs to be further clarified.

At present, we are interested in different effects of Huangqi on macrophages in different states: activated or resting and normal or compromised, as shown in the existing literature. Huangqi has different effects on the generation of NO by normal and compromised macrophages upon iNOS activation; it also has different effects on iNOS of macrophages in a resting state and an activated state. Based on the biphasic regulating effects of Huangqi on the generation of NO by macrophages *in vitro*, a second hypothesis is proposed: the therapeutic effects of Huangqi on different stages of DN may be due to effective intervention with some processes of the disease, such as activation or inactivation of iNOS in macrophages and increase or decrease in NO production, at different stages of DN, and at different states of the patient, thereby reversing the imbalances of the disease condition.

The existing preliminary studies support the clinically therapeutic effects of Huangqi on different stages of DN. On the basis of the accumulation of clinical data, clinicians in China are exploring how to obtain high-quality RCT research reports according to the CONSORT standard including standardizing randomized studies, establishing endpoint measures, and particularly strengthening the flowchart of subjects [69].

The pharmacological studies on treatment of DN with Huangqi by regulating the iNOS activity of macrophages are mostly based on *in vitro* experiments, and more *in vivo* studies are required. A recent *in vivo* study suggested that Astragalus polysaccharides may modulate the immunity of the host organism through activation of toll-like receptor- (TLR-) 4 mediated myeloid differentiation factor 88-dependent signaling pathway [70]. Another report suggests that Astragaloside IV might have anti-inflammatory effects *in vivo* by inhibiting the TLR4 signaling pathway [71]. The research showed that blocking TLR4 suppressed LPS-induced iNOS expression, and its role in kidney disease is being explored [72]. Based on this related research, further *in vivo* studies on iNOS/TLR4 pathway involvement might assist our understanding of the role of macrophages in the clinical mechanism of the effect of Huangqi on DN.

Following renal injury and repair in the different stages of DN, macrophages have been shown to exhibit critical regulatory activity. Disturbances in macrophage function can lead to aberrant repair, with uncontrolled inflammatory mediator and growth factor production [73]. Along with the development of phenotypic and functional changes in macrophages, clinical observations, and pharmacological research into Huangqi, further evidence is required to understand the possible biphasic or antagonistic effect of Huangqi on the regulation of macrophage differentiation and polarization, to clarify the roles of Huangqi and macrophages and their interaction in kidney disease.

Conflicts of Interest

All the authors, including Hui Liao, Ling Hu, Xingnuo Cheng, Xiaocheng Wang, Jiarui Li, Linda Banbury, and Rongshan Li, of this manuscript declare that there is no conflict of interest regarding the publication of this paper.

Acknowledgments

This study was supported by Basic Research Project (key fund project) of Shanxi Province (no. 201701D111001) and Shanxi Scholarship Council of China (no. 2015-105).

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