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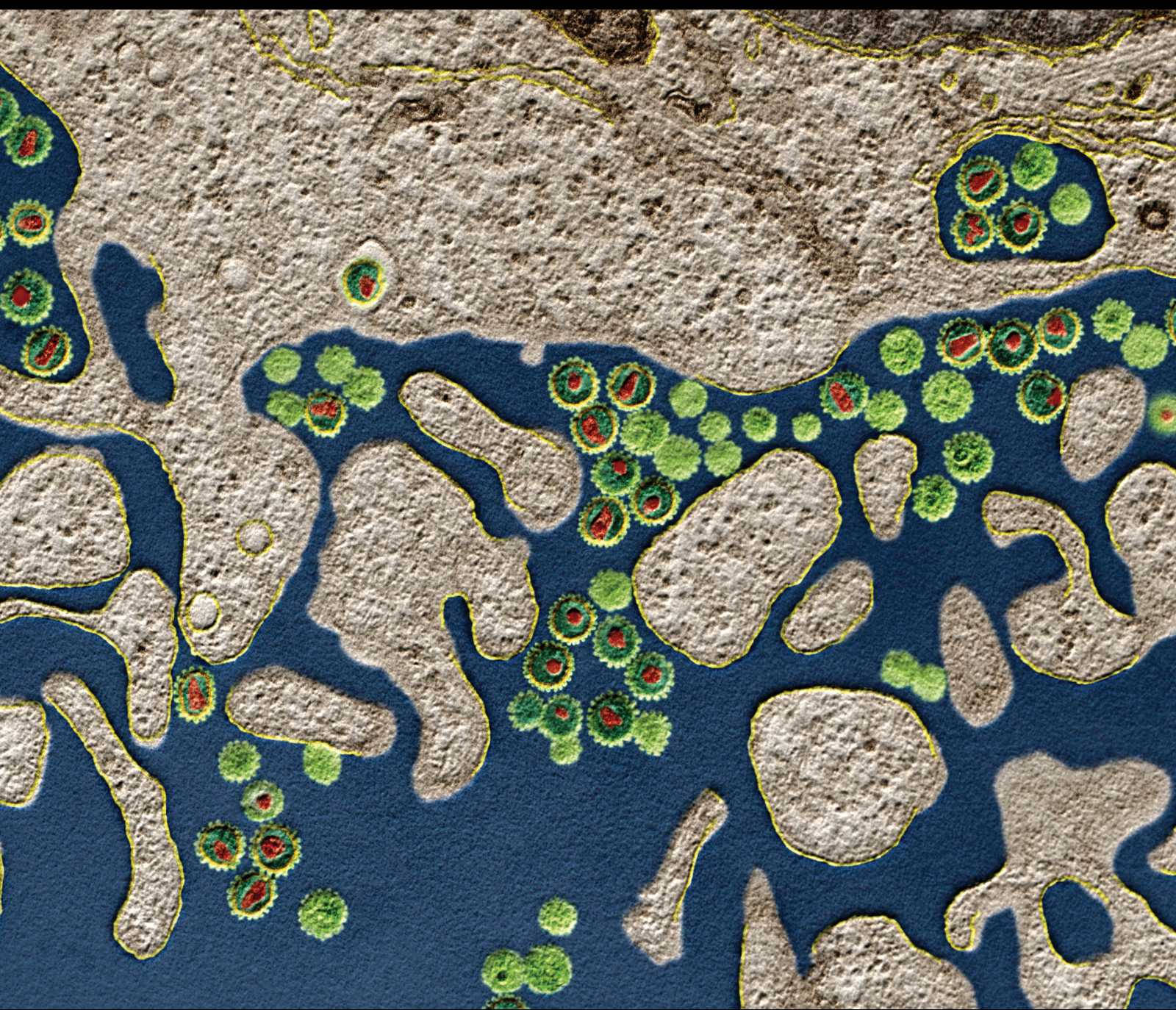
Neutrophils: Their Role in Innate and Adaptive Immunity

Guest Editors: Carlos Rosales, Nicolas Demaurex, Clifford A. Lowell, and Eileen Uribe-Querol



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Editorial Board

Contents

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Editorial

Neutrophils: Their Role in Innate and Adaptive Immunity

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Polymorphonuclear neutrophils (PMNs) are the most abundant leukocytes in the blood and constitute the first line of host defense against numerous infectious pathogens, including bacteria, fungi, and protozoa. Neutrophils are the first leukocytes to migrate from the blood to injured or infected sites for killing pathogens and removing cellular debris. Neutrophils migrate to sites of inflammation and infection where they recognize and phagocytose invading microorganisms, in order to kill them via different cytotoxic mechanisms. This process involves molecular mechanisms that coordinate cell polarization, delivery of receptors, and activation of integrins at the leading edge of neutrophils migrating toward chemoattractants. Once at sites of infection, neutrophils actively phagocytose microorganisms or form neutrophil extracellular traps (NETs) to trap and kill pathogens. Association of the nicotinamide adenine dinucleotide phosphate (NADPH) reduced oxidase complex at the phagosomal membrane for the production of reactive oxygen species (ROS) and delivery of proteolytic enzymes into the phagosome initiate pathogen killing and removal.

In recent years, it has become evident that neutrophils not only have a fundamental role in the acute phase of inflammation when they actively eliminate pathogens, but also are capable of modifying the overall immune response. Neutrophils can do this by exchanging information with macrophages, dendritic cells, and other cells of the adaptive immune system through either soluble mediators or direct cell-cell contact.

To illuminate the complex role of neutrophils in infection, inflammation, and immunity, this special issue has gathered original and review articles that will help us expand our knowledge on neutrophil biology.

As stated before, any neutrophil response begins with migration of these leukocytes to the site of infection or inflammation. Chemotaxis, the directional movement of the cell guided by extracellular chemoattractant gradients, plays an essential role in the recruitment of neutrophils to sites of inflammation. Chemotaxis is mediated by G protein-coupled receptor (GPCR) signaling pathway. The article by X. Xu and T. Jin describes the novel functions of the PLC/PKC/PKD signaling axis in GPCR-mediated chemotaxis of neutrophils. Similarly, the review by J. Gamara et al. explains how the small monomeric GTPases of the Arf family and their guanine exchange factors (GEFs) and GTPase activating proteins (GAPs) participate in GPCR signaling cascades regulating several neutrophil functional responses. The various cell responses to different chemoattractants are highlighted by the studies of R. Vaivoda et al. and M. A. Hidalgo et al. R. Vaivoda et al. describe how the deficiency of CYP4F18, an enzyme that catalyzes hydroxylation of leukotriene B4 (LTB4), does not alter the chemotactic response to LTB4 but causes a twofold increase response to complement component C5a. M. A. Hidalgo et al. describe that although N-formyl-methionyl-leucyl-phenylalanine (fMLF) and Platelet Activating Factor (PAF) induce similar intracellular signaling profiles, only fMLF induces interleukin-8 (IL-8) release and NADPH oxidase activity in neutrophils.

Several microbicidal functions of neutrophils involve the activation of the NADPH oxidase complex for production of reactive oxygen species (ROS) to mediate pathogen killing.

The interesting review by R. C. Allen describes the principles of particle physics and quantum mechanics to develop a fundamental explanation of neutrophil microbicidal metabolism based on ROS atomic properties. Myeloperoxidase, the most abundant neutrophil-granule protein, is known to have potent microbicidal properties. But, recently it has become apparent that myeloperoxidase also participates in regulating adaptive immune responses. The review by D. Odobasic et al. presents an overview on how this enzyme has key roles in various functions of neutrophils in innate and adaptive immunity. When neutrophils cannot kill microorganisms by the classical phagocytosis or degranulation mechanisms, they can also form neutrophil extracellular traps (NETs) to kill microbes. NETs are fibers composed of chromatin and neutrophil-granule proteins and are induced by several pathogens and also some pharmacological stimuli. Antigen-antibody complexes can also induce NET formation. The paper by O. R. Alemán et al. explores direct stimulation of individual Fcγ receptors to induce NET formation and finds that only FcγRIIIb cross-linking induced NET formation in a NADPH oxidase-, PKC-, and ERK-dependent fashion. An intriguing observation on the microbicidal function of neutrophils of older women is reported by J. Bartholomeu-Neto et al. They find that phagocytic and oxidative activities of neutrophils of healthy older women that exercise regularly are higher than those of sedentary older women. The physical condition of each individual was a significant predictor of phagocytosis potential. Clearly, regular exercise contributes to a better innate immune system. However, no mechanism for this beneficial effect is known. This should be an interesting line of future neutrophil research.

Neutrophils are potent regulators of inflammation via the release of proinflammatory factors and several cytokines. The paper by M. R. Tardif et al. describes an alternative secretion pathway in neutrophils for the release of the S100A8/A9 (calprotectin) and S100A12, proinflammatory mediators. The secretion of these cytoplasmic proteins was dependent on the production of ROS and required K^+ exchanges through ATP-sensitive K^+ channels. Because neutrophils can also cause tissue damage, their downregulation at the inflammatory site is required for proper resolution of inflammation. The paper by M. A. Sugimoto et al. describes how Annexin A1, an endogenous glucocorticoid-regulated protein, inhibits neutrophil tissue accumulation by reducing leukocyte infiltration and activating neutrophil apoptosis. Annexin A1 also induced macrophage reprogramming toward a resolving phenotype, resulting in reduced production of proinflammatory cytokines and increased release of immunosuppressive and proresolving molecules. Similarly, the paper by M. Cohen-Mazor et al. describes how heparin binds to activated neutrophils and induces apoptosis. These results provide an explanation for the long-known anti-inflammatory effects of heparin.

Neutrophils seem to have dual roles in promoting and controlling inflammation. The mechanisms that control the final outcome are not completely described, but these opposite functions must be tightly balanced. During sepsis, neutrophils are responsible for both the release of cytokines and the phagocytosis of pathogens. But, in SIRS (systemic inflammatory response syndrome), neutrophils contribute to maintaining of a whole-body inflammatory state. In the review by H. Fang et al., the role of neutrophils in these two clinical conditions is described and the therapeutic effect of G-CSF in sepsis is discussed in relation to its function of regulating neutrophil blood levels. Moreover, because neutrophil-derived products can regulate the action of other immune cells and can contribute to the development and chronicity of inflammatory diseases, I. Naegelen et al. propose in their article an original strategy based on linear fitting, to analyze the link between cytokine release and degranulation time. This method could find correlations between granule proteins and cytokines secreted to the inflammatory site. The idea is to be able in the future to predict the type of inflammatory response that neutrophils could induce under certain conditions. In addition, C. F. M. Morris et al. describe in their review how two apparent opposite models of inflammation may be compatible in the outcome of inflammation. The two-hit model states that a first injury (i.e., hit) can serve as a priming event which sequential insults can build on, culminating in a disproportioned inflammatory response to injury. On the other hand, the ischemic preconditioning (IPC) model states that a mild ischemic event, either remote or local, can be protective and can actually attenuate the inflammatory response to the following insults. This article tries to reconcile both models and presents evidence that each of them brings its own unique perspective onto the biology of inflammation. Finally, the dual role of neutrophil function during inflammation is emphasized again in the review by E. Uribe-Querol et al., where the protumor or antitumor character of the tumor-associated neutrophils (TANs) is revised. Recent findings on the mechanisms for neutrophil recruitment to the tumor, for neutrophils supporting tumor progression, and for neutrophil activation to enhance their antitumor functions are presented.

These articles together represent the fascinating flexible functions of neutrophils not only in fighting infections, but also in shaping the immune response and the consequences this may have to important health issues such as resolution of inflammation, autoimmunity, and cancer.

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Review Article

Neutrophil-Mediated Regulation of Innate and Adaptive Immunity: The Role of Myeloperoxidase

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Neutrophils are no longer seen as leukocytes with a sole function of being the essential first responders in the removal of pathogens at sites of infection. Being armed with numerous pro- and anti-inflammatory mediators, these phagocytes can also contribute to the development of various autoimmune diseases and can positively or negatively regulate the generation of adaptive immune responses. In this review, we will discuss how myeloperoxidase, the most abundant neutrophil granule protein, plays a key role in the various functions of neutrophils in innate and adaptive immunity.

1. Neutrophils in Innate and Adaptive Immunity: The Role of MPO

Neutrophils are capable of affecting many aspects of both innate and adaptive immunity. They are well known to be the first leukocyte to arrive at sites of infection. There, they play a key role in the clearance of pathogens, both by phagocytosis and by subsequent intracellular killing, as well as the release of neutrophil extracellular traps (NETs) into the extracellular space [1]. However, through the release of various inflammatory mediators, neutrophils can also contribute to tissue injury and organ damage in inflammatory and autoimmune diseases. On the other hand, neutrophil proteins are targets in autoimmune anti-neutrophil cytoplasmic antibody- (ANCA-) associated vasculitis (AAV) [2]. In more recent years, evidence has been accumulating to show that not only do neutrophils act at sites of inflammation, but they also infiltrate secondary lymphoid organs where they regulate the development of adaptive immunity [3]. MPO, the major protein in neutrophil granules, has been shown to be one of the key players in the neutrophil functions described above. This paper will review the contribution of MPO to neutrophil-mediated intracellular microbial killing, formation of NETs, and tissue damage, as well as the development of AAV. Particular attention will be given to the more recently described and less well known function of neutrophil MPO as a regulator of adaptive immunity.

2. Biosynthesis, Cellular Sources, Storage, and Release of MPO

MPO, which was originally named verdoperoxidase due to its intense green colour, is a highly cationic, heme-containing, glycosylated enzyme [4] which is found mainly in primary (azurophilic) granules of neutrophils, making up approximately 5% of the total dry cell weight [5]. Human neutrophils contain about 5–10-fold higher levels of MPO than murine neutrophils [6]. MPO is also found, to a lesser extent, in monocytes where it constitutes about 1% of total cell protein [7]. During monocyte-to-macrophage differentiation, MPO expression is generally lost [8]. However, MPO can be found in some macrophage subpopulations including resident tissue macrophages such as Kupffer cells [9], peritoneal macrophages [10], and microglia [11], as well as in organ infiltrating macrophages in various inflammatory diseases including atherosclerosis [12], multiple sclerosis (MS) [13],

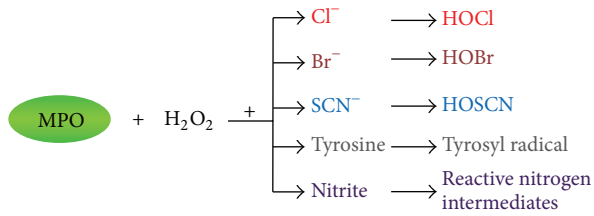

Figure 1: *Reactive intermediates formed by MPO*. In the presence of hydrogen peroxide and chloride, bromide, thiocyanate, tyrosine, or nitrite, MPO catalyses the formation of hypochlorous, hypobromous, and hypothiocyanous acids, tyrosyl radical, and reactive nitrogen intermediates. H_2O_2, hydrogen peroxide; Cl^-, chloride; Br^-, bromide; SCN^-, thiocyanate; HOCl, hypochlorous acid; HOBr, hypobromous acid; HOSCN, hypothiocyanous acid.

and AAV [14]. Macrophages can also acquire neutrophil-derived MPO by phagocytosis of apoptotic neutrophils or uptake of extracellular MPO through the mannose receptor [15].

Although it is possible for MPO transcription to be reinitiated in macrophages under certain conditions [16], MPO synthesis is otherwise restricted to myeloid cells in the bone marrow [17, 18]. During granulocyte/monocyte differentiation in the bone marrow, only promyelocytes and promyelomonocytes actively transcribe the MPO gene [17]. The primary 80 kDa MPO translation product undergoes cleavage of the signal peptide and N-linked glycosylation [17], resulting in a 90 kDa apoproMPO which is heme-free and thus enzymatically inactive [17]. During association with endoplasmic reticulum chaperones, calreticulin and calnexin, apoproMPO acquires heme and becomes the enzymatically active precursor proMPO [19], which then enters the Golgi. After exiting the Golgi, a series of proteolytic steps follow during which the propeptide is removed and the protein is cleaved into a heavy (α) subunit (59 kDa) and a light (β) subunit (13.5 kDa) joined together by disulfide bonds [8]. This heavy-light chain complex dimerizes to generate mature, enzymatically active MPO containing a pair of heavy-light protomers and two heme groups [8]. Human and mouse MPO have molecular weights of 146 kDa and 135 kDa, respectively [8, 20].

Mature MPO is stored in azurophilic granules of fully differentiated neutrophils. However, following priming and activation by inflammatory mediators including TLR ligands and cytokines such as GM-CSF and TNF, as well as Ig/Fc receptor-mediated signals [22–24], MPO can be released via multiple mechanisms. Neutrophils can rapidly release MPO by degranulation and by cell death pathways including apoptosis and necrosis [8, 21, 25]. More recently, it has been shown that MPO can be released from neutrophils via the extrusion of NETs [26]. Interestingly, some proMPO is also constitutively secreted by neutrophils via the Golgi [17], but the function and physiological relevance of extracellular proMPO are still to be elucidated.

3. Production of Reactive Intermediates by MPO

In the presence of hydrogen peroxide (H_2O_2) and a low-molecular-weight intermediate (halide: chloride, bromide, or thiocyanate; tyrosine; or nitrite) MPO catalyses the formation of powerful reactive intermediates including hypochlorous (HOCl), hypobromous (HOBr), and hypothiocyanous (HOSCN) acids, tyrosyl radical, and reactive nitrogen intermediates (Figure 1), all of which can have profound effects on cellular function by modifying proteins, lipids, and/or DNA [8]. The H_2O_2 required for MPO function comes mainly from the phagocyte NADPH oxidase during the respiratory burst [8]. Given its abundance in physiological fluids [27], chloride is believed to be the physiological halide and, therefore, a preferred substrate for MPO and subsequent HOCl production in most circumstances.

HOCl is a short-lived, but very potent chlorinating oxidant [28]. It can oxidize/chlorinate a variety of targets including proteins, lipids, and DNA and thus have significant biological effects [28]. Formation of 3-chlorotyrosine (due to HOCl chlorination of tyrosine on proteins) and, more recently described, glutathione sulfonamide (GSA; a product of HOCl-mediated oxidation of glutathione), serves as specific biomarkers of MPO/HOCl production *in vivo* [8, 28–30]. Taurine, a free amino acid present at high concentrations in neutrophils [31], also readily reacts with HOCl to form taurine chloramine, a less reactive, but long-lived oxidant which can contribute to cell damage [8]. More detailed descriptions of the reactions catalysed by MPO and the oxidants it produces are provided elsewhere [8, 32].

4. The MPO/HOCl System in Intracellular and Extracellular Microbial Killing by Neutrophils

Neutrophils are one of the most important front line defenders involved in microbial ingestion and subsequent killing. Several lines of evidence demonstrate that the MPO/HOCl system plays an important role in optimal intracellular killing of bacteria (e.g., *Pseudomonas aeruginosa*) and fungi (e.g., *Candida albicans*) by neutrophils [8, 33, 34]. It should be noted, though, that the clearance of several pathogens including *Staphylococcus aureus* and *Candida glabrata* is not affected by the absence of MPO [8, 34]. In addition, the majority of MPO-deficient patients do not suffer from chronic infections despite the demonstration of a neutrophil microbicidal defect *in vitro* [8]. This suggests the existence of MPO-independent antimicrobial systems such as

reactive nitrogen intermediates and proteases which have been shown to contribute to microbicidal activity of neutrophils in the presence as well as in the absence of MPO [32, 35]. In addition, the reduction of microbial killing due to the absence of MPO in humans may be compensated by an enhancement of protective (i.e., antimicrobial) adaptive immunity, as discussed in more detail below. For in-depth discussion about the well recognised and extensively studied role of MPO in intracellular microbial killing by neutrophils, we suggest references to more comprehensive past reviews [32].

Neutrophils are also well known to release NETs, structures composed of decondensed chromatin, histones, and various antimicrobial molecules including elastase and MPO, into the extracellular space [1], thus aiding in the overall elimination and spread of pathogens. NETs can trap extracellular microbes that they come in contact with and, although limited, evidence also exists to suggest that NETs can kill some, but not all, extracellular microbes [36, 37]. Together with elastase, MPO has been demonstrated to associate with nuclear DNA/histones and play a role in NET formation as well as NET-mediated bacterial killing [38–40]. Studies with normal and MPO-deficient human neutrophils showed that MPO contributes to the formation/release of NETs in response to stimulation with PMA or *Candida albicans* [38]. Similarly, the induction of NETs in neutrophils from healthy donors via TNF, IL-8, or IL-1β, in the absence of any infectious stimuli, required the presence of active MPO [41]. A recent study has added to our understanding of how MPO contributes to NET formation inside human neutrophils by showing that MPO activates elastase allowing it to enter the nucleus where it can then associate with DNA/histones [42].

However, MPO is not required for NET formation with all stimuli. For example, in human neutrophils stimulated with *S. aureus* or *E. coli*, inhibition of MPO had no effect on NETs [43]. Reports in which human neutrophils were stimulated with *Pseudomonas aeruginosa* have yielded conflicting results [43, 44], the explanation for which is still to be provided. Furthermore, studies using cells from MPO-deficient animals or inhibitors of MPO activity showed that MPO is not involved in PMA/bacteria-induced NETosis by murine neutrophils [44]. Mouse neutrophils do contain less MPO than their human counterparts [6], which may provide a partial explanation for the discrepancy between the human and murine studies.

In addition to playing a role in NET formation, MPO has been demonstrated to contribute to NET-mediated killing of extracellular microbes. NET-associated MPO is enzymatically active and can produce HOCl in the presence of its substrate, H_2O_2 [39]. Production of HOCl by MPO bound to NETs resulted in the killing of *S. aureus in vitro* and inhibition of MPO or addition of a strong HOCl scavenger methionine reversed this effect [39]. This suggests that HOCl generated by NET-associated MPO may also play an important role in extracellular bacterial killing *in vivo* at sites of inflammation.

5. MPO-Derived Oxidants Cause Tissue Injury in Inflammatory/Autoimmune Diseases

Through the release of various mediators including reactive oxygen species (ROS), proinflammatory cytokines, and proteases, neutrophils play an important role as effector cells in many inflammatory and autoimmune diseases including cardiovascular disease and atherosclerosis, rheumatoid arthritis (RA), and inflammatory diseases of the lung and kidney [1]. Through the formation of reactive oxidating/chlorinating agents, MPO is one of the key neutrophil-derived mediators contributing to organ inflammation and fibrosis in many immune-mediated diseases. As this classical effector function of MPO has been comprehensively described in many other reviews [8, 45], it is not discussed here in detail. Active MPO and/or its products such as HOCl-modified proteins, 3-chlorotyrosine and GSA, are upregulated at sites of inflammation in cardiovascular disease and atherosclerotic lesions, RA joints, and lungs of patients with cystic fibrosis and inflammatory and fibrotic kidney disease [12, 29, 46–49]. This indicates MPO-mediated damage by reactive oxidants, mainly HOCl. Reports showing that there is significant attenuation or exacerbation of disease due to the absence of endogenous or administration of exogenous MPO, respectively, in models of these conditions [50–58], further support the hypothesis that MPO is an important local mediator of inflammation and subsequent organ damage.

MPO-containing NETs have also been implicated in the pathogenesis of several inflammatory diseases including systemic lupus erythematosus (SLE), atherosclerosis, and RA. For example, glomerular NET deposition positively correlates with anti-dsDNA autoantibody levels and the severity of lupus nephritis [59]. Recently, NET-induced macrophage activation and cytokine production have been reported to contribute to the development of atherosclerotic lesions [60]. In addition, lipid oxidation/chlorination by the MPO/HOCl system is suggested to contribute to the pathogenesis of atherosclerosis and SLE [61, 62]. Enhanced formation of NETs, providing a source of citrullinated autoantigens, is also observed in joints of patients with RA [63]. MPO is present on NETs in target organs in SLE, atherosclerosis, and renal vasculitis [14, 59, 60] and it is therefore plausible, although not yet experimentally confirmed, that NET-bound MPO contributes to organ inflammation and injury in those conditions given that NET-associated MPO is enzymatically active and can produce tissue damaging HOCl in the presence of H_2O_2 [39].

6. MPO as an Autoantigen in AAV

In addition to neutrophil-derived inflammatory mediators contributing to tissue damage in inflammatory conditions as effector molecules, neutrophil proteins such as proteinase-3 and MPO play a key role in the development of autoimmune AAV, acting as targets (i.e., autoantigens) against which the pathogenic immune response has been generated. MPO is a common autoantigen in AAV, a disease characterised by inflammation of small blood vessels including glomerular capillaries in the kidney and commonly associated with

the presence of pathogenic neutrophil-activating MPO-ANCA [64]. Renal biopsies from vasculitis patients show a prominence of glomerular delayed-type hypersensitivity effectors (T cells, macrophages, and fibrin) and neutrophils, suggesting that cellular immunity, together with MPO-ANCA, plays a significant part in the disease process [65].

Evidence from animal studies, which is supported by human observations and *in vitro* experiments [65–72], suggests that the pathogenesis of MPO-AAV involves 4 major steps, as outlined below. First, MPO-specific autoimmunity develops in secondary lymphoid organs, resulting in the emergence of autoreactive effector CD4 T cells and MPO-ANCA-producing B cells. Although it is not known how autoimmunity to MPO is generated in humans, evidence from animal studies shows that activation of myeloid DCs by NETotic, but not apoptotic or necrotic, neutrophils can result in the generation of MPO-specific autoimmunity and development of renal vasculitis [73]. Of note, the induction of autoimmunity by NET-activated DCs in the animal studies was not restricted to MPO but also resulted in the generation of anti-dsDNA antibodies [73] which are associated with SLE. This is not surprising since NET release by neutrophils would expose a variety of intracellular autoantigens for presentation to DCs. Recently, the immunodominant MPO T cell epitope ($MPO_{409–428}$) was defined in mice [72], and, interestingly, it showed significant overlap with the dominant B cell epitope in AAV patients [74], indicating its relevance to human disease. Importantly, $MPO_{409–428}$ was shown to be nephritogenic since immunisation of mice with $MPO_{409–428}$ resulted in the generation of pathogenic MPO-specific CD4 T cells and ANCA [72].

Second, neutrophil priming by cytokines (e.g., TNF), which may occur after infection-related stimuli, leads to MPO exposure on the cell surface, allowing ANCA to bind and fully activate the neutrophils. Third, ANCA-activated neutrophils lodge in glomeruli [75], causing injury [76] and depositing the autoantigen, MPO [69]. Finally, MPO-specific effector CD4 T cells migrate to inflamed glomeruli where they recognise MPO and direct accumulation of macrophages and fibrin, causing, together with ANCA-induced neutrophil responses, severe and proliferative renal vasculitis (glomerulonephritis; GN) [69, 72]. The antigen presenting cells exposing MPO peptides/MHC-II for recognition by effector CD4 T cells in glomeruli are yet to be identified; however several potential candidates exist including intrinsic glomerular cells such as endothelial cells and podocytes, as well as kidney-infiltrating MPO-positive macrophages and neutrophils. All these cell types can upregulate surface MHC-II and costimulatory molecules in response to proinflammatory stimuli and have been shown to contain MPO in biopsies from patients with AAV [14, 77–80].

In addition to acting as an autoantigen in AAV, MPO may contribute to disease pathogenesis through its enzymatic activity and production of oxidative/chlorinating radicals, although this remains to be confirmed. Extracellular, including NET-associated, MPO is pronounced in glomeruli of AAV patients [14, 26]. Future studies are yet to demonstrate the presence of specific biomarkers of MPO activity such as 3-chlorotyrosine, HOCl-modified proteins, or GSA, to suggest MPO-mediated damage in AAV. Moreover, recent advancements in the development of specific MPO inhibitors for *in vivo* use [81–83] should make it more feasible to investigate whether MPO contributes to renal injury in models of AAV via its enzymatic activity.

7. Nonenzymatic Functions of MPO in Inflammation

MPO actions are mediated predominantly via its enzymatic activity and generation of reactive intermediates. However, evidence exists demonstrating that it can also regulate the function of immune and nonimmune cells via its nonenzymatic effects. For example, by binding to CD11b/CD18 (Mac-1), MPO can induce neutrophil activation in an autocrine fashion including MAPK and NFκB activation, ROS production, surface integrin upregulation, and degranulation [84], as well as decreased apoptosis leading to enhanced inflammation in the lung [85]. In addition, human leukocytes can adhere to MPO via binding to CD11b/CD18 [86] which may also contribute to the proinflammatory effects of MPO by further augmenting leukocyte accumulation at sites of inflammation. Inactive MPO has also been shown to increase macrophage activation such as cytokine production and induction of respiratory burst *in vitro* [87]. These observations are likely to be relevant *in vivo* as well since injection of inactivated MPO into the joints of rats exacerbated symptoms of arthritis [52]. Here, the proinflammatory effects of MPO were reversed by injection of mannan, thus most likely blocking the interaction between extracellular MPO and mannose receptor on macrophages [52]. Furthermore, enzymatically inactive MPO can activate endothelial cells to produce cytokines such as IL-6 and IL-8 [88]. The exact mechanisms by which this occurs are unknown, but MPO-mediated endothelial cell activation is likely to add to the proinflammatory effects of MPO, since the leukocyte-endothelial cell interaction is one of the critical processes in inflammatory responses within tissues.

8. MPO Suppresses the Generation of Adaptive Immunity

In addition to playing an important role at sites of inflammation, neutrophils have been shown to contribute to the development of adaptive immunity. A number of studies have shown that neutrophils can attract and activate immature DCs at sites of inflammation, as well as promote DC trafficking to draining lymph nodes, thus augmenting adaptive immune responses [89–92]. Neutrophils can also rapidly migrate to lymph nodes after antigen injection, mainly via the lymphatics, in a CD11b-, CXCR4-, and, in some cases, CCR7-dependent manner, where they either enhance or suppress the subsequent induction of T cell responses [3, 92–94]. Neutrophil-mediated inhibition of adaptive immunity in mice is supported by studies in humans showing that a subset of human neutrophils can attenuate T cell responses [95]. However, the mechanisms and mediators by which neutrophils inhibit the generation of adaptive immunity in lymph nodes are not well known. Recent studies demonstrating that

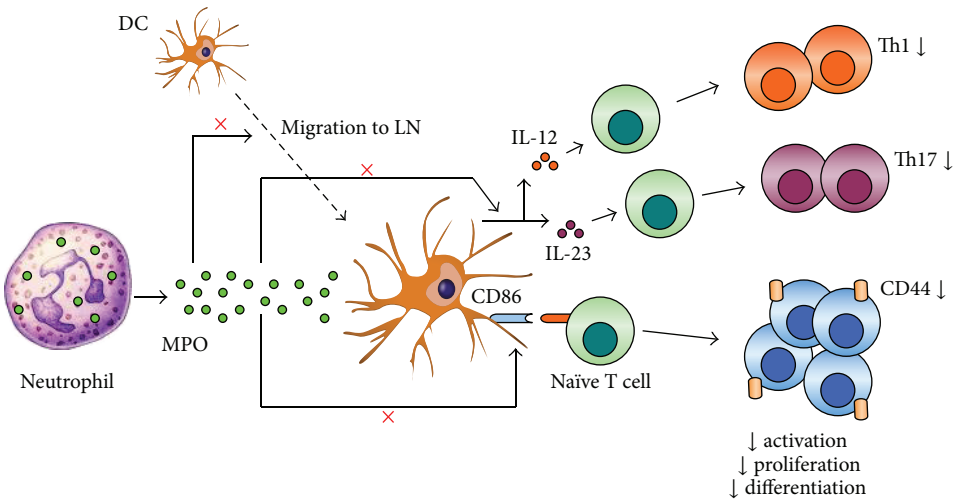

Figure 2: *Neutrophil MPO suppresses DC function and adaptive immunity*. Odobasic et al. [21] showed that rapidly infiltrating neutrophils release MPO in draining lymph nodes (LN) after antigen/adjuvant injection. The deposited MPO suppresses various aspects of DC function including costimulatory molecule (e.g., CD86) expression and cytokine (IL-12, IL-23) production and migration, resulting in decreased generation of CD4 T cell responses including T cell activation (CD44 expression), proliferation, and differentiation into Th1 (IFNγ-producing) and Th17 (IL-17A-releasing) effectors.

MPO plays a key role in neutrophil-mediated suppression of adaptive immunity have provided important insights into these important, but underexplored issues [21, 56, 57, 96].

Our group has shown that neutrophils rapidly and transiently infiltrate draining lymph nodes after antigen injection [21], as observed in other models [97]. Four hours after OVA/LPS injection, neutrophils degranulated and deposited MPO in lymph nodes, where it interacted with DCs [21], suggesting that extracellular MPO may affect DC function and subsequent induction of adaptive immunity. Further experiments using MPO-deficient mice or a specific MPO inhibitor, 4-aminobenzoic acid hydrazide (ABAH), showed that MPO, via its enzymatic activity, suppresses various aspects of DC function *in vivo* (Figure 2) including their activation (costimulatory molecule and MHC-II expression, cytokine production), antigen uptake/processing and migration to lymph nodes (by decreasing CCR7 expression), without affecting their apoptosis [21]. MPO-mediated suppression of DC function correlated with decreased generation of adaptive CD4 T cell (particularly Th1) immunity [21]. The suppressive effects of MPO on DCs were confirmed *in vitro* by culturing bone marrow-derived DCs with LPS and either supernatant from wild type (WT) or MPO−/− neutrophils degranulated in the presence or absence of ABAH, or purified enzymatically active native mouse MPO with or without ABAH. Importantly, *in vitro* studies using monocyte-derived DCs and supernatant from degranulated human neutrophils (±ABAH) or purified human MPO showed that MPO has similar inhibitory effects on DCs in humans [21]. Further mechanistic experiments indicated that HOCl and, to a lesser degree, HOBr are the main products involved in MPO-mediated suppression of DC activation *in vitro* [21], consistent with previous studies showing that taurine chloramine (a product formed by the reaction of HOCl and the amino acid taurine) can decrease DC maturation [98]. HOSCN also had inhibitory effects on DCs, but to a much lesser extent than HOCl and HOBr [21], consistent with HOSCN being a much less reactive oxidant [99]. In contrast, MPO-mediated consumption of nitric oxide, which itself can reduce DC maturation [100], reversed the effects of HOCl on DC activation *in vitro*. Interestingly, Mac-1, an inhibitory receptor on DCs [101], was shown to be involved in the enzymatic MPO-mediated suppression of DC IL-12 production [21]. Although the exact pathways involved in this process are still to be identified, these observations may be explained, in part, by previous reports showing that oxidants (thus potentially MPO-derived products) can induce activating conformational changes in Mac-1 [102], which would be expected to inhibit DC function.

The inhibition of DC function and subsequent generation of adaptive immunity by MPO are relevant to immune-driven diseases since it can result in attenuation of certain T cell-mediated inflammatory conditions. For example, in a model of lupus nephritis, dependent on both autoreactive T cells and humoral immunity [103, 104], we showed that MPO-deficient mice develop more severe renal injury in association with enhanced accumulation of cellular effectors, CD4 T cells, macrophages, and neutrophils [96]. This in turn correlated with enhanced activation of DCs and increased T cell autoimmunity in lymph nodes and spleen. Of note, augmented renal injury was observed in MPO−/− mice despite reduced deposition of humoral mediators of injury (antibody and complement) in glomeruli and decreased presence of markers of oxidative damage, 8-hydroxydeoxyguanosine and GSA [96]. Therefore, in experimental lupus nephritis, MPO-mediated suppression of pathogenic T cell autoimmunity overrides the local damaging effects of MPO in the kidney. These results are concordant with observations in humans

showing an increased incidence of lupus nephritis in patients with a polymorphism causing reduced MPO expression [105]. Similarly, in antigen-induced arthritis (AIA), which is very T cell-driven [106], MPO−/− mice developed more severe joint inflammation and damage in association with augmented CD4 T cell responses in the spleen [21]. Furthermore, Brennan et al. demonstrated that MPO−/− mice develop more severe disease in experimental autoimmune encephalomyelitis (EAE), a model of MS, correlating with higher antigen-specific lymphocyte proliferation in draining lymph nodes [13]. Collectively, these studies are supported by a report showing increased incidence of chronic inflammatory conditions in MPO-deficient patients [107], indicating their relevance to humans.

In another murine model of GN induced by a planted foreign antigen (sheep globulin) against the glomerular basement membrane (GBM), our group reported that MPO−/− mice are protected from renal injury in the initial (heterologous) phase of the disease [56] which is mediated by neutrophils but is independent of T cells [108], showing that neutrophil-derived MPO contributes to kidney damage locally. However, during the later (autologous) T cell/macrophage-mediated phase of the disease [109, 110], renal injury was similar between WT and MPO−/− mice, despite enhanced adaptive immunity in the spleen and increased glomerular accumulation of T cells and macrophages in MPO-deficient animals [56]. Together, these experiments suggested that the inhibitory effects of MPO on adaptive immunity in secondary lymphoid organs can also be counterbalanced by the local pathogenic effects of MPO in the target organ.

In other inflammatory conditions though, the injurious local effects of MPO can dominate over its inhibitory effects on immune responses in lymph nodes and spleen, leading to exacerbation of disease, as shown in some models of RA. For example, in collagen-induced arthritis (CIA), mediated by autoreactive T cells and antibody, but also neutrophils [111–113], we demonstrated that disease is attenuated due to MPO deficiency despite enhanced T cell autoimmunity in secondary lymphoid tissues, without an effect on autoantibody levels [57]. This suggested that MPO has dominant proinflammatory local effects in the joints which was confirmed in an acute neutrophil-mediated, T cell-independent KB × N model [114] by showing that joint inflammation and damage were significantly reduced in MPO−/− mice without an effect on circulating cytokines [57].

Overall, these studies indicate that the net impact of MPO on disease development depends on the balance between its local injurious effects in the target organ and inhibitory effects on adaptive immunity in secondary lymphoid tissue and that this balance varies in different autoimmune diseases. Although it is not clearly understood which factors tip this balance in either direction, the above studies do suggest that under conditions where neutrophils play a very important role as effectors of injury in the inflamed organs (e.g., CIA, KB × N arthritis, heterologous anti-GBM GN), the local pathogenic effects of MPO predominate. In contrast, in situations where T cell immunity is the main driver of disease with lesser involvement of neutrophils as effectors (e.g., AIA, lupus nephritis, autologous anti-GBM GN, and EAE), the immunosuppressive effects of MPO in secondary lymphoid organs predominate, tipping the balance towards MPO-mediated attenuation of disease.

9. MPO Deficiency in Humans

Hereditary MPO deficiency in humans is not rare, with reported prevalence in the United States and Europe ranging from 1 : 1000 to 1 : 4000 [7, 115–118]. Patients lacking MPO are more susceptible to fungal infections, particularly those caused by *Candida albicans* [118–120]. This is in line with murine studies [33] and the MPO/HOCl system being critical for the direct killing of the fungus and *C. albicans*-induced NET formation [38, 53, 118]. Although reports exist demonstrating that MPO-deficient patients can have a higher incidence of severe infections [107, 121], the majority of patients lacking MPO have been shown not to be particularly susceptible to chronic infections [8]. Similar to humans, MPO knockout mice exhibit higher susceptibility to some, but not all infections including those caused by *Candida glabrata*, *S. aureus*, and *S. pneumoniae* [8, 34]. This may be due to several factors: (i) MPO-deficiency increases the expression and activity of inducible NO synthase resulting in augmented levels of NO and reactive nitrogen intermediates which have been shown to play a role in the killing of microbes by neutrophils in the presence and in the absence of MPO [32, 35], (ii) the lack of the MPO/HOCl system results in increased activity of antimicrobial neutrophil granule proteases such as elastase and cathepsin-G [122–124], (iii) neutrophils from MPO-deficient patients have increased phagocytosis and degranulation [125, 126], and (iv) the absence of MPO augments the generation of adaptive immunity, as shown by us and others [13, 21, 56, 96]. Therefore, decreased microbial killing due to the absence of MPO/HOCl may be compensated for by other systems involved in pathogen clearance, including increased expression reactive nitrogen intermediates, enhanced activity and release of proteases from neutrophil granules, augmented phagocytic activity of neutrophils, and increased adaptive immunity against the invading microbes.

Although the clinical consequences of MPO deficiency in humans have not been thoroughly investigated, some studies have found that patients with total or subtotal lack of MPO have an increased incidence of chronic inflammatory conditions [107, 121] which are known to be mediated by the adaptive immune system. Similarly, patients with a genetic polymorphism resulting in decreased expression of MPO have a higher risk of developing autoimmune lupus nephritis [105], and MS and diabetes patients have been reported to have lower MPO activity in their blood leukocytes [127, 128]. These observations may be, in part, explained by studies showing increased development of adaptive immune responses and T cell-driven inflammatory conditions in MPO-deficient animals [13, 21, 56, 96], as discussed above.

10. Conclusion

Neutrophils use MPO to mediate many of their multifaceted functions that they have in the immune system (summarised

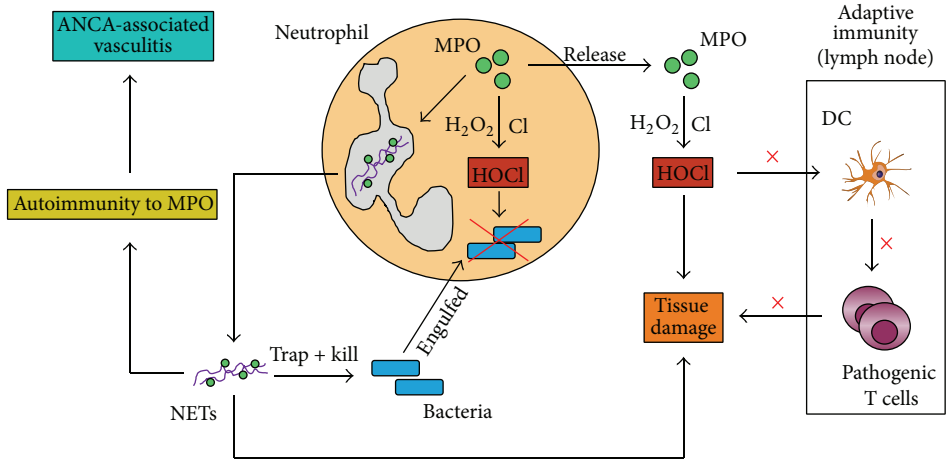

Figure 3: *Summary of MPO involvement in neutrophil functions in innate and adaptive immunity*. MPO is involved in microbe clearance by neutrophils both intracellularly (via the production of HOCl) and extracellularly (via the release of NETs). On the other hand, the release of MPO-containing NETs can result in the generation of autoimmunity against MPO and subsequent development of ANCA-associated vasculitis. HOCl that is produced outside of activated neutrophils following MPO release can cause significant tissue damage. In contrast, MPO that is released by neutrophils in lymph nodes can inhibit DC activation and thus generation of adaptive T cell responses, thus attenuating organ injury. HOCl, hypochlorous acid; H_2O_2, hydrogen peroxide; Cl, chloride; NETs, neutrophil extracellular traps; DC, dendritic cell; ANCA, anti-neutrophil cytoplasmic antibody.

in Figure 3). Through the production of HOCl in the presence of H_2O_2 and chloride, MPO plays an important role in the killing of microbes which have been engulfed by neutrophils. MPO can also associate with nuclear DNA/histones and contribute to the formation/release of NETs which trap and sometimes kill (via MPO-produced HOCl) extracellular bacteria. On the other hand, stimulation of DCs by NET-bound MPO can result in the generation of MPO-specific autoimmunity and subsequent development of AAV. MPO release and subsequent formation of HOCl in the extracellular environment following neutrophil activation have been shown to contribute to tissue inflammation and damage. Importantly, MPO deposited by neutrophils in lymph nodes can inhibit DC activation and subsequent generation of adaptive T cell immunity thus leading to attenuation of immune-mediated tissue injury. Future studies will not only further our understanding about these already described functions of MPO but are also likely to uncover novel roles of this neutrophil enzyme in the regulation of cellular events that take place during the course of innate and adaptive immune responses.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Differential Use of Human Neutrophil Fcγ Receptors for Inducing Neutrophil Extracellular Trap Formation

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Neutrophils (PMN) are the most abundant leukocytes in the blood. PMN migrate from the circulation to sites of infection, where they are responsible for antimicrobial functions. PMN use phagocytosis, degranulation, and formation of neutrophil extracellular traps (NETs) to kill microbes. NETs are fibers composed of chromatin and neutrophil-granule proteins. Several pathogens, including bacteria, fungi, and parasites, and also some pharmacological stimuli such as phorbol 12-myristate 13-acetate (PMA) are efficient inducers of NETs. Antigen-antibody complexes are also capable of inducing NET formation. However the particular Fcγ receptor involved in triggering this function is a matter of controversy. In order to provide some insight into what Fcγ receptor is responsible for NET formation, each of the two human Fcγ receptors was stimulated individually by specific monoclonal antibodies and NET formation was evaluated. FcγRIIa cross-linking did not promote NET formation. Cross-linking other receptors such as integrins also did not promote NET formation. In contrast FcγRIIIb cross-linking induced NET formation similarly to PMA stimulation. NET formation was dependent on NADPH-oxidase, PKC, and ERK activation. These data show that cross-linking FcγRIIIb is responsible for NET formation by the human neutrophil.

1. Introduction

Neutrophils (PMN) are the most abundant leukocytes in the blood. PMN are innate immune cells that migrate from the circulation to sites of infection, where they are responsible for antimicrobial functions [1]. PMN use phagocytosis, degranulation, and formation of neutrophil extracellular traps (NETs) to kill microbes [2, 3]. NETs are formed through a unique cell death program named "NETosis" that involves activation in most cases of nicotinamide adenine dinucleotide phosphate- (NADPH-) oxidase, which produces reactive oxygen species (ROS) [4–6]. During NETosis, the characteristic lobular nucleus of neutrophils disappears, and the chromatin expands in the cytosol, while the cell membrane remains intact. Three or four hours after stimulation, the cell membrane breaks and the chromatin fibers get released forming a netlike structure outside the cell. NET fibers are composed of chromatin covered with histones [7] and antimicrobial proteins derived from the neutrophil granules, such as the bactericidal/permeability-increasing protein (BPI), elastase, myeloperoxidase, lactoferrin, and metalloprotease 9 [2, 4]. The requirements for NADPH-oxidase and myeloperoxidase in NET formation differ depending on the stimulus [8, 9]. Besides their antimicrobial capacity, NETs seem to act as a physical barrier where microorganisms get trapped and consequently prevent further spread of pathogens. Thus, NETs bind, block, and kill microorganisms extracellularly and independently of phagocytosis [10].

Binding of receptors for the Fc portion of antibodies (Fc receptors) to opsonized microorganisms is one of the most important mechanisms for pathogen recognition and activation of neutrophils [11]. Human neutrophils express

constitutively two antibody receptors that are members of the Fc receptor family for IgG molecules, namely, FcγRIIa (CD32a) and FcγRIIIb (CD16b) [12]. These receptors are exclusive human receptors. FcγRIIa is composed of a single polypeptide chain bearing an ITAM on its cytoplasmic domain [11]. This ITAM confers on FcγRIIa the ability to initiate signaling events that regulate cell responses, including phagocytosis, cytokine production, antibody-dependent cell-mediated cytotoxicity, and the respiratory burst [13]. FcγRIIIb is present exclusively on neutrophils and it is a glycophosphatidylinositol- (GPI-) linked receptor, lacking transmembrane and cytoplasmic domains [14]. Although signaling molecules associated with FcγRIIIb are still unknown and its signaling mechanism remains unidentified, several reports show that FcγRIIIb can initiate signaling events leading to calcium transients [15], actin polymerization [16], activation of integrins [17], and NF-κB activation [18].

Several pathogens, including virus, bacteria, fungi, and parasites, have all been reported to be inducers of NET formation [10]. In addition, proinflammatory stimuli such as lipopolysaccharide (LPS) [19], interleukin- (IL-) 8, and tumor necrosis factor (TNF) [20, 21] and also some pharmacological stimuli such as phorbol 12-myristate 13-acetate (PMA), an activator of protein kinase C (PKC), are efficient inducers of NETs [2]. Some reports indicate that NET formation was increased when microorganisms were opsonized with autologous serum [3]. This suggested a possible role for FcγR in NET formation.

Recent reports indicated that antigen-antibody complexes seem capable of inducing NET formation [22, 23]. In one report, a B cell-deficient mouse was used to show NETs could not be formed, thus suggesting a direct role for Fc receptors in this function [23]. In this study, however no particular receptor was identified. In another report, soluble immune complexes were used to activate neutrophils and induce NET formation. It was determined that FcγRIIIb promoted endocytosis of soluble immune complexes and that FcγRIIa promoted NET formation in vivo [22]. However, more recently, it was reported that FcγRIIIb is the receptor responsible for NET formation in response to immobilized immune complexes [24]. Thus, in order to provide some insight into this controversy, each of the two human Fcγ receptors was stimulated individually by specific monoclonal antibodies and NET formation was evaluated.

FcγRIIa cross-linking did not promote NET formation. Cross-linking other receptors such as integrins also did not promote NET formation. In contrast FcγRIIIb cross-linking induced efficient NET formation similarly to PMA stimulation. NET formation was dependent on NADPH-oxidase, PKC, and extracellular signal-regulated kinase (ERK) activation. These data support the idea that different Fc receptors promote independent cell functions.

2. Materials and Methods

2.1. Neutrophils. Neutrophils (PMN) were purified from blood collected from adult healthy volunteers exactly as previously described [25]. All volunteers provided a written informed consent for their participation in this study. The informed consent form and all experimental procedures were approved by the Bioethics Committee at Instituto de Investigaciones Biomédicas, UNAM.

2.2. Reagents. Bovine serum albumin (BSA) was from F. Hoffmann-La Roche Ltd. (Mannheim, Germany). Piceatannol, a spleen tyrosine kinase (Syk) inhibitor, was from Acros Organics (New Jersey, USA). Wortmannin, a phosphatidylinositol 3-kinase (PI-3K) inhibitor; GÖ6976, a protein kinase C (PKC) inhibitor; GÖ6983, another PKC inhibitor; 3-(1-methyl-1H-indol-3-yl-methylene)-2-oxo-2,3-dihydro-1H-indole-5-sulfonamide (iSyk), another Syk inhibitor (catalog number 574711); and the antibleaching mounting medium FluorSave (catalog number 345789) were from Calbiochem/EMD Millipore (Billerica, MA). UO126, a MEK (ERK kinase) inhibitor, was from Promega (Madison, WI, USA). Diphenyleneiodonium (DPI), an NADPH-oxidase inhibitor; (E)-3-[4-methylphenylsulfonyl]-2-propenenitrile (BAY 117082), an NF-κB inhibitor; phorbol 12-myristate 13-acetate (PMA); and all other chemicals were from Sigma Aldrich (St. Louis, MO). The following antibodies were used: anti-human FcγRIIa (CD32) mAb IV.3 [26] (ATCC HB-217) was from American Type Culture Collection (Manassas, VA). Anti-β1 integrin mAb TS2/16 was donated by Martin Hemler (Dana Farber Cancer Research Institute, Boston, MA). Anti-β2 integrin (CD18) mAb IB4 and anti-human FcγRIIIb (CD16) mAb 3G8 [27] were donated by Dr. Eric J. Brown (University of California in San Francisco, San Francisco, CA). Mouse monoclonal anti-neutrophil elastase (D-7; catalog number sc-365950), rabbit polyclonal anti-histone H2B (FL-126; catalog number sc-10808), rabbit polyclonal anti-ERK 1 (catalog number sc-94), and mouse monoclonal anti-phospho-ERK 1 (pTyr204) (catalog number sc-7383) were from Santa Cruz Biotechnology (Santa Cruz, CA). Alexa Fluor 555-conjugated donkey anti-rabbit IgG (catalog number A-31572), Alexa Fluor 488-conjugated donkey anti-mouse IgG (catalog number A-21202), and FITC-conjugated $F(ab')_2$ goat anti-mouse IgG (catalog number A-10683) were from Invitrogen Molecular Probes (Eugene, OR). $F(ab')_2$ goat anti-mouse IgG (catalog number 0855468), HRP-conjugated $F(ab')_2$ goat anti-mouse IgG (catalog number 0855572), and HRP-conjugated $F(ab')_2$ goat anti-rabbit IgG (catalog number 0855686) were from MP Biomedicals (Santa Ana, CA).

2.3. Preparation of Specific Monoclonal Antibodies. Hybridoma cells were grown in DMEM (Gibco; Grand Island, NY) containing 10% fetal bovine serum (FBS) also from Gibco (Grand Island, NY). Antibodies were purified from saturated (8-day-old) tissue culture supernatants with Protein-G Sepharose 4 Fast Flow from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). After elution from the Sepharose with 0.1 M glycine-HCl, pH 2.7, antibodies were dialyzed against PBS, adjusted to 1 mg/mL, and filter-sterilized. Finally, antibodies were stored in small aliquots at −80°C. The functionality of antibodies was confirmed by their binding to neutrophil receptors (Supplemental Figure 1S in Supplementary Material available online at http://dx.doi.org/10.1155/2016/2908034).

2.4. Labeling of Neutrophil Receptors with Specific Monoclonal Antibodies. For receptor stimulation, PMN were treated with specific anti-receptor monoclonal antibodies as follows. PMN (1×10^6) in 500 μL PBS were placed into a 1.5 mL Eppendorf tube, and 10 μg/mL of the corresponding anti-receptor monoclonal antibody was added. Cells were incubated at 4°C for 20 minutes and then washed twice with 1 mL of cold PBS (4°C) centrifuged at 1,743 ×g, 1 minute each time. This centrifugation protocol did not preactivate cells as long as they were maintained cold. Next, PMN were resuspended in 500 μL cold (4°C) RPMI-1640 medium (Gibco; Grand Island, NY) containing 5% fetal bovine serum (FBS) also from Gibco (Grand Island, NY).

2.5. NET Formation Assay. Neutrophils were left untreated for PMA stimulation or previously treated with anti-receptor antibodies (as described above) for receptor stimulation. Neutrophils (1×10^6) in 500 μL RPMI-1640 medium were added to each well of a 24-well plate, containing a 12 mm coverslip, and then incubated in a humidified incubator with 5% CO_2 at 37°C for 30 minutes. Then 100 μL of 120 nM PMA in PBS or 100 μL of 450 μg/mL $F(ab')_2$ goat anti-mouse IgG (for receptor stimulation) was added to each well. Plates were incubated in 5% CO_2 at 37°C for 4 hours. Next, 600 μL of 2% paraformaldehyde in PBS was added to each well, and the plates were incubated overnight in 5% CO_2 at 37°C.

In selected experiments, PMN were incubated for 30 minutes before stimulation, with the inhibitors piceatannol (50 μM), wortmannin (50 nM), UO126 (50 μM), GÖ6983 (1 μM), GÖ6976 (1 μM), DPI (10 μM), BAY 117082 (2.5 μM), or the vehicle dimethyl sulfoxide (DMSO) alone.

2.6. NET Visualization and Immunofluorescence. All washes and incubations were done at room temperature by placing the coverslip upside down over a 250 μL drop of each solution formed on a well of Parafilm placed on a tube rack, exactly as previously described [28]. Coverslips were taken out of the 24-well plate one at a time and washed four times with water for 5 minutes each. Next, they were placed over 0.1% Triton X-100 in 4% paraformaldehyde for 10 minutes, then on PBS for 5 minutes, and then on 10 μg/mL of the corresponding primary antibody (anti-neutrophil elastase or anti-histone) in 5% BSA in PBS for 60 minutes. Coverslips were then washed four times with PBS for 5 minutes each and placed on 8 μg/mL of the corresponding secondary antibody (Alexa Fluor-conjugated anti-rabbit IgG or anti-mouse IgG) in 5% BSA in PBS containing 300 nM DAPI. Coverslips were incubated in the dark for 60 minutes. Finally, coverslips were mounted on a microscope slide with one drop of FluorSave. Slides were observed with a fluorescence inverted microscope model IX-70 from Olympus (Center Valley, PA). Images were captured with an Evolution-VF Cooled Color camera from Media Cybernetics (Rockville, MD) and the computer program QCapture Pro 6.0 from QImaging, Surrey (British Columbia, Canada). Images were processed with the computer program ImageJ 1.47v from the National Institutes of Health (Bethesda, MD).

2.7. NET Formation with Opsonized Particles. Opsonization of 4.8 μm fluorescent (catalog number 16592) or nonfluorescent (catalog number 17135) latex particles from Polysciences, Inc. (Warrington, PA), was performed exactly as described [29]. These particles were used in phagocytosis assays as described [30] or in NET formation assays as follows. PMN (1×10^6 cells) in 500 μL were centrifuged in a 1.5 mL Eppendorf tube at 1,743 ×g for 1 min. After removing the supernatant, the cell pellet was disaggregated by tapping the tube against a rack, and 80 μL of opsonized latex particles (1.25×10^8 particles/mL) resuspended in RPMI-1640 medium with 5% FBS was added. The PMN-particle mixture was incubated at 4°C for 20 min. Then, 1 mL of cold PBS was added, the tube was centrifuged at 1,743 ×g for 1 min, and the cell pellet was resuspended in 500 μL RPMI-1640 medium with 5% FBS. Cell suspension was transferred into a well of a 24-well plate, containing a 12 mm coverslip, and then incubated in a humidified incubator with 5% CO_2 at 37°C for 4 h. Then 500 μL of 2% paraformaldehyde in PBS was added to each well, and the plate was kept in the incubator overnight. Finally, the coverslip was used for NET visualization as described above.

2.8. Quantification of NETs. A 96-well plate was previously covered with 25 μg/mL poly-D-lysine for three hours at room temperature. Each well was then washed three times with 50 μL PBS for 5 minutes each time, and the plate was allowed to air dry inside a flow laminar hood for two hours. Neutrophils were resuspended at 1.25×10^6 cells/mL in RPMI-1640 medium, containing 500 nM SYTOX Green (Molecular Probes, Inc.; Eugene, OR), and 80 μL of this cell suspension (1×10^5 PMN) was added to each well of the 96-well plate. Next, the plate was incubated at 37°C in a 5% CO_2 incubator for 20 minutes. For FcγR stimulation, the supernatant was removed gently with a micropippetor and 50 μL of 10 μg/mL of the corresponding anti-Fc receptor antibody was added to each well. The plate was placed in a 35°C prewarmed microplate reader model Synergy HT from BioTek Instruments (Winooski, VT) and incubated there for 20 minutes. Next, the supernatant was removed gently with a micropippetor and 100 μL of 75 μg/mL of $F(ab')_2$ goat anti-mouse IgG containing 500 nM SYTOX Green was added to each corresponding well. At this time, for PMA stimulation 20 μL of 100 nM PMA was added to each corresponding well. The plate was then incubated for up to 4 hours. For this assay, cells were not fixed. Finally the fluorescence from the bottom of the plate was read, using the 485 nm excitation and 528 emission filters.

For NET formation induced with opsonized latex particles, PMN (1×10^6 cells) in 500 μL RPMI-1640 medium with 5% FBS and 500 nM SYTOX Green were mixed with 80 μL of opsonized latex particles (1.25×10^8 particles/mL). Then 100 μL of the PMN-particle mixture was transferred into a well of a 96-well plate and incubated in a 35°C prewarmed microplate reader for 4 hours. Fluorescence from the bottom of the plate was read using the 485 nm excitation and 528 emission filters.

2.9. Western Blotting. Western blots were performed exactly as previously described [31]. Cells were lysed in RIPA buffer (150 mM NaCl, 5 mM EDTA, 50 mM HEPES, 0.5% sodium deoxycholate, 1% Nonidet P-40, and 10 mM 2-mercaptoethanol, pH 7.5) containing cOmplete protease inhibitor cocktail from Roche (Basel, Switzerland), for 15 minutes at 4°C. Cell lysates were then cleared by centrifugation and proteins resolved on SDS 12% PAGE. Proteins were then electrotransferred onto polyvinylidene fluoride (PVDF) membranes (Immobilon-P; Millipore, Bedford, MA). Membranes were incubated in blocking buffer (1% BSA and 5% nonfat dry milk (Carnation; Nestle, Glendale, CA) and 0.1% Tween 20 in PBS) overnight at 4°C. Membranes were subsequently probed with the corresponding antibody in blocking buffer, for 1 hour at room temperature, anti-ERK 1 (1/1000 dilution) or anti-phospho-ERK 1 (1/500 dilution). Membranes were washed with PBS six times and incubated with a 1/3000 dilution of HRP-conjugated $F(ab')_2$ goat anti-rabbit IgG or HRP-conjugated $F(ab')_2$ goat anti-mouse IgG for 1 hour at room temperature. After washing six more times, the membrane was developed with a chemiluminescence substrate (SuperSignal; Pierce, Rockford, IL) according to the manufacturer's instructions.

2.10. Determination of Apoptosis. Apoptosis was assayed with the FITC annexin V and propidium iodide (PI) dead cell apoptosis kit for flow cytometry (catalog number V13242) from Molecular Probes, Inc. (Eugene, OR), following the manufacturer's instructions. Briefly, PMN were treated with nothing, PMA, or the antibodies against each of the Fcγ receptors as described above. After a two-hour incubation at 37°C, PMN (1×10^5) were washed in PBS and resuspended in annexin-binding buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM Ca^{2+}, pH 7.4), and 2 μL of FITC annexin V and 1 μL of 1.5 mM PI were added. Cells were incubated for 15 min at room temperature and then 400 μL of annexin-binding buffer was added. Cells were immediately analyzed by flow cytometry. PMN apoptosis (positive control) was induced by UV-light irradiation as previously described [32].

2.11. Reactive Oxygen Species (ROS). ROS production was assessed with the DCFDA-Cellular Reactive Oxygen Species Detection Assay Kit (catalog number ab113851) from Abcam, Inc. (Cambridge, MA), following the manufacturer's instructions. Briefly, PMN were treated with mAb IV.3 or mAb 3G8 at 10 μg/mL for 20 minutes on ice. PMN were washed with 1x buffer and then incubated with 15 μM DCFDA in 1x buffer for 30 minutes at 37°C. After one wash in 1x buffer, 5×10^4 PMN were placed in each well of a 96-well clear-bottom black plate from Corning Inc. (New York, NY) and incubated for 20 minutes at 35°C in a plate reader, model Synergy HT from BioTek Instruments (Winooski, VT). Then, for antibody treatment 50 μL of goat anti-mouse IgG (150 μg/mL) was added and for PMA treatment, 50 μL of PMA (40 nM) was added. Fluorescence was read for two hours at excitation 485 nm and emission 535 nm.

2.12. Phagocytosis Assays. Neutrophil phagocytosis was determined as previously described [29]. Briefly, PMN (1×10^5 cells) were resuspended in 100 μL cold phagocytosis buffer (2 mM calcium chloride, 1.5 mM magnesium chloride, and 1% human serum albumin in PBS) and mixed with 3.5 μL of a suspension (1×10^8 beads/mL) of IV.3-opsonized, or 3G8-opsonized or control-opsonized (no antibody) fluorescent latex beads. PMN and beads were incubated at 37°C for 30 min, centrifuged at 6000 rpm for 1 min, and resuspended in 100 μL of ice-cold trypsin-EDTA solution (0.05% trypsin, 1 mM EDTA in PBS) to detach uninternalized beads from the cells. After a 15 min incubation on ice, PMN were washed with 1 mL cold PBS plus 0.5% BSA plus 2 mM EDTA and resuspended in 500 μL cold 1% paraformaldehyde in PBS. To analyze phagocytosis by flow cytometry, latex particles were gated out during sample acquisition and 10,000 cells were acquired per sample. Phagocytosis was reported as percent of fluorescence-positive cells (cells internalizing at least one fluorescent particle). Phagocytosis was also analyzed by microscopy and reported as phagocytic index, the number of beads internalized by 100 cells.

2.13. Statistical Analysis. Quantitative data were expressed as mean ± standard error of mean (SEM). Single variable data were compared by unpaired-sample Student's t-tests using the computer program KaleidaGraph version 3.6.2 for Mac (Synergy Software; Reading, PA). Also, variance homogeneity was checked by using Levene's test, and multiple pair-comparisons were performed using Tukey's test after ordinary one-way analysis of variance (ANOVA) [33]. Post hoc differences were considered statistically different at a value $p < 0.05$. Analyses were done using the SAS software version 9.0 (2012) from SAS Institute Inc. (Cary, NC).

3. Results

Several types of pathogens have been reported to induce NET formation, but there are not reports on particular receptors used by neutrophils to recognize these pathogens and to induce NETosis. Most studies on NETs have used PMA, a potent activator of PKC, and efficient inducer of NETs [2]. In this case, no receptor is involved since PMA directly activates intracellular signaling. Some reports indicated that NET formation was increased when microorganisms were opsonized with autologous serum and also that antigen-antibody complexes seemed to be capable of inducing NET formation. These studies suggested a possible role for IgG Fc receptors (FcγR) in NET formation. However the particular Fcγ receptor involved in triggering this function is a matter of controversy. Thus, in order to explore what particular Fc receptor could induce NET formation, PMN were stimulated by cross-linking individual receptors with specific monoclonal antibodies.

When PMN were stained with DAPI, the typical lobular nuclei were clearly seen (Figure 1(a)). Immunolabeling of histones also showed the localization of these proteins within the PMN nucleus (Figure 1(b)). When PMN were treated with PMA, nuclei lost their typical morphology and long NETs were formed (Figure 1(d)). Also, the cell morphology was altered; PMN appeared larger and diffuse (Supplemental

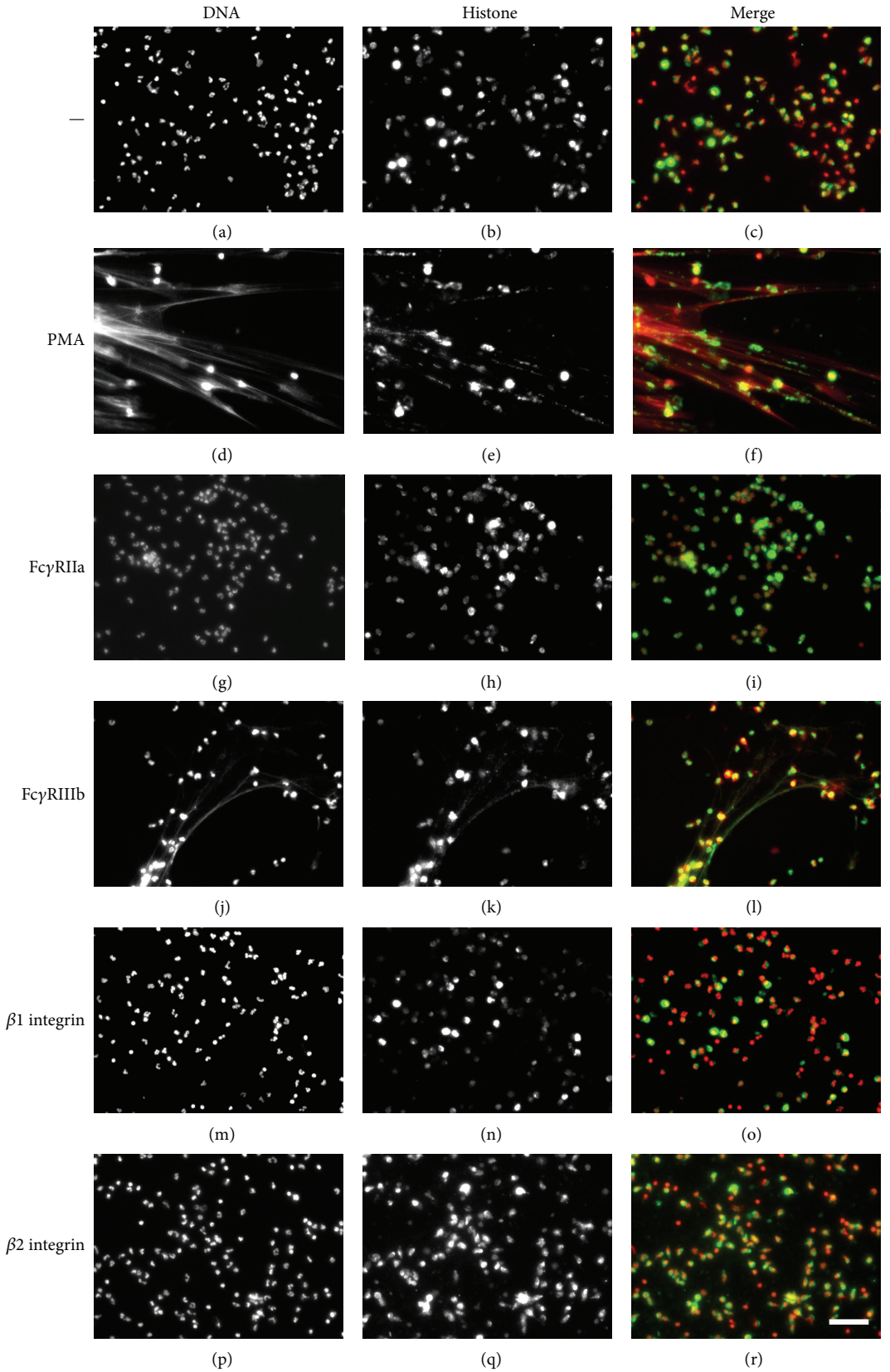

FIGURE 1: FcγRIIIb induces NET formation. (a) Human neutrophils (PMN) were left untreated (—) or were stimulated with 20 nM phorbol 12-myristate 13-acetate (PMA), by cross-linking FcγRIIa with mAb IV.3, by cross-linking FcγRIIIb with mAb 3G8, by cross-linking β1 integrins with mAb TS2/16, or by cross-linking β1 integrins with mAb IB4. After four hours, PMN were fixed and stained for DNA (DAPI, red) and for histone (green). Microphotographs were taken at 200x magnification and are representative of more than 10 experiments. Bar is 50 μm.

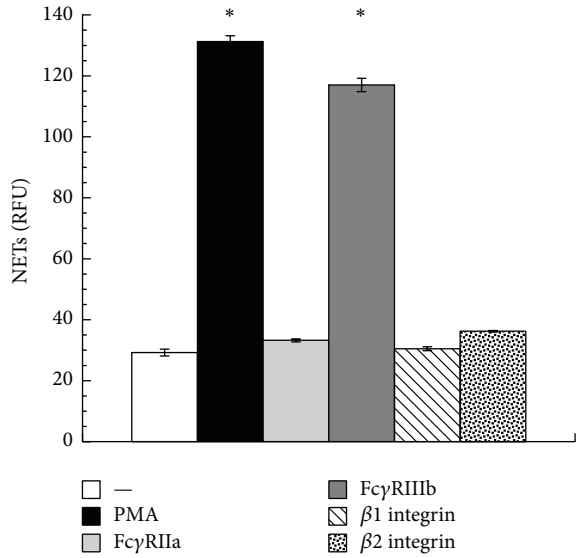

Figure 2: NETs are induced by PMA and FcγRIIIb cross-linking. Human neutrophils (PMN) were left untreated (—) or were stimulated with 20 nM phorbol 12-myristate 13-acetate (PMA), by cross-linking FcγRIIa with mAb IV.3, by cross-linking FcγRIIIb with mAb 3G8, by cross-linking β1 integrins with mAb TS2/16, or by cross-linking β2 integrins with mAb IB4. The relative amount of NETs, as extracellular DNA of nonfixed cells, was estimated from SYTOX Green fluorescence in relative fluorescent units (RFU) at 4 hours after stimulation, as described in Materials and Methods. Data are mean ± SEM of 4 experiments. Asterisks denote conditions that are statistically different from control ($p < 0.05$).

Figure 2S). Histones were also present along the extracellular DNA fibers (Figure 1(e)). Cross-linking FcγRIIa with the specific mAb IV.3 did not induce NET formation and PMN retained intact nuclei with typical lobular morphology (Figures 1(g) and 1(h)). Similarly, cross-linking β1 integrins (Figures 1(m) and 1(n)) or β2 integrins (Figures 1(p) and 1(q)) did not induce any NET formation (Supplemental Figure 2S). In contrast, cross-linking FcγRIIIb with the specific mAb 3G8 induced strong NET formation (Figure 1(j)) similar to the one induced by PMA (Figure 2). These FcγRIIIb-induced extracellular DNA fibers were also covered with histones (Figure 1(k) and Supplemental Figure 3S). Cross-linking of FcγRIIa together with FcγRIIIb or β2 integrins together with FcγRIIIb did not induce changes in the amount of NET formation induced by FcγRIIIb alone (not shown). An important characteristic of NETs is that they are covered with antimicrobial proteins from the PMN granules. The presence of neutrophil elastase on NETs was confirmed for NETs induced both by PMA and by FcγRIIIb (Figure 3 and Supplemental Figure 4S). These data indicated that cross-linking FcγRIIIb is an efficient stimulus for NET formation. NETosis [4] is a form of cell death different from apoptosis [34]. In neutrophils, apoptosis appears spontaneously when these cells get older or after they have activated their proinflammatory functions [35]. Because the NET quantification method is related to detection of extracellular DNA, it was important to determine whether PMN were in apoptosis after stimulation of Fcγ receptors. After PMA stimulation or Fcγ receptor cross-linking, PMN did not have an increase in annexin V-binding, indicating that PMN were not in apoptosis [34, 36] (Supplemental Figure 5S).

Because PMA is an activator of PKC, the involvement of this kinase in NET formation induced by FcγRIIIb was tested with two specific PKC inhibitors. PMN treated with PMA formed NETs as expected (Figure 4). However, when PMN were treated previously with GÖ6983, an inhibitor of PKCα, PKCβ, and PKCγ isozymes (Figure 4), or with GÖ6976, a conventional PKC inhibitor (Figure 4), NETs were not formed after PMA stimulation. Similarly, NET formation after FcγRIIIb cross-linking (Figure 4) was inhibited by these PKC inhibitors (Figure 4). In addition, downstream of PKC, the MEK, ERK pathway has been reported to participate in NET formation after PMA stimulation [37]. When PMN were treated with UO126, a potent specific MEK inhibitor, NETs were not formed after PMA stimulation (Figure 5). Also, UO126 treatment blocked NET formation after FcγRIIIb stimulation (Figure 5). These data suggested that FcγRIIIb stimulation led to NET formation using PKC and ERK. To confirm that ERK 1 was activated after PMA or Fc receptor stimulation as previously reported [38], PMN were stimulated in the presence or absence of the MEK inhibitor and ERK 1 activation was detected by Western blotting. PMA induced ERK phosphorylation in PMN (Figure 6(a)), and this ERK activation was completely blocked by the MEK inhibitor UO126 (Figure 6(a)). Similarly, FcγRIIa cross-linking (Figure 6(b)) or FcγRIIIb cross-linking (Figure 6(c)) resulted in efficient ERK 1 phosphorylation. This ERK 1 activation was completely blocked in both cases by the MEK inhibitor (Figures 6(b) and 6(c)). These data suggested that both Fcγ receptors can induce ERK activation, but this enzyme is not sufficient for NET formation, since only FcγRIIIb led to release of NETs.

Other signaling molecules that are important for Fc receptor signaling via ITAM are Syk and PI-3K. Although, FcγRIIIb does not have an ITAM, it has been suggested that FcγRIIIb might signal in cooperation with FcγRIIa [39]. Thus, to explore the possible involvement of these molecules in FcγRIIIb-induced NET formation, PMN were treated with piceatannol (Figure 7) or iSyk (Supplemental Figure 6S), selective inhibitors of Syk, or with wortmannin (Figure 7), a selective inhibitor of PI-3K, before stimulation. These inhibitors prevented NET formation when PMN were stimulated by PMA (Figure 7). Similarly, NET formation was inhibited after cross-linking of FcγRIIIb in the presence of piceatannol (Figure 7) but proceeded normally in the presence of wortmannin (Figure 7). Interestingly, iSyk only caused small but statistically significant inhibition of NET formation after PMA stimulation, while it did not block FcγRIIIb-induced NET formation (Supplemental Figure 6S). These data suggested that FcγRIIIb-induced NET formation involves Syk, but it is independent of PI-3K.

NETs formed after PMA stimulation require activation of NADPH-oxidase and formation of ROS [40] and also activation of NF-κB [41]. Thus, we explored the involvement of these molecules in FcγRIIIb-induced NET formation.

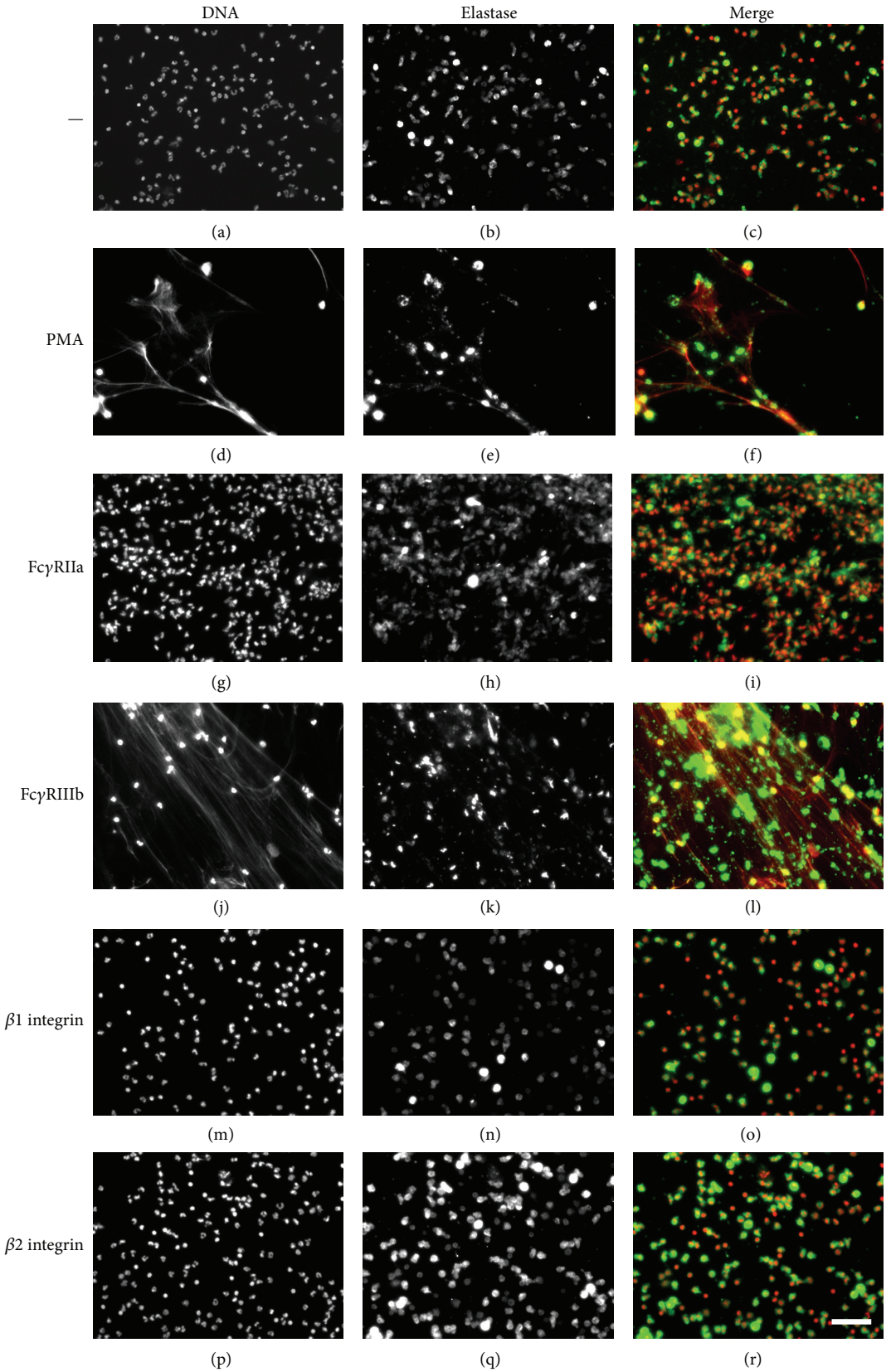

Figure 3: NETs are decorated with neutrophil elastase. Human neutrophils (PMN) were stimulated with 20 nM phorbol 12-myristate 13-acetate (PMA), by cross-linking FcγRIIa with mAb IV.3, by cross-linking FcγRIIIb with mAb 3G8, by cross-linking β1 integrins with mAb TS2/16, or by cross-linking β1 integrins with mAb IB4. After four hours, PMN were fixed and stained for DNA (DAPI, red) or for elastase (green). Microphotographs were taken at 200x magnification and are representative of five experiments. Bar is 50 μm.

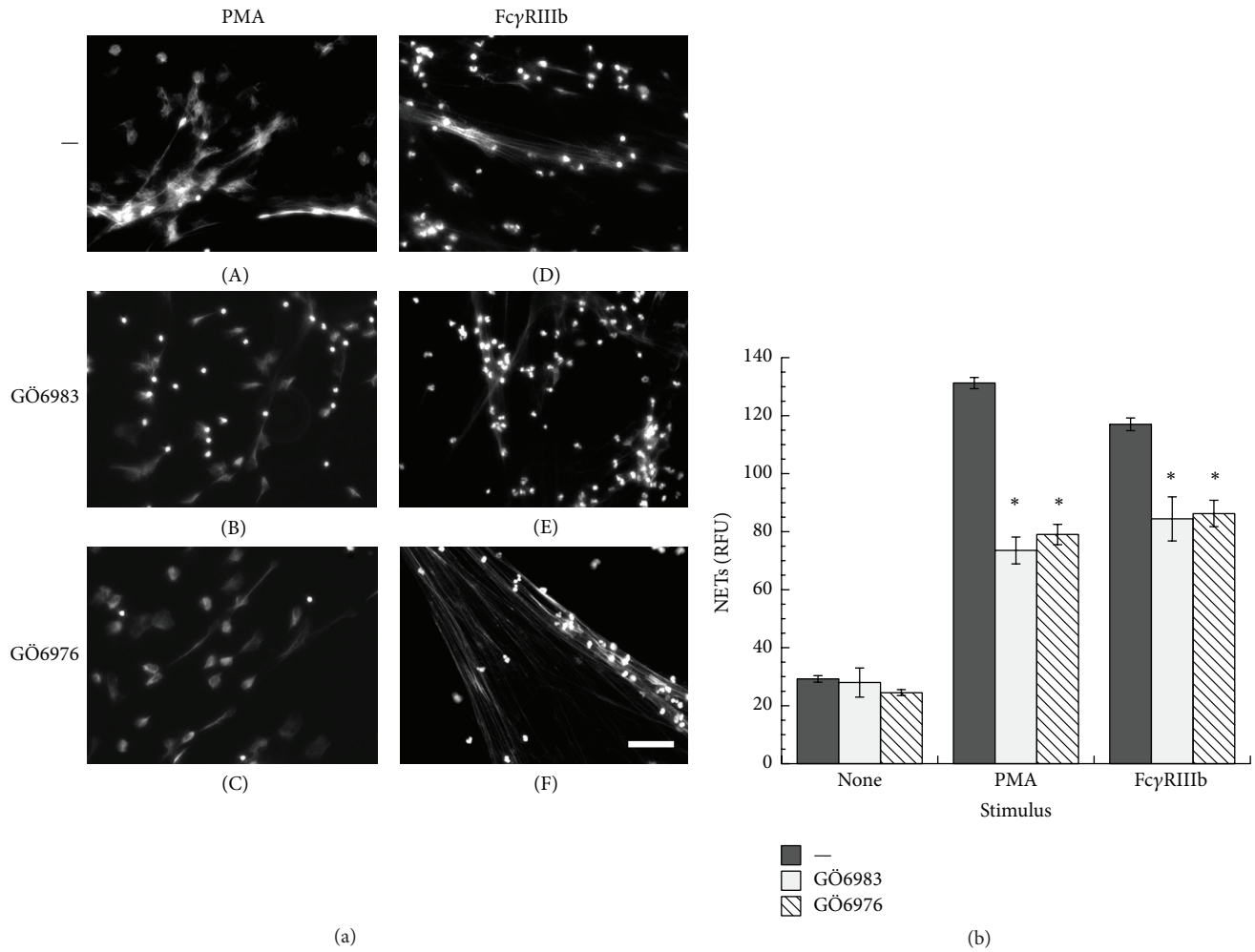

Figure 4: FcγRIIIb-induced NET formation is dependent on PKC. (a) Human neutrophils (PMN) were stimulated with 20 nM phorbol 12-myristate 13-acetate (PMA) or by cross-linking FcγRIIIb with mAb 3G8. PMN were previously treated with solvent alone (—) or with the PKC inhibitors GÖ6983 (1 μM) or GÖ6976 (1 μM). After four hours, PMN were fixed and stained for DNA (DAPI). Microphotographs were taken at 200x magnification and are representative of five experiments. Bar is 50 μm. (b) The relative amount of NETs was estimated from SYTOX Green fluorescence in relative fluorescent units (RFU) at 4 hours after stimulation. Data are mean ± SEM of 11 experiments. Asterisks denote conditions that are statistically different from control ($p < 0.001$).

PMN treated with diphenyleneiodonium (DPI), an NADPH-oxidase inhibitor, were not able to form NETs after PMA stimulation (Figure 8). Similarly, DPI-treated PMN could not form NETs after cross-linking of FcγRIIIb (Figure 8). PMA treatment, as well as cross-linking of both FcγRIIa and FcγRIIIb, indeed induced ROS production that was completely blocked by DPI (Supplemental Figure 7S). In addition, PMN treated with BAY 117082, an NF-κB inhibitor, were not able to form NETs after PMA stimulation (Figure 9). In contrast, PMN treated with BAY 117082 at two different concentrations formed NETs efficiently after cross-linking of FcγRIIIb (Figure 9). These data suggested that FcγRIIIb could indeed induce the formation of NETs via NADPH-oxidase activation, but independently of NF-κB activation.

Clearly, selective activation of FcγRIIIb on the PMN membrane was enough to induce the formation of NETs. In order to explore whether cross-linking of Fc receptors by a more natural stimulus could also induce NET formation, PMN were mixed with opsonized latex particles. These particles covered with Protein A and then opsonized with selective anti-Fc receptor antibodies can be recognized by only one or the other of the Fc receptors. As shown previously [30], PMN were capable of efficient phagocytosis of latex beads opsonized with mAb IV.3 (anti-FcγRIIa) and of very poor phagocytosis of latex beads opsonized with mAb 3G8 (anti-FcγRIIIb) (Figure 10). These beads were opsonized at similar levels with both anti-Fcγ receptor antibodies (Supplemental Figure 8S). PMN and fluorescent beads can be easily separated as two distinct populations in a flow cytometer. Thus, by gating on cells an increase in fluorescence indicates efficient phagocytosis (Supplemental Figure 9S). The efficient FcγRIIa-mediated phagocytosis was dependent on ERK activation [30], since the MEK inhibitor UO126 prevented it (Figure 10), and it was independent of NF-κB activation,

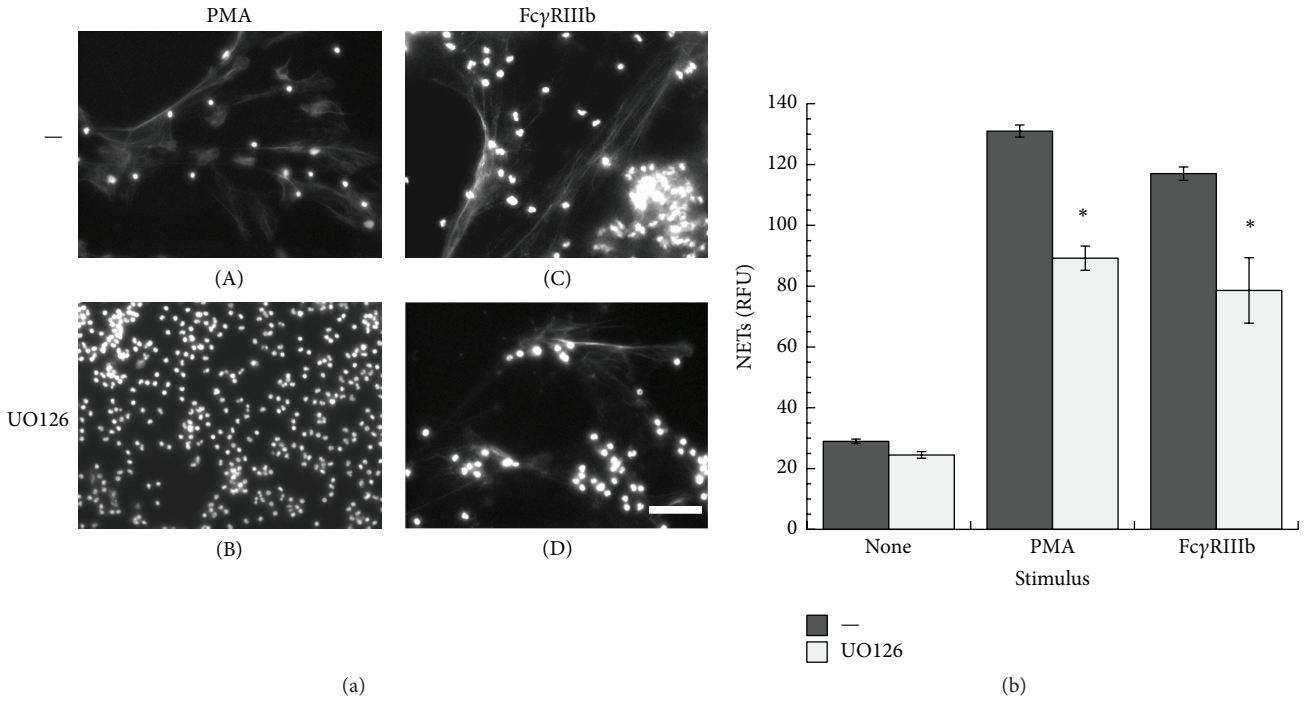

Figure 5: FcγRIIIb-induced NET formation is dependent on MEK. (a) Human neutrophils (PMN) were not stimulated (None) or were stimulated with 20 nM phorbol 12-myristate 13-acetate (PMA) or by cross-linking FcγRIIIb with mAb 3G8. PMN were previously treated with solvent alone (—) or with the MEK inhibitor UO126 (50 μM). After four hours, PMN were fixed and stained for DNA (DAPI). Microphotographs were taken at 200x magnification and are representative of five experiments. Bar is 50 μm. (b) The relative amount of NETs was estimated from SYTOX Green fluorescence in relative fluorescent units (RFU) at 4 hours after stimulation. Data are mean ± SEM of 8 experiments. Asterisks denote conditions that are statistically different from control ($p < 0.001$).

since the inhibitor BAY 117082 did not affect it (Figure 10). These data were also confirmed by evaluating phagocytosis by microscopy (Supplemental Figure 10S). In contrast, the poor phagocytic response of FcγRIIIb was independent of both MEK and NF-κB (Figure 11). These beads when not opsonized (Figure 11(a)(A)) or when opsonized with anti-FcγRIIa antibodies (Figure 11(a)(B)) could not induce the formation of NETs. However, beads opsonized with anti-FcγRIIIb antibodies efficiently induced the formation of NETs (Figure 11(a)(C and D)). In addition, a mixture of beads opsonized with either anti-FcγRIIa antibodies or anti-FcγRIIIb antibodies also induced NET formation to the same level as anti-FcγRIIIb beads alone (Figure 11(b)). These data strongly suggested that FcγRIIa can efficiently promote phagocytosis, while it cannot induce the formation of NETs. In contrast, FcγRIIIb poorly promotes phagocytosis, but it can efficiently induce the formation of NETs.

4. Discussion

Neutrophils are the most abundant circulating leukocytes in mammals and they are rapidly recruited to sites of infection, where they act as the first line of defense against invading pathogens [42]. Neutrophil activation, through various membrane receptors, is also required for the initiation of the several defense mechanisms displayed by these cells [43], including phagocytosis, respiratory burst, release of various microbicidal molecules by degranulation [44], and the recently described formation of neutrophil extracellular traps (NETs) [3]. NETs are extracellular fibers formed by chromatin covered with histones [7] and antimicrobial proteins derived from neutrophil granules [2]. NETs seem to act as a physical barrier where microorganisms get trapped [10] and display antimicrobial activity that is independent of phagocytosis [45]. Despite the fact that many pathogens, including virus, bacteria, fungi, and parasites [10], have all been reported to induce NET formation, no particular receptors on the neutrophil membrane leading to release of NETs have been identified until very recently. IgA-opsonized bacteria or IgA-opsonized beads activated the FcαRI (CD89) leading to release of NETs [46]. Other previous reports indicated that NET formation was increased when microorganisms were opsonized with autologous serum [3], and also antigen-antibody complexes seemed capable of inducing NET formation [22, 23]. These reports thus suggested a role for IgG Fc receptors (FcγRs) in NET formation.

In the neutrophil two types of FcγR are constitutively expressed, namely, FcγRIIa and FcγRIIIb [12, 13]. This fact has made it difficult to establish which functions are initiated by each of these two FcγRs. For phagocytosis, there is no doubt that FcγRIIa is an important receptor [30]. In contrast, FcγRIIIb is an important receptor for signaling to the

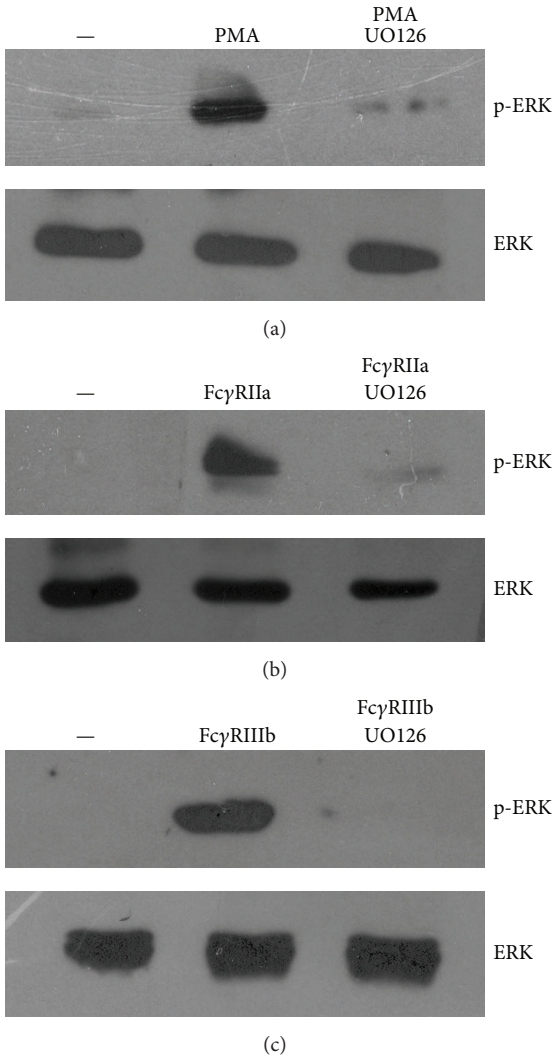

Figure 6: FcγR-cross-linking induces activation of ERK. Human neutrophils (PMN) were left untreated (—) or were stimulated (a) with 20 nM phorbol 12-myristate 13-acetate (PMA) or (b) by cross-linking FcγRIIa with mAb IV.3 or (c) by cross-linking FcγRIIIb with mAb 3G8. PMN were also stimulated in the presence of the MEK inhibitor UO126 (50 μM). PMN cell lysates were prepared after 30 min stimulation. Proteins were resolved by SDS-PAGE and then Western blotted for phosphorylated-ERK (p-ERK) (upper panel) or for total ERK (lower panel). Data are representative of three separate experiments.

nucleus [38]. In the case of NET formation, it is not clear which FcγR is preferentially responsible for this function. It was previously reported that FcγRIIIb promoted endocytosis of soluble immune complexes and that FcγRIIa promoted NET formation in vivo [22]. However, more recently, it was reported that FcγRIIIb is the receptor responsible for NET formation in response to immobilized immune complexes [24]. In addition, neutrophil stimulation by IgG antineutrophil cytoplasmic antibodies (ANCA) led to degranulation and neutrophil extracellular trap formation in an FcγRIIIb allele-specific manner [47]. Here, we have found that indeed FcγRIIIb, but not FcγRIIa, induced significant amounts of NETs.

Selective FcγRIIIb cross-linking with specific monoclonal antibodies on human neutrophils induced NET formation. The release of these NETs was detected 3-4 hours after stimulation and was dependent on ROS, since the NADPH-oxidase inhibitor DPI abrogated trap release. This NET release was similar to the one induced by cross-linking FcαRI [46] or by phorbol 12-myristate 13-acetate (PMA) stimulation [2] but different from the rapid, oxidant-independent NET release recently described [48]. FcγRIIIb-induced NETs consisted of long DNA fibers decorated with histones and neutrophil elastase showing a bona fide neutrophil extracellular trap structure. Although both FcγRIIa and FcγRIIIb induced a strong respiratory burst as shown by activated ROS production, cross-linking of FcγRIIa alone did not induce NET formation. ROS are required for NET formation in most cases [2, 4, 8], but they are not sufficient, since ROS production induced by phagocytosis cannot initiate NET formation [9].

FcγRIIIb-induced NETs are similar in shape and molecular structure to those induced by PMA [9], and the molecular mechanism leading to their release seems to be also similar. Most studies on NET formation have been conducted with PMA stimulation [2]. PMA is a direct activator of protein kinase C (PKC); thus any possible receptor involved in NET formation is bypassed. Several inhibitors of PKC have been shown to block NET formation [49]. In agreement with those reports, we found that two different inhibitors of PKC indeed blocked NET formation after PMA and FcγRIIIb stimulation. In addition, inhibition of Syk with piceatannol blocked the release of NETs induced either by PMA or by FcγRIIIb (Figure 7). However, inhibition of Syk with iSyk slightly reduced only PMA-induced NETosis (Figure 6S). The differential inhibition of NET formation with two reported Syk inhibitors suggests that some of the discrepancies in the literature regarding signaling pathways regulating NETosis may be due to the use of various pharmacological inhibitors. It is necessary to revise these pathways more carefully in future studies. Despite this caveat, inhibition of Syk with piceatannol points to an important role for this kinase in NET formation induced by specific cross-linking of FcγRIIIb. Syk was also found to participate in NET formation induced by soluble immune complexes [22, 50], by insoluble immune complexes [24], and by PMA [24]. Syk is normally associated with initial signaling events at the level of cell surface receptors, but PMA can bypass these receptors to directly activate PKC [51]. Yet activation of Syk by PMA has been previously described in neutrophils. PMA induced PKC-dependent phosphorylation of Syk [52], and piceatannol reduced ROS production in response to PMA [53]. Together these reports and our data support the idea that Syk activation is involved in both PMA- and also FcγRIIIb-induced ROS-dependent NETosis.

Downstream of PKC, a role for the MEK, ERK pathway [54] and for NF-κB [41] in PMA-induced NET formation has been suggested. MEK inhibition blocked PMA- and FcγRIIIb-induced NETosis indicating that ERK activation is required in this process. ERK was also found to be required for NET formation in response to soluble immune

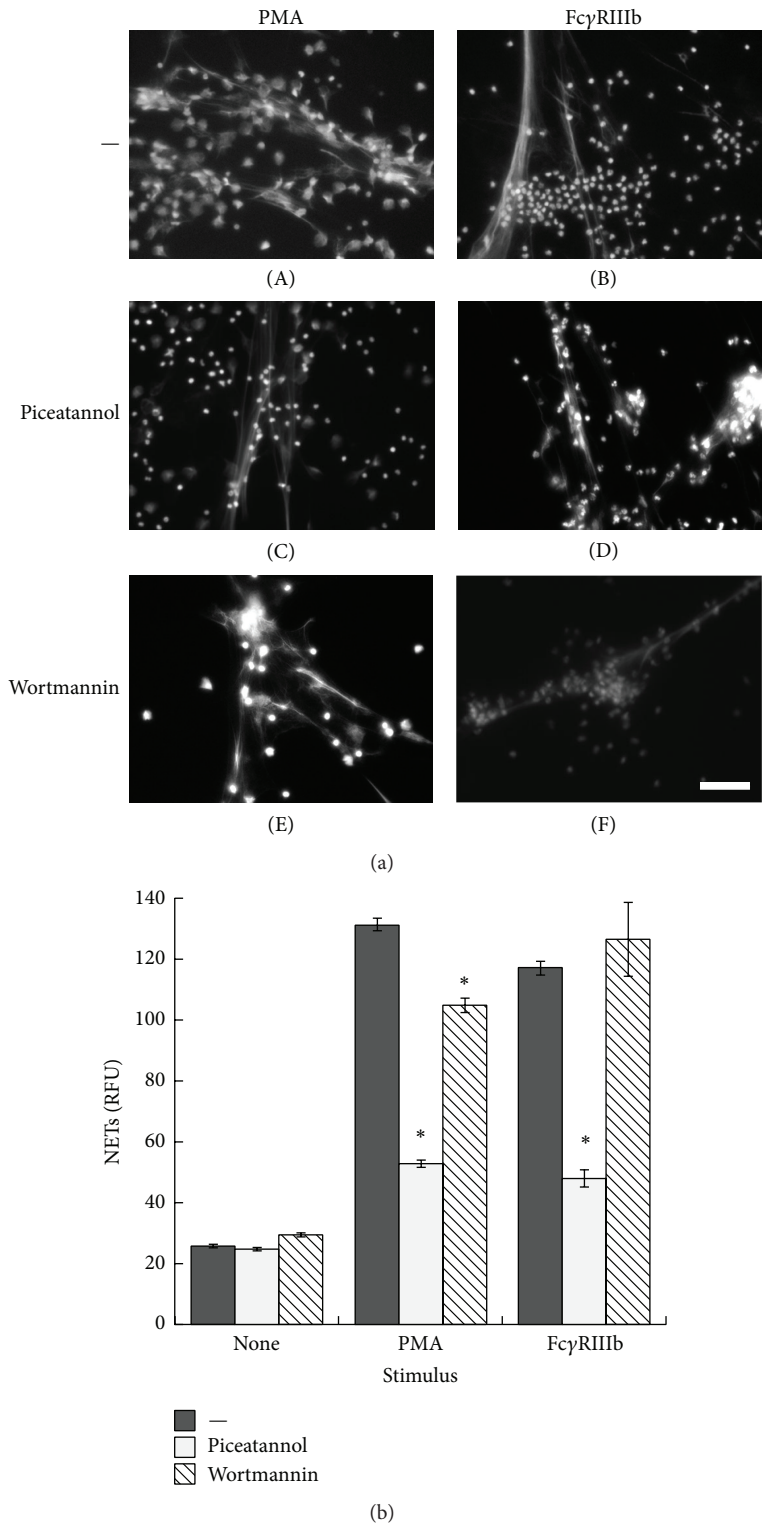

Figure 7: FcγRIIIb-induced NET formation requires Syk but is independent of PI-3K. (a) Human neutrophils (PMN) were not stimulated (None) or were restimulated with 20 nM phorbol 12-myristate 13-acetate (PMA) or by cross-linking FcγRIIIb with mAb 3G8. PMN were previously treated with solvent alone (—) or with the Syk inhibitor piceatannol (50 μM) or with the PI-3K inhibitor wortmannin (50 nM). After four hours, PMN were fixed and stained for DNA (DAPI). Microphotographs were taken at 200x magnification and are representative of three experiments. Bar is 50 μm. (b) The relative amount of NETs was estimated from SYTOX Green fluorescence in relative fluorescent units (RFU) at 4 hours after stimulation. Data are mean ± SEM of 5 experiments. Asterisks denote conditions that are statistically different from control ($p < 0.05$).

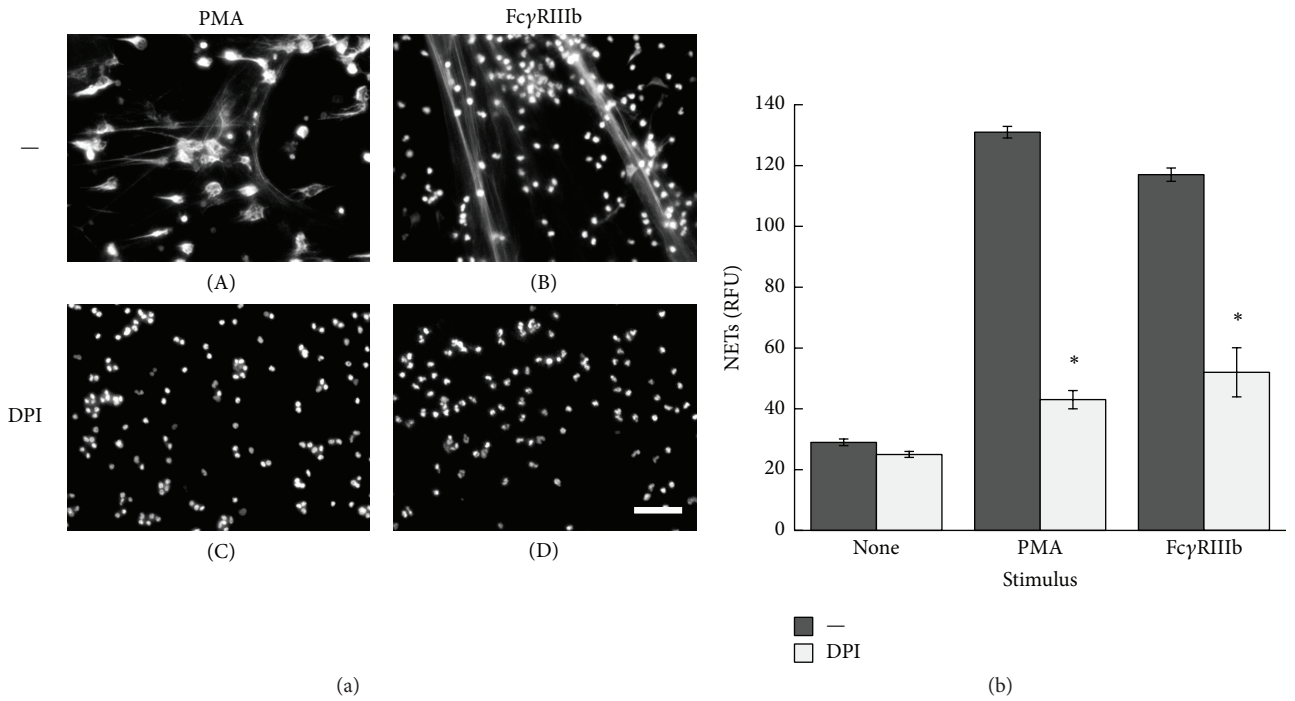

Figure 8: FcγRIIIb-induced NET formation is dependent on NADPH-oxidase. (a) Human neutrophils (PMN) were not stimulated (None) or were stimulated with 20 nM phorbol 12-myristate 13-acetate (PMA) or by cross-linking FcγRIIIb with mAb 3G8. PMN were previously treated with solvent alone (—) or with the NADPH-oxidase inhibitor diphenyleneiodonium (DPI) (10 μM). After four hours, PMN were fixed and stained for DNA (DAPI). Microphotographs were taken at 200x magnification and are representative of three experiments. Bar is 50 μm. (b) The relative amount of NETs was estimated from SYTOX Green fluorescence in relative fluorescent units (RFU) at 4 hours after stimulation. Data are mean ± SEM of 5 experiments. Asterisks denote conditions that are statistically different from control ($p < 0.001$).

complexes [22] and immobilized immune complexes [24]. However, the role of ERK in NET formation remains unclear. A previous report indicated that ERK is required for NADPH-oxidase activation [37], placing ERK upstream of ROS production, while another report suggested that ROS are downstream of ERK activation [54]. Therefore, it seems that NADPH-oxidase activation for NET formation may proceed not only through an ERK pathway, but also independently of ERK activation, depending on the stimulus [19, 20]. As previously reported, NF-κB inhibition reduced PMA-induced NET formation [41]. However, FcγRIIIb-induced NETosis was unaffected when neutrophils were treated with the same inhibitor for NF-κB (Figure 9). Similarly, inhibition of PI-3K by wortmannin reduced NET formation by PMA but had no effect on FcγRIIIb-induced NET formation (Figure 7). A possible role for PI-3K involvement in NET formation induced by immobilized immune complexes was also found using the inhibitor LY29004 [24]. We did not find the same result, but as mentioned above for Syk the particular inhibitor used may be responsible for these different results. It was proposed that PI-3K could influence NF-κB activation via phosphatidylinositol-trisphosphate and in turn NF-κB activate genes important for signaling to NET formation [41]. These ideas, however, have not been proven experimentally and the role of PI-3K and NF-κB in FcγRIIIb-mediated NETosis needs further exploration.

FcγRIIIb has been suggested to signal in cooperation with other molecules such as integrin Mac-1 (CD11b/CD18), also known as complement receptor 3 [55]. However, complement receptor ligands are not sufficient to induce NET formation in isolated neutrophils [9]. Similarly, in our case selective cross-linking of β2 integrins with mAb IB4 also did not induce any NET formation. In contrast, blocking Mac-1 with antibodies against both CD11b and CD18 chains prevented NET formation by LPS [19], by β-glucan [56], and by immobilized immune complexes [24]. These reports and our data suggest that β2 integrins cooperate with other receptors to induce NETosis, but they cannot by themselves cause NET formation. The involvement of β2 integrins in NET formation might be more related to the adhesion requirement of neutrophils to form NETs [28] than to a signaling capacity of the integrin. Along the same line of thought, cross-linking of other receptors such as β1 integrins also did not promote any NET formation (Figure 1), although the same procedure was capable of activating NF-κB in neutrophils [25]. Recently, it was also reported that NET formation in response to *Candida albicans* required fibronectin via β1 integrins. However, β1 integrin engagement alone was not sufficient to activate NETosis [56]. Similarly, the adhesive protein invasin from *Yersinia pseudotuberculosis* promotes bacteria crossing the intestine epithelium by binding to β1 integrins on M-cells. Invasin was also shown to induce

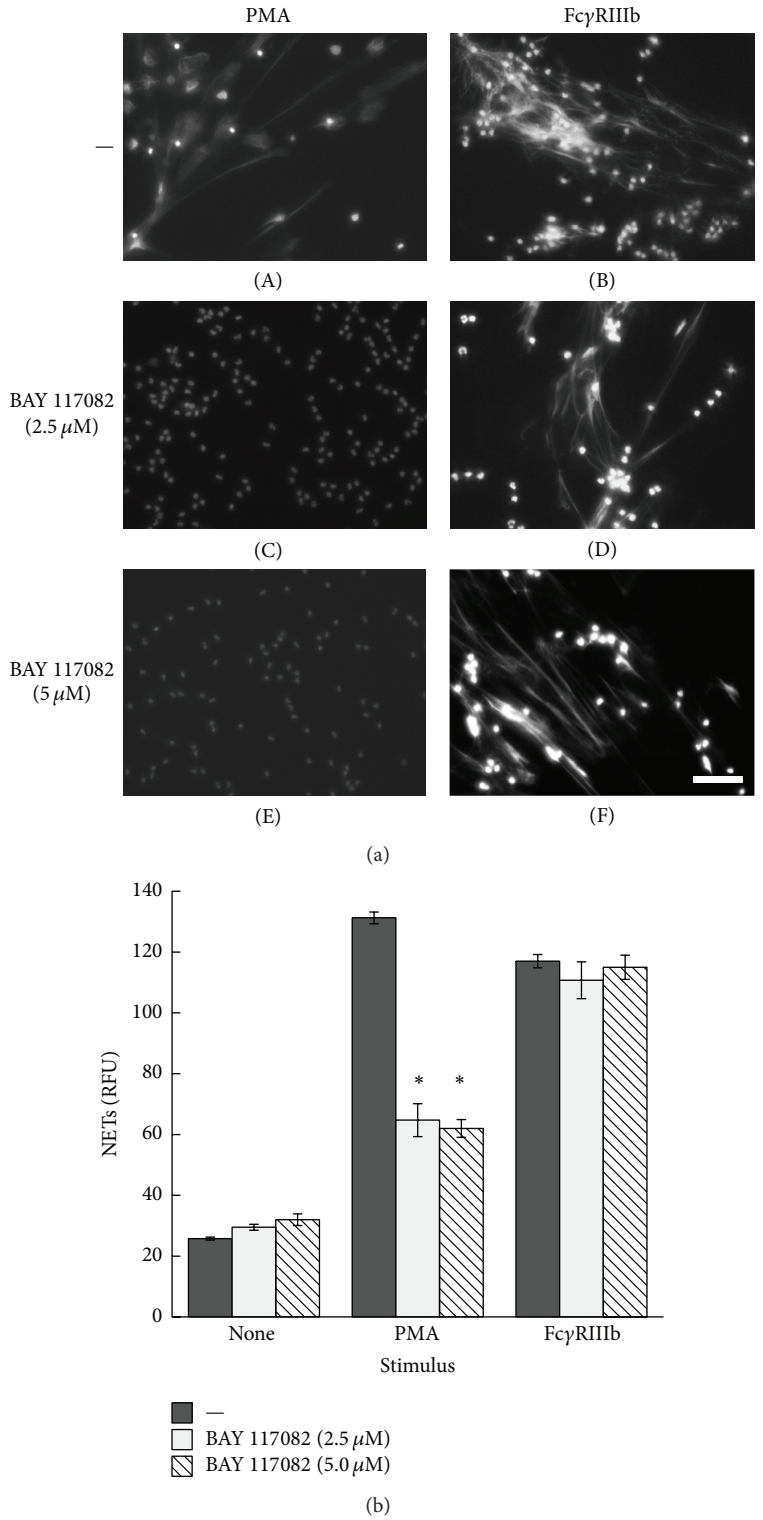

Figure 9: FcγRIIIb-induced NET formation is independent of NF-κB. (a) Human neutrophils (PMN) were not stimulated (None) or were stimulated with 20 nM phorbol 12-myristate 13-acetate (PMA) or by cross-linking FcγRIIIb with mAb 3G8. PMN were previously treated with solvent alone (—) or with the NF-κB inhibitor BAY 117082 at 2.5 μM and 5 μM. After four hours, PMN were fixed and stained for DNA (DAPI). Microphotographs were taken at 200x magnification and are representative of three experiments. Bar is 50 μm. (b) The relative amount of NETs was estimated from SYTOX Green fluorescence in relative fluorescent units (RFU) at 4 hours after stimulation. Data are mean ± SEM of 4 experiments. Asterisks denote conditions that are statistically different from control ($p < 0.05$).

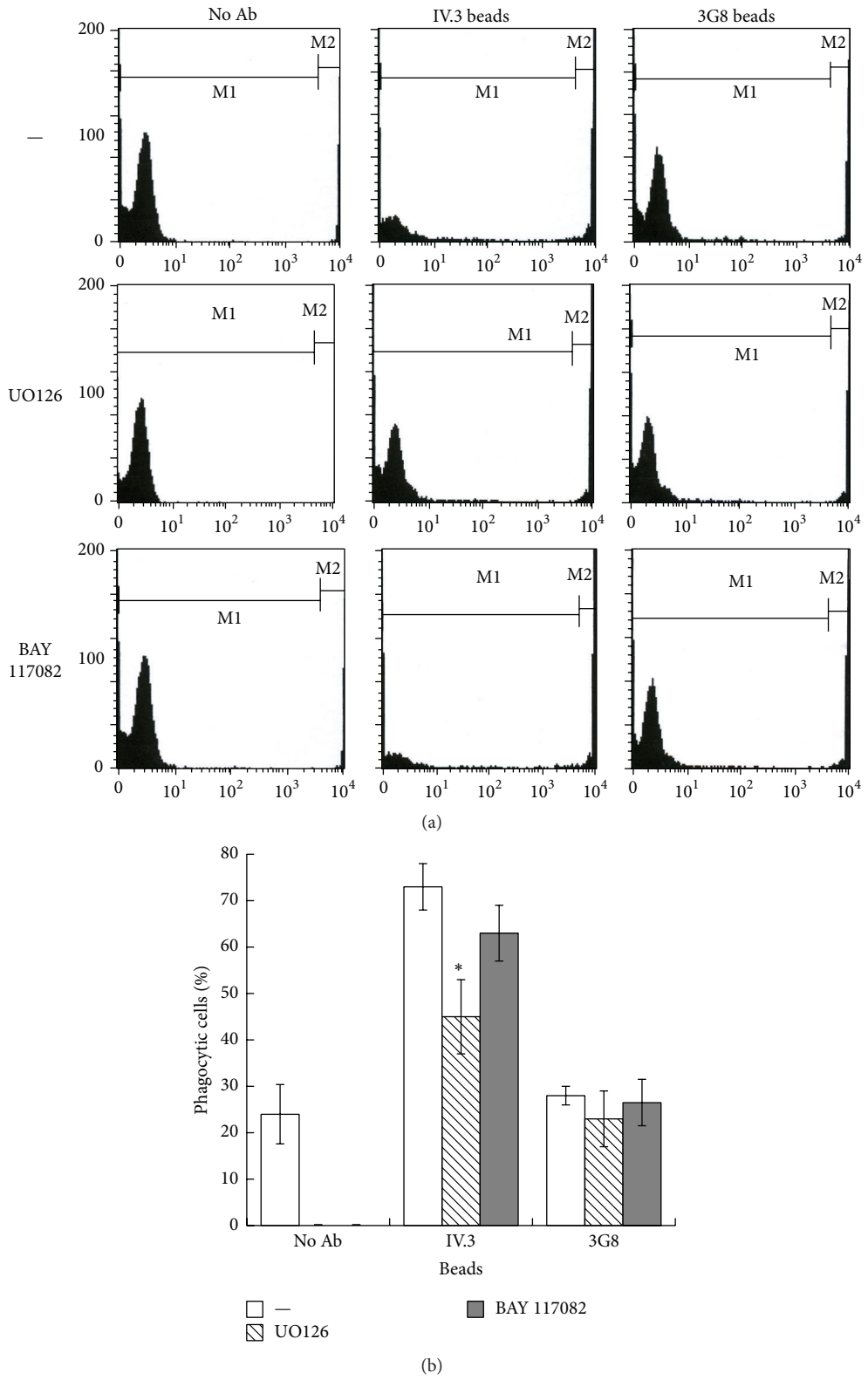

Figure 10: FcγRIIa induces efficient phagocytosis. (a) Human neutrophils were mixed with fluorescence latex particles. Particles were nonopsonized (no Ab) or opsonized with monoclonal antibody (mAb) IV.3 anti-FcγRIIa or with mAb 3G8 anti-FcγRIIIb. Cells were allowed to ingest the particles for 30 min. Phagocytosis of latex beads was also evaluated in the absence (None) or the presence of 50 μM UO126 (MEK inhibitor) or 2.5 μM BAY 117082 (NF-κB inhibitor). Phagocytosis was assessed by flow cytometry, detecting the reduced number of cells with low fluorescence (M1 marker), and the appearance of cells with high fluorescence in the far right side (M2 marker) of the histogram of gated PMN. (b) Phagocytosis was quantified by flow cytometry, as the percentage of high-fluorescence cells (marker M2) in the histogram of gated PMN. Data are representative of four separate experiments.

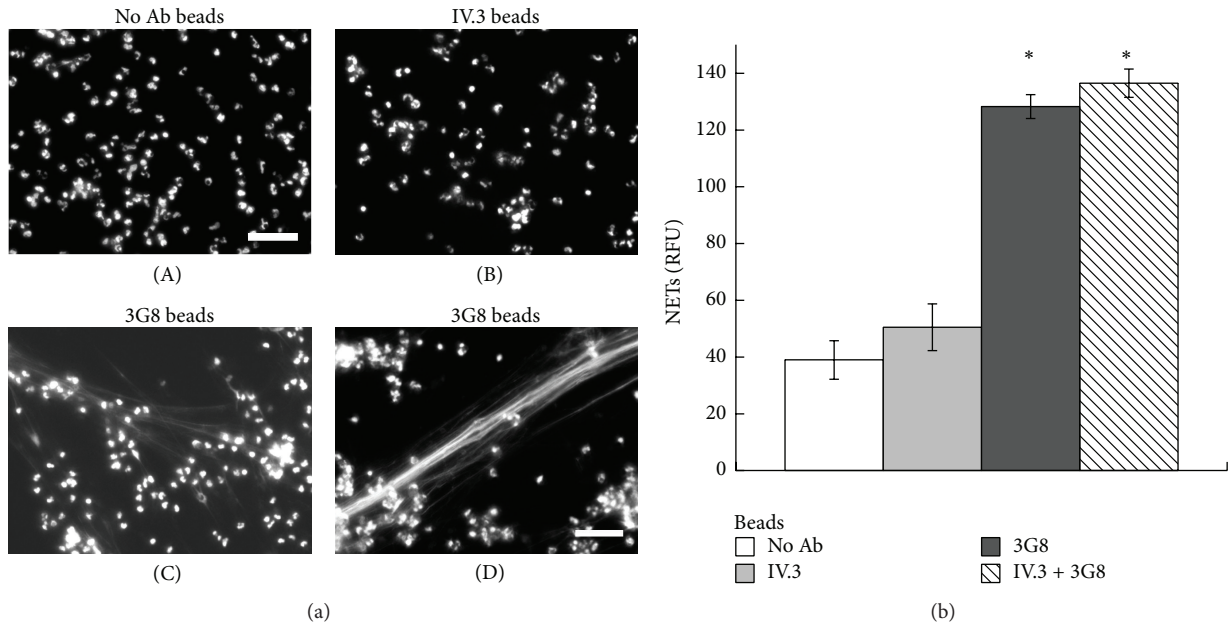

Figure 11: Anti-FcγRIIIb-opsonized particles induce NET formation. (a) Human neutrophils (PMN) mixed with latex particles nonopsonized (no Ab beads) or opsonized with monoclonal antibody IV.3, anti-FcγRIIa (IV.3 beads), or with monoclonal antibody 3G8, anti-FcγRIIIb (3G8 beads), were incubated for four hours and fixed and stained for DNA (DAPI). Microphotographs were taken at 200x magnification and are representative of three experiments. Bar is 50 μm. (b) The relative amount of NETs was estimated from SYTOX Green fluorescence in relative fluorescent units (RFU) at 4 hours after stimulation, as described in Materials and Methods. Data are mean ± SEM of four experiments. Asterisks denote conditions that are statistically different from control ($p < 0.02$).

ROS and NET formation [57]. However, invasin-mediated triggering of β1 integrin was essential but not sufficient for NET production [57]. Additional, so far uncharacterized costimuli were required for NET formation. Clearly, integrins cooperate in different scenarios to activate NETosis after various stimuli including immune complexes, but the exact role they play in this process remains elusive.

Moreover, as mentioned above, selective cross-linking of FcγRIIa also did not promote NET formation. This was not due to a defect in FcγRIIa signaling because the same cross-linking procedure led to robust activation of ERK (Figure 6). In addition, latex beads opsonized with the specific anti-FcγRIIa mAb IV.3 were efficiently phagocytosed by neutrophils [30] (Figure 10). The same opsonized latex beads were not capable of inducing any NET formation. In contrast, latex beads opsonized with the specific anti-FcγRIIIb mAb 3G8 were poorly phagocytosed by neutrophils [30] (Figures 10 and 10S) but efficiently induced NET formation. Our results support the idea presented in a recent report showing that neutrophils sensed microbe size and selectively released NETs in response to large pathogens, such as *Candida albicans* hyphae and extracellular aggregates of *Mycobacterium bovis*, but not in response to small yeast or single bacteria [58]. In this study, phagocytosis via the receptor dectin-1 acted as a sensor of microbe size and prevented NET release by downregulating the translocation of neutrophil elastase to the nucleus [58]. Similarly, we present here that neutrophils responded via FcγRIIa with efficient phagocytosis; however NET formation was absent. In contrast, stimulation via FcγRIIIb led to poor phagocytosis but to significant NET formation. Thus we conclude that NETs are not formed when an opsonized target can be efficiently phagocytosed via FcγRIIa. However, upon inefficient phagocytosis via FcγRIIIb engagement, NET formation is induced strongly. Together, these data support the idea that indeed each FcγR on the human neutrophil is capable of triggering specific responses. FcγRIIa promotes efficient phagocytosis, while FcγRIIIb induces NET formation instead. The inflammatory environment may be responsible for what receptor FcγRIIa or FcγRIIIb may predominate and initiate a particular cell response [11]. FcγRIIIb is expressed 4- to 5-fold more abundantly and has a higher affinity for IgG than FcγRIIa [59], thus probably becoming the preferred receptor to first engage immune complexes. At the same time, inflammatory stimuli can lead to FcγRIIIb shedding from the cell, favoring now immune complex interactions with FcγRIIa [60] to induce phagocytosis and cytotoxicity [13]. We believe that when a strong activating threshold is achieved by cross-linking FcγRIIIb an efficient induction of NET formation takes place.

In conclusion, our data show that FcγRIIIb governs Fc receptor-induced NET formation in human neutrophils. The signaling pathway used by FcγRIIIb to induce NETs involves PKC, PKC, and ERK 1. Our results also support the idea that different Fc receptors promote independent cell functions.

Conflict of Interests

The authors do not have any conflict of interests related to this paper.

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Review Article

Annexin A1 and the Resolution of Inflammation: Modulation of Neutrophil Recruitment, Apoptosis, and Clearance

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Neutrophils (also named polymorphonuclear leukocytes or PMN) are essential components of the immune system, rapidly recruited to sites of inflammation, providing the first line of defense against invading pathogens. Since neutrophils can also cause tissue damage, their fine-tuned regulation at the inflammatory site is required for proper resolution of inflammation. Annexin A1 (AnxA1), also known as lipocortin-1, is an endogenous glucocorticoid-regulated protein, which is able to counterregulate the inflammatory events restoring homeostasis. AnxA1 and its mimetic peptides inhibit neutrophil tissue accumulation by reducing leukocyte infiltration and activating neutrophil apoptosis. AnxA1 also promotes monocyte recruitment and clearance of apoptotic leukocytes by macrophages. More recently, some evidence has suggested the ability of AnxA1 to induce macrophage reprogramming toward a resolving phenotype, resulting in reduced production of proinflammatory cytokines and increased release of immunosuppressive and proresolving molecules. The combination of these mechanisms results in an effective resolution of inflammation, pointing to AnxA1 as a promising tool for the development of new therapeutic strategies to treat inflammatory diseases.

1. Introduction

Inflammation is a crucial physiological response for the maintenance of tissue homeostasis, protecting the host against invading microorganisms, foreign substances, or host self-disturbers, such as the molecules derived from damaged cells [1]. After the host has been incited, important microcirculatory events occur in response to local release of proinflammatory mediators, such as histamine, prostaglandins, leukotrienes, cytokines, and chemokines, leading to higher vascular permeability and increased leukocyte recruitment [1]. Leukocytes, such as neutrophils and macrophages, play a key role in inflammatory response, by releasing further inflammatory mediators and acting as effector cells and phagocytes to remove the inflammatory agent/stimuli [2].

Despite the important roles of neutrophils for effective host defense, these cells can also cause tissue damage requiring appropriate regulation [3, 4]. Continuous inflammatory stimuli can lead to aggressive and/or prolonged inflammatory responses, which may be detrimental to the host, leading to chronic inflammation [5]. The efficient removal of the inciting agent by phagocytes is the first signal for triggering proper resolution, through inhibition of proinflammatory mediators production and activation of their catabolism,

resulting in the ceasing of further leukocyte recruitment [6]. After that, proresolving pathways are activated in order to restore tissue structure, function, and homeostasis [7, 8]. In this context, anti-inflammatory and proresolving molecules such as specialized lipid mediators (lipoxin A4, resolvins, maresins, and protectins), peptides/proteins (melanocortins, galectins, and annexin A1), and several other substances of different natures are released at the site of inflammation [7, 9, 10]. These endogenous mediators are known for their ability to decrease endothelial activation, reduce leukocyte infiltration, and activate neutrophil apoptosis, which ensures their secure removal by scavenger macrophages through a process called efferocytosis (phagocytosis of apoptotic cells) [4].

Annexin A1 (AnxA1) is an important glucocorticoid- (GC-) regulated protein, which contributes to the resolution of inflammation through various ways (Figure 1). AnxA1 limits neutrophil recruitment and production of proinflammatory mediators. Moreover, AnxA1 acts by inducing neutrophil apoptosis, modulating monocyte recruitment, and enhancing the clearance of apoptotic cells by macrophages. Emerging evidence suggests that AnxA1 also induces macrophage reprogramming toward a resolving phenotype, another key event to restore tissue homeostasis. In this review, we summarize several physiological and potential therapeutic actions of AnxA1 on inflammation resolution. In particular, this review highlights recent advances on the actions of this endogenous mediator and its potential clinical utility.

2. Annexin A1: General Aspects

Endogenous mediators of inflammation, such as AnxA1, are potential therapeutic tools to control inflammatory diseases. Although whether clinical use of proresolving strategies will be useful for treating inflammatory maladies or will show significant undesirable effects remains to be elucidated, it is believed these will be effective and have fewer side effects due to their ability to mimic or induce natural pathways of the resolution phase of inflammation [8, 12].

Annexin superfamily is composed of 13 members, grouped in view of their unique Ca^{2+}-binding-site architecture, which enables them to peripherally attach to negatively charged membrane surfaces [13–15]. AnxA1, also known as annexin I or lipocortin I, was originally identified as a GC-induced protein active on phospholipase- (PL-) A2 inhibition and prevention of eicosanoid synthesis [16–18]. It was subsequently recognized as an endogenous modulator of the inflammatory response, through several studies, mainly those led by Dr. Flower and Dr. Perretti [19, 20]. This 37 kDa protein consists in a homologous core region of 310 amino acid residues, representing almost 90% of the structure, attached to a unique N-terminal region [15]. In addition to mediating membrane binding, Ca^{2+} ions can also induce a conformational change that leads to the exposure of the bioactive N-terminal domain [15, 21, 22]. In fact, studies on the anti-inflammatory activity of AnxA1 revealed not only that the different functions of the protein lie within the unique N-terminus, but also that synthetic peptides from the N-terminal domain may mimic the pharmacological property of the whole protein, specifically binding to formyl peptide receptors (FPRs) [12].

In inflammatory conditions intact AnxA1 (37 kDa) can be cleaved by proteinase-3 and neutrophil elastase generating the 33 kDa cleaved isoform, which is believed to be inactive, and peptides derived from the AnxA1 N-terminus [23–25]. The main cleavage sites on AnxA1 are located at A^{11}, V^{22}, and V^{36}, as identified by cleavage assays coupled to mass spectrometric analyses [25]. Investigation of the $AnxA1_{2–50}$ peptide revealed a novel cleavage site at position 25, probably unmasked due to the simpler conformation of the peptide, compared with the full-length AnxA1 [26]. In fact, the substitution of the mentioned cleavage sites allowed the generation of metabolically stable forms of AnxA1 and its peptide, respectively, named SuperAnxA1 (SAnxA1) [27] and cleavage-resistant $AnxA1_{2–50}$ (CR-$AnxA1_{2–50}$) [26]. The proinflammatory nature of AnxA1 cleavage products is supported by reports of increased levels of the 33 kDa fragment in human and animal inflammatory samples, including bronchoalveolar lavage fluids [28–30] and exudates [11, 25, 31, 32]. For instance, using a model of acute pleurisy, our research group has shown increased levels of the 33 kDa breakdown product of AnxA1 during the time points of high neutrophil infiltration into the pleural cavity followed by regain of the intact form during the resolving phase of the pleurisy [11]. However, what the biological functions of this and other AnxA1-generated peptides are is still unclear, and this matter deserves further investigation.

Evidence for physiological function of AnxA1 in modulating inflammation emerged from studies involving AnxA1-null mice and AnxA1 neutralization strategies. AnxA1-null mice are viable and have a normal phenotype until they are challenged with inflammatory stimuli when they show stronger and more prolonged inflammatory reaction when compared to the wild-type (WT) [33–40]. Resistance to glucocorticoid treatment and aberrant inflammation in AnxA1-deficient mice provided initial evidence for the physiological relevance of the protein [33]. In the absence of AnxA1, the inflammatory response is exacerbated as demonstrated by increased neutrophil extravasation following zymosan-induced peritonitis [35] and endotoxin-induced uveitis [37]. In addition, animals lacking this protein exhibited exacerbated arthritis severity [34] and allergic response in ovalbumin-induced conjunctivitis [39]. AnxA1 KO mice also showed increased atherosclerotic lesion size with an overall increase in lesional macrophages and neutrophils [40]. Moreover, our research group has shown the prevention of spontaneous and dexamethasone-driven resolution of inflammation by using an AnxA1 neutralizing strategy [11]. Aside from the physiological role of the endogenous protein, pharmacological treatment with both human recombinant AnxA1 and its N-terminal peptides exerts anti-inflammatory and proresolving effects in a variety of experimental models, highlighting their therapeutic potential for inflammation resolution [11, 26, 27, 41] and wound repair [42].

AnxA1 exerts many of its anti-inflammatory and proresolving actions through the formyl peptide receptor type

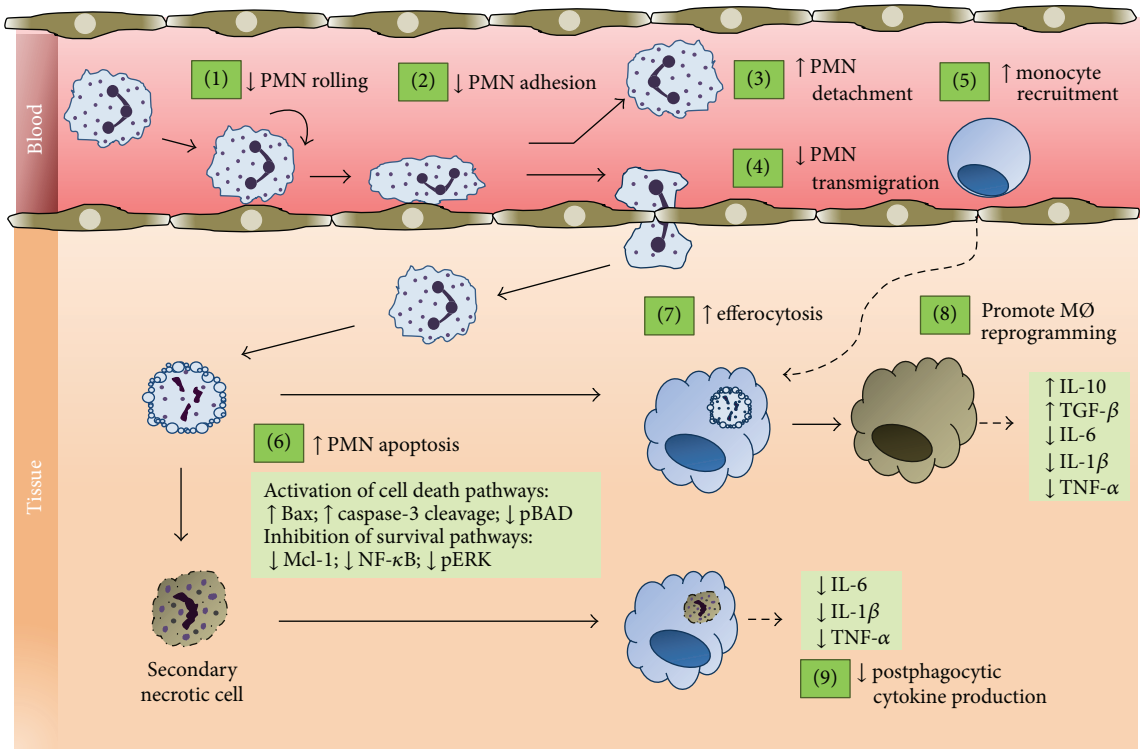

Figure 1: Cellular events associated with the anti-inflammatory and proresolving effects of annexin A1 (AnxA1) and its mimetic N-terminal peptides. AnxA1 modulates a wide range of cellular and molecular steps of the inflammatory response and is deeply involved in the endogenous mechanisms that are activated to bring about proper resolution. Pharmacological administration of AnxA1 results in decreased neutrophil rolling (1) and adhesion (2) to endothelium, increased detachment of adherent cells (3), and inhibition of neutrophil transmigration (4). In addition, AnxA1 is able to induce apoptosis, overriding the prosurvival signals that cause prolonged lifespan of neutrophils at the inflammatory site (6). Endogenous and exogenous AnxA1 also promote monocyte recruitment (5) and clearance of apoptotic neutrophils by macrophages (7). Phagocytosis of apoptotic neutrophils by macrophages is coupled with release of anti-inflammatory signals, including transforming growth factor-β, and lower levels of proinflammatory cytokines (8). Besides, AnxA1 is related to macrophage reprogramming toward a proresolving phenotype (8). Initial *in vitro* studies using AnxA1 knock-down leucocytes demonstrate that AnxA1 prevents proinflammatory cytokine production after phagocytosis of secondary necrotic cells. This effect provides an important fail-safe mechanism counteracting inflammatory responses when the timely clearance of apoptotic cells has failed (9).

2/lipoxin A4 receptor (FPR2/ALX). This receptor, along with FPR1 and FPR3, composes a family of seven-transmembrane domain G protein-coupled receptors which share significant sequence homology [43]. FPR2/ALX receptor is shared by a variety of other peptide/protein and lipid ligands, mediating diverse biological functions of relevance for host defence and inflammation. Interestingly, FPR2/ALX agonists are associated with both proinflammatory (e.g., serum amyloid A and cathelicidin) and proresolving (e.g., AnxA1 and LXA_4) signalling pathways [43, 44]. However, how FPR2/ALX can promote both inflammatory response and limit its duration and intensity still remains to be fully elucidated. It is noteworthy that distinct FPR2/ALX domains are required for signalling by different agonists [45]. Using FPR2/ALX transfected cells and chimeric FPR1 and FPR2 clones, Bena and col. (2012) identified that while AnxA1-mediated signalling involves the N-terminal region and extracellular loop II of FPR2/ALX, SAA interacts with the extracellular loops I and II of the same receptor [45]. Otherwise, LXA_4 has been shown to activate FPR2/ALX by interacting with extracellular loop III and the associated transmembrane domain [46].

The versatility of FPR2/ALX receptors also seems to rely on the activation of receptor dimmers in a biased fashion. AnxA1 was found to activate FPR2/ALX homodimerization but not the proinflammatory SAA [47]. In contrast to the full-length AnxA1, the short AnxA1 derived peptide Ac2–26 is able to activate all members of the human FPR family [48] and induce FPR2/ALX-FPR1 heterodimerization [47]. These observations suggest that short AnxA1 mimetic peptides might fulfill other functions at variance to those reported for the parental protein [49]. However, a good degree of selectivity was retained by longer AnxA1 derived anti-inflammatory sequences such as $AnxA1_{2-50}$ [26].

Interestingly, the promiscuity of FPR2/ALX seems to be linked to a network of resolution mediators as discussed by Brancaleone and col. (2011) [50]. In fact, the authors provide strong evidence that the engagement of FPR2/ALX by selective agonists (such as LXA_4 and antiflammin 2) would induce AnxA1 phosphorylation and mobilization in human PMN [50]. In a similar vein, the proresolving mediator Resolvin E1 (RvE1) stimulates endogenous LXA_4 production [51, 52]. Moreover, it has been shown that proresolving

mediators such as resolvins and LXA_4 induce further anti-inflammatory molecules *in vivo*, such as interleukin- (IL-) 10 [41]. Taken together, these data suggest that a proresolving cascade may be operating during resolution with FPR2/ALX playing a central role in this process.

3. Anti-Inflammatory and Proresolving Actions of AnxA1

3.1. AnxA1 Regulates Neutrophil Recruitment to the Inflammatory Site. During inflammation neutrophils are rapidly recruited to the infected or injured tissue. However, due to the potential tissue-damaging effects of PMN, their fine-tuned regulation at the inflammatory site is required [53]. Indeed, exacerbated or overshooting inflammatory response with high neutrophil influx may account for chronic inflammatory diseases [5]. Thus, restricting leukocyte infiltration to the tissue is an essential process for spontaneous or pharmacological-induced resolution of inflammation [4, 8].

Neutrophil trafficking to the site of inflammation requires adhesion and transmigration through blood vessels, which is orchestrated by molecules on leukocytes (e.g., β1, β2 integrins, and L-selectin) and on endothelial cells (e.g., vascular cell adhesion molecule-1, intercellular adhesion molecule-1, and E-selectin). The leukocyte adhesion cascade is a tightly regulated process, subjected to both positive and negative regulators [71]. For example, anti-inflammatory and proresolving mediators, such as AnxA1, are well documented to counterregulate excessive neutrophil accumulation (an anti-inflammatory action). Human PMN interaction with endothelial cells during the early stage of inflammation promotes modulation of AnxA1 in several ways, such as induction of gene expression [35] and mobilization and cell surface externalization of intracellular AnxA1 [72, 73]. In turn, the externalized protein acts as a brake for PMN adhesion to the microvascular wall, preventing overexuberant cell transmigration to the inflammatory site [4, 27, 72, 74]. Dalli and col. (2008) [75] reinforced the anti-inflammatory properties of PMN-derived microparticles containing functionally active AnxA1. Released upon adhesion to endothelial cells, these microparticles inhibit neutrophil/endothelium interaction under flow, *in vitro*, and PMN recruitment to an air pouch inflamed with IL-1β, *in vivo* [75]. Moreover, microparticles derived from WT but not from AnxA1-deficient neutrophils were able to inhibit IL-1β-induced leukocyte trafficking [75].

Several studies using exogenously administrated AnxA1 have provided further evidence for the modulating role of AnxA1 on neutrophil trafficking. *In vivo* observations produced through intravital microscopy techniques indicated that AnxA1 and Ac2–26 administration to mice during zymosan-induced peritonitis produced detachment of adherent neutrophils from the vascular wall with consequent inhibition of neutrophil extravasation across mouse mesenteric postcapillary venules (Table 1) [56]. Supporting these first findings, *in vitro* studies showed that recombinant AnxA1 and its mimetic peptides display inhibitory effects on neutrophil rolling [26, 27, 54, 55] adhesion to endothelial monolayer [26, 27, 40, 48, 54, 55] and transmigration [48].

Shedding of L-selectin appears to be one of the molecular mechanisms that mediate the effects of AnxA1 and its N-terminal peptides on neutrophil recruitment. Walther and col. (2000) [48] have described the ability of the AnxA1 peptide Ac9–25 to cause transient calcium fluxes and L-selectin shedding in human neutrophils. After that, the same mechanism was linked to the inhibitory effects of Ac2–26 on PMN capture and rolling in a flow chamber assay [54]. Similarly, promotion of L-selectin shedding was demonstrated for human recombinant AnxA1 [57, 58], an effect mediated by cell surface metalloprotease ("sheddase") [58]. Recently, Drechsler and col. (2015) [40] brought further insights into the mechanisms behind the antimigratory effects of Ac2–26. According to the authors, the peptide dose dependently reduces the affinity of activated neutrophils for vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1), a response abrogated in cells harvested from FPR2 knockout mice. They demonstrated that Ac2–26 inhibits the adhesiveness of β1 and β2 integrins by downmodulating their affinity and valency, but without changing their cell surface expression. It was also demonstrated that Ac2–26 interferes with the chemokine-driven activation of Rap1, an essential step in integrin activation [76, 77].

Pederzoli-Ribeil and col. (2010) [27] combined *in vitro* and *in vivo* experimental strategies to show that AnxA1 and its mutant cleavage-resistant form, SAnxA1, are able to augment rolling velocity and reduce adhesion of PMN to endothelial cells through FPR2 receptors. Furthermore, Dalli and col. (2013) [26] demonstrated the anti-inflammatory actions of the longer acetylated AnxA1 peptide $AnxA1_{2-50}$ and its cleavage-resistant form, CR-$AnxA1_{2-50}$. Both displayed antimigratory effects *in vivo*, reducing leukocyte adhesion to inflamed cremaster venule, neutrophil migration into dermal air pouches in response to IL-1β, and neutrophil migration into peritoneum in response to zymosan.

In vivo anti-inflammatory and antimigratory properties of the short AnxA1 peptide Ac2–26 have also been extensively demonstrated, as exemplified by its ability to inhibit carrageenan-induced PMN adhesion to the vasculature and extravasation into the peritoneal fluid [74]. The peptide was also able to prevent neutrophil recruitment in myotoxin-induced peritonitis [78] and during lung inflammation induced by intestinal ischemia/reperfusion [79]. Moreover, Ac2–26 showed potential benefits in an ocular model by inhibiting neutrophil influx, protein leak, chemical mediator release, and COX-2 expression during endotoxin-induced uveitis [37]. The Ac2–26 peptide also demonstrated antimigratory effects in a model of ovalbumin-induced allergic conjunctivitis, significantly reducing the clinical signs of conjunctivitis through the inhibition of leukocyte influx and cytokines and chemokines release, effects correlated with inhibition of the ERK pathway [39]. Interestingly, increased levels of ERK phosphorylation were associated with exacerbated allergic response observed in AnxA1-deficient mice in comparison to WT animals [39]. Reinforcing the involvement of AnxA1 pathway in neutrophil recruitment, AnxA1-null mice demonstrated a higher extent of neutrophil extravasation in animal models of peritonitis [35, 74], allergic conjunctivitis [39], and uveitis [37].

Table 1: *In vitro* and *in vivo* evidence for anti-inflammatory and proresolving properties of annexin A1 and its fragments.

Agent	Experimental model	Outcome/effect on resolution	References
	Inhibition of neutrophil recruitment		
AnxA1	Neutrophil/endothelial interaction (*in vitro*)	↓ PMN capture, rolling, and adhesion ↓ PMN transmigration	[27, 48, 54, 55]
	Neutrophil/endothelial interaction (*in vivo*)	↓ PMN rolling, adhesion, and emigration ↑ Detachment of adherent PMN	[27, 56]
	Human PMN	↑ L-selectin shedding	[57, 58]
	IL-1β inflamed air pouch	↓ PMN migration	[26, 59]
	Carrageenan-induced paw edema	↓ edema ↓ leukocyte infiltration	[27]
SAnxA1	Neutrophil/endothelial interaction (*in vitro*)	↓ PMN capture, rolling, and adhesion	[27]
	Neutrophil/endothelial interaction (*in vivo*)	↓ PMN rolling and adhesion	[27]
	fMLP induced skin edema	↓ MPO activity	[27]
	Carrageenan-induced paw edema	↓ edema ↓ leukocyte infiltration	[27]
$AnxA1_{2-50}$	Neutrophil/endothelial interaction (*in vitro*)	↓ PMN rolling and adhesion	[26]
	Neutrophil/endothelial interaction (*in vivo*)	↓ PMN adhesion	[26]
	IL-1β inflamed air pouch	↓ PMN recruitment	[26]
Ac2–26	Neutrophil/endothelial interaction (*in vitro*)	↓ PMN capture, rolling, and adhesion ↑ L-selectin shedding	[40, 54]
	Human PMN activated with CCL5	↓ β integrin activation	[40]
	Neutrophil/endothelial interaction (*in vivo*)	↓ PMN adhesion and emigration ↑ detachment of adherent PMN	[56]
Ac1–26	Neutrophil/endothelial interaction (*in vitro*)	↓ PMN transmigration	[48]
Ac9–25	Neutrophil/endothelial interaction (*in vitro*)	↓ PMN adhesion and transmigration ↑ L-selectin shedding	[48]
AF-2	Neutrophil/endothelial interaction (*in vitro*)	↓ PMN adhesion ↓ β2 integrin expression	[60, 61]
	Induction of neutrophil apoptosis		
AnxA1	Human PMN	↑ apoptosis (↓ pBAD)	[57]
$AnxA1_{2-50}$	Human neutrophils stimulated with SAA	↑ apoptosis	[26]
Ac2–26	Human neutrophils stimulated with SAA	↑ apoptosis (↑ caspase-3 cleavage; JNK dependent)	[47]
	Acute pleurisy	↑ apoptosis (↑ Bax; ↑ caspase-3 cleavage; ↓ Mcl-1; ↓ NF-κB; ↓ pERK)	[11]
	Skin allograft model	↑ skin allograft survival ↑ apoptosis ↓ neutrophil transmigration	[62]
	Enhancement of monocyte recruitment and efferocytosis		
Ac2–7	Transmigration assay (*in vitro*)	Stimulating human monocyte chemotaxis	[63]
AnxA1	Chemotaxis assays	Human monocyte chemoattractant	[64]
	Administration to mouse peritoneum	↑ monocyte recruitment	[64]
	Phagocytosis of apoptotic leukocytes	↑ efferocytosis ↑ binding of apoptotic cells to MØ	[65, 66]
Ac2–26	Phagocytosis of apoptotic neutrophils	↑ phagocytosis Inducing actin reorganization ↑ TGF-β release ↓ IL-8 release	[67]
$AnxA1_{2-50}$	Zymosan-induced peritonitis	↑ efferocytosis	[26]

Table 1: Continued.

Agent	Experimental model	Outcome/effect on resolution	References
	Macrophage reprogramming		
AnxA1	Human MØ cell line	Induced M2-like polarization	[44]
	Human monocytes	↑ IL-10	[47]
	LPS stimulated THP-1 MØ	↓ IL-6, TNF, and IL-1β	[66]
	MØ from NASH livers	↓ M1 polarization (↓ iNOS, IL-12p40) ↑ IL-10	[68]
	Intraperitoneal injection	↑ IL-10	[47]
	Phagocytosis of apoptotic neurons by microglial cells	↓ phagocytosis of healthy cells ↓ NO production	[69]
Ac2–26	Endotoxin-challenged monocytes	↓ IL-6 signalling ↓ TNF-α release	[70]

AnxA1: annexin A1; fMLP: N-Formyl-Met-Leu-Phe; IL: interleukin; MPO: Myeloperoxidase; MØ, macrophage; NASH; nonalcoholic steatohepatitis; PMN: polymorphonuclear; NO: nitric oxide; SAA: serum amyloid A; SAnxA1: SuperAnxA1 (proteinase-3 resistant); TGF-β: transforming growth factor-β; TNF-α: tumor necrosis factor alpha.

AnxA1 may also be tightly coupled to the anti-inflammatory properties of other FPR2/ALX agonists such as LXA_4 and antiflammin 2 (AF-2) [50]. The nonapeptide AF-2, which corresponds to region 246–254 of AnxA1 [80], is known to interfere with PMN activation, chemotaxis, and adhesion to endothelial cells [60, 61], via FPR2/ALX receptor [81]. Also, LXA_4 is a potent regulator of PMN trafficking in experimental inflammation [9, 82]. Interestingly, recent data indicated a crucial role for endogenous AnxA1 in the detachment phenomenon promoted by both compounds [50]. For instance, LXA_4 and AF-2 lost their antimigratory effects in AnxA1 KO mice suggesting AnxA1 as a downstream mediator of other proresolving and anti-inflammatory molecules [50].

3.2. AnxA1 Induces Neutrophil Apoptosis. Neutrophils are produced in the bone marrow from myeloid stem cells, which in turn proliferate, differentiate into mature neutrophils, and are delivered into circulation [83]. Although the circulatory half-life of neutrophils is now thought to be longer than previously estimated (days instead of hours) [84], at inflammatory sites the constitutive apoptotic pathway is delayed by the action of local inflammatory mediators, resulting in increased neutrophil half-life [85], an effect that can be opposed by proresolving mediators including AnxA1 and lipoxins [86].

In addition to affecting the migration of leukocytes through FPR activation, strong evidence of the involvement of AnxA1 on neutrophil apoptosis has emerged. Proapoptotic effect of AnxA1 on neutrophils was first described *in vitro* associated with transient calcium fluxes and dephosphorylation of BAD, an intracellular protein whose proapoptotic function is lost upon phosphorylation [57]. Our group [11] demonstrated the *in vivo* proapoptotic functions of endogenous AnxA1 during self-resolving inflammation. In an acute pleurisy model, blockage of the AnxA1 pathway by using a specific anti-AnxA1 antiserum prevented dexamethasone-(dexa-) induced resolution of neutrophilic inflammation, abolishing morphological and biochemical apoptotic events in the pleural cavity. AnxA1 neutralization also hampered dexa-induced decrease of ERK1/2 and IκB-α phosphorylation and Bax accumulation. In addition, anti-AnxA1 treatment prevented spontaneous resolution of neutrophilic inflammation, suggesting an important role of endogenously produced AnxA1 in the proresolutive program [11]. Furthermore, pharmacological administration of Ac2–26 peptide promoted active resolution and augmented the extent of neutrophil apoptosis. These effects were prevented by the pan-caspase inhibitor zVAD-fmk and linked to activation of the cell death pathways Bax and caspase-3 and inhibition of the survival-controlling pathways Mcl-1, ERK1/2, and NF-κB [11] (Figure 2).

In a skin allograft model, pharmacological treatment with Ac2–26 increased transplantation survival related to inhibition of neutrophil transmigration and induction of apoptosis, thereby reducing the tissue damage compared with control animals [62]. *In vitro*, Ac2–26 counteracted the survival signal in SAA-treated neutrophils, an effect associated with caspase-3 cleavage and prevented by the JNK inhibitor [47]. Dalli and col. (2013) also demonstrated that $AnxA1_{2-50}$ and CR-$AnxA1_{2-50}$ peptides can override the antiapoptotic effect of SAA in human neutrophils *in vitro* [26]. This proapoptotic effect may have contributed to the *in vivo* anti-inflammatory and proresolving actions of the peptides characterized by reduced granulocyte counts and enhanced efferocytosis in peptide-treated mice during peritonitis [26].

AnxA1 has also been described as a mediator of drug-induced apoptosis, supporting its involvement in the induction of cell death. The proapoptotic effect described for the histone deacetylase inhibitor (HDCAI) FK228, in leukemia cells, was linked to the induction of AnxA1 expression, externalization, and cleavage. Neutralization with anti-AnxA1 antibody or gene silencing with AnxA1 siRNA inhibited FK228-induced apoptosis, suggesting the involvement of AnxA1 in apoptotic cell death in response to HDCAI [87]. Recently, the *in vitro* ability of HDACIs to promote apoptosis was also demonstrated in bone-marrow neutrophils from WT but not from AnxA1 knockout mice [88]. *In vivo*, HDACIs significantly reduced neutrophil numbers and

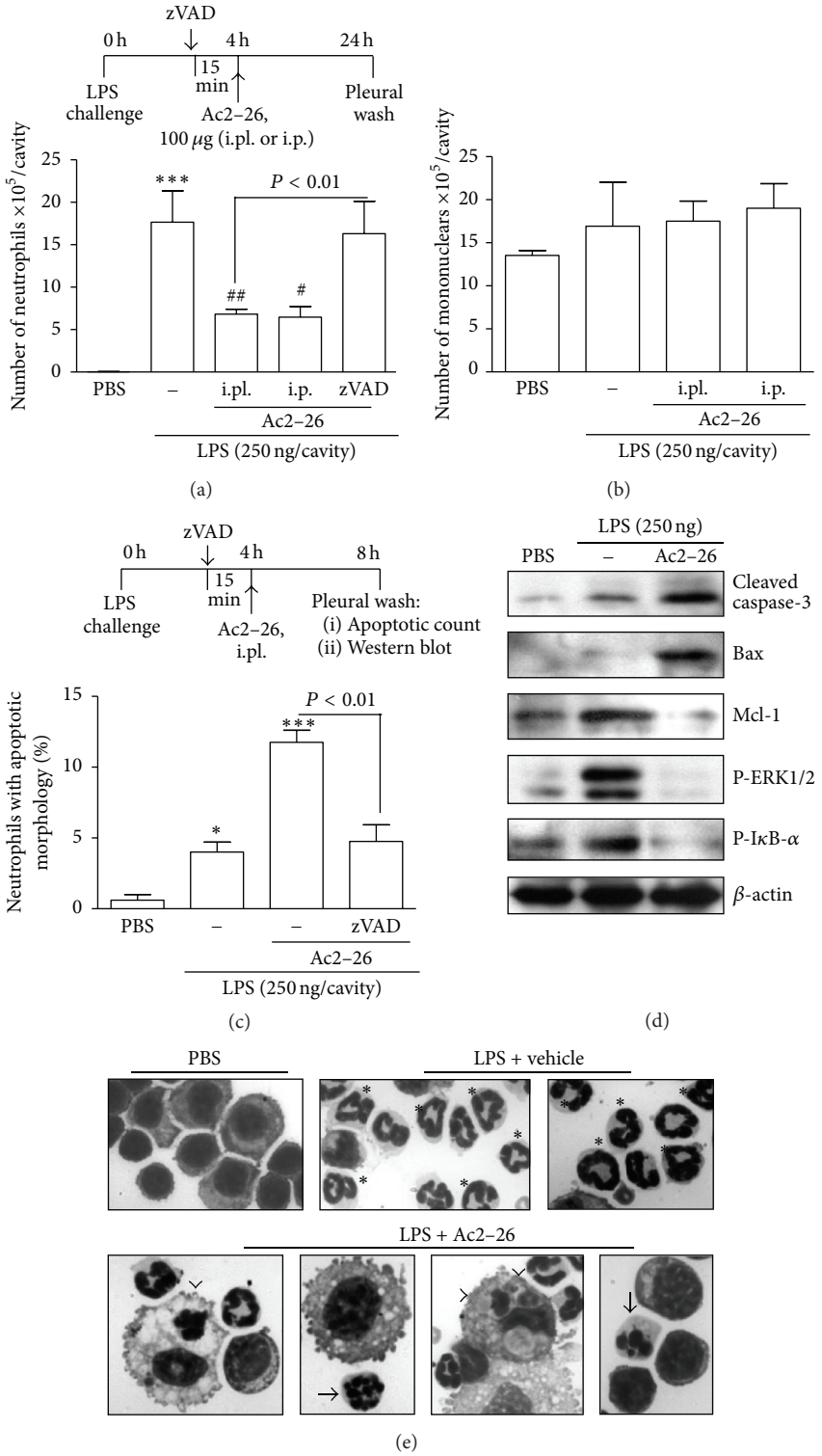

Figure 2: Effect of exogenous administration of AnxA1 derived peptide Ac2–26 on LPS-induced pleurisy. Mice were injected with LPS (250 ng/cavity, i.pl.) and 4 h later received an injection of Ac2–26 (100 μg/mouse, i.pl. or i.p.). The treatment with the pan-caspase inhibitor zVAD-fmk (1 mg/kg, i.p.) was performed 15 min before the injection of peptide. The numbers of neutrophils (a) and mononuclear cells (b) were evaluated 20 h after drug treatment. Cells with distinctive apoptotic morphology (c and e) and Western blot for detection of cleaved caspase-3, Bax, Mcl-1, P-ERK, and P-IκB-α (d) were evaluated 4 h after the peptide treatment. $^{*}P < 0.05$ or $^{***}P < 0.001$ when compared with PBS-injected mice and $^{\#}P < 0.05$ or $^{\#\#}P < 0.01$ when compared with vehicle-treated, LPS-injected mice. (e) Representative figures of nonapoptotic (asterisk) and apoptotic (arrows) neutrophils and apoptotic cells inside macrophages (arrowheads). PBS and vehicle (upper panels) and Ac2–26-treated (lower panels) animals are shown. Original data from Vago et al., 2012 [11].

induced neutrophil apoptosis in a zymosan-induced peritonitis model. Once again, the lack of AnxA1 hampered this *in vivo* proapoptotic effect [88].

It is important to keep in mind that the proapoptotic effect of AnxA1 can be underestimated in dynamic *in vivo* models of inflammation. Regarding other anti-inflammatory drugs, it is documented in a number of diverse experimental and clinical settings that small changes in apoptosis rates can promote dramatic changes in total neutrophil numbers over time. This observation is most likely due to rapid recognition and phagocytosis of apoptotic cells [89–91].

3.3. AnxA1 Induces Monocyte Recruitment and Increases Efferocytosis. Macrophage phagocytic clearance of apoptotic neutrophils plays an important role in the resolution of inflammation since this process prevents excessive neutrophil activation and the exposure of tissues to noxious neutrophil intracellular contents [92, 93]. For this reason, appropriate (nonphlogistic) monocyte recruitment from the bloodstream to inflammatory sites is a critical step in acute inflammation, enabling the clearance of apoptotic neutrophils and orderly progression towards resolution.

It has long been established that extravasation of PMN to the site of inflammation contributes to the launch of monocyte recruitment, with PMN granule proteins being important monocyte attractors [94]. Recent research from Perretti's group [64] indicates apoptotic neutrophils as the principal reservoir of AnxA1, which acts as important recruiting agent for monocytes to orchestrate the second resolving phase of acute inflammation. Associating *in vitro* and *in vivo* experiments, Professor Mauro Perretti's group filled an important gap in our knowledge by demonstrating the central role of the AnxA1–ALX/FPR2 pathway in modulating monocyte recruitment [64]. The authors demonstrated that intraperitoneal administration of AnxA1 induced monocyte migration, an effect absent in FPR2 null mice. Supporting these findings, both AnxA1 and FPR2/ALX null mice challenged with intraperitoneal zymosan exhibited diminished recruitment of monocytes as compared to WT mice, despite the higher levels of chemoattractants [64].

After initial steps of apoptosis, neutrophils lose their functional properties, such as the ability to move by chemotaxis, generate a respiratory burst, or degranulate [95]. Furthermore, they exhibit alterations on their intracellular pathways and cell surface molecules while some externalized molecules, such as phosphatidylserines (PS), facilitate the recognition and removal of apoptotic neutrophils by macrophages [92, 96].

Recent studies have reported that AnxA1 from apoptotic cells is involved in their phagocytic clearance. The first observation that AnxA1 participates in the engulfment of apoptotic cells was described by Arur and col. (2003) [97]. By using a differential proteomics technology, they showed that AnxA1 is exported to the outer plasma membrane of apoptotic lymphocytes, colocalizes with PS, and is required for efficient clearance of apoptotic cells, suggesting a role for AnxA1 as bridging PS molecules on apoptotic cells to phagocytes [97]. Scannell and col. (2007) [65] subsequently demonstrated that apoptotic neutrophils release AnxA1, which acts on macrophages, promoting the removal of effete cells [65]. Noteworthily, not only the intact form of AnxA1 released by apoptotic cells but also the cleavage fragments, under 10 kDa, were effective in stimulating efferocytosis [65].

Studies have also documented macrophages as a source of endogenous AnxA1, which in turn facilitates phagocytic uptake of apoptotic cells. Maderna and col. (2005) showed that human macrophages release AnxA1 upon treatment with GC and that this protein acts in autocrine or paracrine manners to increase the engulfment of apoptotic neutrophils [67]. Additional experiments with AnxA1-null mice provided further evidence for a functional role of AnxA1 in efferocytosis, as macrophages derived from their bone marrow were defective in clearance of apoptotic cells [67]. In fact, the authors demonstrated, *in vitro*, the ability of the AnxA1 mimetic peptide Ac2–26 to promote phagocytosis of apoptotic PMN by human macrophages, an effect associated with actin rearrangement in the phagocytic cells and abrogated in the presence of FPR antagonist [67]. Subsequently, it was clearly demonstrated the nonredundant function of FPR2/ALX receptor in Ac2–26 induced efferocytosis since the peptide failed to exert its proefferocytic action on FPR2/ALX deficient macrophages [98]. Furthermore, Yona and coworkers (2006) associated *in vitro* and *in vivo* strategies that indicated reduced phagocytosis of zymosan particles by AnxA1 knockout macrophages [99].

It has been proposed that AnxA1 released by macrophages can opsonize apoptotic cells, probably by interacting with surface-exposed PS, enhancing their uptake by phagocytes [66]. Interestingly, McArthur's group demonstrated that the binding of microglial-derived AnxA1 to PS on the surface of apoptotic neuronal cells is critically required for phagocytosis [69]. Moreover, Dalli and colleagues (2012) reported that AnxA1 expressed by resident macrophages is a critical determinant for the clearance of senescent neutrophils in the bone marrow [100]. Proefferocytic effects were also observed for $AnxA1_{2-50}$ and its cleavage-resistant form (CR- $AnxA1_{2-50}$), which stimulated efferocytosis *in vitro* by human and mice bone-marrow derived macrophages [26]. This effect was confirmed *in vivo* in a zymosan-induced peritonitis model, when the peptides significantly reduced exudate neutrophil counts and increased the number of macrophages containing ingested PMN [26].

Once phagocytic removal of apoptotic cells has failed, neutrophils undergo secondary postapoptotic necrosis, probably leading to the leakage of cytotoxic and antigenic intracellular contents into the surrounding tissue [63]. Blume and col. (2012) revealed, in two complementary studies, the role of externalized AnxA1 as a fail-safe mechanism after neutrophil transition from apoptosis to secondary necrosis. First, they described AnxA1 externalization during secondary necrosis, which in turn promotes the removal of dying cells and prevents proinflammatory cytokine production [66]. In the second study, they demonstrated that *in vitro* AnxA1 proteolysis during secondary necrosis generates a monocytic "find-me" signal, contributing to the recruitment of monocytes and consequently preventing inflammation [63].

The removal of apoptotic cells has dual importance: prevention of potentially toxic content release and induction

of macrophage reprogramming toward a resolving phenotype [101–103], another key event to restore tissue homeostasis. Accordingly, AnxA1-induced efferocytosis is coupled with increased release of transforming growth factor- (TGF-) β and lower levels of the proinflammatory cytokine IL-6 [65, 67]. In agreement with this observation, impaired phagocytosis in AnxA1-deficient macrophages is mirrored by increased release of tumor necrosis factor- (TNF-) α and IL-6 [99]. Supporting an immunomodulatory effect of AnxA1 on cytokine production, AnxA1-null mice showed increased mortality in a model of LPS-induced endotoxic shock which was correlated with increased activation of inflammatory cells [104]. The authors detected delayed and more prolonged increase in the levels of TNF-α, IL-1, and IL-6 in the blood of AnxA1-null mice, as well as increased production of these cytokines by AnxA1 KO macrophages [104]. This data is consistent with the increased production of IL-6 and TNF by stimulated AnxA1 KO peritoneal macrophages in comparison to WT cells [105]. Moreover, *in vitro* studies linked AnxA1 to brain homeostasis, demonstrating that exogenous AnxA1 can suppress microglial activation, limiting indiscriminate phagocytosis of healthy neurones and nitric oxide (NO) production during the phagocytic reaction [69]. Recently, the functional role of macrophage-derived AnxA1 in modulating hepatic inflammation and fibrogenesis during nonalcoholic steatohepatitis (NASH) progression was documented [68]. NASH in AnxA1 KO mice was characterized by enhanced lobular inflammation resulting from increased macrophage recruitment and exacerbation of the proinflammatory M1 phenotype [68]. In line with these results, AnxA1 administration to liver macrophages suppressed M1 activation, characterized by reduced expression of iNOS and IL-12p40, and increased IL-10 expression. Interestingly, activation of FPR2 by AnxA1 skewed M1 macrophages to anti-inflammatory M2-like cells, attenuating the expression of IL-6, IL-1β, and TNF-α [44]. Furthermore, Cooray and col. (2013) revealed an AnxA1-specific FPR2/ALX proresolving signal pathway centered in p38, leading to the production of IL-10 by human monocytes, an effect replicated *in vivo* after intraperitoneal AnxA1 injection [47].

Although uptake of secondary necrotic leukocytes was shown to be AnxA1 independent, the protein has an anti-inflammatory action on macrophages, since phagocytosis of AnxA1 knock-down necrotic cells induced increased release of proinflammatory cytokines TNF, IL-6, and IL-1β by phagocytic cells [66]. Pupjalis and col. (2011) added knowledge to the immunosuppressive actions of AnxA1 derived from apoptotic PMN. According to the authors, the treatment of human monocytes with AnxA1-containing supernatant of apoptotic granulocytes or Ac2–26 peptide results in a significantly diminished release of proinflammatory cytokines when the monocytes are subsequently challenged with endotoxin [70].

Taken together, these findings indicate that AnxA1-induced efferocytosis collaborates with the resolution of inflammation by promoting the elimination of effete neutrophils allied to an alternative macrophage activation that downregulates the production of proinflammatory mediators. Such events pave the way to the resolution of inflammation.

4. Concluding Remarks

AnxA1 is a GC-regulated protein that modulates a wide range of cellular and molecular steps of the inflammatory response and is deeply involved in the endogenous mechanisms that are activated to bring about proper resolution. So, it is reasonable to suppose that AnxA1-based pharmacologic strategies could be as effective as steroids, without their metabolic side effects. We have discussed here the ability of AnxA1 and its mimetic peptides to limit neutrophil accumulation in the tissue. Besides limiting neutrophil recruitment and increasing neutrophil apoptosis, AnxA1 promotes apoptotic neutrophil clearance by modulating monocyte recruitment and enhancing efferocytosis. Indeed, AnxA1 contributes to tissue homeostasis by inducing macrophage reprogramming toward a resolving phenotype. The combination of these mechanisms results in an effective resolution of inflammation, pointing to AnxA1 and its mimetic peptides as promising therapeutic agents for treating inflammatory diseases.

The promising findings on the potential therapeutic use of AnxA1 in inflammatory diseases have stimulated the development of pharmaceutical formulations containing AnxA1 mimetic peptides, such as the controlled-release hydrogels for dermal wound repair application [106] and targeted polymeric nanoparticles [107]. The latter demonstrated ability to enhance resolution in zymosan-induced peritonitis [107], promote colonic wounds healing [42], and protect hypercholesterolemic mice against advanced atherosclerosis [108]. These pharmaceutical strategies offer further benefits, overcoming the critical pharmacokinetics of short peptides *in vivo*, protecting them from proteolysis during pharmacological treatment, and facilitating the delivery to injury sites.

Abbreviations

Ac2–26:	Peptide from N-terminal portion of annexin A1 (residues 2–26)
AnxA1:	Annexin A1
FPR2/ALXR:	Formyl peptide receptor 2/lipoxin A4 receptor
Boc-1:	N-t-Boc-Met-Leu-Phe
Dexa:	Dexamethasone
ERK1/2:	Extracellular signal-regulated kinase
FPR:	Formyl peptide receptor
GC:	Glucocorticoid
ICAM-1:	Intercellular adhesion molecule-1
i.pl.:	Intrapleural
IL:	Interleukin
IκB-α:	Inhibitory kappa B alpha
KO:	Knockout
LPS:	Lipopolysaccharide
Mcl-1:	Myeloid cell leukemia-1
NF-κB:	Nuclear factor kappa B
PMN:	Polymorphonuclear
SAnxA1:	SuperAnxA1 or cleavage-resistant AnxA1
TGF-β:	Transforming growth factor-β
TNF:	Tumor necrosis factor

VCAM-1: Vascular cell adhesion molecule-1
WT: Wild-type
zVAD-fmk: Benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Secretion of S100A8, S100A9, and S100A12 by Neutrophils Involves Reactive Oxygen Species and Potassium Efflux

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S100A8/A9 (calprotectin) and S100A12 proinflammatory mediators are found at inflammatory sites and in the serum of patients with inflammatory or autoimmune diseases. These cytoplasmic proteins are secreted by neutrophils at sites of inflammation via alternative secretion pathways of which little is known. This study examined the nature of the stimuli leading to S100A8/A9 and S100A12 secretion as well as the mechanism involved in this alternative secretion pathway. Chemotactic agents, cytokines, and particulate molecules were used to stimulate human neutrophils. MSU crystals, PMA, and H_2O_2 induced the release of S100A8, S100A9, and S100A12 homodimers, as well as S100A8/A9 heterodimer. High concentrations of S100A8/A9 and S100A12 were secreted in response to nanoparticles like MSU, silica, TiO_2, fullerene, and single-wall carbon nanotubes as well as in response to microbe-derived molecules, such as zymosan or HKCA. However, neutrophils exposed to the chemotactic factors fMLP failed to secrete S100A8/A9 or S100A12. Secretion of S100A8/A9 was dependent on the production of reactive oxygen species and required K^+ exchanges through the ATP-sensitive K^+ channel. Altogether, these findings suggest that S100A12 and S100A8/A9 are secreted independently either via distinct mechanisms of secretion or following the activation of different signal transduction pathways.

1. Introduction

S100A8, S100A9, and S100A12 are small calcium-binding proteins abundantly expressed by neutrophils. S100A8 and S100A9 represent up to 40% of neutrophil cytosolic proteins, whereas close to 5% are S100A12 [1]. These proteins are also expressed by monocytes, macrophages, platelets, and epithelial and endothelial cells following cell stimulation [2–6]. S100A8, S100A9, and S100A12 exist as homodimers, but S100A8 and S100A9 associate in presence of calcium to form the noncovalently bound heterodimer S100A8/A9 (calprotectin).

S100A8, S100A9, and S100A12 induce neutrophil recruitment, adhesion, and release from the bone marrow and are crucial for neutrophil migration to inflammatory sites in response to bacterial infection, LPS, and monosodium urate (MSU) crystals (the causative agent of gout) [7–10]. S100A9 is a potent proinflammatory factor, stimulating neutrophil migration, phagocytosis, and degranulation [9, 11, 12]. S100A12, on the other hand, is a mild activator of granulocyte functions and a potent inducer of mast cell functions [7, 13, 14], and S100A8 is a chemotactic factor for neutrophils [15]. In addition, S100A8 is extremely sensitive to oxidation and nitrosylation, which transforms it into an anti-inflammatory factor [16, 17]. The S100A8/A9 complex has been reported to activate monocyte migration and cytokine secretion [18, 19]. Thus, these damage-associated molecular patterns (DAMPs) control inflammation through distinct but overlapping proinflammatory activities.

S100 proteins lack signal peptides required for the classical Golgi-mediated secretion pathway. Consequently, their release is mediated by alternative secretion pathways [4]. The mechanisms underlying these alternative pathways are unclear, but secretion of S100A8/A9 from monocytes is known to be tubulin-dependent [20]. Moreover, stimulation of neutrophils with MSU crystals leads to the release of

S100A8/A9 in a Src kinase-, syk-, and tubulin-dependent manner [8, 10, 21].

High concentrations of S100A8/A9 and S100A12 are found in the serum and at inflammatory sites of patients with acute and chronic inflammation. However, the source of S100A8/A9 and S100A12 remains largely unknown. In some situations, S100A8/A9 release correlates with neutrophil necrosis [22], and this might contribute significantly to the high concentrations found in acute inflammatory lesions or chronic conditions where neutrophil infiltration is significant, such as cystic fibrosis and rheumatoid arthritis [23]. Monocytes secrete S100A9 and S100A8/A9, but not S100A8 alone, upon stimulation with pokeweed mitogen [24]. In addition, GM-CSF, TNF-α, IL-1β, LPS, and PMA induce the release of S100A8/A9 from monocytes [20, 25]. Likewise, S100A8/A9 was shown to be secreted by neutrophils stimulated with LPS, TNF-α, and IL-1β [25, 26]. The stress response modulator norepinephrine also induces S100A8 and S100A9 expression in human monocytic cells, suggesting that these proteins may be regulated by stress [27]. Activation of protein kinase C by proinflammatory stimuli and elevation of intracellular [Ca^{2+}] following contact with activated endothelium, collagen, or fibronectin can also stimulate S100A8/A9 release from phagocytes [28, 29].

Most of the studies to date have concentrated on the secretion of S100A8/A9, and thus little is known about the mechanisms of secretion of S100A8, S100A9, and S100A12. In order to decipher the type of stimuli triggering the release of S100 proteins from human neutrophils, we exposed neutrophils to a variety of stimuli, including inflammatory mediators, microbes or their derived products, and particulates. Altogether, our results demonstrate that S100A8, S100A9, and S100A8/A9 can be released without the secretion of S100A12 and that the secretion of S100A12 is induced by stimuli promoting the presence of oxidative stress and activation of the NLRP3 inflammasome. The results also demonstrate that the proteins are secreted independently either via distinct mechanisms or following activation of different signal transduction pathways.

2. Material and Methods

2.1. Ethics Statement. These studies were approved by the CHU de Québec Research Center ethical committee, and all participants gave written informed consent.

2.2. Reagents. Triclinic monosodium urate (MSU) crystal preparation was a generous gift from Dr. Paul H. Naccache and generated as described previously [21]. Fullerenes (C_{60}) and single-wall carbon nanotubes (60–100 nm in diameter, 1-2 μm in length) were obtained from SES research (Houston, TX, USA). Silica (1.6 μm in diameter) was purchased from US Silica Company (Berkeley Springs, WV, USA). Titanium dioxide (TiO_2, diameter $\leq$5 μm), phorbol 12-myristate 13-acetate (PMA), the chemotactic peptide N-formyl-methionyl-leucylphenylalanine (fMLP), H_2O_2, protease inhibitors cocktail, sodium orthovanadate (Na_3VO_4), and DMSO were purchased from Sigma-Aldrich (St. Louis, MO, USA). IL-8 was obtained from Peprotech Inc. (Rocky Hill, NJ, USA). Leukotriene B_4 (LTB_4) and platelet-activating factor (PAF) were purchased from Calbiochem (Gibbstown, NJ, USA). Complement factor 5a (C5a) was obtained from Biovision (Mountain View, CA, USA). Heat-killed *Candida albicans* (HKCA) and Pam3CSK4 were purchased from Invivogen (San Diego, CA, USA). Unpurified autologous decomplemented serum was used as a source of opsonins when indicated. Serum was heat-inactivated for 30 min at 56°C and stored at −20°C. The zymosan, HKCA, or MSU suspensions were opsonized by incubation with 50% autologous serum for 30 minutes at 37°C before being washed and resuspended in Hank's balanced saline solution (HBSS). The monoclonal antibody 27E10 was purchased from HyCult Biotechnology (Canton, MA, USA). The HRP-conjugated antibodies were purchased from Jackson ImmunoResearch. The cOmplete Ultra protease inhibitor cocktail was obtained from Roche (Laval, Canada).

2.3. Production and Purification of Recombinant Proteins. Cloning, expression, and purification of human S100A8, S100A9, and S100A12 were described previously [8]. Endotoxins were removed from the protein solutions using Acticlean Etox column (Sterogene Bioseparation). Contamination by endotoxins was always less than 1 pg of LPS/μg of recombinant proteins as measured by the *Limulus* amebocyte assay (Lonza). The proteins were kept at −80°C for up to 6 months until used.

2.4. Sandwich ELISA. High-binding 96-well plates were incubated overnight at 4°C with 100 μL of a solution of mAbs clone 1F8 (anti-S100A8; 0.5 μg/mL), 6B4 (anti-S100A9; 2.5 μg/mL), 2A10 (anti-S100A12; 1 μg/mL), or the polyclonal anti-S100A9 (2.5 μg/mL) diluted in 0.1 M carbonate buffer (pH 9.6). The plates were washed three times with PBS/0.1% Tween and the nonspecific binding sites were blocked by the addition of PBS/0.1% Tween/2%BSA for 45 min at room temperature. One hundred μL of samples (diluted in blocking buffer) and recombinant proteins or purified calprotectin (used as standard curve) diluted in blocking buffer was then added. The plates were extensively washed 45 min later and 100 μL of the polyclonal anti-S100A8 (4 μg/mL), anti-S100A9 (0.5 μg/mL), anti-S100A12 (0.25 μg/mL), or the monoclonal anti-calprotectin clone 27E10 (0.075 μg/mL) diluted in blocking buffer was added. After 45 min, the wells were washed three times and 100 μL of HRP-conjugated anti-rabbit or mouse IgGs was added to the wells for 45 min. The wells were washed three times and TMB substrate was added. The reaction was stopped by the addition of H_2SO_4 0.18 M and the optical density was read at 450 nm.

2.5. Isolation of Human Neutrophils. Peripheral blood was collected in heparinized tubes from healthy adult volunteers and neutrophils were isolated as described previously [21]. Cells were resuspended in HBSS1x containing physiological concentrations of Ca^{2+} (1.3 mM) and Mg^{2+} (0.8 mM) and 10 mM HEPES, pH 7.4. The purity and cell viability of

neutrophils preparations were always ≥98% as assessed by acetic blue staining and trypan blue exclusion, respectively.

2.6. Isolation and Purification of Calprotectin from Human Neutrophils. Calprotectin was purified from human neutrophils as described previously [21]. The purity of calprotectin was confirmed by SDS-PAGE under nonreducing conditions. Less than 20% of the S100A8 and S100A9 were in homodimer forms. The calprotectin was aliquoted and kept in −20°C until used.

2.7. Stimulation of Human Neutrophil. Neutrophils (10^6 cell/mL in a final volume of 500 μL) were preincubated with protease inhibitors to avoid proteolysis for 5 min and then incubated in presence or absence of stimuli like LTB4 (50 nM), C5a (100 nM), PAF (10 μM), IL-8 (10 ng/mL), MSU crystals (1 mg/mL), PMA (10 nM), fMLP (1 μM), TNF (50 ng/mL), GM-CSF (20 nM), TiO_2 (1.5 mg/mL), silica (3 mg/mL), single-wall carbon nanotubes (1.5 mg/mL), fullerenes (1.5 mg/mL), heat-killed *C. albicans* (10^8 cells/mL), zymosan (1 mg/mL, i.e., 60×10^6 particles/mL), Pam3Csk4 (10 μg/mL), H_2O_2 (250 μM), or their respective vehicle (HBSS1X or DMSO (<0.1%)) for 60 min at 37°C under gentle agitation (300 rpm). Stimulations were stopped by centrifugation at 1,500 ×g for 15 s, and the supernatants were harvested and frozen at −20°C until analysed. Cell viability was determined at the end of the stimulation by trypan blue staining. Crude neutrophil extracts were obtained by lysis of 40×10^6 cells in 400 μL of RIPA lysis buffer (Tris 50 mM pH 8.0, 150 mM NaCl, 0.1% SDS, 1% NP-40, and Roche protease inhibitor cocktail). Samples were kept at −20°C until used.

2.8. Sequential Solubilisation of Intracellular Compartments from Human Neutrophils. Neutrophil pellets recovered after stimulation were solubilised with 500 μL of hypotonic buffer (20 mM Tris-HCl pH 7.5, 10 mM NaCl, 1 mM EDTA, 0.1% NP-40, protease inhibitors, and 2 mM Na_3VO_4), kept on ice for 30 min, and centrifuged at 13,000 ×g for 10 min at 4°C. The soluble fractions, which contained cytosolic proteins, were transferred to fresh tubes and frozen at −20°C. The pellets were dissolved in 500 μL of hypertonic buffer (20 mM Tris-HCl pH 7.5, 400 mM NaCl, 1 mM EDTA, 1% NP-40, 0.1% SDS, protease inhibitors, 2 mM Na_3VO_4) known to solubilise membrane-bound proteins and centrifuged at 13,000 ×g for 10 min at 4°C. The soluble material of this second solubilisation step was transferred into fresh tubes and frozen at −20°C. The pellets were next dissolved into 500 μL of 10 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Triton X-100, 0.1% SDS, protease inhibitors, 2 mM Na_3VO_4 to solubilise cytoskeleton and lipid raft-bound proteins and kept frozen at −20°C until used for ELISA and SDS-PAGE/western blotting under nonreductive conditions.

2.9. Isolation of Microvesicles (MV). PMNs were stimulated with 1.5 mg/mL of the particulate agonists MSU crystal for 60 min at 37°C. Cell activation was stopped by centrifugation (10 min, 400 ×g at 4°C) and supernatants were collected. Supernatants (S1) were then centrifuged at 10,000 ×g/10 min and the supernatant and pellet (S2, P2) were collected. MV from PMNs were concentrated by ultracentrifugation for 45 min at 100,000 ×g (S3, P3), and the resulting supernatant (S3) was then centrifugated again for 60 min at 160,000 ×g (S4, P4) at 4°C. Calprotectin secretion was estimated by measuring the protein in the different fractions by ELISA. Immunoblotting of proteins S100A8 and S100A9 in the fractions with MV of neutrophils treated with MSU or HBSS was performed.

2.10. Proteomic Analysis. Neutrophils from 10 different blood donors were stimulated with fMLP (10^{-7} M), MSU crystals (1.5 mg/mL), fullerenes (1.5 mg/mL), or their diluents for 30 min at 37°C. Equal volumes of supernatants were pooled, and then 1 μg of recombinant papaya mosaic virus core protein (internal control, generous gift from Denis Leclerc, Université Laval) was added to the pooled samples (as an internal standard). One hundred μg of the pooled supernatants was precipitated overnight with 5 vol of acetone and resuspended in triethylammonium bicarbonate 0.5 M containing 0.5% sodium deoxycholate. Proteins were then reduced and alkylated according to the isobaric tags for relative and absolute quantitation (iTRAQ) kit manufacturer's instructions (AB SCIEX). Samples were digested with trypsin (Sequence grade Modified, Promega) using 1 : 30 ratio overnight at 37°C. After digestion, peptides were acidified to precipitate sodium deoxycholate and then purified with an oasis HLB cartridge (1 cc, 10 mg, Water Corp.) and lyophilized. Dried peptides were dissolved in 30 μL 0.5 M triethylammonium bicarbonate 0.5 M and labeled with iTRAQ label reagent (AB SCIEX). 4-plex labeling was performed for 2 h at room temperature in the dark. Labeled peptides were combined in one tube and dried with the SpeedVac. Samples were cleaned up using HLB cartridge (Water Corp.). Samples were dried and reconstituted in 200 μL HPLC water and 1/100 ampholytes pH 3–10 (Biorad). The peptides were then fractionated on two 18 cm immobilized pH gradient strips pH 3–6 and pH 3–10 using isoelectric focusing. Immobilized pH gradient strips were passively rehydrated 5 hours. Focusing was performed according to the following protocol: 0–250 V (gradient over 15 min); 250–4000 V (gradient over 2 h); 4000 V (fixed, until a total of 10000 Vh). Strips were cut in 72 fractions and peptides were extracted in 2% ACN, 1%FA solution followed by 50% ACN, 1% FA. Finally, fractions were dried with the SpeedVac.

The MS analysis was performed on a QSTAR XL (AB SCIEX, Foster City, CA) in an information-dependent acquisition (IDA) mode. Mass spectra were acquired across 400–1600 m/z for 1 s followed by 3 MS/MS of 3 s per cycle. A dynamic exclusion window of 15 s was used. Rolling collision energy was on with CE adjustment for iTRAQ reagent analysis.

Protein/peptide identification and quantification were performed using Protein Pilot Software version 3.0 searching a Uniprot database containing human proteins and common contaminants. Only proteins identified by at least three peptides and with a P value less than 0.05 were used for quantitation. Relative concentrations of the 123 proteins identified

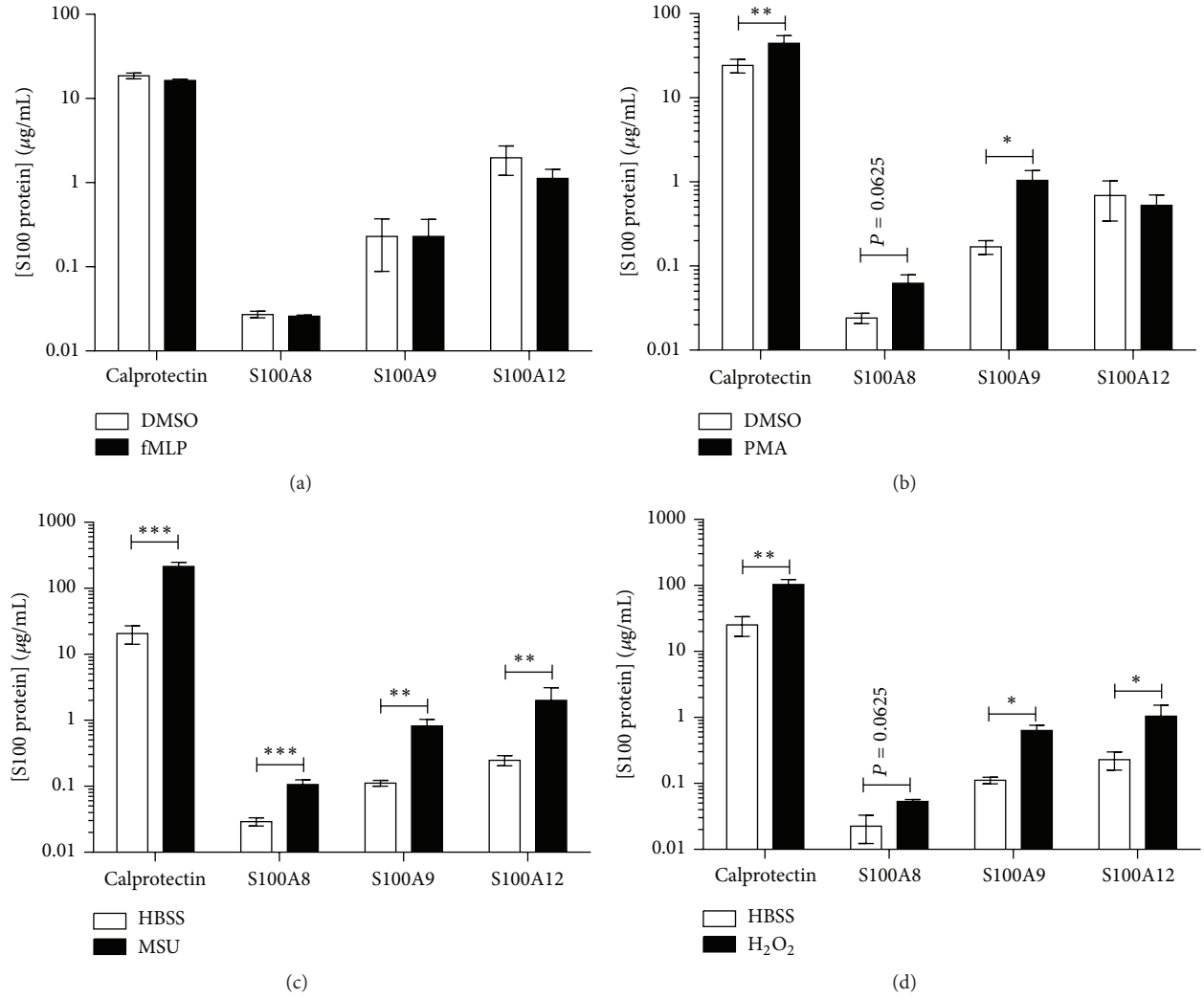


Figure 1: Secretion of S100A8, S100A9, S100A12, and S100A8/A9 by human neutrophils. Neutrophils were stimulated with (a) 10^{-7} M fMLP, (b) 10 nM PMA, (c) 250 μM H_2O_2, or (d) 1.5 mg/mL MSU crystals for 1 h as described in Section 2. Cells were then centrifuged and supernatants were harvested to perform ELISA for S100A8, S100A9, S100A12, or S100A8/A9. Results represent the means ± SEM of 4 donors. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

in the supernatants were then normalized according to the relative concentration of the internal control (papaya mosaic virus core protein).

2.11. Statistical Analyses. All experiments were performed three to six times using neutrophils from different donors. The results are expressed as mean ± SEM of separate experiments. Statistical analyses were performed using Bonferroni multiple comparison test except when stated in figure legend. The results were considered significant if P values were less than 0.05. All statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1. Neutrophils Exposed to Stress Release S100A8, S100A9, Calprotectin, and S100A12. Using specific ELISA described in Supplementary Data, in Supplementary Material available online at http://dx.doi.org/10.1155/2015/296149 (Supplementary Figures 1–3), we investigated the secretion of S100A8, S100A9, and S100A12 by neutrophils. Neutrophils were stimulated with fMLP, MSU, PMA, or H_2O_2 to study the secretion of S100A8, S100A9, and S100A12. fMLP, a powerful activator of neutrophils triggering degranulation, failed to induce the secretion of calprotectin, S100A8, S100A9, or S100A12 (Figure 1(a)). Other chemotactic agents tested, including C5a, PAF, IL-8, and LTB_4, also failed to induce the release of calprotectin and S100A12 (Supplementary Figure 4). Absence of S100 proteins in the supernatants was not due to degradation by proteases as addition of protease inhibitors only modestly increased detected concentrations in unstimulated and stimulated supernatants. PMA, which has been reported to promote the secretion of calprotectin from human monocytes, weakly stimulated the secretion of calprotectin (1.8-fold), S100A9 (7-fold), and S100A8 (2.6-fold)

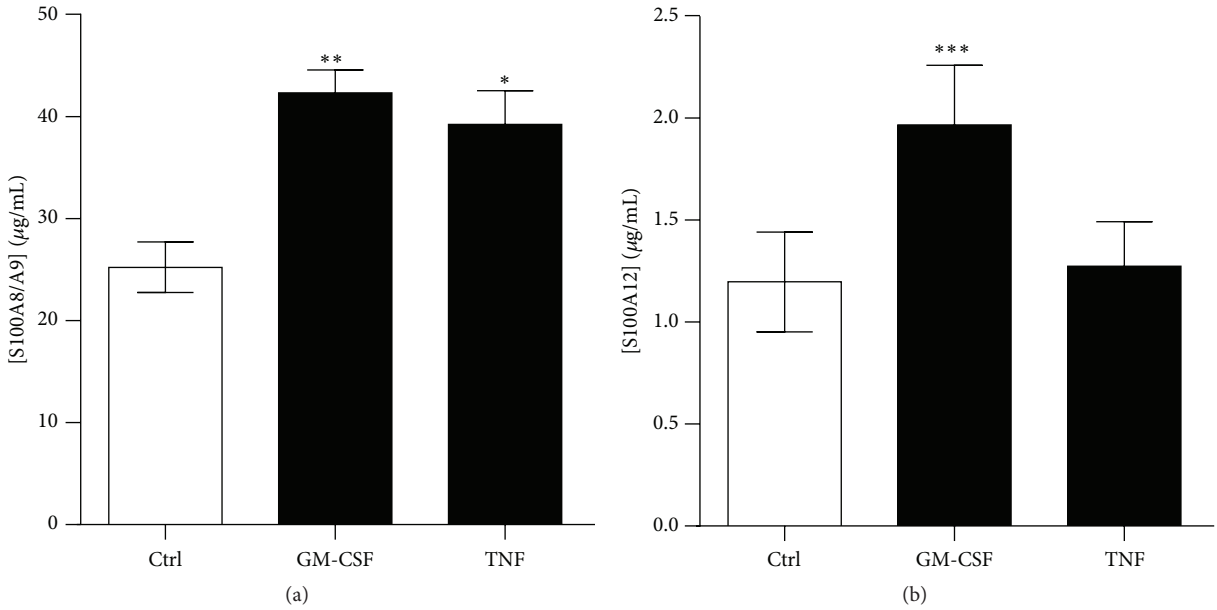


Figure 2: Secretion of calgranulins by neutrophils stimulated with GM-CSF and TNF. Neutrophils were stimulated with 20 nM GM-CSF or 50 ng/mL TNF for 60 minutes. (a) S100A8/A9 and (b) S100A12 were then quantified by ELISA. Results represent the means ± SEM of 5 donors. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

(although the latter was not significant), but not consistently of S100A12 from human neutrophils (Figure 1(b)). Similarly, cells exposed to H_2O_2, which mimics oxidative stress found in inflammatory environment, released calprotectin, S100A9, S100A8, and S100A12 with 7-, 5.4-, 2.4-, and 5.6-fold increases, respectively (Figure 1(c)). MSU crystals, which we previously reported to induce the release S100A8/A9 [21], led to S100A8, S100A9, and S100A12 secretion from neutrophils with 3.6-, 7-, and 8-fold increases, respectively, compared to vehicle (Figure 1(d)). These results indicate that S100A8 and S100A9 can be secreted independently of each other by neutrophils, and not exclusively in a heterodimer form (calprotectin). As S100A8 and S100A9 homodimers were secreted together with calprotectin, we focused the rest of our studies on S100A8/A9 and S100A12.

3.2. GM-CSF Induces Secretion of Calprotectin and S100A12. Various neutrophil functions, such as adhesion, superoxide release, and phagocytosis, are known to be activated or potentiated by hematopoietic growth factors or inflammatory cytokines, including GM-CSF and TNF-α. These cytokines may contribute not only to host defense against invading microorganisms but also to tissue damage at inflammatory sites [30]. Stimulation of neutrophils with TNF-α or GM-CSF led to 1.56- and 1.68-fold increases in the secretion of S100A8/A9 (Figure 2). TNF-α did not induce secretion of S100A12, while GM-CSF led to a 1.64-fold increase in its release.

3.3. Dectin-1 Ligand Stimulates the Release of S100A8/A9 and S100A12. After recruitment to the site of infection, neutrophils first detect microbes through different pathogen-recognizing receptors (PRR), including Toll-like receptors (TLR) and C-type lectins, such as dectin-1. Because S100A8/A9 and S100A12 exhibit antimicrobial proprieties, particularly against yeast and fungi [22, 25, 26], we investigated the effect of microbe-derived products or whole microorganisms on the secretion of these proteins. For this purpose, neutrophils were incubated with Pam3CSK4 (TLR2 ligand), zymosan (yeast cell wall, TLR2/dectin-1 ligand), and heat-killed *C. albicans* (HKCA; dectin-1 ligand). Stimulation with HKCA led to the secretion of S100A8/A9 and S100A12 (Figure 3). In contrast, zymosan or the TLR2 ligand Pam3CSK4 induced the secretion of S100A8/A9 but not S100A12. Thus, S100A8/A9 secretion is increased in response to activation of TLR2 and dectin-1 signaling by pathogen-associated molecular patterns (PAMPs), but only a full activation of dectin-1 induces the release of S100A12.

3.4. Particulates Stimulate the Secretion of S100A8/A9 and S100A12 by Neutrophils. Of all the stimuli tested, MSU crystals were the most powerful trigger of S100 proteins secretion. We hypothesized that their spindle-like shape could participate in neutrophil stimulation. Therefore, we examined whether other agonists of similar shape also stimulated the secretion of S100A8/A9 and S100A12. Neutrophils were exposed to MSU crystals (1–40 μm), silica (1.6 μm), TiO_2 (<5 μm), or single-wall carbon nanotube (100 nm in diameter; 1-2 μm in length), and these agonists share a common spindle-like structure. Fullerenes were used as controls because they have the same chemical composition as carbon nanotubes (i.e., pure carbon) but in a spherical conformation. All particulate agonists induced the secretion of S100A8/A9

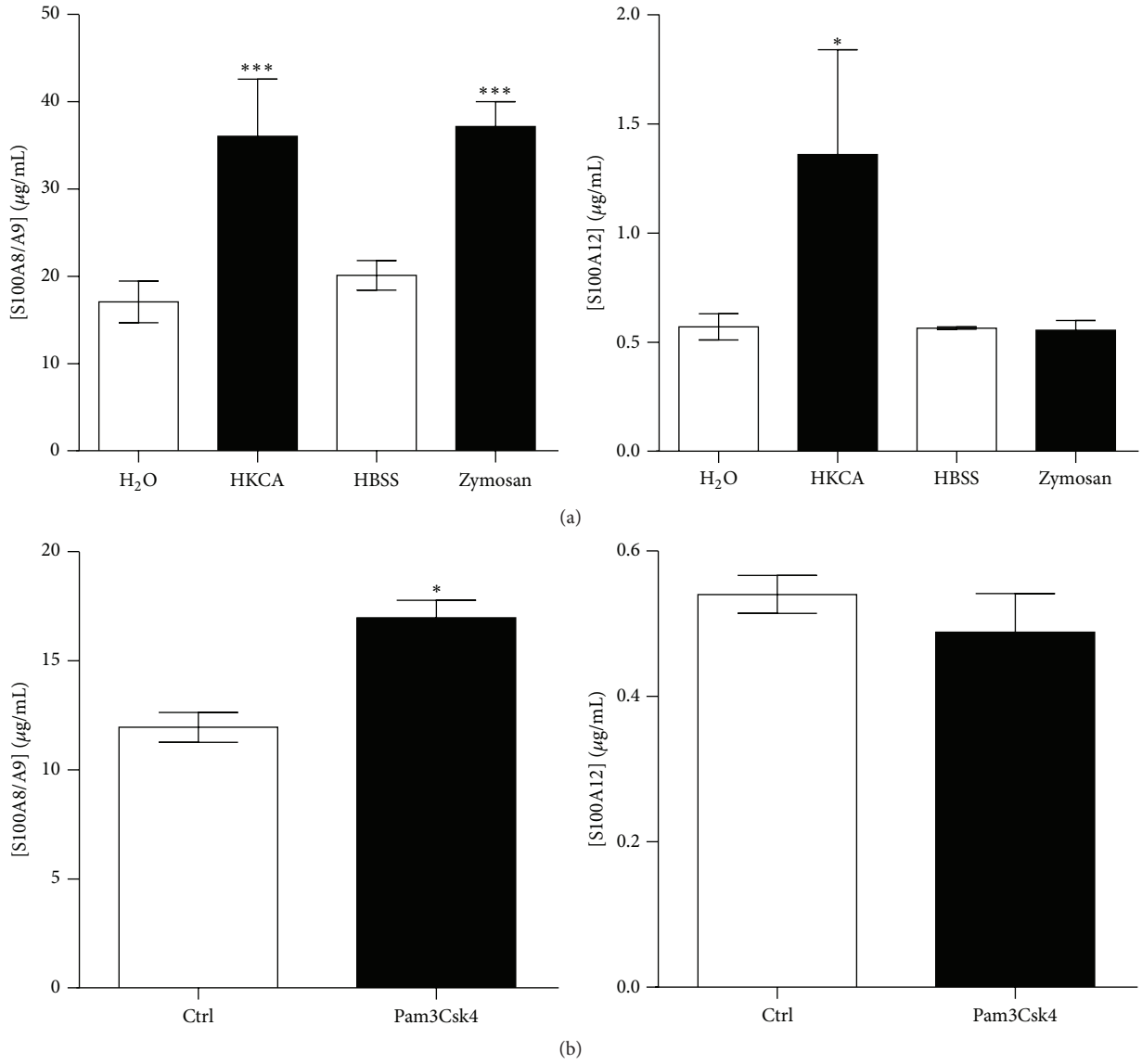


Figure 3: Effects of microbe-derived products and whole yeasts on the release of S100A8/A9 and S100A12. (a) *C. albicans* (10^8 cells/mL) enhances the secretion of S100A8/A9 and S100A12, whereas zymosan (1.5 mg/mL) induces only the release of S100A8/A9. (b) *Pam3CSK4* (TLR2 ligand, 10 μg/mL) potentiates the release of S100A8/A9 but not S100A12. Neutrophils were incubated with whole microorganisms or microbe-derived product for 60 min at 37°C. The concentrations of secreted S100A8/A9 and S100A12 were determined by ELISA. Results are the mean ± SEM of at least three independent experiments performed on neutrophils from different donors. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with control by paired t-test.

and S100A12, MSU and TiO_2 being the most efficient at promoting secretion (Figure 4). Taken together, these results indicate that the nature of the stimulus, but not its shape, determines the release of S100A8, S100A9, calprotectin, and S100A12 by neutrophils.

3.5. Role of Redox in the Secretion of Calprotectin and S100A12. Damage associated-molecular patterns (PAMPs) like MSU crystals trigger the alternative secretion of IL-1β through the activation of inflammasome and caspase-1, a phenomenon linked to K^+ efflux and ROS production [31]. As MSU crystals and H_2O_2 induced the release of S100A8/A9 and S100A12, we examined the role of redox in the secretion of S100 proteins. Neutrophils were stimulated with MSU crystals or PMA in presence of the NADPH oxidase inhibitor diphenyleneiodonium sulfate (DPI) (Figure 5). As expected, DPI reduced ROS production in neutrophils (Supplementary Figure 5). DPI mildly reduced the secretion of S100A8/A9 and S100A12 by unstimulated cells. In contrast, DPI inhibited S100A8/A9 and S100A12 secretion by 36% and 50%, respectively, when neutrophils were stimulated with MSU (Figures 5(a) and 5(b)). Similarly, DPI decreased S100A8/A9 and S100A12 secretion by neutrophils stimulated with PMA (Figures 5(c) and 5(d)). Altogether, the results suggest that redox status

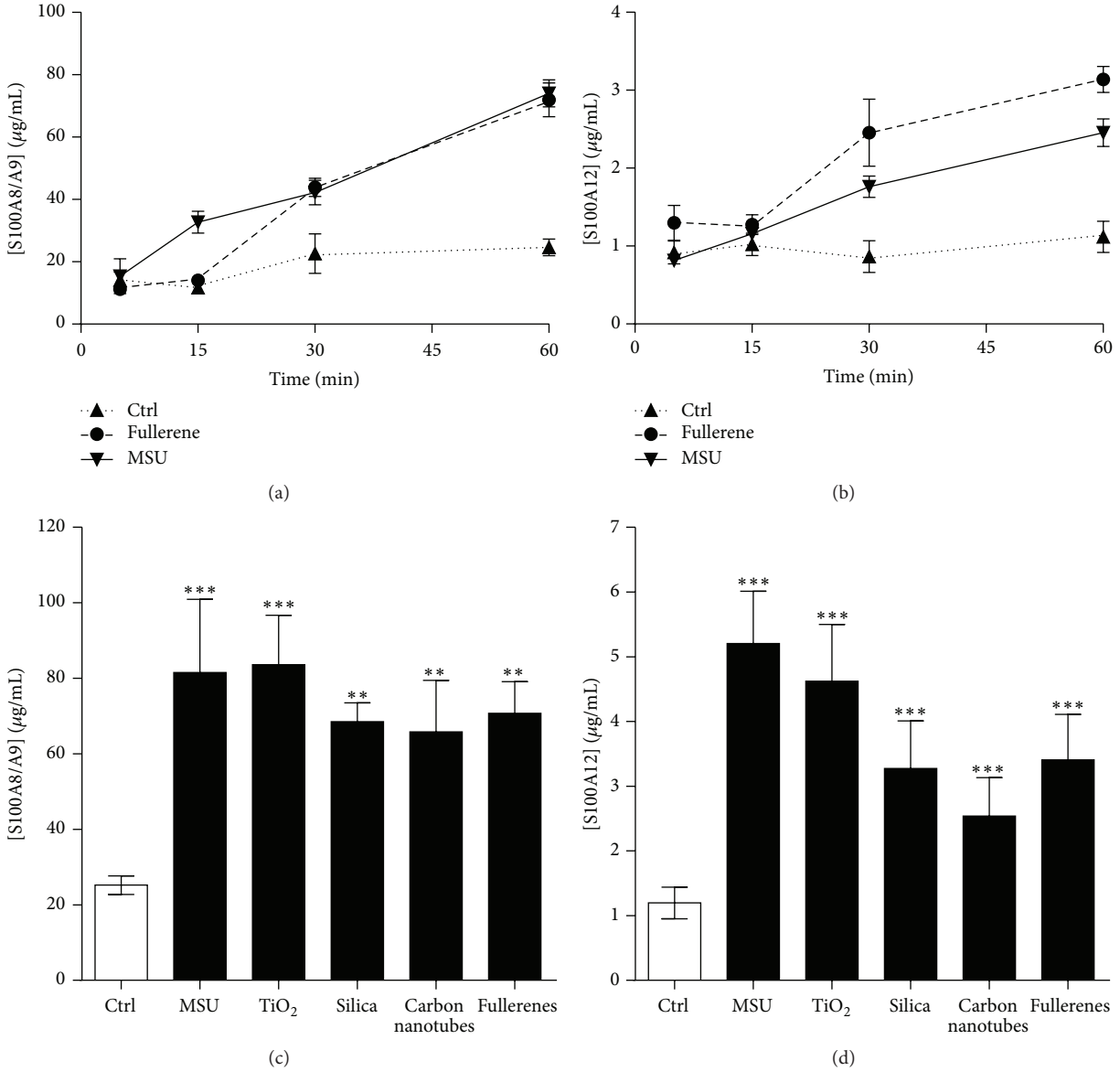


Figure 4: Phagocytic particles induce the secretion of S100A8/A9 and S100A12. Neutrophils were stimulated with 1.5 mg/mL MSU crystals or fullerenes for increasing periods of time. (a) S100A8/A9 and (b) S100A12 in the supernatants were then quantified by ELISA. (c and d) Neutrophils were stimulated with 1.5 mg/mL MSU crystals, 1.5 mg/mL TiO_2, 3 mg/mL silica, 1.5 mg/mL carbon nanotubes, or 1.5 mg/mL fullerenes for 60 minutes. (c) S100A8/A9 and (d) S100A12 in the supernatants were then quantified by ELISA. Results represent the means ± SEM of 5 donors. $^{**}P < 0.01$; $^{***}P < 0.001$.

plays a role in the secretion of both S100A8/A9 and S100A12 as it is the case for IL-1β in human monocytes/macrophages.

3.6. K^+ Efflux from ATP-Sensitive K^+ Channels Is Involved in S100A8/A9 and S100A12 Secretion. The role of K^+ efflux from ATP-sensitive K^+ channels in the secretion of S100A8/A9 and/or S100A12 by neutrophils exposed to inflammatory conditions was investigated as it regulates the alternative secretion of IL-1β by macrophages stimulated with LPS and ATP [32–34]. Neutrophils were treated with glibenclamide or DMSO (vehicle control) before the addition of MSU. Preincubation of cells with glibenclamide reduced the secretion of S100A8/A9 by MSU-stimulated neutrophils by 42% compared to cells pretreated with DMSO (Figure 6). A similar inhibition (44%) was observed in unstimulated cells. Secretion of S100A12 was reduced by 64% and 73%, respectively, in unstimulated and MSU crystal-stimulated neutrophils. Similar inhibition was observed when cells were stimulated with PMA. Glibenclamide had no apparent cytotoxic effect on neutrophils during the course of stimulation (data not shown). To confirm the effect of glibenclamide, neutrophils were incubated in medium containing high K^+ concentrations. As with glibenclamide, inhibition of the K^+ efflux led to a reduction of S100A8/A9 secretion induced by

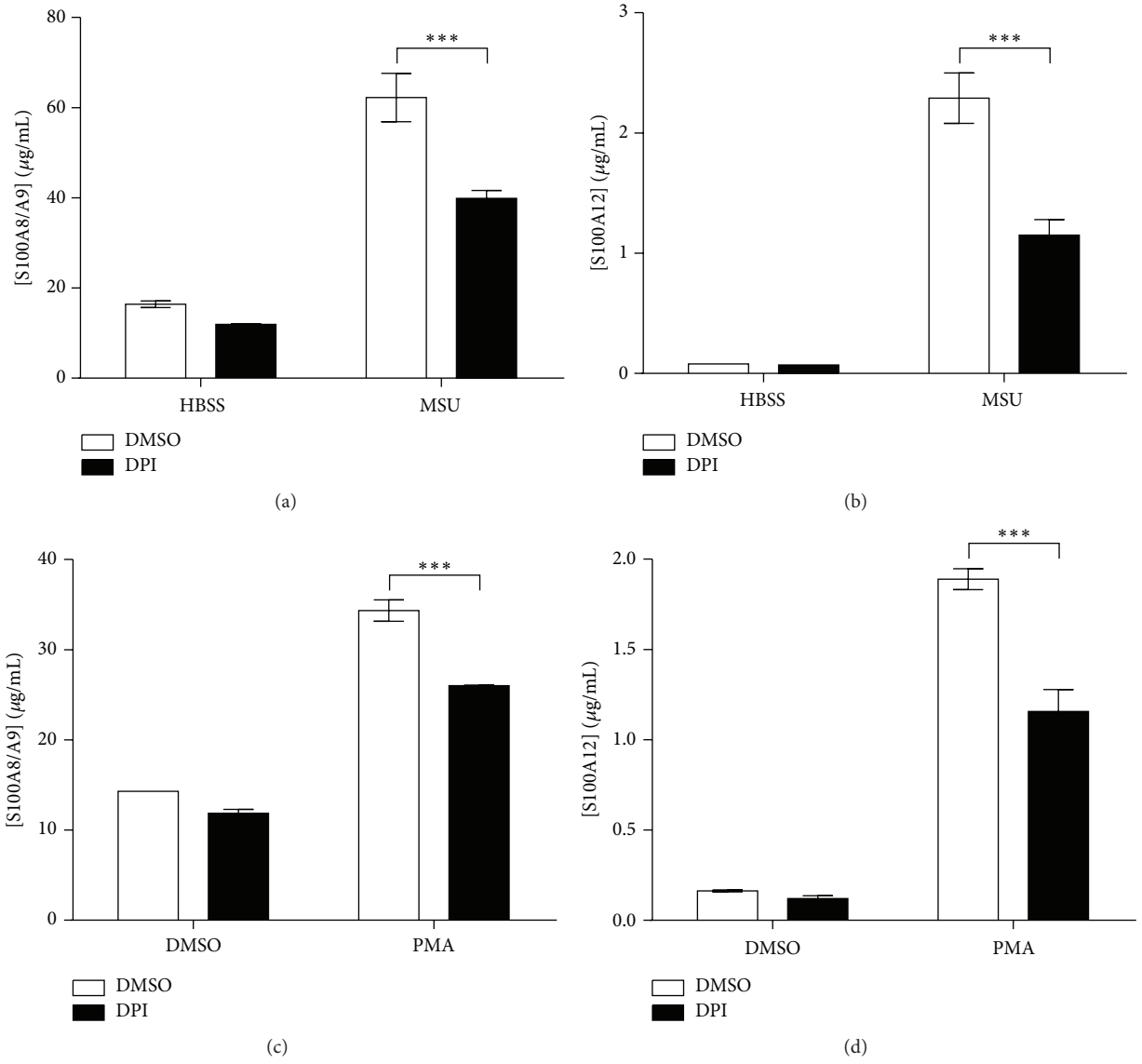


Figure 5: Inhibition of calgranulin secretion by DPI. Neutrophils were preincubated with the NADPH oxidase inhibitor DPI (10 μM) and then stimulated with 1.5 mg/mL MSU crystals or 10 nM PMA for 60 minutes. (a and b) S100A8/A9 and (c and d) S100A12 in the supernatants were then quantified by ELISA. Results represent the means ± SEM of 5 donors. $^{***}P < 0.001$.

MSU crystals (Supplementary Figure 6). Neutrophils were then incubated with ouabain, an inhibitor of the activity of the Na^+/K^+ ATPase. Inhibition of the Na^+/K^+ ATPase by ouabain had no effect on S100A8/A9 and S100A12 secretion from neutrophils exposed or not to MSU or PMA (data not shown). These results suggest that K^+ exchange through the ATP-sensitive K^+ channels participates in the stimulated secretion of S100A8/A9 and S100A12, as is the case for IL-1β in monocytes/macrophages.

3.7. S100A8 and S100A9 Move toward Cytoskeleton/Lipid Rafts Fractions upon Neutrophils Activation by MSU Crystals. Calprotectin translocates into tubulin-enriched compartments in PMA-stimulated monocytes [20], a phenomenon preceding its release. Considering that we found a significant secretion of calprotectin, S100A8, S100A9, and S100A12 we expected that similar phenomenon occurred in neutrophils. To confirm this hypothesis, neutrophils were subjected to sequential lysis in buffers of increasing tonicity to determine the forms and localization of calprotectin, S100A8, S100A9, and S100A12. Beta actin, alpha tubulin, and lipid raft marker flotillin were used as markers of cytosol, cytoskeleton, and membranes (Figure 7(a)). The majority of calprotectin was detected in dimer forms, mainly in the cytosol fraction (soluble hypotonic) of resting neutrophils (Figure 7(b)). However, calprotectin was also retained in the stacking gels of soluble hypotonic fractions of resting neutrophils (data not shown) suggesting the presence of heterooligomeric forms in unstimulated neutrophils. Interestingly, the larger forms of calprotectin within cytoplasm decreased following

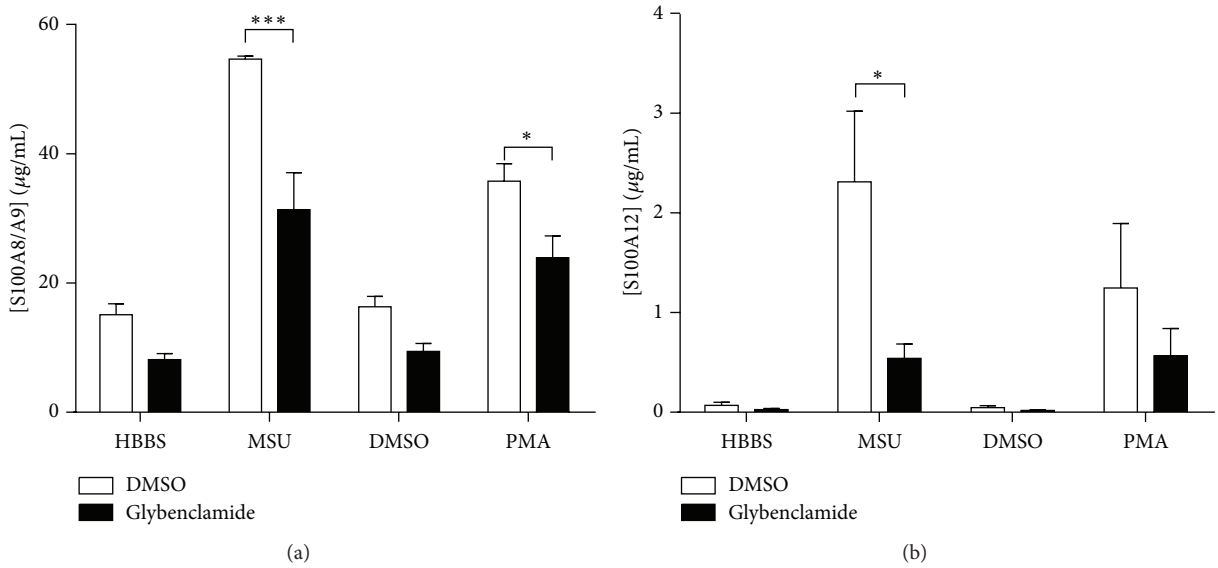
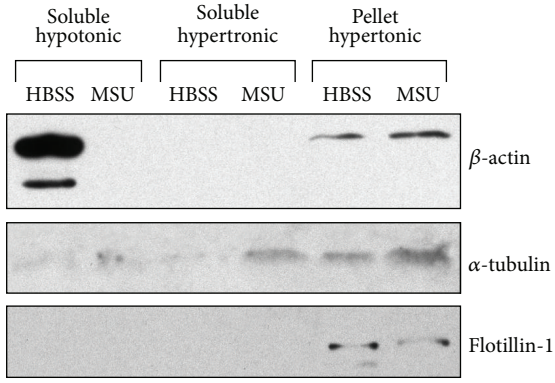


Figure 6: Inhibition of calgranulin secretion by glibenclamide. Neutrophils were preincubated with the ATP-sensitive K^+ channel inhibitor glibenclamide (50 μM) and then stimulated with 1.5 mg/mL MSU crystals or 10 nM PMA for 60 minutes. (a) S100A8/A9 and (b) S100A12 in the supernatants were then quantified by ELISA. Results represent the means ± SEM of 5 donors. $^*P < 0.05$; $^{***}P < 0.001$.

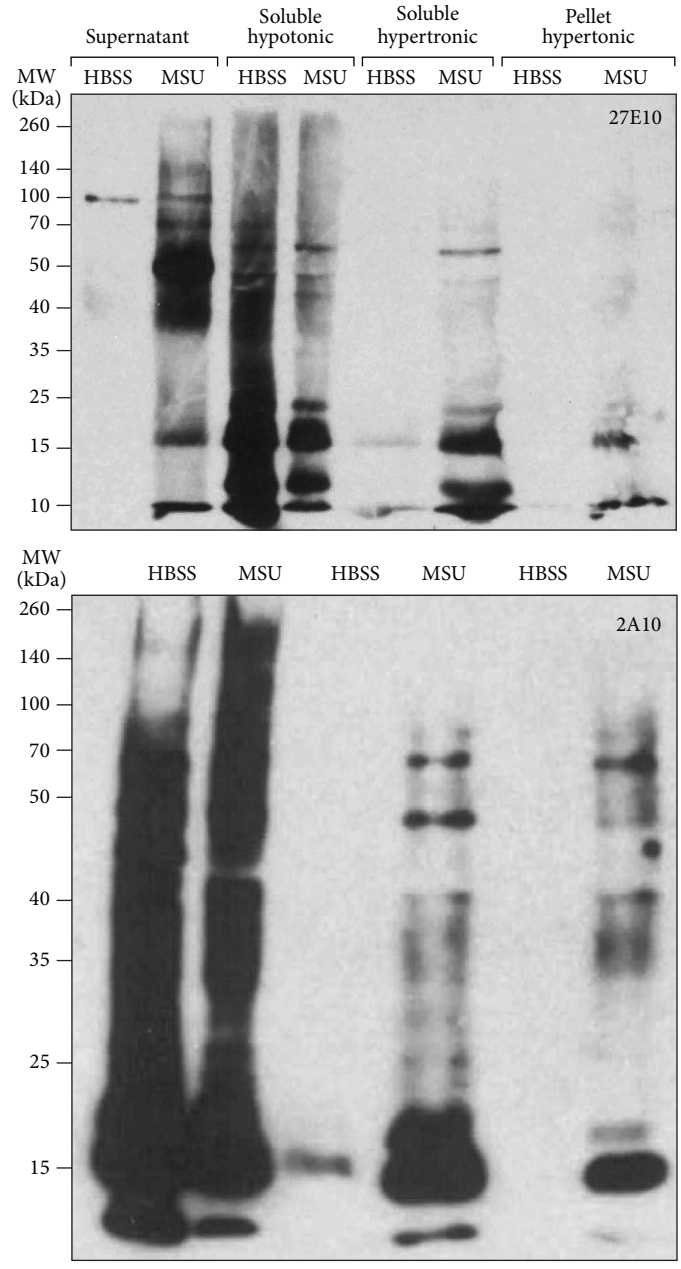
stimulation with MSU crystals. This could be related to the massive secretion of the heterocomplex forms. Moreover, the heterodimers, and to a lesser extent the high molecular weight heterocomplex forms, moved toward soluble and insoluble hypertonic fractions (membrane and cytoskeleton/lipid raft compartments) in neutrophils exposed to MSU. Thus, stimulation of neutrophils with MSU crystals leads to the redistribution of high molecular weight forms of calprotectin from the cytosol to the membranes and lipid rafts, leading to their secretion.

The different forms of S100A proteins were next quantified in each fraction by ELISA to confirm their localization. Homomeric forms of S100A8 and S100A9 were detected in the cytosol of neutrophils (approximately 10 times less S100A8, S100A9, and S100A12 than calprotectin, Figure 7(c)). As expected, the subcellular localization of the S100A proteins changed following neutrophil stimulation and the proteins moved toward membrane and cytoskeleton/lipid raft fractions. This phenomenon was more pronounced for S100A8 and S100A9 when neutrophils were exposed to MSU and to a lesser extent H_2O_2 (18x and 2x increases, resp., for S100A8 and 22x and 3x for S100A9). Similar trends were observed for S100A12 (with a 12-fold increase following MSU stimulation) but fewer proteins were associated with cytoskeleton and/or lipid rafts compared to S100A8 and S100A9. Relocalization of calprotectin to cytoskeleton/lipid rafts fraction was not as marked (with 3-fold increase). Altogether, these results indicate that calprotectin, S100A8, S100A9, and S100A12 localize to cytoskeleton/lipid rafts in neutrophils following activation.

3.8. S100 Proteins Are Not Secreted through Degranulation or Netosis. S100A8 and S100A9 have been reported in gelatinase and specific granules while S100A12 and S100A8/A9 were reported in specific granules [35]. Others have reported S100A8/A9 secretion through netosis [36]. To decipher the mechanism of secretion, we compared relative protein concentrations in supernatants from neutrophils stimulated with MSU crystals and fullerenes (both inducing degranulation and the release of S100A8/A9 and S100A12) with those of neutrophils stimulated with fMLP (inducing degranulation, but not the secretion of S100A8/A9) to confirm that secretion of S100A8/A9 was not linked to degranulation. A 30 min stimulation was also chosen as we could detect secretion of S100A8/A9 at this early time point, but not netosis by microscopy (data not shown). Concentrations of catalase were slightly increased in MSU- (2.5-fold) and fullerene-stimulated (1.1-fold) supernatants (Table 1). However, concentrations of histone 1 and 2B were similar or slightly lower in MSU and fullerene-stimulated supernatants, compared to supernatants of neutrophils stimulated with fMLP. Thus, we found no relationship between relative concentrations of the netosis associated proteins catalase and histones 1 and 2 and secretion of S100A8, S100A9, and S100A12, suggesting that netosis may not be involved as a mechanism of secretion. In addition, concentrations of antimicrobial proteins and enzymes like metalloproteinase-9, elastase, and lactoferroxin C (found in granules) were increased in MSU-stimulated supernatants compared to fMLP (ratios of 4.5, 4.5, and 5.7, resp.), but not in fullerenes-stimulated supernatants (Table 1), indicating no correlation between degranulation and secretion of S100A8/A9 or S100A12. These results were confirmed by examining PMN degranulation induced by MSU crystals or fMLP in absence or presence of cytochalasin B, known to potentiate neutrophil degranulation [37]. Complete degranulation induced by fMLP and cytochalasin B did not increase calprotectin (Supplementary Figure 7) and



(a)



(b)

Figure 7: Continued.

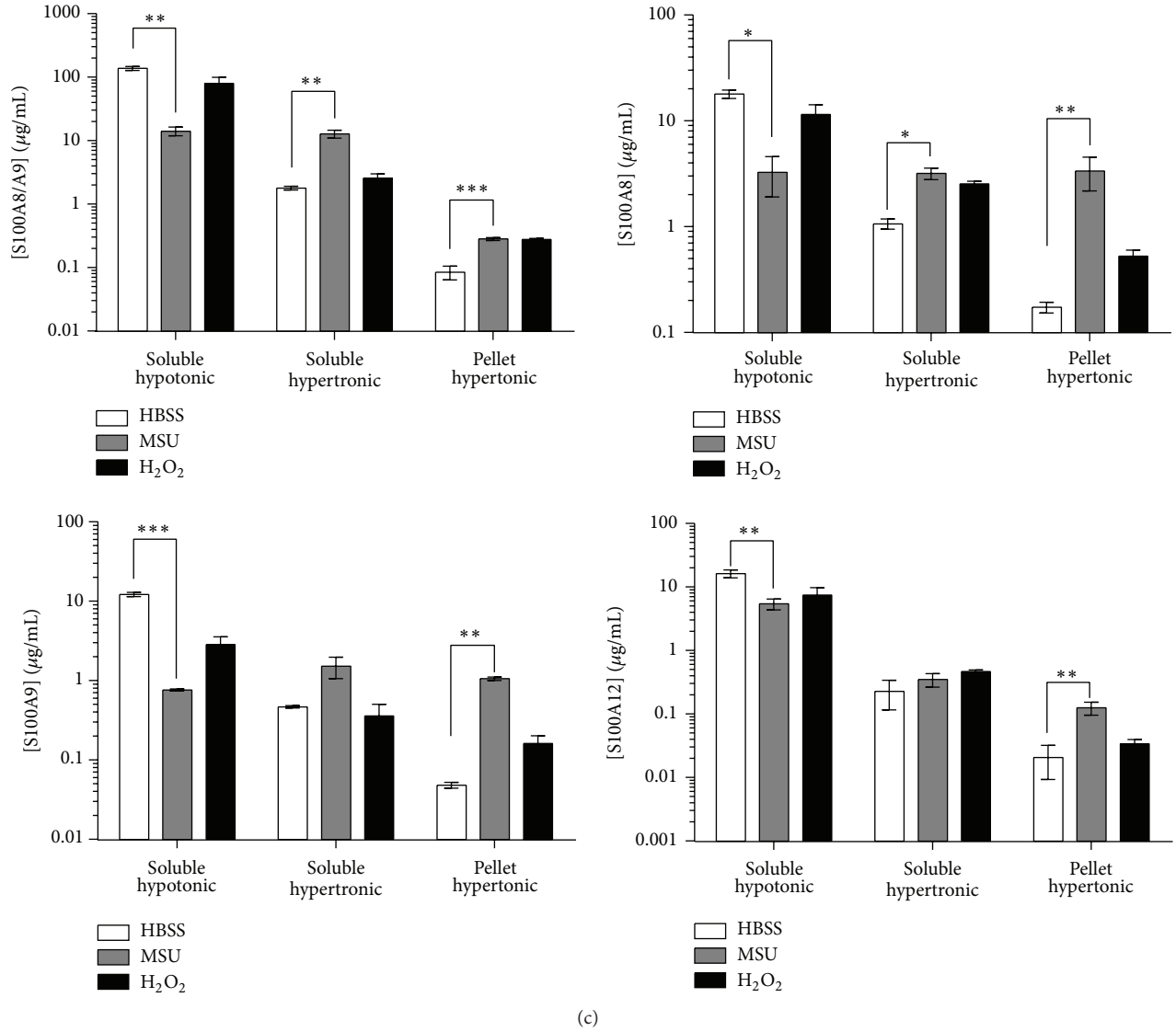


Figure 7: S100A8, S100A9, S100A12, and calprotectin move toward cytoskeleton/lipid raft fractions following neutrophils stimulation by H_2O_2 or MSU. Neutrophils were stimulated with 1.5 mg/mL MSU crystals or 250 μM H_2O_2 and supernatants harvested before sequential cells lysis as described in Section 2. Fractions were loaded onto SDS-PAGE and (a) β actin, α tubulin, and flotillin-1 or (b) S100A8/A9 or S100A12 was detected by immunoblotting. Results are from one experiment are representative of 2 others. (c) Fractions were subjected to sandwiches ELISA against S100A8, S100A9, S100A12, or S100A8/A9. Results represent the mean ± SEM of 4 donors quantified in duplicate. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, MSU versus HBSS.

S100A12 secretion. Relative concentrations of lactate dehydrogenase, myosin, and beta actin, which were not increased in MSU or fullerene-stimulated supernatants, confirmed that secretion of S100A8, S100A9, and S100A12 was not due to cell leakage (Table 1). Together, these results strongly suggest that S100A8/A9 and S100A12 are not secreted by degranulation, netosis, or cell necrosis.

3.9. Calprotectin and S100A12 Are Mainly Secreted in a Nonvesicular Form. Alternative secretion occurs via vesicular and nonvesicular pathways. We therefore investigated whether calprotectin and S100A12 were secreted in a vesicular-dependent manner from neutrophils stimulated with MSU. Microvesicles (MV) were isolated after sequential centrifugation from the supernatants of neutrophils stimulated with MSU crystals. Microvesicles were separated in large (P2), intermediate (P3), and small microvesicles (ectosomes, P4) by sequential centrifugation. Calprotectin and S100A12 were mostly found in the soluble fractions (Figures 8(a) and 8(b), S1 to S4). Almost no S100A12 was detected in vesicular fractions (P2 to P4). However, some calprotectin was detected in the large microvesicles in unstimulated and stimulated PMN and its presence in smaller vesicles was enhanced by cell stimulation. Disruption of vesicular

Table 1: Proteomic analysis of proteins secreted by neutrophils.

Accession number	Name	Number of peptides[a]	MSU/fMLP[b]	P value[c]	Fullerenes/fMLP[d]	P value[c]
NETosis-associated proteins						
P04040	Catalase	26	2.46	$6,71E-14$	1,123677	$4,59E-05$
P16401	Histone H1.5	3	1,020058	0,973432	0,745488	0,050647
Q99880	Histone H2B type 1-L	3	0,51384	0,677915	0,386307	0,237565
Granule's antimicrobial proteins and enzymes						
P14780	Metalloproteinase-9	27	1,632532	0,285746	0,757033	0,475546
P05164-3	Myeloperoxidase	46	4,544349	$5,89E-20$	0,934431	0,071378
P08246	Neutrophil elastase	8	4,532902	0,001009	1,151382	0,1147
P02788	Lactoferroxin-C	70	5,713208	0,014768	1,489547	0,117136
O75594	Peptidoglycan recognition protein 1	6	3,00764	0,00035	0,892349	0,189002
Cytoplasmic/intracellular proteins						
P07195	Lactate dehydrogenase B chain	3	1,563257	0,274256	1,324116	0,095393
P04083	Annexin A1	6	1,199672	0,307777	0,695102	0,001041
P12429	Annexin A3	3	1,917971	0,038612	0,974026	0,701223
P26447	S100A4	3	1,885171	0,033789	1,114386	0,027142
P31949	S100A11	2	1,926023	0,159743	1,063338	0,363006
P35579	Myosin-9	45	1,276348	0,097059	1,114768	$6,50E-05$
Q53GK6	Beta actin variant	12	1,649348	0,235332	1,09936	0,612781
Calgranulins						
P05109	S100A8	15	5,706729	$1,04E-06$	2,008216	0,001211
P06702	S100A9	17	5,775399	$1,83E-05$	1,982834	0,000234
P80511	S100A12	7	2,426018	0,042156	1,293581	0,001423

[a]Number of peptides identified by isobaric tags for relative and absolute quantitation.
[b]Ratio of the different proteins in supernatants from neutrophils stimulated with MSU crystals over neutrophils stimulated with fMLP (which does not induce secretion of calgranulins).
[c]P value for the identification of the peptides.
[d]Ratio of the different proteins in supernatants from neutrophils stimulated with fullerenes over neutrophils stimulated with fMLP (which does not induce secretion of calgranulins).

membranes by adding 0.5% triton did not improve the detection of S100A8/A9 or S100A12, confirming that most of the S100 proteins found in the supernatant were not trapped into microvesicles. These results were confirmed by western blot (Figure 8(c)).

4. Discussion

The secretion of proteins through alternative pathways is a complex process involving multiple signals and protein-protein interactions. These interactions differ according to the cell type and the nature of the stimulus. In this study, we examined the secretion of 4 related proteins (S100A8/A9, S100A8, S100A9, and S100A12) from freshly isolated human neutrophils in response to inflammatory stimuli. Stimulation with MSU crystals and other phagocytic particles increased the secretion of all calgranulins while secretion was restricted to S100A8/A9, S100A8, and S100A9 when cells were activated by PMA. In contrast, chemotactic factors like fMLP, C5a, and IL-8 did not induce the secretion of calgranulins. Activation of neutrophils led to the translocation of heterodimers (S100A8/A9) and homodimers (S100A8, S100A9, and S100A12) from the cytosol to the cytoskeleton and membrane before secretion. Similar to IL-1β secretion, the alternative secretion pathway used by calgranulins was dependent on ROS production and K^+ fluxes. The secretion was not associated with netosis or degranulation, and the majority of secreted calgranulin was found in soluble form, but some was associated with large vesicles. Thus, stimulation of neutrophils leads to the translocation of calgranulins to the cytoskeleton/membrane before their secretion.

S100A8/A9, S100A8, S100A9, and S100A12 have been found at inflammatory sites and in the serum in acute and chronic inflammation [38], and several reports indicate that they exert proinflammatory activities and are involved in phagocyte migration. S100A8, S100A9, and S100A8/A9 have been associated with granulocyte and monocyte adhesion to endothelium, as well as with their migration across endothelial cells [8, 28, 39, 40]. In addition, a report suggests that S100A8/A9 promotes the expression of adhesion molecules such as ICAM-1 and VCAM-1 and downregulates the expression of tight junction proteins on endothelial cells [41]. S100A12, on the other hand, stimulates granulocyte adhesion [7]. More recently, Wei et al. described mast cell and monocyte recruitment induced by S100A12 [13], while Yang et al. described mast cell degranulation induced by S100A12 [14]. S100A8/A9 and S100A12 have also been reported to inhibit microbial growth [42–44]. These data suggest distinct

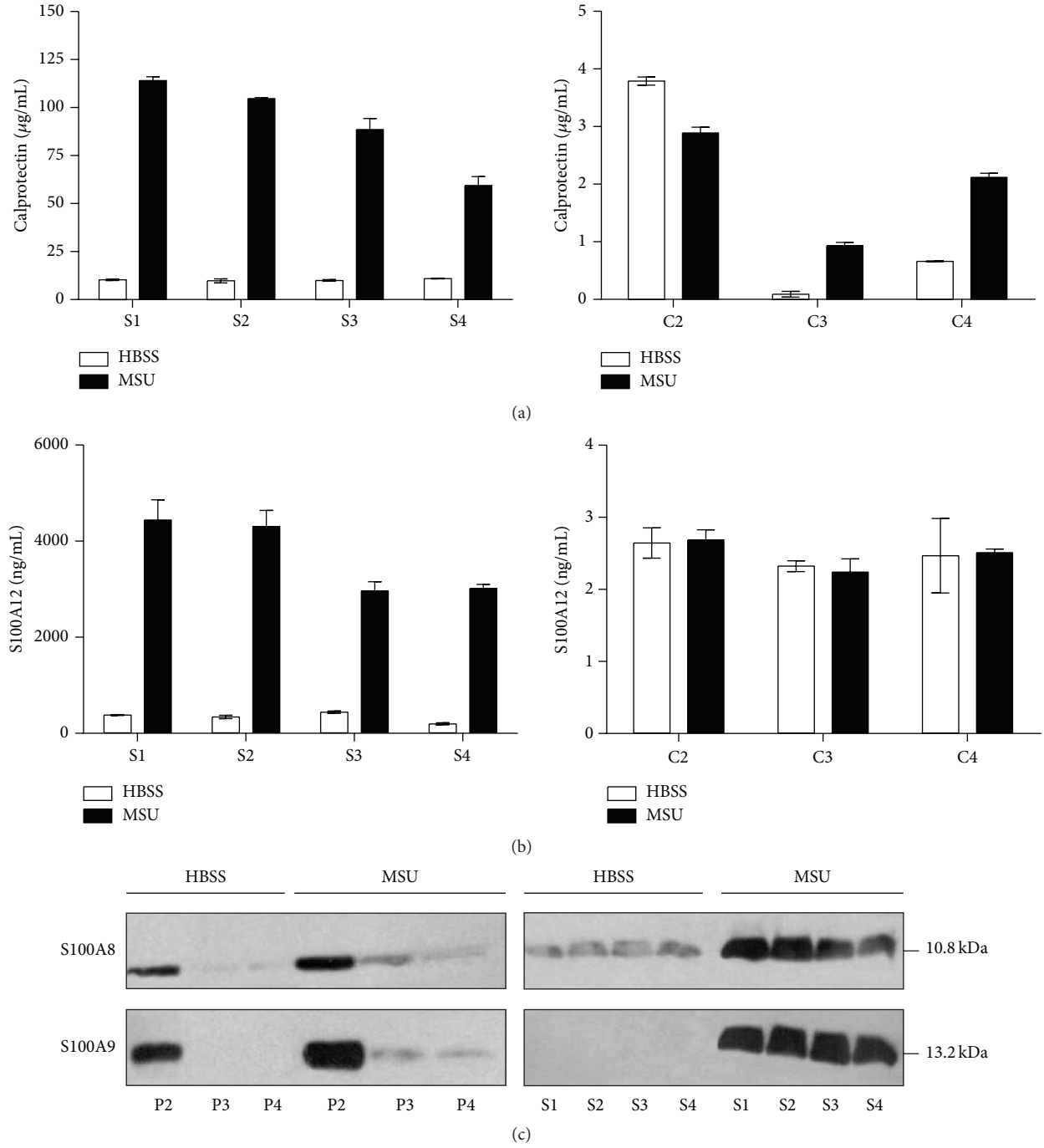


Figure 8: S100A8/A9 and S100A12 are almost absent from microvesicles. Neutrophils were incubated with MSU crystals (1.5 mg/mL) or its diluent for 60 min at 37°C. The MV were isolated after sequential centrifugation. The concentrations of (a) S100A8/A9 and (b) S100A12 were determined by ELISA in the supernatant fractions (S1, S2, S3, and S4) and the pellet fractions (P1, P2, and P3) containing the MV. Data are the mean ± SEM of three separate experiments. (c) The presence of S100A8 and S100A9 in the supernatants and MV was determined by western blot. Results are from one experiment representative of 2 others.

biological activities for these proteins, which should be reflected in their patterns of secretion. Indeed, we found that stimuli usually found near or on endothelium predominantly favored the secretion of S100A8/A9 over that of S100A12. For example, TNFα and PAF (which are produced by or near endothelial cells during inflammatory reactions) induced the secretion of S100A8/A9 but had almost no effect on S100A12 secretion. This contrasts with the myeloid growth factor GM-CSF, which promotes the release of both S100A8/A9 and S100A12. This suggests that stimuli found

near endothelium induce the release of S100A8/A9, which could then promote phagocyte migration through the expression of adhesion molecules and downregulation of tight junction proteins by endothelial cells [41]. Interestingly, secretion of S100A8/A9 was always associated with the secretion of S100A8 and S100A9 homodimers.

Most of S100A8 and S100A9 were secreted in the heteromeric form (calprotectin) by resting and stimulated neutrophils. As reported by others [45], western blot analyses in nonreductive conditions suggested that the major forms of secreted S100A8/A9 were heterotetramers and, to a lesser extent, heterooctamers followed by heterodimers (data not shown). Interestingly, monomeric/homocomplex forms of S100A9 were secreted by neutrophils stimulated with H_2O_2, MSU crystals, and PMA with concentrations reaching 0.5 to 2 μg/mL whereas very little S100A8 was released, only in response to MSU crystals (approximately 100 ng/mL). Nonetheless, such concentrations are sufficient to induce chemotaxis and cytokine secretion in response to S100A8 and S100A9 [9]. The secretion of S100A12 by neutrophils was at least 20-fold inferior to S100A8/A9 (0.5 to 5 μg/mL) and was significantly triggered once cells were exposed to MSU crystals or H_2O_2, but not PMA.

S100A8/A9 and S100A12 have been reported in granules [35], suggesting that they could be released following neutrophil degranulation. In addition, Hetland et al. previously reported the release of S100A8/A9 by neutrophils stimulated with fMLP [46], a potent inducer of degranulation. However, the main conclusion of this study was based on the disappearance of intracellular S100A8/A9 after stimulation, and the authors of the study were not able to detect S100A8/A9 in the supernatants of stimulated neutrophils. We did not detect any S100A8/A9 secretion by neutrophils stimulated with fMLP even when cells were primed with cytochalasin B, a well-known priming agent promoting fMLP-mediated neutrophil effector responses (data not shown). This confirms that degranulation is not involved in the secretion of S100A8/A9 and S100A12 from neutrophils. C5a and fMLP induce calcium mobilisation, which in turn activates the translocation of S100A8/A9 to the plasma membrane or cytoskeleton [47, 48] which could explain the disappearance of S100A8/A9 in the cytosol reported by Hetland et al. [46], although this translocation is not sufficient to allow its secretion. Other signals are therefore necessary to induce the secretion of calgranulins. Another possible route of secretion for calgranulins is vesicular secretion which depends on intracellular membrane-bound intermediates that need to fuse with plasma membranes to release cargo into the extracellular space. Such mechanisms involve either secretory lysosomes, exosomes derived from multivesicular bodies, or microvesicle shedding from cell surfaces [49]. Since very low levels of S100A8/A9 and S100A12 are detected in the vesicular fractions from differential centrifugation (data not shown), we conclude that the vesicular secretion pathway is not the main route used by S100A proteins to reach the extracellular environment.

IL-1β is perhaps the most studied protein secreted through an alternative secretion pathway. Although neutrophils are weak producers of IL-1β, DAMPs like MSU crystals and phagocytic particles are potent inducers of IL-1β secretion by monocytes [50]. The same stimuli induced S100A8/A9 and S100A12 secretion by neutrophils, suggesting common mechanisms of secretion between IL-1β and calgranulins. Secretion of IL-1β necessitates inflammasome activation consecutive to decreased intracellular K^+ concentrations and the generation of oxidative stress [34, 51]. Closure of ATP-sensitive K^+ channels by glibenclamide or inhibition of NOX-2 activation by DPI prevented the release of both calprotectin and S100A12 from resting and stimulated neutrophils without affecting cell viability. This suggests that, as for IL-1β, calgranulin secretion requires ROS production and K^+ fluxes and that secretion of calgranulins and IL-1β might be linked. This hypothesis is supported by the close association of high concentrations of IL-1β and calgranulins in the serum of patients with autoinflammatory diseases harboring mutations in pyrin or pyrin-associated proteins [38].

In summary, we have identified a number of inflammatory stimuli that induce the secretion of both S100A12 and S100A8/A9 from human neutrophils. The secretion of each protein is not intimately linked with that of the other; some agonists that induce the release of S100A8/A9 are not able to induce the exocytosis of S100A12. Therefore, different signal transduction pathways may participate in the secretion of both proteins. The identification of events leading to S100A8/A9 and S100A12 secretion might help us better understand the inflammatory conditions leading to secretion of these proteins and consequently the role of neutrophil in innate immunity and inflammation.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Research Article

Heparin Interaction with the Primed Polymorphonuclear Leukocyte CD11b Induces Apoptosis and Prevents Cell Activation

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Heparin is known to have anti-inflammatory effects, yet the mechanisms are not completely understood. In this study, we tested the hypothesis that heparin has a direct effect on activated polymorphonuclear leukocytes (PMNLs), changing their activation state, and can explain its anti-inflammatory effect. To test our hypothesis, we designed both *in vitro* and *ex vivo* studies to elucidate the mechanism by which heparin modulates PMNL functions and therefore the inflammatory response. We specifically tested the hypothesis that priming of PMNLs renders them more susceptible to heparin. Amplified levels of CD11b and increased rate of superoxide release manifested PMNL priming. Increase in cell priming resulted in a dose-dependent increase in heparin binding to PMNLs followed by augmented apoptosis. Blocking antibodies to CD11b inhibited heparin binding and abolished the apoptotic response. Moreover, heparin caused a significant dose-dependent decrease in the rate of superoxide release from PMNLs, which was blunted by blocking antibodies to CD11b. Altogether, this study shows that the interaction of heparin with the PMNL CD11b results in cell apoptosis and explains heparin's anti-inflammatory effects.

1. Introduction

In many inflammatory responses, polymorphonuclear leukocytes (PMNLs) are among the first cells to exit the blood stream and migrate to an inflammatory site, where they become fully activated. This activation is a two-stage process: PMNLs first encounter a stimulus that does not activate the cells directly but leaves them in a "primed" state. Then, upon encountering a second stimulus in the inflamed site, the transition into a fully activated state will occur [1, 2]. This process involves the production of free radicals and release of granule enzymes into the surrounding milieu. Therefore, tight regulation of PMNL activation is needed throughout the steps of infiltration from the blood stream to the inflamed site in order to prevent damage to the vascular wall and the extracellular matrix (ECM).

One of the ECM components is heparin (in the form of heparan sulfate), a soluble molecule that plays a major role in defining the physical and chemical properties of the ECM [3]. Heparin, which is commonly used as a blood anticoagulant, is also known to have anti-inflammatory effects; however, the mechanism of these biological activities remains largely unknown [4, 5]. Some of heparin's anti-inflammatory effects are mediated through the modulation of cellular activation, particularly of PMNLs [6–9]. Heparin decreases phorbol myristate acetate (PMA), *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), and opsonized zymosan-induced superoxide production [7], a decrease which is even greater

when the PMNLs are primed by platelet activating factor (PAF) [9]. Heparin reduces fMLP-stimulated PMNL adhesion to endothelial cells and decreases the release of beta-glucuronidase and lysozyme from stimulated PMNLs [6]. In addition, heparin has been shown to inhibit leukocyte recruitment and chemotaxis in response to zymosan-activated serum [10].

Recently, it was shown that immobilized heparin can mediate cell adhesion via interaction with the PMNL integrin Mac-1 (CD11b/CD18, $\alpha_M\beta_2$) [11]. Mac-1 is one of the most versatile adhesion molecules, with ligands of various biological functions. One of these functions is the induction of a signal transduction cascade that substantially augments apoptosis of activated PMNLs [12].

The above data, especially the known apoptotic effect of heparin on PMNLs [13, 14], led us to hypothesize that priming of PMNLs renders them more susceptible to the apoptotic effects of heparin and that apoptosis induced by heparin is mediated in part by heparin interactions with CD11b, which is highly expressed on the surface of primed PMNLs [15]. In order to test our hypothesis, we used PMNLs isolated from hemodialysis (HD) patients as a model of *in vivo* primed cells [16] and PMNLs isolated from healthy controls (NC) primed *ex vivo* with PAF. Our results indicate that primed PMNLs, regardless of their priming origin (*ex vivo* or *in vivo*), are more susceptible to the apoptotic effect of heparin compared to nonprimed PMNLs. We also show that heparin binds to CD11b, leading to apoptosis that can be blocked with neutralizing antibodies against CD11b.

2. Methods

2.1. Patients and Blood Samples. Blood was drawn from 17 patients on chronic hemodialysis and 24 age- and gender-matched healthy control subjects (NC). Blood for the determination of biochemical and hematological parameters and for the isolation of PMNLs was drawn after an overnight fast. Blood was collected into citrate tubes from the arterial line of all the HD patients immediately before a dialysis session. All patients underwent hemodialysis three times a week; each dialysis treatment lasted 4 hours and was carried out with low flux polysulfone membranes (F8, Fresenius Medical Care, Bad Homburg, Germany). The water for dialysis met the standards of the Association for the Advancement of Medical Instrumentation (AAMI). Patients with evidence of acute or chronic infection or malignancy or who had received blood transfusion within 3 months prior to blood sampling were excluded. All participants signed an informed consent for blood sampling, and the study was approved by the Institutional Committee in accordance with the Helsinki declaration.

2.2. PMNL Isolation and Analysis. PMNLs were isolated as described previously [17]. Isolated PMNLs (>98% pure, approximately 10^7 cells per isolation) were resuspended and counted in phosphate buffered saline (PBS, Beit Haemek, Israel) containing 0.1% glucose. PMNL priming was assessed by the rate of superoxide release [17] and by the surface levels of CD11b, as described previously [15]. The rate of superoxide release was determined after cell stimulation with 0.32×10^{-7} M phorbol 12-myristate 13-acetate (PMA; Sigma, St. Louis, MO). The assay is based on superoxide dismutase (SOD) inhibitable reduction of 80 μM cytochrome C (Sigma, St. Louis, MO) to its ferrous form. The change in optical density was monitored at 549 nm, as described previously [17]. Expression of CD11b on PMNLs in whole blood was determined using the FC500 flow cytometer (Beckman-Coulter). 50 μL of blood was incubated for 10 min with anti-CD11b-PE conjugated monoclonal antibody (Immunotech, Marseille, France), followed by red blood cell lysis (Q-prep method; Coulter Corporation, Hialeah, FL, USA). To enable gating on the PMNL population, anti-CD16, conjugated to PC5 monoclonal antibody (IQ Products), was used. Surface levels of CD11b on PMNLs are expressed as mean fluorescence intensity (MFI), after subtracting the nonspecific background.

2.3. Priming of PMNLs by Platelet Activating Factor (PAF). PAF (Sigma, USA), a known dose-dependent priming agent of PMNLs [18], was used for the *in vitro* priming of NC PMNLs in three concentrations: 1 pM, 1 nM, and 1 μM. Cells were incubated with PAF for 15 minutes at room temperature and then washed with PBS. In PAF-stimulated PMNLs where CD11b intensity was measured and in experiments where CD11b was blocked, 5 minutes after the initiation of incubation with PAF, mouse anti-human-CD11b-PE was added. CD11b expression was measured as described above for whole blood, but without the lysis step.

2.4. Effect of Heparin on PMNLs. The effect of heparin was studied on three types of isolated PMNLs: normal control (NC) PMNLs, PAF-stimulated NC PMNLs, and HD PMNLs. All cells were incubated for 30 min at room temperature with 25, 50, and 100 U/mL sodium heparin (Kamada, Beit Kama, Israel) diluted in PBS. In order to measure time-dependent effects of heparin, NC and HD PMNLs were incubated with 100 U/mL sodium heparin for 300 min at room temperature.

2.5. Analysis of PMNL Apoptosis. The percentage of apoptotic PMNLs was based on Annexin-V-FITC binding according to the manufacturer's recommendations (Annexin-V/FITC Kit, Bender MedSystems Diagnostics GmbH, Vienna, Austria). Annexin-V-FITC and propidium iodide (PI) were added to the cell suspension and incubated for 10 min in the dark at room temperature. PI staining is a dye-exclusion assay that discriminates between cells with intact membranes (PI negative) and permeabilized membranes (PI positive) [19]. The cell population showing Annexin-V−/PI− was considered viable; Annexin-V+/PI− was considered as an early apoptotic population. Late stage apoptotic or necrotic cells were represented by the Annexin-V+/PI+ population [19].

2.6. Analysis of PMNL Apoptosis after Preincubation with Anti-CD11b. Separated PMNLs (5×10^5) were incubated with anti-CD11b-PE or its isotype control- (IC-) PE for 10 minutes, washed, and incubated for 30 minutes with 25 U/mL heparin. This dose of heparin was chosen since it is the minimal dose

that caused a statistically significant difference in apoptosis between primed and nonprimed PMNLs. Apoptosis in PMNLs was detected using the following: (A) Annexin-V-FITC binding was measured as mentioned above without the addition of PI. The percentage of apoptotic cells was compared with apoptosis detected using FITC-labeled Annexin-V, added after preincubation with IC-PE (Immunotech, Marseille, France). The positive cutoff point was determined by FITC-isotype controls (IQ Products, Netherlands), added after preincubation with IC-PE. (B) PMNLs (5×10^6) were cytospinned and apoptosis was detected with an Axioscop 2 upright fluorescence microscope, using the *In Situ* Cell Death detection kit (TUNEL staining, Roche Molecular Biochemicals). Nuclear staining was done with 5 μg/mL Hoechst (Calbiochem).

2.7. PMNL Viability. Cells (2×10^6) were incubated at room temperature for 0, 30, and 90 minutes in PBS with and without heparin (100 U/mL), stained with trypan blue solution (0.4% in HBSS), and counted.

2.8. Heparin Binding to PMNLs. Separated PMNLs (5×10^5) were incubated with anti-CD11b-PE or its isotype control for 10 minutes, washed, and assayed for heparin binding by two different methods: (A) the PMNLs were further incubated with 25 U/mL sodium heparin. The percentage of PMNLs that bound heparin and the intensity of heparin binding to PMNLs were determined by flow cytometry using mouse anti-human heparan sulfate-FITC (United States Biological, Massachusetts). The Ab was generated by immunization with liposome-intercalated membrane HS proteoglycans and recognizes an epitope present in many types of human heparan sulfate including heparin. The epitope includes N-sulfated glucosamine residues that are critical for the reactivity of the antibody. The Ab does not react with hyaluronan, chondroitin sulfate, dermatan sulfate, keratan sulfate, or DNA [20, 21]. The positive cutoff point was determined by FITC-isotype control (IQ Products, Netherlands), added after preincubation with anti-CD11b-PE. The percentage of heparin bound cells and the intensity of heparin binding to PMNLs detected in this experiment were compared with cells detected using FITC-labeled anti-heparan sulfate, added after preincubation with IC-PE (Immunotech, Marseille, France). The positive cutoff point was determined by FITC-isotype controls (IQ Products, Netherlands), added after preincubation with IC-PE. (B) The PMNLs were further incubated with 25 U/mL FITC-labeled heparin (Molecular Probes, Eugene, OR) in PBS for 30 minutes at room temperature. The intensity of heparin binding to PMNLs was determined by FC500 flow cytometer (Beckman-Coulter).

2.9. Statistical Analysis. Data are expressed as mean ± SD. In the box and whiskers presentations, the horizontal line in the middle shows the median (50th percentile), the top and bottom of the box indicate the 75th and 25th percentiles, respectively, and the whiskers show the maximum and the minimum values. The nonparametric Mann-Whitney test was used for comparing two independent groups. The two-paired Wilcoxon Signed Ranks test was used for comparing two dependent groups. Statistical significance was considered at $P < 0.05$.

3. Results

3.1. PMNL Priming. PMNL priming was manifested by increased rates of superoxide release and amplified levels of membrane CD11b [16–18]. Preincubation of isolated normal control (NC) PMNLs with increasing concentrations of PAF caused a dose-dependent increase in the rate of superoxide release from PMA-stimulated PMNLs ($^*P < 0.05$, Figure 1(a)). In addition, the expression of CD11b was higher in PAF-treated NC PMNLs compared to nontreated NC PMNLs ($^*P < 0.05$, Figure 1(b)).

We reported previously that PMNLs from hemodialysis (HD) patients are primed [15]. To confirm these results, we isolated HD PMNLs and measured their priming. The rate of superoxide release following PMA stimulation was higher in PMNLs isolated from HD patients compared to PAF untreated NC cells (33.5 ± 4 versus 24.7 ± 5 nmoles/10^6 cells/10 min, resp., $^*P < 0.05$, Figure 1(a)) and was similar to the rate achieved by stimulation with the highest concentration of PAF. The expression of CD11b was also higher in HD PMNLs than in NC PMNLs (61 ± 25 versus 29 ± 11 MFI, resp., $^*P < 0.05$, Figure 1(b)) and comparable to the levels measured in NC cells stimulated with the highest concentration of PAF.

3.2. Dose-Dependent Effect of Heparin on PMNL Apoptosis and Priming. We have previously reported that heparin exerts an apoptotic effect on PMNLs [13]. To determine whether primed PMNLs are differently affected by heparin compared to nonprimed cells, we exposed 3 groups of PMNLs: NC, 1 μM PAF-stimulated NC, and HD PMNLs, to increasing concentrations of heparin for 30 min (Figure 2). We used 1 μM PAF-stimulated NC since the rate of superoxide release and the expression of CD11b on these *ex vivo* primed cells were similar to *in vivo* primed cells, isolated from HD patients (Figure 1). Incubation of PMNLs with increasing concentrations of heparin resulted in an increase in early apoptosis in all three groups, however to a much greater extent in HD PMNLs (Figure 2(b)). The increase in HD PMNL apoptosis was significant at all heparin concentrations versus without heparin ($P < 0.05$), while in NC PMNLs and PAF-stimulated NC PMNLs significance was achieved only at 100 U/mL of heparin ($P < 0.05$). Moreover, the maximal percentage of early apoptotic PMNLs was 8% in NC and 10% in PAF-stimulated NC whereas 17% apoptotic cells were detected in HD PMNLs.

Incubation of PMNLs with increasing concentrations of heparin caused a significant increase in apoptosis (early + late) in all three groups of PMNLs, however to a much greater extent in PAF-stimulated NC and HD PMNLs with both groups showing similar dose-dependent responses (Figure 2(c)). The increased degree of apoptosis in PAF-stimulated NC and HD PMNLs was significant at all heparin concentrations versus without heparin ($P < 0.05$), while in

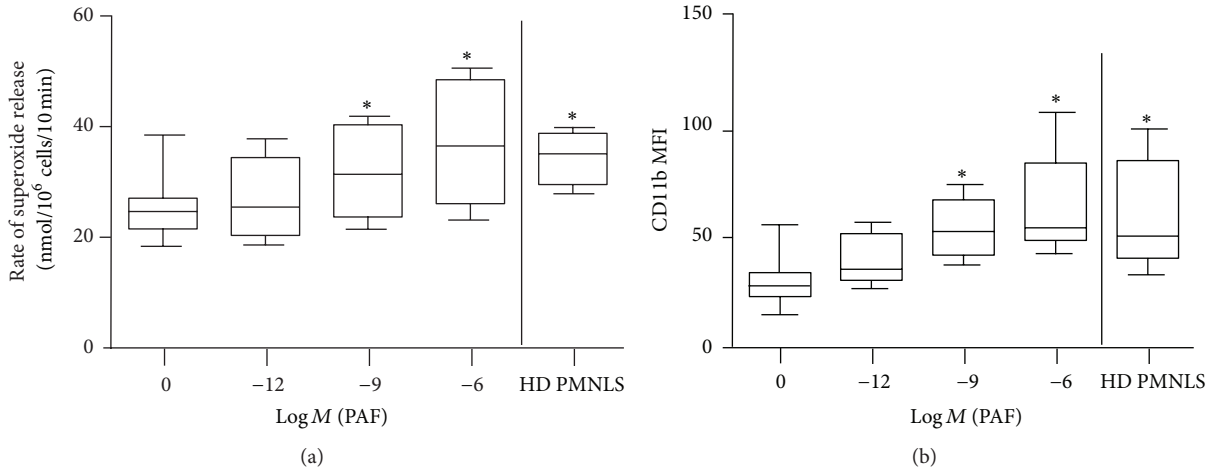

Figure 1: PMNL priming. (a) Rates of superoxide release from separated NC PMNLs after 15 min of stimulation by 1 pM, 1 nM, and 1 μM PAF and HD PMNLs activated with 0.32×10^{-7} M PMA. The changes in optical density were monitored at 549 nm continuously up to 10 min in the presence of 0.08 mM cytochrome C. Data are expressed as nmoles/10^6 cells/10 min; $^*P < 0.05$ for HD and PAF stimulated (10^{-9} M and 10^{-6} M) versus nonstimulated NC PMNLs (no PAF), $n = 10$. (b) Relative expression of surface CD11b on PMNLs measured by flow cytometry using specific PE-labeled antibody, as described in Section 2. Data are expressed as mean fluorescent intensity (MFI); $^*P < 0.05$ for HD and PAF stimulated (10^{-9} M and 10^{-6} M) versus nonstimulated NC PMNLs (no PAF), $n = 10$.

NC PMNLs significance was detected only at 100 U/mL of heparin ($P < 0.05$).

The effect of heparin on PAF-stimulated NC and HD PMNLs was much greater than its effect on NC PMNLs ($P < 0.05$ NC versus HD and PAF-stimulated NC at all heparin concentrations). The maximal percentage of apoptotic PMNLs in NC was only 14%, whereas 22% apoptotic cells were detected in PAF-stimulated NC and HD PMNLs.

Priming is also reflected by the rate of superoxide release from PMA-activated PMNLs. To determine whether apoptosis induced by heparin modulates activation of primed PMNLs, we investigated the dose-dependent effect of heparin on the rate of superoxide release. A significant reduction in the rate of superoxide release was observed in all groups of PMNLs ($P < 0.05$ for all heparin concentrations versus no heparin; Figure 2(d)). Moreover, PAF-stimulated NC and HD PMNLs showed lower rates of superoxide release versus NC PMNLs.

The decreased rate of superoxide release was maximized at heparin concentrations of 25 U/mL for NC PMNLs and 50 U/mL for 1 μM PAF-stimulated NC and HD PMNLs. This result suggests that the effect of heparin is greater with increased priming; the maximal inhibition in the rate of superoxide release from primed PMNLs was approximately three times higher than in unprimed cells.

3.3. Time-Dependent Effect of Heparin on PMNLs. To determine whether heparin exerts a similar time-dependent apoptotic effect on primed versus quiescent cells, we incubated NC and HD PMNLs with 100 U/mL of heparin up to 300 min (Figure 3). We used 100 U/mL of heparin as it was the only heparin concentration that induced apoptosis in NC PMNLs (Figures 2(b) and 2(c)). Heparin induced early apoptosis in both HD and NC PMNLs at all time points during the incubation, while without heparin, these cells did not exhibit enhanced early apoptosis at any time point (Figure 3(a)). Moreover, heparin caused an oscillatory apoptotic pattern of HD and NC PMNLs reaching apoptotic peaks at 30 and 210 min for HD PMNLs and 90 and 210 min for NC PMNLs. When detecting total apoptosis (early + late), the oscillatory apoptotic patterns of HD and NC PMNLs were similar regarding the apoptotic peaks, but with an augmented percentage of apoptotic cells at all points (Figure 3(b)). To test whether the oscillatory pattern could be explained by the loss of apoptotic cells in the *ex vivo* incubation, we counted the NC and HD PMNLs with and without heparin up to 90 min. This assay revealed that during the 30 min of incubation most of the cells (NC, HD) were still alive, while after 90 min a significant decrease in HD PMNL count was found (Figure 3(c)).

Based on these results, all incubation experiments were for 30 minutes to avoid significant cell disintegration *in vitro*.

3.4. The Effect of Priming on Heparin Binding to PMNLs. PAF stimulation resulted in a dose-dependent increase in heparin binding to PMNLs (Figures 4(a)–4(c)). Pretreatment of PAF-stimulated PMNLs with anti-CD11b antibodies completely prevented heparin binding to PMNLs, indicating that heparin binds to PMNLs, at least partially, via CD11b (Figure 4(c)). These results were confirmed by additional studies using heparin that was conjugated to FITC. Heparin binding intensity to unstimulated NC PMNLs was 6.22 ± 0.19 MFI and increased to 13.15 ± 1.76 MFI after stimulation with 1 μM PAF, demonstrating increased heparin binding with increased priming. Pretreatment of PAF-stimulated PMNLs with anti-CD11b antibodies completely prevented heparin binding to PMNLs with heparin-FITC intensity reduced to 6.48 ± 0.57 MFI.

In a second set of experiments depicted in Figure 4(d), FITC-conjugated anti-heparin antibodies were used to detect

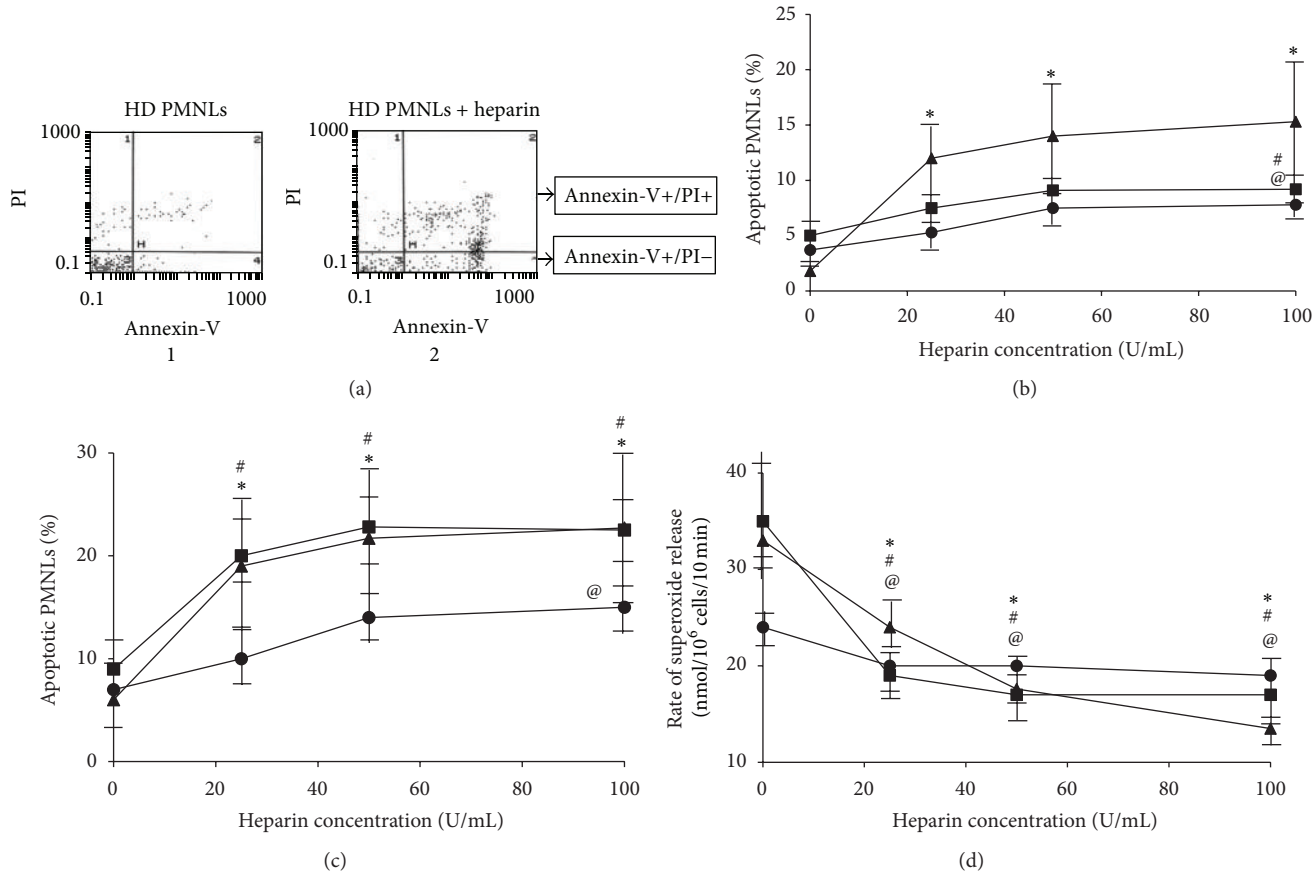

Figure 2: Dose-dependent effect of heparin on PMNL apoptosis and priming. (a) Representative histograms for apoptosis of HD PMNLs without heparin (1) and with 100 U/mL heparin (2) based on Annexin-V-FITC and PI binding. Cell populations: Annexin-V−/PI−, Annexin-V+/PI−, and Annexin-V+/PI+ were regarded as alive, early apoptotic, and late apoptotic or necrotic, respectively. (b) Early apoptosis (Annexin-V+/PI−) in NC PMNLs without stimulation (●) and after 15 min stimulation with 1 μM PAF (■) and HD PMNLs (▲). Cells were preincubated with PAF, washed, and incubated with increasing concentrations of heparin for 30 min. Apoptosis was determined by flow cytometer analysis, using a commercial kit for detection of Annexin-V as described in Section 2. Data are expressed as percentage of apoptotic PMNLs; $n = 10$. $^{*}P < 0.05$ for HD at 25, 50, and 100 U/mL heparin versus no heparin. $^{\#}P < 0.05$ for NC pretreated with 1 μM PAF incubated with 100 U/mL heparin versus no heparin. $^{@}P < 0.05$ for NC at 100 U/mL heparin versus no heparin. (c) Early and late (total) apoptosis (sum of Annexin-V+/PI− and Annexin-V+/PI+) in NC PMNLs without stimulation (●) and after 15 min stimulation with 1 μM PAF (■) and HD PMNLs (▲). $^{*}P < 0.05$ for HD at 25, 50, and 100 U/mL heparin versus no heparin. $^{\#}P < 0.05$ for NC pretreated with 1 μM PAF incubated with 25, 50, and 100 U/mL heparin versus no heparin. $^{@}P < 0.05$ for NC at 100 U/mL heparin versus no heparin. (d) Superoxide release from NC PMNLs without stimulation (●) and after 15 min stimulation with 1 μM PAF (■) and HD PMNLs (▲). Following preincubation with PAF, cells were washed and incubated with increasing concentrations of heparin for 30 min. The rate of superoxide release was determined after cells activation with 0.32×10^{-7} M PMA. The changes in optical density were monitored at 549 nm continuously in the presence of 0.08 mM cytochrome C. Data are represented by nmoles/10^6 cells/10 min; $n = 10$. $^{*}P < 0.05$ for HD at 25, 50, and 100 U/mL heparin versus no heparin. $^{\#}P < 0.05$ for NC pretreated with 1 μM PAF incubated with 25, 50, and 100 U/mL heparin versus no heparin. $^{@}P < 0.05$ for NC at 25, 50, and 100 U/mL heparin versus no heparin.

the percentage of PAF-stimulated PMNLs which bound heparin. The more primed the PMNLs, the higher the percentage of cells that bound heparin. This binding was also prevented by preincubation with anti-CD11b antibodies applied before the addition of heparin (Figure 4(d)). These results indicate that heparin binding is mediated by CD11b and that the higher the priming state is, the more the heparin binds to PMNLs.

3.5. The Effect of Priming on Apoptosis Induced by Heparin. PAF-stimulated NC PMNLs showed increased apoptosis dependent on the cell priming state when incubated with heparin (Figures 5(a) and 5(b)) and apoptosis was increased concomitantly with increases in cell priming. Anti-CD11b which prevents the binding of heparin to PMNLs abolished the apoptotic effect of heparin at all PAF concentrations (Figure 5(b)). In addition, anti-CD11b also prevented the binding of heparin to primed PMNLs isolated from HD patients and almost completely abolished the apoptosis induced by heparin (Figure 5(b)).

The effect of 25 U/mL heparin on apoptosis of NC PMNLs was also examined before and following the stimulation with 1 μM PAF, using the TUNEL assay. Representative results are shown in Figure 5(c). Very few apoptotic cells (with green

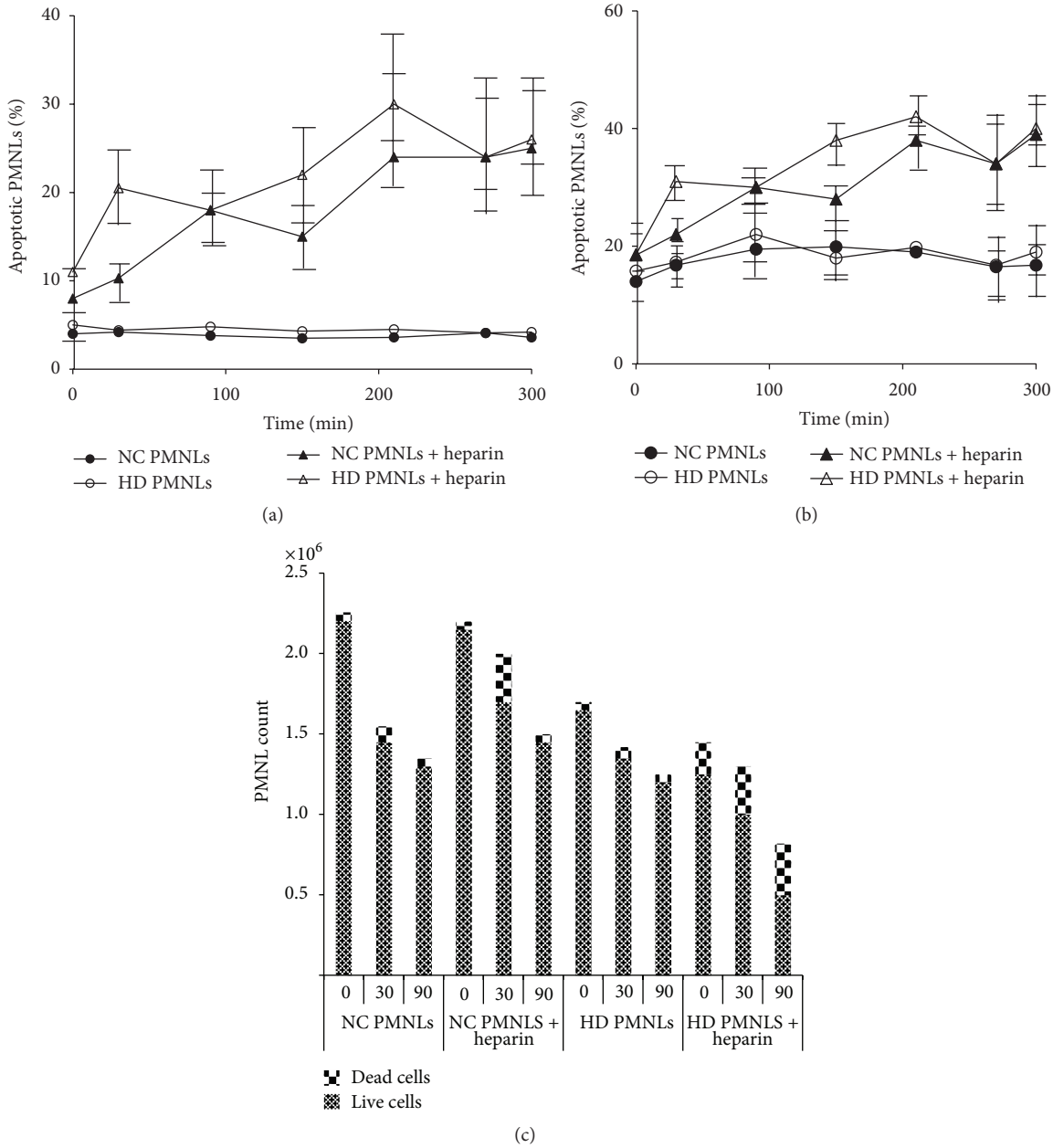

Figure 3: ((a), (b)) Early and total apoptosis (resp.) in PMNLs separated from NC (▲) and HD (△) after incubation with 100 U/mL of heparin for 300 min. The results of NC (●) and HD (○) represent the apoptotic levels throughout 300 min without heparin. Apoptosis was determined by flow cytometry using specific FITC-labeled Annexin-V, as described in Section 2. Data are expressed as percentage of apoptotic PMNLs; $n = 7$. (c) PMNL count using trypan blue exclusion assay: NC and HD PMNLs were incubated with 0 and 100 U/mL of heparin for 0, 30, and 90 minutes. Data is expressed as cells counts; $n = 3$.

nuclei, ~2%) were observed in NC PMNLs (Figure 5(c)(1)) and NC PMNLs stimulated by PAF (Figure 5(c)(2)) without exposure to heparin. When NC PMNLs were exposed to 25 U/mL heparin, the percentage of apoptotic cells increased to 6 ± 2%, together with 25% cell loss (Figure 5(c)(3)). When PAF-stimulated NC PMNLs were exposed to 25 U/mL heparin, the percentage of apoptotic cells increased to approximately 22 ± 5% (Figure 5(c)(4)), concomitantly with 65% cell disappearance. Exposure of the cells to anti-CD11b-PE antibodies prior to the addition of heparin completely abolished the apoptotic effects of heparin and cell disappearance (Figures 5(c)(5) and 5(c)(6)) on both NC PMNLs and NC PMNLs stimulated by PAF. Under the same conditions, exposure to isotype-controls- (IC-) PE (instead of to anti-CD11b-PE) did not prevent the apoptotic effects of heparin and cell disappearance (Figures 5(c)(7) and 5(c)(8)). These results clearly demonstrate that heparin-mediated apoptosis depends on PMNL priming.

In order to detect whether exposure of the cells to heparin causes loss of primed PMNLs, we further stained the

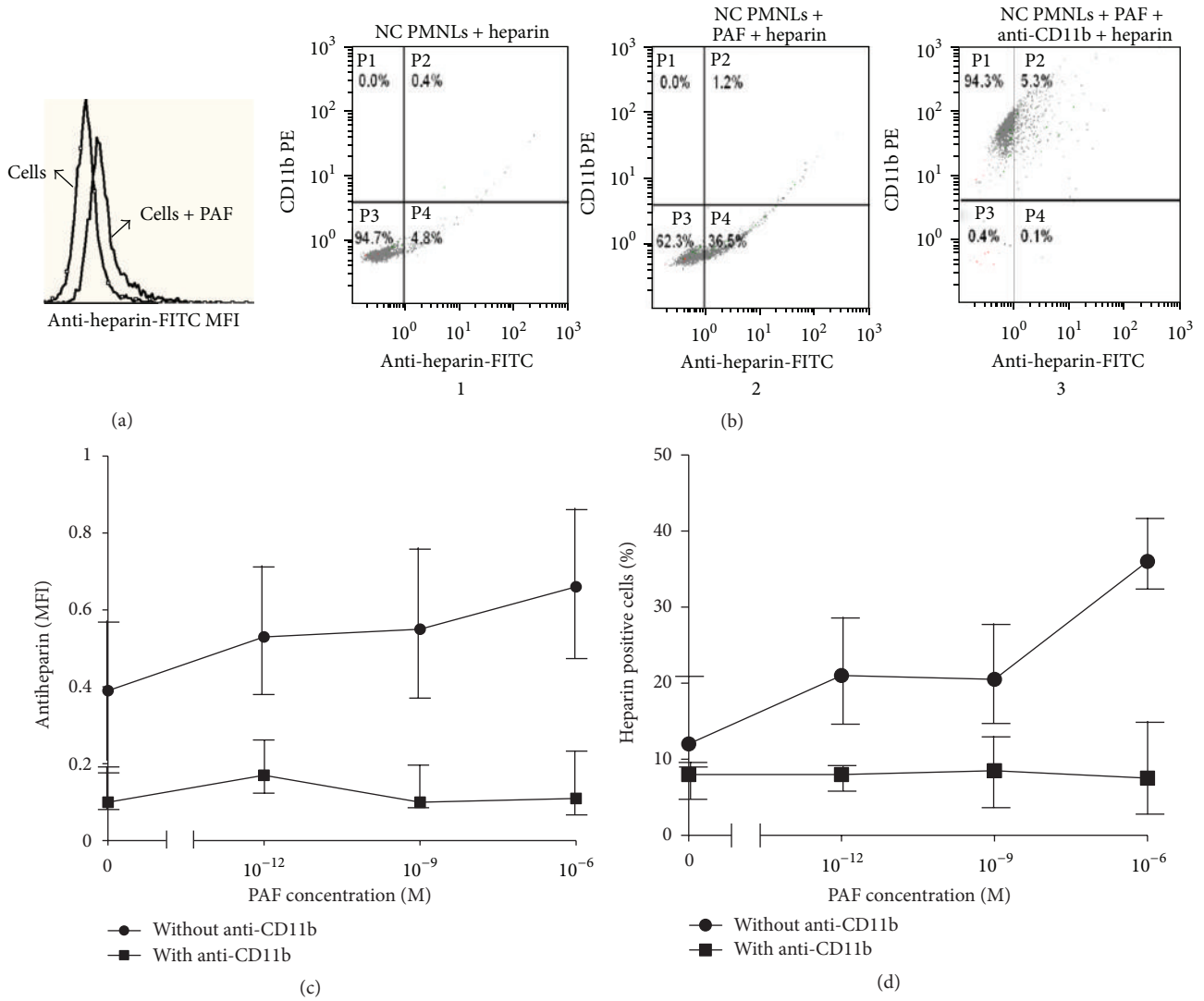

Figure 4: The effect of priming on heparin binding to PMNLs. (a) A representative histogram of flow cytometry, showing heparin binding intensity in NC PMNLs and NC PMNLs stimulated with 10^{-6} M PAF. (b) Representative histograms of flow cytometry showing percentage of heparin binding cells after incubation with 25 U/mL heparin for 30 min: NC PMNLs without PAF stimulation (1), NC PMNLs with 10^{-6} M PAF stimulation (2), and NC PMNLs with 10^{-6} M PAF stimulation and exposure to anti-CD11b-PE antibody (3). ((c), (d)) Levels of heparin bound to NC PMNLs (MFI) and percent of NC PMNLs that bind heparin, respectively, after preincubation with increasing concentration of PAF, determined by flow cytometry using specific FITC-labeled anti-heparin antibody, as described in Section 2 (●). Levels of heparin bound to PMNLs were also measured after preincubation of the cells with anti-CD11b antibodies (■) before the incubation with heparin ($n = 3$).

PAF-primed PMNLs with anti-CD11b-PE (Figure 6). Thirty percent of the PAF-treated cells that were not exposed to heparin showed elevated levels of CD11b, supporting their primed state. When these cells were exposed to heparin, no primed PMNLs or decreases in cell count could be seen.

3.6. The Effect of Anti-CD11b on Superoxide Release in the Presence of Heparin. Since heparin causes a decrease in superoxide release (Figure 2(d)) and at the same time it induces apoptosis via CD11b, it was interesting to find out whether blocking CD11b would have an effect on superoxide release. PAF-stimulated NC PMNLs incubated with IC showed increased rates of superoxide release which were dependent on the extent of the cell priming: the greater the priming state, the greater the rate of superoxide release (PMNLs; Figure 7). Heparin (25 U/mL) prevented this increase in superoxide release (PMNLs + heparin; Figure 7). PAF-stimulated NC PMNLs, incubated with anti-CD11b but not with heparin, showed a similar increased rate of superoxide release as with IC, which was also dependent on the extent of the cell priming (PMNLs + anti-CD11b; Figure 7). When anti-CD11b was added to PMNLs prior to the incubation of the cells with heparin (PMNLs + anti-CD11b + heparin), the effect of heparin in terms of inhibition of superoxide release was abolished (Figure 7).

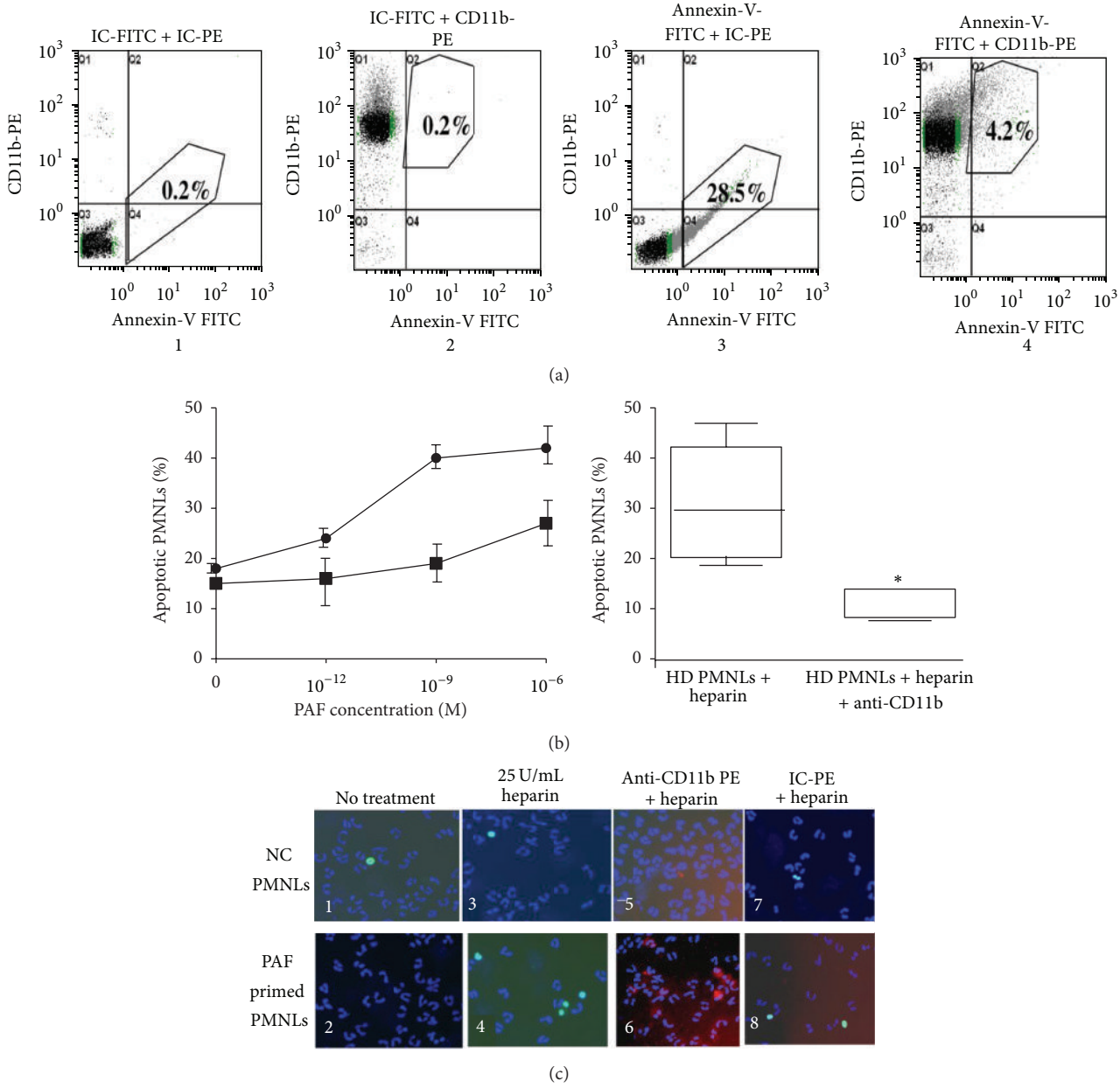

Figure 5: The effect of priming on apoptosis induced by heparin. (a) Representative histograms of flow cytometry showing the percentage of apoptotic cells after incubation with 25 U/mL heparin for 30 min: HD PMNLs with IC-FITC and IC-PE (1), HD PMNLs with IC-FITC and anti-CD11b-PE antibody (2), HD PMNLs with Annexin-V-FITC and IC-PE (3), and HD PMNLs with Annexin-V-FITC and anti-CD11b-PE antibody (4). (b) Apoptosis in NC PMNLs (●) after 15 min stimulation with increasing concentrations of PAF. Cells were preincubated with PAF, washed, and subjected to 30 min incubation with 25 U/mL heparin. Apoptosis was determined by flow cytometer analysis, using Annexin-V commercial kit as described in Section 2. Data are expressed as percentage of apoptotic PMNLs; $n = 3$. In addition, the percentage of apoptotic NC PMNLs that bind heparin was also measured after preincubation of NC separated PMNLs with PAF and anti-CD11b antibodies (■), before heparin incubation. The boxes and whiskers presentations represent apoptosis in HD PMNLs incubated with 25 U/mL heparin for 30 min, with and without incubation with anti-CD11b antibodies. (c) Apoptosis in NC PMNLs (upper left panel 1) and 15 min after stimulation with 10^{-6} M PAF (lower left panel 2). These cells were treated with 25 U/mL heparin for 30 min (panels 3 and 4). Apoptosis was evaluated by the TUNEL method (FITC green staining). Nuclei were stained with Hoechst reagent (blue staining). The percent of apoptotic NC PMNLs that bind heparin was measured after preincubation of the PMNLs with anti-CD11b antibodies (red staining) (panels 5 and 6) or IC-PE antibodies (panels 7 and 8) before incubation with heparin. Quantification of apoptosis: over 300 cells were counted, from at least three independent experiments.

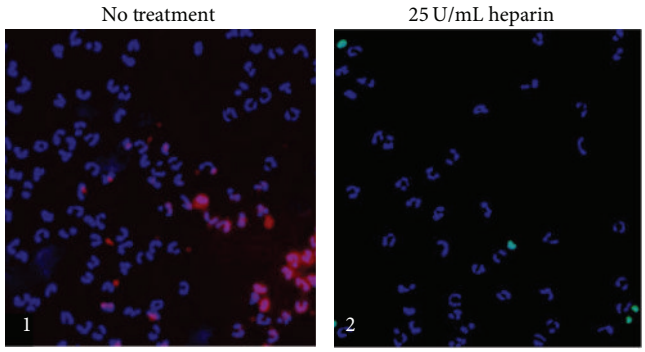

Figure 6: CD11b expression on primed PMNLs. CD11b expression on NC PMNLs after 15 min stimulation with 10^{-6} M PAF without an additional treatment (1) and with 30 min incubation with 25 U/mL heparin (2). Apoptosis was evaluated by the TUNEL method (FITC green staining). Nuclei were stained with Hoechst reagent (blue staining).

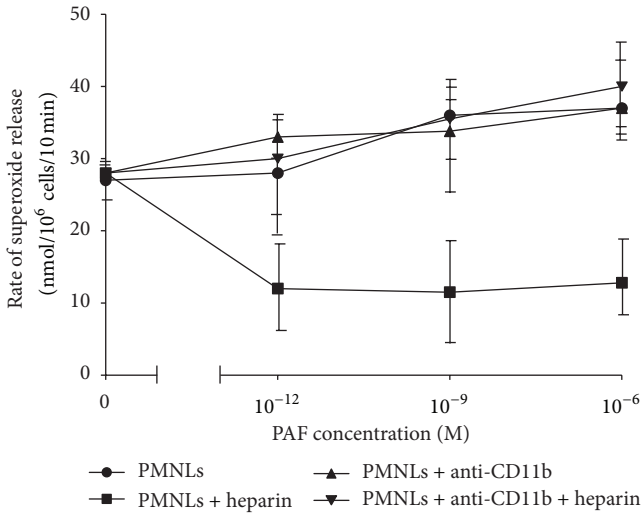

Figure 7: The effect of anti-CD11b on superoxide release in the presence of heparin. Superoxide release from NC PMNLs preincubated with increasing concentrations of PAF and IC-PE antibodies (●). In addition, superoxide release from NC PMNLs was also measured after 15 min preincubation with PAF and anti-CD11b-PE antibodies (▲). Furthermore, cells were washed and subjected to 30 min incubation with 25 U/mL of heparin (■). The rate of superoxide release from NC PMNLs was also measured after preincubation of NC PMNLs with PAF + anti-CD11b antibodies, before heparin incubation (▼). The rate of superoxide release was determined with 0.32×10^{-7} M PMA-stimulated PMNLs. The changes in optical density were monitored at 549 nm continuously in the presence of 0.08 mM cytochrome C. Data are represented as nmoles/10^6 cells/10 min; $n = 3$.

4. Discussion

In this study, we examined the effect of heparin on PMNLs. Our novel finding is that heparin exerts its apoptotic effect on primed PMNLs by binding to their CD11b. Moreover, heparin causes a significant dose-dependent decrease in the rate of superoxide release from PMNLs, blocking primed cells from further activation, partially explaining the anti-inflammatory effects of heparin.

It has been previously reported that heparin induces apoptosis in a dose-dependent manner in NC PMNLs [13]. In the present study, 30 min incubation with heparin enhanced apoptosis in separated human PMNLs in a dose-dependent fashion, however to a much greater extent in primed PAF-stimulated NC and HD PMNLs. In NC PMNLs, the increase in apoptosis was mild and became significant only at 100 U/mL of heparin. Interestingly, apoptosis time courses for NC or HD PMNLs incubated with heparin displayed an oscillatory pattern, with NC apoptosis lagging that of the HD PMNLs. This oscillatory behavior can be explained by the fact that 100 U/mL of heparin caused a time-dependent increase in apoptosis both in NC and in HD PMNLs (however faster and to a much greater extent in HD). The decline in apoptosis, for example, after 90 min for HD PMNLs and only after 150 min for NC PMNLs, can be explained by disappearance of the apoptotic cells as shown in our *ex vivo* survival studies: almost 40–50% of the HD PMNLs disappeared after 90 min, probably by disintegration. Why do we see another increase after the nadirs? We suggest that the next apoptotic peak is the result of an increase in CD11b expression *in vitro* during incubation in PBS (data not shown), which in the presence of heparin will result in apoptosis. Nevertheless, in the absence of heparin, such an increase in CD11b is not accompanied by enhanced apoptosis. Another possible explanation for increased apoptosis after 30 minutes can arise from a toxic effect. While we specifically measured priming and apoptosis, we do recognize that cell counts were decreasing over time (an effect we referred to as cell disintegration), which may suggest toxicity. Yet, when measuring cell activation, we made sure to count the same number of cells for each time point. Moreover, other markers of activation and priming were measured only on viable cells. Thus, even if cell toxicity is possible due to prolonged activation, the rate of superoxide release and levels of CD11b were measured on viable cells and support our conclusion that heparin can increase apoptosis through its actions mediated by CD11b.

Increases in PMNL priming as expressed by increased levels of CD11b and superoxide release resulted in dose-dependent increases of heparin binding to PMNLs. This increased binding is demonstrated both by the augmented intensity of heparin bound to the PMNLs and by the percentage of cells binding anti-heparin antibodies following incubation with heparin. In all experiments anti-CD11b antibodies competed with heparin for binding to CD11b, supporting the proposed mechanism of heparin-CD11b interactions as previously reported [11, 12]. Moreover, the percentage of PMNLs that bind heparin depends on the initial priming state of the cells; that is, a higher priming state results in a higher percentage of cells binding heparin. Finally, increases in cell priming resulted in augmented apoptosis by heparin in PMNLs, which was abolished by anti-CD11b antibodies added prior to the addition of heparin.

Heparin inhibited superoxide release from PMNLs and to a greater extent when cells were primed, such as HD and PAF-stimulated NC PMNLs. This indicates that heparin exerts its effect mainly on the subpopulation of cells that are

primed and are more abundant in HD and PAF-stimulated PMNLs. Since the population of primed PMNLs is the major contributor to superoxide release, rendering them apoptotic by heparin results in a decreased rate of superoxide release. These important findings indicate that the rates of superoxide release are increased due to a primed subpopulation of cells. In addition, the inhibitory effect of heparin on superoxide release from PMNLs was also abolished by anti-CD11b antibodies added prior to the addition of heparin.

Altogether, this study suggests a novel role for the interaction of heparin and PMNL CD11b, resulting in cell apoptosis. Apoptosis induced by heparin occurs when CD11b levels are increased or, in other words, when a subpopulation of primed PMNLs exists. As a result, heparin plays an anti-inflammatory role by making the primed cells apoptotic and preventing leakage of PMNL contents into the surrounding milieu. Heparan sulfate, a less sulfated form of heparin, is also known to bind to the PMNL CD11b [11], and recently we found it can promote apoptosis in separated PMNLs (data not shown), a characteristic that is dose-dependently affecting superoxide release from stimulated PMNLs [22]. The higher the heparan sulfate concentration, the lower the rate of superoxide release. More interesting, however, is the *in vivo* presence of heparan sulfate in the ECM and on the endothelial cell surface of vascular walls. Our studies on heparin raise the intriguing possibility that heparan sulfate may limit and/or regulate, to a certain degree, tissue injury and vascular damage by uncontrolled degranulation and release of reactive oxygen species and toxic contents into the surrounding milieu by activated transmigrating PMNLs.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Meital Cohen-Mazor and Rafi Mazor equally contributed to the preparation of this paper.

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Review Article

Neutrophils in Cancer: Two Sides of the Same Coin

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Neutrophils are the most abundant leukocytes in blood and are considered to be the first line of defense during inflammation and infections. In addition, neutrophils are also found infiltrating many types of tumors. Tumor-associated neutrophils (TANs) have relevant roles in malignant disease. Indeed neutrophils may be potent antitumor effector cells. However, increasing clinical evidence shows TANs correlate with poor prognosis. The tumor microenvironment controls neutrophil recruitment and in turn TANs help tumor progression. Hence, TANs can be beneficial or detrimental to the host. It is the purpose of this review to highlight these two sides of the neutrophil coin in cancer and to describe recent studies that provide some light on the mechanisms for neutrophil recruitment to the tumor, for neutrophils supporting tumor progression, and for neutrophil activation to enhance their antitumor functions.

1. Introduction

Neutrophils are the most abundant leukocytes in blood and are considered to be the first line of defense during inflammation and infections [1]. Invading microorganisms evoke an inflammatory response that recruits neutrophils from the circulation into the tissues. There, neutrophils destroy the microorganism by a series of mechanisms, mainly phagocytosis, release of antimicrobial substances, and the formation of neutrophil extracellular traps (NETs) [2]. Activated neutrophils also release proteinases into the surrounding tissue, causing damage to the host [3]. In addition, neutrophils are capable of producing many cytokines and chemokines, which can influence the inflammatory response, as well as the immune response [4, 5].

Besides this classical role in antimicrobial functions, neutrophils are also found infiltrating many types of tumors. Early studies suggested that these tumor-associated neutrophils (TANs) were mere bystanders because it was hard to imagine that neutrophils, being short-lived cells, could have an effect on chronic and progressive diseases such as cancer. However, more recently it is becoming clear that TANs have relevant roles in malignant disease. This renewed interest comes in part from the recognition that cancer-related inflammation is an important feature for the development of many tumors [6] and it is a hallmark of cancer [7]. Indeed, neutrophils may be potent antitumor effector cells [8]. The various antimicrobial and cytotoxic compounds contained in granules can destroy malignant cells, and cytokines and chemokines secreted by neutrophils can also recruit other cells with antitumor activity [5, 9].

However, an increasing number of clinical observations and laboratory studies have shown that presence of neutrophils in tumors correlates with poor prognosis. This has been well documented for bronchoalveolar carcinoma [10], melanoma [11], renal carcinoma [12], and head and neck squamous cell carcinoma (HNSCC) [13]. In all these cases, neutrophils display a protumor phenotype that could be adverse to the host. The tumor microenvironment controls neutrophil recruitment and in turn TANs help tumor progression. TANs are different from circulating neutrophils (as discussed later), and, in untreated tumors of murine models, they can display a protumorigenic phenotype. The mechanisms for this phenotype are just beginning to be

elucidated, but some of them involve genotoxicity, angiogenesis, and immunosuppression [8]. Hence, tumor-associated neutrophils can be beneficial or detrimental to the host [14]. These two types of TANs described in mice have been named N1 and N2 [15] in a similar manner as antitumor and protumor macrophages (TAMs) [16].

It is the purpose of this review to highlight these two sides of the neutrophil coin in cancer and to describe recent studies that provide some light on the mechanisms for neutrophil recruitment to the tumor, for neutrophils support to the tumor, and for neutrophil activation to enhance their antitumor functions and in the future improve cancer immunotherapy.

2. Neutrophils in Cancer

Our knowledge on the role of neutrophils in human cancers is relatively small. From an initial interest in the 1980s, the number of publications on neutrophils in cancer-related studies has been steadily going down [14]. However, this trend is now beginning to change with the realization that neutrophils are indeed important players in cancer development, as reflected by several recent reviews [16–18], and as we will see next.

In many patients with advanced cancer, elevated counts of neutrophils in blood are found. How tumors induce neutrophilia is uncertain, but production of granulocyte-macrophage colony-stimulating factor (GM-CSF) is a possible mechanism in several types of cancer [19]. In addition, other cytokines such as granulocyte colony-stimulating factor (G-CSF), interleukin- (IL-) 1, and IL-6 produced by tumors seem to contribute to elevated neutrophil numbers in blood [20]. This neutrophilia is associated with poor prognosis in several types of cancers, such as lung, melanoma, and renal carcinomas [11, 21, 22]. In agreement with this, the presence of neutrophils within certain tumors seems also to be an indicator of poor prognosis. Reduced recurrence-free time and overall survival were reported for neutrophil-infiltrated tumors in renal carcinomas [12], HNSCC [13], pancreatic adenocarcinomas [23], and liver carcinoma [24]. Because neutrophilia is frequently associated with inflammatory responses to infections and tissue damage, neutrophilia represents evidence for the concept of cancer-related inflammation inducing tumor progression [7].

2.1. The Neutrophil-to-Lymphocyte Ratio (NLR). The relation of neutrophil numbers in blood to other leukocyte counts has been suggested to serve as a prognostic factor for cancer. Thus, the neutrophil-to-lymphocyte ratio (NLR) was introduced as prognostic factor for colorectal cancer [25]. Due to its simplicity, NLR has shown to be a readily available and inexpensive biomarker for many types of tumors including non-small-cell lung cancer [26], hepatocellular carcinoma [24], nasopharyngeal carcinoma [27], colorectal cancer [28], melanoma [11], and breast cancer [29, 30]. In general, the blood NLR is elevated in patients with more advanced or aggressive disease, as indicated by increased tumor size, nodal stage, and number of metastatic lesions [31]. Also, a high NLR correlates with adverse overall survival in many solid tumors [32, 33].

Despite the clinical evidence from the many studies mentioned above, neutrophilia (larger numbers of neutrophils in blood as a consequence of elevated egress of cells from the bone marrow) is not always a bad indicator for cancer progression. In some types of tumors, for example, gastric cancer, an elevated neutrophil blood count is indicative of positive prognosis [34]. This means that neutrophils can control cancer in some instances. In fact, the capacity of neutrophils to directly kill tumor cells both *in vitro* and *in vivo* was reported long time ago [35–37]. Also, neutrophils from tumor-bearing animals were reported to have enhanced cytotoxic activity [38, 39]. And recently, neutrophils isolated from blood of some healthy individuals presented direct cytotoxicity against several tumor cell lines [40]. Therefore, the exact role of neutrophils within the tumor is a controversial matter [14, 41].

2.2. Myeloid-Derived Suppressor Cells (MDSCs). In addition to the elevated number of neutrophils in blood, an increase in the frequency of immature myeloid cells at earlier stages of differentiation has also been detected in several types of tumors [42], including terminal patients with lung, breast, and gastrointestinal cancer [43]. These immature cells consist of a heterogeneous population of immunosuppressive cells defined as myeloid-derived suppressor cells (MDSCs) [44]. These MDSCs can be divided phenotypically into granulocytic (G-MDSC) and monocytic (Mo-MDSC) subgroups [45, 46] and are found in great numbers in the spleens of tumor-bearing animals, where they display an immunosuppressive phenotype helping tumor progression [47, 48]. The G-MDSCs have immature neutrophil morphology and the consensus phenotype $CD33^+/CD11b^+/HLA\text{-}DR^{lo/-}/CD15^+$ in humans [49]. They have been found in peripheral blood of patients with glioblastoma [50], multiple myeloma [49], Hodgkin lymphoma [51], or head and neck cancer [52].

These MDSCs present various mechanisms of immunosuppression. The main mechanism involves production of reactive oxygen species (ROS) by the respiratory burst of these cells. In advanced cancer patients, the hydrogen peroxide (H_2O_2) produced by activated granulocytes reduced expression of the T cell receptor (TCR) CD3 ζ chain and decreased cytokine production by patients' T cells [53]. These oxidized human T cells had defective chemotaxis and presented impaired F-actin remodeling. The effect was found to be mediated by oxidation of the actin-remodeling protein cofilin [54]. Cofilin is activated through dephosphorylation at Ser3, and then it mediates severing and depolymerization of F-actin for formation of the immune synapse and T cell activation. Cofilin oxidation induced formation of an intramolecular disulfide bridge that prevents its activation, thus leading to impaired T cell activation [54]. Also, long-term oxidative stress leads to translocation of cofilin into the mitochondria and necrotic-like programmed cell death takes place in human T cells [55]. In addition, exposure of ROS to memory/effector $CD45RO^+$ T cells results in inhibition of NF-κB activation and reduction in Th1 cytokines production

[56]. Furthermore, MDSC-produced ROS can lead to $CD8^+$ T cell tolerance by another mechanism involving peroxynitrite [57]. ROS can combine with nitric oxide and form peroxynitrite, which is highly reactive at short distances. During MDSC-T cell contact, peroxynitrite induces nitration of the T cell receptor and CD8 molecules. This process makes CD8-expressing T cells unable to bind peptide-MHC complexes and to respond to the specific peptide [57].

Another mechanism for T cell suppression is production of Arginase 1 (ARG1) by MDSCs. ARG1 inhibits T cell proliferation by degrading extracellular arginine, which results in decreased responsiveness of T cells to CD3/TCR stimulation [58]. For example, in patients with non-small cell lung cancer, TANs had reduced intracellular ARG1 and tumor-infiltrating lymphocytes showed reduced proliferation in response to CD3/TCR stimulation. All non-small cell lung cancer cell lines secreted IL-8, and IL-8 was effective in triggering ARG1 release [59]. Also, in patients with glioblastoma, degranulated neutrophils associated with elevated levels of serum ARG1 correlated with decreased T cell CD3 zeta chain expression in peripheral blood T cells, resulting in immunosuppression [60]. Together, these mechanisms explain how MDSC-produced ROS and ARG1 mediate T cell suppression in cancer settings.

Because, G-MDSCs share many properties with neutrophils [61] but seem to be functionally different from mature neutrophils [13, 62], a transcriptomic analysis was conducted to compare in tumor-bearing animals circulating neutrophils with TANs and with MDSCs [63]. It was concluded that indeed TANs are not "tissue-based" G-MDSC but a distinct population of neutrophils [63]. However, at present it is not clear whether TANs are mature neutrophils or represent a special category of cells such as immature neutrophils with protumor properties.

2.3. Phenotypes of Tumor-Associated Neutrophils (TANs). Depending on the phenotype displayed by TANs, they have been classified in tumor-bearing mice as N1 or N2 [15]. Similarly to antitumor tumor-infiltrating macrophages (M1), N1 cells display proinflammatory and antitumorigenic functions. In contrast, M2 and N2 cells display protumorigenic activity [16]. TANs seem to be different from circulating neutrophils and also from G-MDSC in the bone marrow and spleen [44, 63]. Upon transforming growth factor-beta (TGF-β) blockade, murine $CD11b^+/Ly6G^+$ neutrophils recruited to tumors were hypersegmented and more cytotoxic to tumor cells and expressed higher levels of proinflammatory cytokines [15]. In contrast, depletion of these neutrophils decreased tumor growth and resulted in more activated $CD8^+$ T cells intratumorally. Thus, it seems that TGF-β within the tumor microenvironment induces a population of TANs with a protumor phenotype [15]. In support of this idea, in two models of murine tumor cancer cell lines (Lewis lung carcinoma and AB12 mesothelioma), neutrophils were found primarily at the periphery of the tumor at early stages of tumor development. These TANs were more cytotoxic toward tumor cells and produced higher levels of tumor necrosis factor-alpha (TNF-α), NO, and H_2O_2. In contrast, TANs in established tumors had these functions downregulated and presented a more protumorigenic phenotype [64]. These results showed that neutrophils enter the tumor and become more protumor with tumor progression [64]. Therefore, murine TANs can have an antitumorigenic (N1) phenotype but also a protumorigenic (N2) phenotype capable of supporting tumor growth and suppressing the antitumor immune responses [14, 41], depending on the tumor microenvironment [17].

Despite this classification, the nature and function of TANs in the cancer microenvironment remain largely unknown, particularly with human tumors. However, two recent publications describe the phenotype of neutrophils infiltrated into human tumors. In one study of surgically resected lung cancer patients, TANs were isolated from digested human lung tumors and constituted 5%–25% of the cells in the tumor. These TANs presented an activated phenotype ($CD62L^{lo}/CD54^{hi}$) with expression of a distinct repertoire of chemokine receptors that included CCR5, CCR7, CXCR3, and CXCR4 [65]. In addition, TANs produced larger quantities of the proinflammatory factors MCP-1, IL-8, MIP-1α, and IL-6 than blood neutrophils did. TANs could also stimulate T cell proliferation and interferon gamma (IFN-γ) release. These results indicate that, in the earliest stages of lung cancer, TANs are not immunosuppressive but rather stimulate T cell responses [65]. In the second study, the role of chronic inflammation, particularly via IL-23 and IL-17, in developing human colorectal cancer was investigated. Authors found that innate $\gamma\delta$T ($\gamma\delta$T17) cells were the major cellular source of IL-17 in colorectal cancer. Tumor growth led to epithelial barrier disruption allowing microbial products to induce inflammatory dendritic cell accumulation and $\gamma\delta$T17 polarization in human tumors. These activated dendritic cells induced $\gamma\delta$T17 cells to secrete IL-8, TNF-α, and GM-CSF, thus leading to accumulation of neutrophils in the tumor. These TANs were characterized by $CD45^+/Lin^-/HLADR^-/CD11b^+/CD33^+/CD66b^+$ and displayed typical polymorphonuclear morphology. Thus, they were described as G-MDSC [66]. These TANs (G-MDSC) produced much more ARG1 and ROS than autologous neutrophils and inhibited proliferation of activated autologous T cells and IFN-γ production [66].

The TANs described in these reports show that in human tumors the dual role of neutrophils is also observed. In early tumors, TANs seem to be able to stimulate T cell responses [65], but later in established tumors TANs are immunosuppressive [66]. These important reports are just "the tip of the iceberg" in our understanding of the origin and function of TANs. Many questions remain—for example, are TANs in early tumors mature neutrophils with antitumor properties and TANs in established tumors immature cells (G-MDSCs) with immunosuppressive properties directly recruited from the circulation? Or are TANs mature neutrophils that develop a more protumor phenotype with tumor progression?—as suggested by several tumor animal models and cancer patients [17, 64]. A very recent publication identifies several subpopulations of neutrophils in the blood of tumor-bearing mice and in human cancer patients and describes several

relationships of these cells in connection to cancer progression [67].

In this study, subpopulations of circulating neutrophils in cancer animals could be distinguished according to their densities. One subpopulation is composed of "normal" high-density neutrophils (HDNs). The other subpopulation has lower density neutrophils (LDNs) that copurify with the low-density mononuclear cells layer formed when separating leukocytes by density gradient centrifugation [68]. In tumor-free mice, most neutrophils were HDNs, but in tumor-bearing animals LDNs increased progressively and often became the dominant neutrophil type in circulation [67]. The HDNs from cancer animals, which were previously reported as tumor-entrained neutrophils (TENs) [69], displayed high cytotoxicity toward tumor cells in culture, whereas LDNs were not cytotoxic [67]. Also, the LDNs had reduced expression of various chemokines (CXCL1, CXCL2, CXCL10, CCL2, and CCL3) and chemokine receptors (CXCR2 and CCR5), consistent with a reduced inflammatory state. The LDN subpopulation consists of large mature (fully formed lobulated nucleus) neutrophils and also of immature neutrophils, similar to G-MDSC. The authors then showed by BrdU labeling that LDNs rapidly accumulate in the circulation, whereas HDNs appear in the circulation much later. This is consistent with the idea that some of the LDNs are indeed immature neutrophils. In addition, authors showed that HDNs are capable of becoming LDNs upon treatment with TGF-β [67]. It is interesting to note that TGF-β was able to induce the change of HDNs from tumor-bearing mice into LDNs, but it had no effect on HDNs from tumor-free mice [67]. This indicates that other stimuli are also needed for this change in animals with cancer. For example, treatment of naïve healthy mice with recombinant G-CSF protein elicited G-MDSC similar to those induced in tumor-bearing animals [70]. Together, all these results support a model proposed by the authors, in which neutrophils are present in three subpopulations in cancer: normal high-density neutrophils, immature low-density neutrophils (G-MDSC), and large mature low-density neutrophils. These cell types present diversity in function and plasticity. While the HDNs are antitumor and the LDNs are protumor [67], they can change under the influence of the different chemokines and cytokines in the tumor microenvironment [17].

3. Recruitment

Solid tumors are composed of several cell types, including tumor cells and stromal cells. The tumor stroma contains fibroblasts, endothelial cells of blood vessels, and in many cases immune cells. These tumor-infiltrating immune cells highlight the inflammatory microenvironment that is commonly associated with tumor progression [7]. In addition to lymphocytes and macrophages, neutrophils are found in great numbers in a wide variety of tumors [12, 13, 23, 24]. Clearly, neutrophils are recruited to the tumor by the action of neutrophil-attracting chemokines that can be produced not only by other immune cells but also directly by several tumor cells (Figure 1). The most effective neutrophil chemokine is interleukin-8 (IL-8/CXCL8). It was found that oncogenic Ras induced IL-8 expression [71], and Ras-expressing mouse adenomas produced KC/CXCL1 and MIP-2/CXCL2, the murine equivalents of IL-8, to attract TANs [72]. These findings suggested that TANs are recruited to help the tumor. Accordingly, increased IL-8 levels were found in HNSCC patients [13], and elimination of neutrophils in cancer murine models reduced tumor burden [73] and metastasis [74]. Deleting IL-8 receptors also reduced tumor growth [75]. These findings support the notion that tumor-produced IL-8 is important for neutrophil recruitment to help tumor progression [76]. However, the IL-8 receptors CXCR1 and CXCR2 are also expressed on other cell types including endothelial cells and tumor cells. Thus, determining the extent of neutrophil involvement in IL-8-mediated tumor progression will require future studies.

Using the same CXCR1 and CXCR2 receptors, neutrophils can also respond to other chemokines such as CXCL1, CXCL2, CXCL5, CXCL6, and CXCL7 [77] (Figure 1). CXCL2 can induce neutrophil infiltration in tumors, and it was suggested that this is an autocrine effect [78]. Supporting this idea, it was also found that in TANs the expression of CXCL2, and also CXCL1, was upregulated more than 150-fold [63]. Therefore, it seems that neutrophils activate a positive feedback mechanism by releasing neutrophil chemokines that attract more neutrophils into the tumor, similarly to neutrophil recruitment into sites of infection [79]. The role of ENA-78/CXCL5 in appearance of TANs in carcinoma of the liver was investigated in 919 patients with hepatocellular carcinoma. CXCL5 was found to be overexpressed in patients with recurrent tumors, and the levels of CXCL5 correlated with greater appearance of TANs and with shorter overall survival [80]. Another chemokine that also participates in neutrophil recruitment to tumors is GCP-2/CXCL6. In a melanoma mouse model, specific anti-CXCL6 monoclonal antibodies reduced the number of TANs and also tumor size [81]. In addition, migration inhibitory factor (MIF), another tumor-derived chemokine for neutrophils, was identified in HNSCC tumors. MIF was described as an inhibitor of macrophage migration *in vitro*, but it is now known that it also binds CXCR2 [82] (Figure 1). Tumor-derived MIF levels correlated with higher TANs levels and poor survival of these patients [83].

Many tumor cells can directly produce chemokines for neutrophils, but various other cells within the tumor may also be the source for these chemokines and other cytokines. In particular, activated T cells are known to produce GM-CSF, CXCL1, CXCL2, TNF-α, and IFN-γ [84]. These factors could directly or indirectly recruit more neutrophils to the tumor. Although the influence of activated T cells in neutrophil recruitment to tumors is not known, regulatory T cells (Treg) seem to be important for neutrophil infiltrating tumors. In one study, Treg were found to inhibit neutrophil recruitment to a tumor site by reducing the expression of CXCL1 and CXCL2 [85]. In contrast, in another study, Treg promoted neutrophil infiltration to tumors by producing IL-8 [86]. Thus, the influence of T cell function on the appearance of TANs needs to be further explored. In addition, TANs can also recruit more Treg. Murine TANs secrete CCL17,

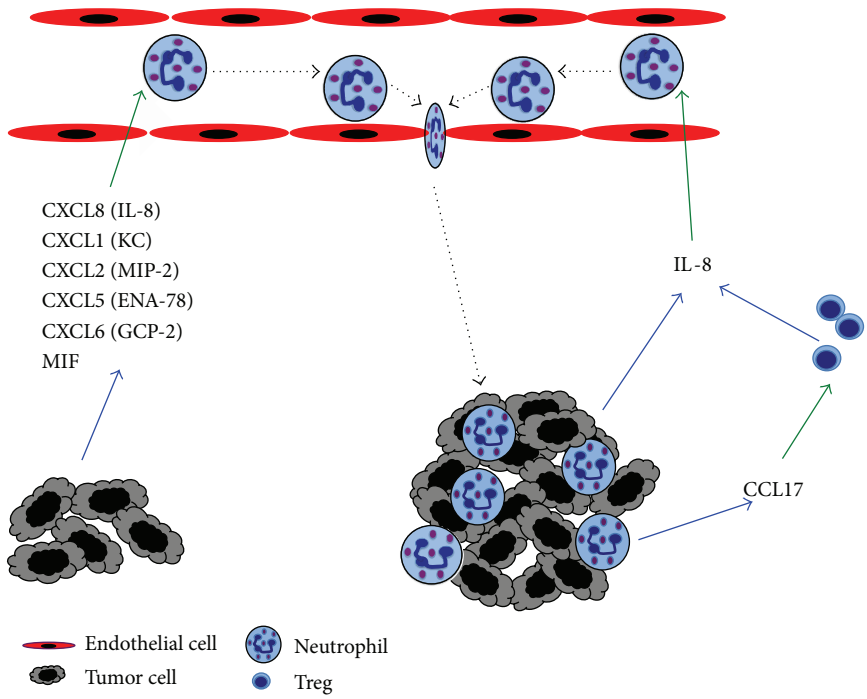

Figure 1: Mechanisms of neutrophil recruitment to tumors. Tumor cells produce many chemokines, such as CXCL1 (KC), CXCL2 (MIP-2), CXCL5 (ENA-78), CXCL6 (GCP-2), CXCL8 (IL-8), and MIF, which are chemoattractants for neutrophils. These cells then migrate out of the blood circulation into the tumor. Tumor-associated neutrophils can also produce CCL17, an important chemoattractant for regulatory T cells (Treg). These inhibitory Treg in turn produce more IL-8, the most potent chemoattractant for neutrophils, creating a positive loop for more neutrophil infiltration into the growing tumor. Blue arrows denote molecules secreted by cells. Green arrows denote the action of molecules on cells. Dotted lines denote cell movement.

a potent chemokine for Treg, at higher levels than circulating or splenic neutrophils [87] (Figure 1). Moreover, the amounts of CCL17 increased progressively during tumor progression. It seems then that TANs and Treg act together to impair antitumor immunity [87].

4. Protumor Function of Neutrophils

A large body of clinical evidence indicates that neutrophils are involved in cancer development and tumor progression. In most cases, large numbers of TANs are associated with advanced disease and poor prognosis for cancer patients. This negative association has been reported for several solid tumors, such as melanoma, hepatocellular carcinoma, non-small cell lung carcinoma, glioma, HNSCC, adenocarcinoma, and colon cancer [41, 88].

Neutrophils display several protumor functions. Most of them have just recently begun to be revealed. These functions involve the same molecules neutrophils use to destroy microorganisms and to modulate inflammation. Important molecules that can modify growth and invasiveness of tumors involve granule proteins, matrix-degrading proteinases, reactive oxygen species (ROS), chemokines, and cytokines. Recent reports describe how TANs use these molecules to affect cell proliferation, angiogenesis, metastasis, and immune surveillance (Figure 2).

4.1. Neutrophil Molecules

4.1.1. Neutrophil Elastase. Neutrophil elastase (NE) is a major protein of azurophilic granules that is released upon cell degranulation. The main physiologic function of NE seems to be elimination of invading microorganisms [89], but it also has important inflammatory effects [3]. NE is a serine protease with a broad range of substrates; among them are neutrophil-derived antibacterial proteins, extracellular matrix proteins, integrins, cytokines, and cytokine receptors. In addition to its roles in inflammation and bacteria destruction, NE has presented various protumor effects both *in vivo* and *in vitro* [90]. NE was found to directly promote A459 tumor cell proliferation when murine neutrophils were cocultured with this lung carcinoma cell line [91]. This effect was markedly reduced when tumor cells were cocultured with $NE^{-/-}$ neutrophils, or in the presence of an NE inhibitor. The effect of NE on tumor growth was dependent on phosphatidylinositol 3-kinase (PI-3K), since it was also reduced in the presence of a PI-3K inhibitor [91]. Staining experiments showed that NE got inside the tumor cells via clathrin-coated pits and localized at early endosomes [92]. Once inside the cell, NE acted on insulin receptor substrate-1 (IRS-1). Because IRS-1 binds to the regulatory unit of PI-3K, its degradation by NE led to more PI-3K available to enhance the proliferation pathway [93]. Similar results have been found with other types of tumor cells, including esophageal cancer [94], gastric cancer [95], and breast cancer [96]. In these cases,

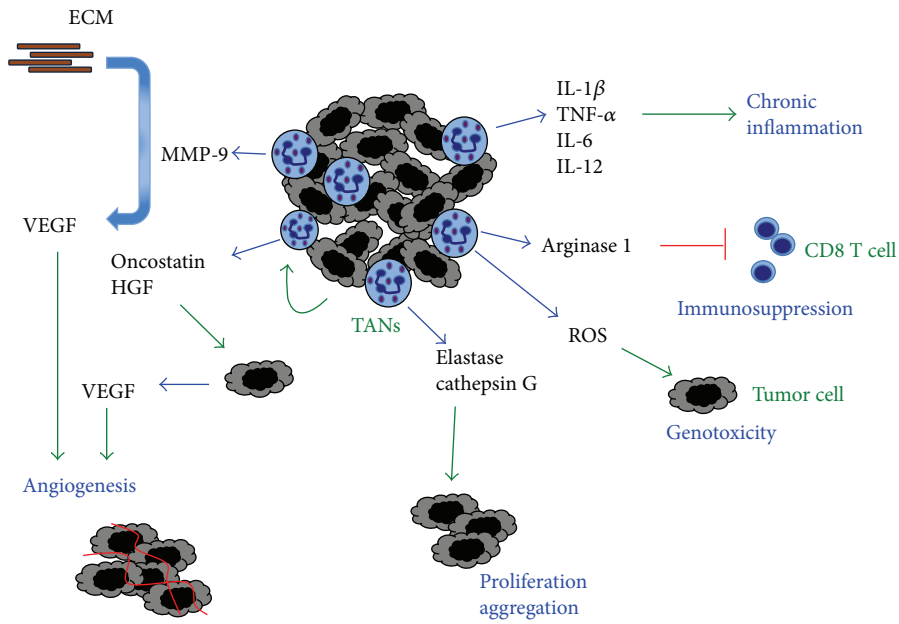

Figure 2: Protumor activity of neutrophils. Tumor-associated neutrophils (TANs) help tumor progression in several ways. TANs can secrete matrix metalloproteinase-9 (MMP-9) that releases vascular endothelial growth factor (VEGF) from the extracellular matrix (ECM) to promote angiogenesis. TAN can secrete cytokines (IL-1β, TNF-α, IL-6, and IL-12) that induce a chronic inflammatory state and arginase 1, which inhibits CD8 T cells, creating an immunosuppressive state. TANs also produce reactive oxygen species (ROS) that can damage DNA, inducing genotoxic effects on tumor cells. Serine proteases, such as elastase and cathepsin G, from neutrophil granules seem to have a direct effect on tumor cells for inducing proliferation. Certain tumors, like breast cancer cells, induce neutrophils to produce Oncostatin, an IL-6-like cytokine that then stimulates breast cancer cells to secrete vascular endothelial growth factor to promote angiogenesis (red lines represent new blood vessels). Also, hepatocellular carcinoma cells induce neutrophils to release hepatocyte growth factor (HGF), which activates tumor cells to become more invasive. Blue arrows denote molecules secreted by cells. Green arrows denote the action of molecules on cells.

NE mediated release of transforming growth factor-alpha (TGF-α) from the cell surface. Furthermore, NE has also been found to promote migration of tumor cells. Coculture of human neutrophils with pancreatic ductal adenocarcinoma cells (PDAC) resulted in dyshesion of cells from the monolayer. The same effect was observed by adding NE to PDAC cultures and correlated with loss of surface expression of E-cadherin [97]. NE also enhanced the migratory capacity of esophageal cancer cells [94].

4.1.2. Cathepsin G. Cathepsin G is a peptidase from azurophilic granules that participates in degradation of phagocytosed microorganisms and in remodeling of extracellular matrix (ECM) proteins [98]. Also, cathepsin G can promote angiogenesis and tumor cell migration [99–101]. Breast cancer MCF-7 cells form spherical cell aggregates when incubated with neutrophils. This process involves cell adhesion via E-cadherin and requires cathepsin G [99]. Moreover, the process has been shown to involve two steps. First cathepsin G binds to the tumor cell surface, independently of its catalytic site, and then induces cell aggregation, which is dependent on its enzymatic activity [99] (Figure 2). Cathepsin G degrades ECM molecules such as fibronectin and attenuates binding between integrins and fibronectin. This leads to E-cadherin-mediated homotypic cell-cell adhesion, which is protease-resistant [101]. The formation of these tumor cell aggregates would allow tumor cells to disseminate via the circulation to distant sites and establish new metastases. Once at the new site, tumor cells would need new vasculature. In a model of breast cancer metastasis to the bone, it was also found that cathepsin G enhanced TGF-β signaling and upregulated vascular endothelial growth factor (VEGF) to promote angiogenesis [100]. Together, these reports indicate that TANs-derived cathepsin G may induce ECM remodeling and promote tumor progression and metastasis [102, 103].

4.1.3. Matrix Metalloproteinase-9. Matrix metalloproteinase-9 (MMP-9/gelatinase B) is released from secondary (specific) granules and is believed to help neutrophils in the process of extravasation via degradation of ECM proteins. MMP-9 was found to promote tumor proliferation in a human papilloma virus- (HPV-) 16 skin carcinogenesis model. MMP-$9^{-/-}$ mice showed reduced keratinocyte proliferation, but this phenotype was reversed when bone marrow-derived leukocytes were transplanted into irradiated mice [104]. Also, immunostaining of MMP-9 in squamous cell carcinoma tumors showed that MMP-9 was found only in tumor infiltrating leukocytes and not in tumor cells [104]. In addition, MMP-9 has been shown to inhibit apoptosis of tumor cells in the lung [105]. Thus, MMP-9 supplied by bone marrow-derived cells is responsible for enhancing tumor proliferation via both increased proliferation and reduced apoptosis of tumor cells.

Another important effect of MMP-9 that supports tumor growth is angiogenesis. The vascular endothelial growth factor (VEGF) is sequestered in the ECM after it is produced by cells (Figure 2). The proteolytic release of VEGF from tissue ECM via MMPs is regarded as a prerequisite for *in vivo* induced angiogenesis [106, 107]. The angiogenic effect of MMP-9 has been reported in several cancer models. Melanoma cells were transfected to overexpress the GCP-2/CXCL6 chemokine and then implanted into nude mice. The new CXCL6-melanoma tumors grew larger and with a well-developed vasculature than wild type (WT) melanomas [108]. These larger tumors also presented higher levels of MMP-9 and induced a strong influx of TANs [108]. Similarly, in a model of pancreatic adenocarcinoma, new dysplastic lesions that develop into carcinomas are formed with enhanced angiogenesis. This process has been named the angiogenic switch [109]. In these new lesions, MMP-2 and MMP-9 were upregulated. MMP inhibitors and genetic ablation of MMP-9 reduced the angiogenic switching, tumor number, and tumor growth [109], indicating that MMP-9 can render normal islets angiogenic. In addition, malignant keratinocyte transplantation resulted in tumors with neutrophils expressing predominantly MMP-9 and stromal cells expressing mainly MMP-2 and MMP-3 [110]. Depletion of a singular MMP did not affect neovascularization of malignant murine keratinocytes.

These reports suggested a direct role for MMP-9 in tumor angiogenesis, but they did not identify the cell type producing this protease. Reconstitution of tumor-bearing MMP-$9^{-/-}$ mice with wild type, MMP-9-competent hematopoietic cells demonstrated that tumor-infiltrating myeloid cells were the source for MMP-9 [111, 112]. In a murine model of pancreatic islet carcinogenesis, MMP-9-expressing neutrophils were predominantly found inside angiogenic islet dysplasias and tumors, whereas MMP-9-expressing macrophages were localized along the periphery of such lesions. Transient depletion of neutrophils significantly reduced the frequency of initial angiogenic switching in dysplasias [113]. Also TANs in melanoma or fibrosarcoma tumors expressed high levels of MMP-9 and VEGF, and elimination of these TANs resulted in reduced tumor growth [114]. Also, reducing TANs in prostate carcinoma tumors reduced angiogenesis and tumor cell intravasation [115]. Moreover, in cancer patients, neutrophils expressing high levels of MMP-9 have also been found. In HNSCC, expression of MMP-9 was larger by TANs than by any other cell type in the tumor [116], and in hepatocellular carcinoma larger numbers of TANs correlated with more angiogenesis [117, 118]. Direct proof for neutrophils being the major tumor-associated leukocyte type expressing MMP-9 was recently provided in a study employing human xenografts and syngeneic murine tumors [119]. When tumors or isolated TAMs and TANs were double-stained for MMP-9 and for respective macrophage- or neutrophil-specific antigens, only TANs gave a strong signal for MMP-9 [119, 120]. In addition, it was calculated that 1×10^6 neutrophils or TANs could release approximately 100–200 ng proMMP-9 within 1-2 h of incubation. In contrast, 1×10^6 macrophages or TAMs would require several weeks to produce the same amount of proMMP-9 [119, 120]. Hence, neutrophil-derived MMP-9 is responsible for enhancing angiogenesis via release of VEGF from the ECM in many types of tumors (Figure 2).

The unusual angiogenic potency of neutrophil MMP-9 is related to its unique way of production. In other cell types, the zymogen proMMP-9 is released together with the inhibitor of metalloprotease 1 (TIMP-1), which slows the activation of MMP-9 and can also inhibit the proteolytic activity of the once activated enzyme [121]. Therefore, the TIMP-1-free proMMP-9 from neutrophils can be activated easier and function much longer than MMP-9 from other cell types [122, 123].

4.1.4. Reactive Oxygen Species. Neutrophils are efficient producers of reactive oxygen species (ROS) for destruction of microorganisms. ROS can also indirectly promote tumor growth. First, neutrophils generate hydrogen peroxide (H_2O_2), which is next converted to hypochlorous acid (HOCl) by myeloperoxidase (MPO). HOCl can then activate several ECM-degrading MMPs, including MMP-2, MMP-7, MMP-8, and MMP-9. Also, HOCl can block TIMP-1 and in this manner potentiate the proteolytic activity of MMPs [124, 125]. Finally, as indicated above, MMP activity leads to enhanced tumor progression by inducing proliferation and angiogenesis.

Nevertheless, a more potent and direct effect of ROS on tumor cells is genotoxicity, which might lead to carcinogenesis (Figure 2). Although neutrophil-derived ROS and HOCl can directly damage and destroy tumor cells, they can also cause genotoxicity in circumstances when they do not kill cells. ROS-mediated genotoxicity is induced by two major pathways: oxidative DNA damage and MPO catalyzed activation of chemical carcinogens [126]. Point mutations and DNA strand breaks are induced in many different cell types when cocultured with neutrophils [126], and HOCl has been reported to be mutagenic in lung epithelial A549 cells [127].

4.1.5. Arginase 1 (ARG1). Upon release from neutrophil granules, ARG1 gets activated to degrade extracellular arginine, an essential amino acid for proper activation of T cells. Thus, degranulation of neutrophils may exert an immunosuppressive effect in tumors by inhibiting T cells in a similar manner to the one described for G-MDSC [88]. In fact, depletion of TANs in tumor-bearing animals increased the numbers of activated $CD8^+$ T cells and promoted smaller tumors [15]. Similarly, non-small cell lung cancer cells stimulated neutrophils through IL-8 to release ARG1, and in tumors TANs had reduced levels of ARG1 [59]. More recently, the same group found that ARG1 released from gelatinase granules was inactive at physiological pH unless activated by factor(s) stored in azurophil granules [58]. Thus, TANs can induce ARG1-dependent immunosuppression through concomitant exocytosis of gelatinase and azurophil granules (Figure 2).

4.1.6. Cytokines. Neutrophils can also produce cytokines or growth factors, which increase the tumorigenic potential of cancer cells [5]. Two clear examples have been described for

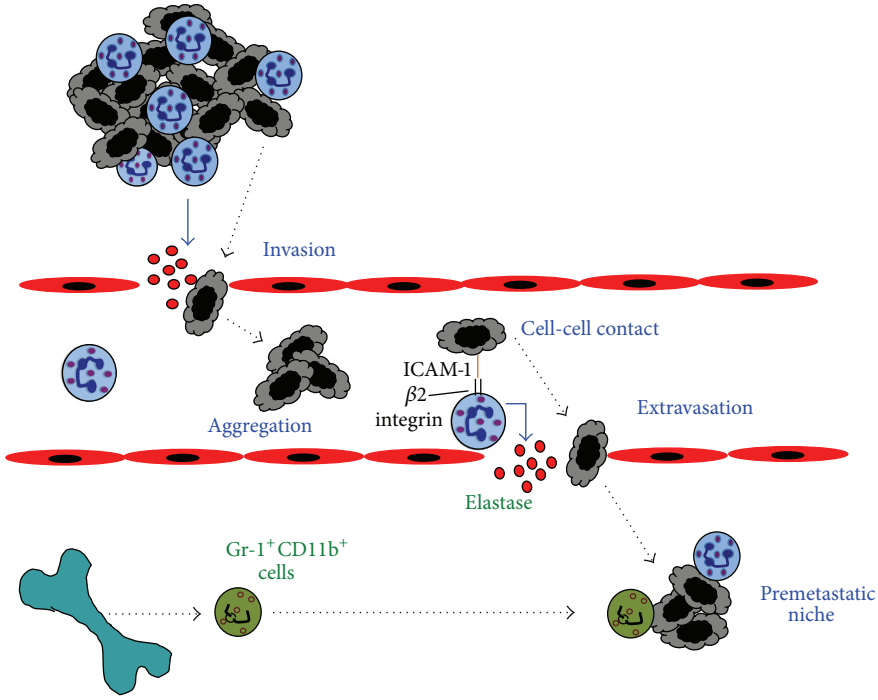

Figure 3: Neutrophils can promote tumor cell invasion and metastasis. Tumor-associated neutrophils (TANs) help tumor invasion in several ways. TANs can secrete enzymes, such as elastase (red dots), that degrade the basement membrane and promote tumor cell invasion through the basement membrane. Once in circulation, neutrophils can also help tumor cells to survive by inducing tumor cell aggregation. Circulating tumor cells can directly adhere to arrested neutrophils via the adhesion molecule ICAM-1 on the tumor cells, and $\beta 2$ integrins on neutrophils. This cell-cell interaction promotes extravasation of the tumor cells. Bone marrow-derived cells including neutrophil precursors ($Gr\text{-}1^+CD11b^+$ cells) migrate to premetastatic niches where they secrete factors that promote tumor cell growth. Blue arrows denote molecules secreted by cells. Dotted lines denote cell movement.

Oncostatin-M [128–130] and for hepatocyte growth factor [10, 131, 132]. Breast cancer cells can stimulate neutrophils to release Oncostatin-M, an IL-6-like cytokine. Oncostatin-M in turn stimulated breast cancer cells to secrete VEGF [133] (Figure 2). Similarly, hepatocellular carcinoma cells stimulated neutrophils to release hepatocyte growth factor (HGF). In turn, HGF stimulated tumor cells to become more invasive [134] (Figure 2).

4.2. Metastasis. Neutrophils can also influence the migration potential of cancer cells. In several types of cancer it has been shown that neutrophils promote metastasis. These tumors include skin squamous cell carcinoma [135], melanoma [136], adenocarcinomas [137], HNSCC [83], and breast cancer [138]. The way neutrophils augment the migratory capacity of tumor cells involves many different mechanisms that are just beginning to be elucidated.

Tumors can induce activation of neutrophils to release inflammatory factors that promote tumor migration. In HNSCC, tumor-derived MIF not only recruits TANs but also induced these cells to display promigratory effects on the tumor cells [83]. Similar responses have been documented for different cancer cell lines but through a different mediator. Various tumor cells release hyaluronan, which can then activate neutrophils via TLR4 and the PI-3K/Akt signaling pathway. In turn, neutrophils induce enhanced migration of the tumor cells [139].

Very early reports suggested that TANs release enzymes that degrade the basement membrane and promote tumor cell invasion through the basement membrane [137] (Figure 3). *In vitro* studies showed that human neutrophils assist the human breast tumor cell line MDA-MB-231 to cross a monolayer of endothelial cells [140]. Tumor cell-conditioned medium downregulated neutrophil cytotoxicity and upregulated expression of adhesion molecules, facilitating tumor cell migration. In contrast, MDA-MB-231 cells alone did not transmigrate [140]. Also, in the presence of neutrophils, melanoma cell adhesion and transmigration through an endothelial cell monolayer were increased [141, 142] (Figure 3). This process seems to involve at least in part the protease NE, which can induce severe tissue damage, and as mentioned before correlates with poor prognosis [90]. Elevated amounts of NE in various types of cancer can induce tumor invasion and metastasis by degrading ECM proteins [143]. In support of this, it was reported that inhibition of NE could reduce metastasis to the liver [144].

Once in circulation, neutrophils can also help tumor cells to survive by inducing tumor cell aggregation (Figure 3). In patients with breast and prostate cancers, tumor cell clusters in blood have been associated with poor survival [145], and in animal models, injection of tumor cell clusters resulted in more metastases than injection of dispersed tumor cells [146]. At least, for breast cancer MCF-7 cells, neutrophils can

promote aggregation *in vitro* [99, 101]. However, metastasis induced by neutrophil-mediated aggregation of tumor cells has not yet been directly demonstrated *in vivo*.

Circulating tumor cells directly adhere to the vascular endothelium promoting extravasation for establishing new metastases. At the site of exit, lung cancer tumor cells have been observed in close association with neutrophils [147]. In this process, neutrophils enhance tumor cell retention and in consequence induce more metastasis [148] (Figure 3). Direct cell-cell interaction of neutrophils with breast carcinoma cells has been shown to involve the adhesion molecule ICAM-1 on the tumor cells and $\beta 2$ integrins on neutrophils. Neutrophils bound tumor cells engaging integrins and inducing ICAM-1 clustering on the tumor cell [148] (Figure 3). This activated in the tumor cell a signaling pathway involving focal adhesion kinase (FAK) and p38-MAPK that resulted in enhanced migration [138]. In addition, this enhanced migration was shown *in vivo* to result in increased metastasis to the liver [149]. Here, the cancer cells adhered directly on top of arrested neutrophils, which acted as a bridge to facilitate interactions between the tumor cells and the liver parenchyma [149].

Moreover, neutrophils seem to participate in facilitating metastasis even before the tumor cells arrive to the new site, the metastatic niche. This is a potential metastatic site where leukocytes create a permissive growth environment prior to the arrival of tumor cells [150, 151]. VEGFR1-positive bone marrow-derived cells are found in premetastatic niches of organs involved in metastasis of particular tumor types [152]. Once at the metastatic niche, these bone marrow-derived cells secrete factors that promote tumor cell growth [152, 153] (Figure 3). In lungs of mice bearing mammary adenocarcinomas, Gr-1$^+$CD11b$^+$ cells were significantly increased before tumor cells arrived. These granulocytic cells had decreased IFN-γ production and increased MMP-9 production, thus promoting angiogenesis [154]. In addition, coinjection of with 4T1 tumor cells with these Gr-1$^+$CD11b$^+$ cells, isolated from tumors and spleens of 4T1 mammary tumor-bearing mice, resulted in increased metastases to lungs [155]. But because these Gr-1$^+$CD11b$^+$ cells are a heterogeneous population of cells, including neutrophils, macrophages, dendritic cells, and other immature myeloid cells, the particular cell type(s) needed to promote metastasis remains unclear. However, neutrophils are a good candidate because it has been reported that circulating neutrophils augment in number with increasing metastatic potential of various rat mammary adenocarcinomas [156], and tumors secreting IL-8 also have an increased metastatic potential [124]. Clearly, the mechanisms TANs use to promote tumor cell migration and metastasis are diverse and complex (Figure 3).

5. Antitumor Function of Neutrophils

Despite the large amount of evidence for a negative role of neutrophils during tumor progression, there is also clear evidence for a positive role of neutrophils in carcinogenesis. As mentioned before, neutrophils can display antitumor activity in different forms. Early murine neutrophils infiltrating tumors have been named N1 since they clearly display an active proinflammatory and an antitumor phenotype [15]. In fact, the antitumor capacity of neutrophils has been recognized for more than three decades. Neutrophils can directly kill tumor cells both *in vitro* [36] and *in vivo* [37].

Neutrophils potentiate this antitumor effect when they have been activated. For example, a colon adenocarcinoma cell line transfected to express G-CSF lost tumorigenic activity after considerable concentration of neutrophils at the tumor site [157]. Interestingly, neutrophils could discriminate between G-CSF-producing and G-CSF-nonproducing cells and directly inhibited only G-CSF-producing tumor cells [157]. This antitumor effect of activated neutrophils can also be transferred to other animals, as demonstrated with spontaneous regression/complete resistance (SR/CR) mice. SR/CR mice resist very high doses of cancer cells that are lethal to WT mice even at low doses. The genetic, cellular, and molecular effector mechanisms in this model are largely unknown. However, purified neutrophils from the SR/CR mice independently killed cancer cells *in vitro* and completely transferred resistance to WT recipient mice [158]. Also, the cancer disappeared gradually following infiltration of a large number of neutrophils and few lymphocytes into the remaining tumor tissues [159]. The importance of N1 type TANs in antitumor responses is also highlighted by reports showing that depletion of murine neutrophils results in enhanced tumor growth [15, 160, 161].

Despite the evidence presented before on neutrophils helping metastasis by preparing the metastatic niche, a complete opposite effect has also been demonstrated for metastatic breast cancer [69] and renal carcinoma [162]. In both models, neutrophils prevented metastasis to the lung. In the breast cancer model, the tumor cells produced CCL2 that induced neutrophil ROS production [69], while, in the renal carcinoma model, tumor-derived IL-8 recruited tumor cytotoxic neutrophils [162]. This goes against the majority of reports implicating IL-8 in protumor functions of neutrophils. Nevertheless, these findings underline the dual antitumor and protumor potential of neutrophils and suggest that neutrophils could be induced to enhance their antitumor responses.

5.1. Mechanisms of Tumor Killing. Neutrophils clearly have the potential of directly killing tumor cells. The mechanisms by which neutrophils accomplish this function are numerous and not completely understood, but they involve many of the same antimicrobial and immune regulatory functions of neutrophils (Figure 4).

5.1.1. ROS. Early reports indicated that neutrophils from tumor-bearing animals displayed enhanced superoxide anion generation and phagocytosis. This led to reduced tumors and less metastatic foci in lungs [38, 39]. Also, it has been shown that indeed ROS produced by neutrophils can induce tumor cell lysis, through HOCl delivered directly at the cell membrane [163]. Although ROS could be genotoxic for tumor cells, it is clear that, in the case of rapidly growing tumors, activated neutrophils producing sufficient singlet oxygen can

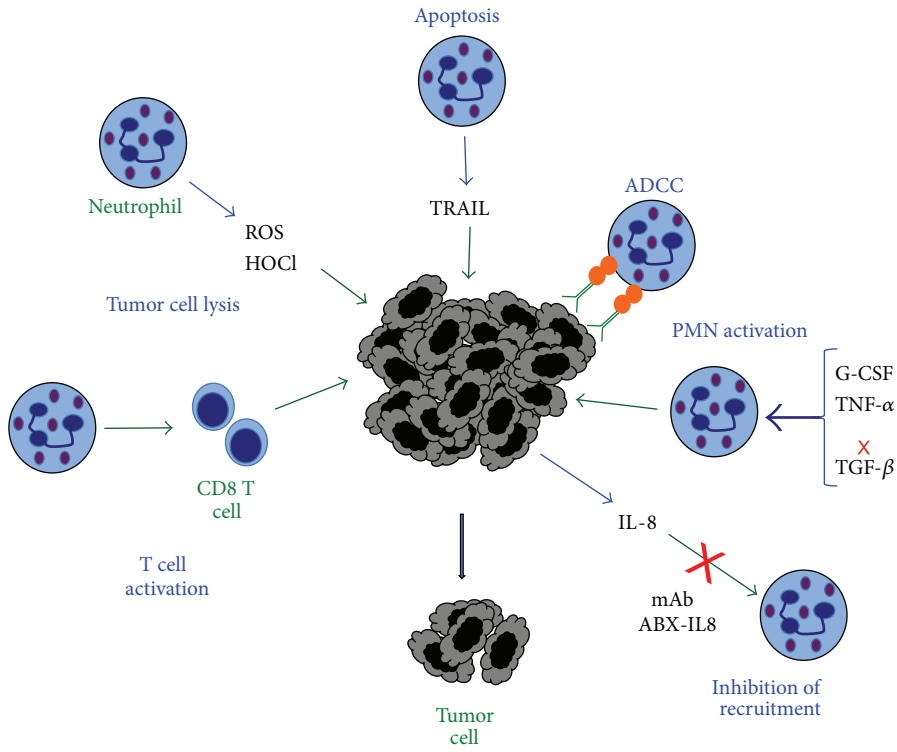

Figure 4: Antitumor activity of neutrophils. Neutrophils produce reactive oxygen species (ROS) and hypochlorous acid (HOCl) that can directly damage and destroy tumor cells. By direct contact or by release of TRAIL, neutrophils can also induce apoptosis of certain tumor cells. The most effective antitumor mechanism is antibody-dependent cell-mediated cytotoxicity (ADCC). Antibody molecules (green) that bind to tumor antigens are recognized by Fc receptors (orange circles) on neutrophils. This binding activates a cytotoxic response against the tumor cell. Neutrophils can be activated to display a stronger antitumor phenotype with granulocyte colony-stimulating factor (G-CSF), transforming growth factor-α (TNF-α), or by blocking (red cross) transforming growth factor-β (TGF-β). Also, the blockage of IL-8, with specific monoclonal antibodies (such as mAb ABX-IL8), can prevent new neutrophil infiltration into growing tumors. Inflammatory neutrophils can also activate cytotoxic (CD8) T cells. All these mechanisms result in smaller tumors. Blue arrows denote molecules secreted by cells. Green arrows denote the action of molecules on cells.

eliminate tumor cells at the early phase of tumor development [164] (Figure 4).

5.1.2. Direct Lysis and Apoptosis. Because neutrophils require close contact mediated by integrins to induce killing, it is also possible that neutrophils may induce direct lysis of tumor cells by a mechanism similar to the one used by NK cells via the enzymes perforin and granzyme [165]. However expression of these enzymes in neutrophils is controversial [166, 167]. Neutrophils can also induce certain tumor cells to undergo apoptosis. Neutrophils induced apoptosis of human breast cancer cells, when stimulated by antibodies targeted to HER-2 [168].

5.1.3. TRAIL. Neutrophils have another way of eliminating tumor cells by inducing apoptosis of the malignant cell. This effect is mediated by the tumor necrosis factor-related apoptosis inducing ligand (TRAIL) (Figure 4). For a long time, carcinoma *in situ* of the bladder has been treated with intravesical administration of *Mycobacterium bovis* bacillus Calmette-Guérin (BCG). This kind of immunotherapy is very effective for treatment of this type of cancer [169], but the mechanism is only partially known [170].

It was then found that neutrophils in urine of patients with carcinoma of the bladder and under BCG immunotherapy expressed high levels of TRAIL [171]. Neutrophils from these patients can selectively induce apoptosis of tumor cells [172]. TRAIL is expressed on these neutrophils at high levels both as a type II membrane protein (intracellular amino terminal portion and carboxyl terminus outside the cell) and as a biologically active soluble form [173], which is released from intracellular stores after interaction with components of the BCG cell wall [174].

TRAIL is a member of the TNF family of molecules, known to have apoptosis-inducing functions [175]. TRAIL binds to target cells through two death receptors (DRs) (DR4/TRAIL-R1 and DR5/TRAIL-R2) and three decoy receptors (DcRs) [DcR1/TRAIL-R3, DcR2/TRAIL-R4, and osteoprotegerin] [176]. DRs activate the formation of a death-inducing signaling complex for caspase activation and initiation of apoptosis [177].

An important feature of neutrophil TRAIL-induced apoptosis is that it can kill tumorigenic and transformed cells but not normal cells and tissues [170, 178]. For this reason, TRAIL is becoming a major physiologic weapon against cancer [172], and several research laboratories and pharmaceutical companies are developing recombinant forms of

TRAIL or TRAIL receptor agonists for therapeutic purposes [178]. In addition, the importance of TRAIL in other clinical conditions, such as infectious diseases, autoimmunity, and cardiovascular diseases, is becoming more apparent. Therefore, understanding the regulatory mechanisms of TRAIL signaling will help in the future to control these health problems [178].

5.1.4. Matrix Metalloproteinase-8. Neutrophils can protect against some tumors by secreting MMP-8. In mice deficient in MMP-8, an increase in skin tumors with an increase in neutrophil infiltrates to the tumors was reported [179]. This protective effect is not clearly defined, but it involves the inhibition of neutrophil migration into the tumor site.

5.1.5. Antibody-Dependent Cell-Mediated Cytotoxicity. Antibodies directed to tumor cells can also bind to Fc receptors on the membrane of immune cells [180]. In many cases, the antibody activates these cells to destroy the tumor cell. This antibody-dependent cell-mediated cytotoxicity (ADCC) is capable of eliminating efficiently various types of tumors. NK cells are particularly efficient in this response via the Fcγ receptors [181] (Figure 4). Neutrophils also present efficient ADCC against cells that have been marked by antibodies [182]. However, the mechanism of killing is not completely described, but it seems to be different from the classic ADCC mechanism used by NK cells.

It is worth noting that an important difference exists between murine and human neutrophils regarding FcγR expression [183]. In addition to FcγRIII, the only Fcγ receptor on murine NK cells, murine neutrophils also express FcγRIV. In contrast, human neutrophils express two unique Fcγ receptors not present in other species: FcγRIIa (CD32a) (homolog to murine FcγRIII) and FcγRIIIb (CD16b) (a glycosylphosphatidylinositol- (GPI-) linked receptor). Human NK cells only express FcγRIIIa (homolog to murine FcγRIV) [183]. Therefore special attention should be paid when interpreting data from murine models on ADCC against tumors. Human neutrophils present a more efficient ADCC when they engage FcγRIIa [184, 185]. Under stimulated conditions mainly with IFN-γ but also with G-CSF, neutrophils can upregulate expression of FcγRI (CD64). This receptor seems also capable of promoting neutrophil ADCC against tumors [186] and in particular with squamous head and neck cancer [187]. However, in other studies, it was shown that immature neutrophils with high expression of FcγRI had reduced ADCC activity via this receptor [188]. In fact, ample reports have demonstrated that the high affinity receptor for IgA, FcαRI (CD89), is a more potent inducer of ADCC by neutrophils [188, 189].

The mechanism for tumor cytotoxicity from neutrophils is not completely known, and it seems to be multifactorial. Both ROS-dependent and ROS-independent mechanisms have been suggested for neutrophil ADCC [190]. For the oxidative mechanism, close cell contact mediated by integrins is required for direct release of HOCl to the tumor cell [163]. However, studies with neutrophils from chronic granulomatous disease (CGD) patients and with ROS scavengers suggest that ROS are not as important for ADCC as they are for antimicrobial functions [191]. Another proposed mechanism is direct cell lysis via perforin and granzyme [165]. However, as mentioned before, expression of these enzymes in neutrophils remains controversial [166, 167].

5.1.6. Regulation of T Cell Function. Neutrophils invading tumors can modify T cell effector functions and in this way instruct T cells to reject tumors. Cytotoxic $CD8^+$ T cells are key contributors in any immune response towards tumors. As mentioned before N2 neutrophils can be inhibitors of T cell functions [15, 192]. However, the proinflammatory N1 neutrophils can recruit and activate $CD8^+$ T cells [15, 193] (Figure 4). Also, after photodynamic therapy, there was a rapid neutrophil infiltration into the treated tumor bed. Neutrophils were necessary for generation of tumor-specific primary and memory $CD8^+$ T cell responses [160]. Together, these reports indicate that neutrophils can influence the outcome of T cell functions depending on the type of cytokines they produce [4, 194].

6. Neutrophil Extracellular Traps

Neutrophil extracellular traps (NETs) constitute a recently described form of the antimicrobial arsenal of neutrophils. NETs are fibers of chromatin released from neutrophils in an active process named NETosis [195]. In this process, neutrophils undergo dramatic changes starting with flattening of the cells. Next, chromatin decondensation with histone modifications takes place. Citrullination of histone H3 by peptidylarginine deiminase 4 (PAD4) is a major modification during NETosis. The nucleus loses its typical lobular morphology and the nuclear membrane disappears. Finally, DNA is released from the cell [196]. DNA fibers in NETs are also decorated with various antimicrobial proteins from the neutrophil granules, including neutrophil elastase, MPO, cathepsin G, proteinase 3, MMP-9, and bactericidal/permeability increasing protein (BPI) [197]. NETs form a mesh-like structure where microorganisms get trapped and are either directly killed on some cases or more often subsequently phagocytosed by other neutrophils [198, 199].

Many microorganisms and various stimuli can directly stimulate NET formation. Bacterial products such as lipo polysaccharide (LPS), formyl-methionyl-leucyl-phenylalanine (fMLF), and also phorbol esters such as phorbol myristate acetate (PMA) are efficient NET inducers [200]. Recent reports also indicated that antigen-antibody complexes are capable of inducing NET formation, thus suggesting a direct role for Fc receptors in this function [201]. In fact both FcαRI [202] and FcγRIIIb [203] have been shown to induce NET formation [204]. Also, some cytokines such as TNF-α and IL-8 can also enhance NET formation [205]. This is interesting because various tumors are known to produce these cytokines and thus it is possible that tumors can enhance NET formation. However, this idea has not yet been proved in any type of cancer.

6.1. NETs in Tumors. The role of NETs in cancer is just beginning to be elucidated. Very little is known about the presence and effect of NETs in different types of tumors. It is also not clear if distinct TANs can make NETs with different efficiency. In an initial study, tumor samples from eight patients with Ewing sarcoma were evaluated for the presence of TANs and NETs, defined as extracellular staining for MOP. In two (25%) patients, intratumoral NETs were found. After surgery these patients presented early relapse. Thus, it was proposed that at least this type of tumor could induce TANs to release NETs [206]. This idea has not been confirmed in other types of cancer. However, in cancer models of chronic myelogenous leukemia and mammary and lung carcinoma, peripheral neutrophils were more prone to NET formation. Neutrophils from tumor-bearing animals responded to platelet-activating factor (PAF) forming more NETs than neutrophils from tumor-free animals. In addition, higher amounts of circulating neutrophils and plasma cell-free DNA were found in tumor-bearing animals [207]. This free DNA is probably in the form of NETs, since a concomitant increase in neutrophils with hypercitrullinated histone H3 was also found [207]. It seems then that some cancers may present a systemic effect on the host that predisposes neutrophils to form NETs.

As discussed earlier, many tumors presenting TANs are associated with poor prognosis. In many of these tumors, free DNA has been found. Thus the presence of NETs in these tumors most certainly would be associated with tumor progression. Supporting this idea, there are studies looking at the phenotype of TANs during tumor development. In one study, neutrophil depletion at 14 days after implantation of Lewis lung carcinoma (LLC) and AB12 mesothelioma tumors resulted in reduced tumor growth. In contrast, neutrophil depletion at 7 days after implantation had no effect on tumor growth. TANs from early tumors were more cytotoxic toward tumor cells, while TANs from established tumors acquired a more protumorigenic phenotype [64]. Moreover, in initial tumors, TANs were found in the periphery of the tumor, but in mature tumors TANs and free DNA were within the tumor [64]. In another study, sparc$^{-/-}$ mice had defective collagen assembly within secondary lymphoid organs. This defect caused an uneven compartmentalization of lymphoid and myeloid populations that led to aberrant interactions between NETs and B cells. Under these conditions, NETs induced B cell proliferation and inhibition of apoptosis, resulting in malignant transformation [208]. Together, these data support a model for primary tumor development. Neutrophils would migrate to the new tumor and there TANs would produce NETs, which would promote tumor growth (Figure 5).

Although evidence strongly indicates that NETs within primary tumors can promote tumor progression, no mechanism for this effect has been revealed yet. However, because NETs are made of chromatin fibers decorated with antimicrobial proteins such as neutrophil elastase, cathepsin G, and MPO, it is very likely that NETs concentrate these factors to high local concentrations within the tumor microenvironment. As discussed above, these factors have all been implicated in tumor promotion. Therefore, NETs may be a way to enhance exposure of tumor cells to these bioactive proteins and in turn increase proliferation, inhibit apoptosis, and induce migration (Figure 5).

7. Therapeutic Approaches

Although in many instances the presence of neutrophils in tumors has a negative effect in cancer disease, these cells clearly have the capacity to destroy tumor cells. Several novel therapeutic approaches are being considered to enhance the antitumor potential of neutrophils or to block the access of TANs into growing tumors. These approaches are briefly described next.

7.1. Activation of Neutrophils. N1 type murine neutrophils display an activated phenotype that leads to tumor control. In consequence, tumor cells modified to express G-CSF induced recruitment of neutrophils that were able to inhibit tumor growth [157]. Activation of neutrophils with G-CSF and IFN-β can generate cells with an antitumor phenotype [114]. Due to the important role of neutrophils in antimicrobial responses, general activation of these cells is not good therapeutic approach since highly activated neutrophils without targeting specificity could cause excessive tissue damage.

The two types of TANs, N1 and N2, suggest that the tumor microenvironment could be manipulated to generate more antitumor TANs. This idea is supported by studies in murine cancer models where inhibition of TGF-β induced the appearance of antitumor neutrophils. These cells produced high levels of proinflammatory cytokines and could kill tumor cells [15].

7.2. Inhibition of Neutrophil Infiltration into Tumors. Another therapeutic approach aims to block infiltration of neutrophils into tumors. As indicated before, several tumors produce chemokines, mainly IL-8, which recruits neutrophils to the tumor. The use of IL-8 antagonists (such as the fully humanized neutralizing monoclonal antibody ABX-IL8) to IL-8 was shown to reduce tumor growth, metastasis, and angiogenesis of melanoma [209] and lung cancer [75]. Because other chemokines also interact with the receptors CXCR1 and CXCR2 [77], a more effective way to block neutrophil migration may be the inhibition of these receptors. Specific inhibitors for these receptors are now being developed with the idea of preventing neutrophil infiltration and retarding tumor progression [210]. For example, the CXCR2 receptor antagonist, GSK135756, is being considered to be used as an anti-inflammatory drug for chronic obstructive pulmonary disease. If GSK135756 is approved, it could have anticancer potential [211]. Another small-molecule inhibitor for CXCR1 is reparixin. This inhibitor has shown to efficiently block neutrophil recruitment into tissues and to selectively target human breast cancer stem cells in xenograft models in mice [212].

7.3. Inhibition of Neutrophil-Specific Enzymes. In addition to blocking neutrophil infiltration, inhibition of particular neutrophil-specific enzymes known to promote tumor progression is another therapeutic avenue being explored.

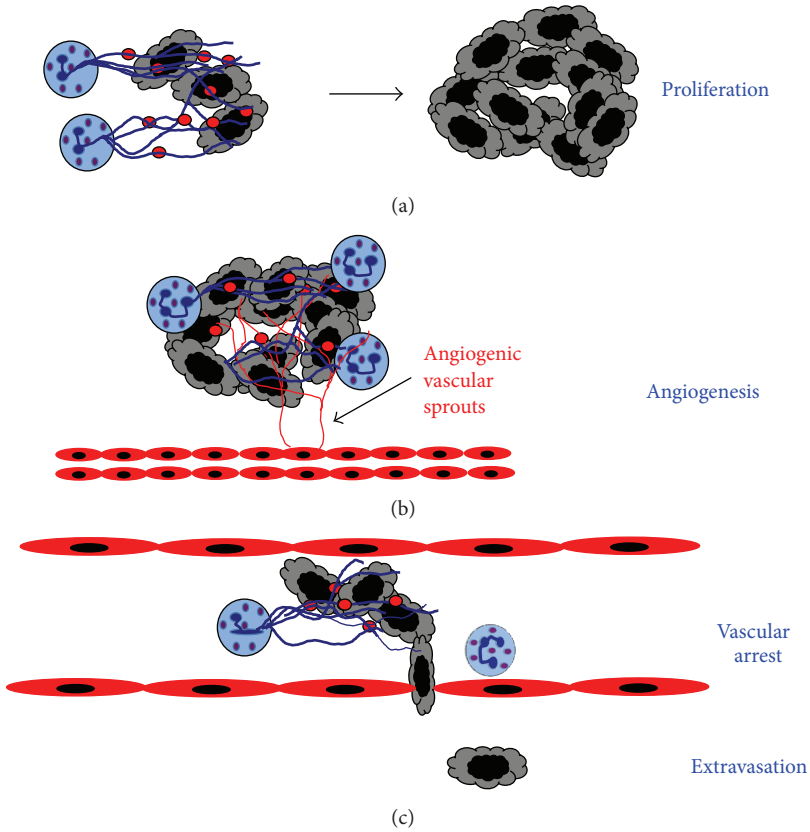

Figure 5: Neutrophil extracellular traps (NETs) can induce tumor progression. Tumor-associated neutrophils can produce NETs (blue lines), which are chromatin fibers decorated with proteins from neutrophil granules (red circles). (a) Tumor cells trapped in these NETs would get exposed to high local concentrations of neutrophil elastase and other factors that induce cell proliferation. (b) NETs could also provide large amounts of matrix metalloproteinase-9 and serine proteases that would release vascular endothelial growth factor to promote angiogenesis. (c) NETs released on the vascular endothelium in response to inflammation could trap tumor cells allowing them to more easily arrest and extravasate the blood circulation into prometastatic sites.

For example, inhibition of NE was able to reduce significantly the growth of lung adenocarcinomas in a mouse model [91]. Also inhibition of MMP has been tried to prevent tumor angiogenesis. The bisphosphonate zoledronic acid, a strong MMP inhibitor, blocked MMP-9 expression and metalloprotease activity reducing angiogenesis and cervical cancer burden [213]. However, in other models and clinical trials, inhibition of MMP-9 was not effective at reducing tumor growth [214, 215].

7.4. Therapeutic Antibodies for ADCC. A more promising approach is the use of antitumor monoclonal antibodies (mAbs) to activate the ADCC potential of neutrophils. Upon Fc receptor activation, neutrophils produce ROS and release mediators with direct antitumor potential [216]. Today most mAbs used in immunotherapy belong to the IgG1 class, and they are effective at activating NK cells via FcγRIIIa (CD16a) [181]. In contrast, neutrophils activate ADCC via FcγRIIa (CD32) by preferentially engaging IgG2 class antibodies [185]. This IgG2-mediated ADCC was influenced by the functional FcγRIIa-R131H polymorphism and was induced more effectively by neutrophils from FcγRIIa-131H homozygous donors than from FcγRIIa-131R individuals [185]. Based on these findings, it has been proposed that Fc receptor polymorphisms could be biomarkers for EGFR antibodies such as Panitumumab, the only human IgG2 antibody approved for immunotherapy and inhibition of EGFR [217]. Therefore, there is a big interest in developing new improved antibodies through Fc engineering technologies in order to potentiate FcγR-mediated functions [218]. Based on this methodology, it was possible to change the ability of an FcγRIII-optimized (for NK cell) anti-EGFR antibody to efficiently activate neutrophil ADCC against EGFR-expressing tumors [219].

In addition to FcγRIIa, IFN-γ-activated neutrophils can perform ADCC against tumors [186]. However, it seems that FcαRI (CD89) is a more potent inducer of ADCC by neutrophils [188, 189]. Thus, it has been proposed that a new generation of cancer therapeutic mAb should include IgA class antibodies to fully take advantage of the cytotoxic potential of neutrophils [220]. Indeed, this idea is supported by a new IgA2 anti-EGFR antibody derived from the IgG anti-EGFR mAb cetuximab. IgA2 EGFR was more effective than cetuximab *in vivo* against EGFR-transfected Ba/F3 target cells [221]. Very recently, it was also shown that the combination of IgG and IgA mAbs to two different tumor targets (EGFR

and HER2) led to enhanced cytotoxicity compared with each isotype alone [222].

8. Conclusion

Tumor development is influenced by many different host cell types. It has become clear that many tumors present infiltrating neutrophils. The exact role for these tumor-associated neutrophils (TANs) has yet to be completely elucidated. Early reports showed that neutrophils could be cytotoxic to tumor cells. However, a tremendous body of clinical evidence has shown that neutrophils promote tumor progression in various ways. Neutrophils can induce tumor proliferation and angiogenesis and can enhance tumor cell migration and metastasis. Yet, a type of TANs, named N1, can indeed display antitumor functions. New therapeutic ways to recruit and activate these N1 type neutrophils are being investigated in order to turn protumorigenic neutrophils into antitumor effector cells. Blocking neutrophil-derived components known to help tumor growth is a field of active research. Also, very promising results have been found with the use of therapeutic antibodies, which induce neutrophils to perform ADCC and to release cytokines that modulate the immune response against tumors. New antibodies are being designed so that they have better affinity for particular Fc receptors and induce stronger antitumor responses. Learning how to flip the neutrophil coin to "the winning side," namely, functioning as antitumor effector cells, is a challenge for future research that will certainly provide us with new therapeutic options for cancer treatment.

Conflict of Interests

The authors declare that they do not have any conflict of interests in the subject discussed in this review.

Acknowledgments

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Review Article

Balancing Innate Immunity and Inflammatory State via Modulation of Neutrophil Function: A Novel Strategy to Fight Sepsis

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Sepsis and SIRS (systemic inflammatory response syndrome) belong to a severe disease complex characterized by infection and/or a whole-body inflammatory state. There is a growing body of evidence that neutrophils are actively involved in sepsis and are responsible for both release of cytokines and phagocytosis of pathogens. The neutrophil level is mainly regulated by G-CSF, a cytokine and drug, which is widely used in the septic patient with neutropenia. This review will briefly summarize the role of neutrophils and the therapeutic effect of G-CSF in sepsis. We further suggest that targeting neutrophil function to modulate the balance between innate immunity and inflammatory injury could be a worthwhile therapeutic strategy for sepsis.

1. Introduction

SIRS (systemic inflammatory response syndrome) and sepsis are two different entities of the same disease complex both leading to multiorgan dysfunction and eventually death of the patient.

SIRS is defined as an overwhelming systemic inflammation without infectious component. In contrast, sepsis is a potential fatal medical condition that is characterized by a severe systemic infection accompanied by a dysregulated systemic inflammation [1]. Experimentally, SIRS is often induced by injection of LPS, whereas the frequently used model of sepsis is based on the intra-abdominal inoculation of fecal suspension.

The causes for SIRS and sepsis could be manifold. SIRS can develop as sterile complication of severe trauma, extensive burns, shock, or severe local inflammation such as pancreatitis. The concomitant inflammatory response to the increasing endotoxin levels may result in a vicious cycle leading to SIRS. Sepsis can result from any local or systemic infection and is frequently associated with increased blood levels of endotoxin.

Severe sepsis is a major cause of death in the intensive care unit (ICU) of hospitals and affects millions of people around the world each year. Despite an overwhelming increase in our knowledge regarding the pathogenesis of sepsis and the subsequent advances in clinical care, sepsis still accounts for an unacceptable high mortality ranging from 25 to 30% [2].

2. Neutrophils Are the Major Cell Type Involved in SIRS and Sepsis

2.1. Neutrophil Is the Primary Line of Defense against Infection. The neutrophils are the major cell type of the innate

immune system, which acts as primary line of defense against invading microbial pathogens [3]. Neutrophils are terminally differentiated hemopoietic cells with a short life span, which respond to infection by migrating from the bloodstream into the infectious site [3].

Efficiency of bacterial elimination is dependent on the rapid recruitment of neutrophils from the circulation, which is promoted by the release of chemotactic agents [4]. Once at the site of infection, neutrophils capture the microbe into a phagosome, which then fuses within transcellular granules forming a phagolysosome. In the phagolysosome, the microbe is destroyed by a combination of oxidative (reactive oxygen species; ROS) and nonoxidative (enzymes, proteases, and antimicrobial peptides) mechanisms [3].

2.2. The Activation of Neutrophils Induces an Overt Inflammatory Response and Causes Tissue Damage. Although neutrophils are important for pathogen clearance, the activation of neutrophils also causes an overt inflammatory response and tissue injury. The migration of neutrophils could potentially extend neutrophil-endothelial cell interactions and enhance vascular damage [4]. Local secretion of cytokines by the neutrophils might change the nonthrombogenic properties of endothelial cells to a procoagulant state with the initiation of disseminated intravascular coagulation (DIC) and induce the production of nitric oxide in both endothelial and smooth muscle cells [4]. The inducible nitric oxide (iNOs) is mainly released by neutrophils and has received considerable attention as a mediator of the tissue response to sepsis [5]. The key function of iNOs is to induce the synthesis of nitric oxide (NO), which leads to vasodilation, cytotoxicity, and inflammation [6].

3. G-CSF Mobilizes Neutrophils and Enhances the Innate Immunity

3.1. Mobilization of Neutrophils Is Induced by Granulocyte Colony-Stimulating Factor (G-CSF). G-CSF is the principal granulopoietic growth factor regulating the maturation, proliferation, and differentiation of neutrophil precursors and has been used in patients with neutropenia [7]. It enhances maturation of neutrophil functions such as chemotaxis, phagocytes, and bactericidal clearance. It suppresses production of tumor necrosis factor-α (TNF-α) and promotes the release of IL-1ra and soluble TNF receptor (sTNFr) [8]. It is formed at the site of infection or inflammation but exerts its primary action at a remote organ, that is, bone marrow [8]. G-CSF is able to stimulate the proliferation of neutrophils and plays an important role in modulating the release of inflammatory mediators in acute inflammation.

3.2. G-CSF Is Modulating the Innate Immune Response. Several studies have documented the neutrophil-activating effect of G-CSF [9], indicating that it should be considered a potent activator of mature circulating neutrophils. It is capable of priming the respiratory burst, inducing the release of secretory vesicles, and modulating the expression of surface adhesion molecules. The polymorphonuclear surface antigen CD11b/CD18 expression and the plasma elastase-α1AT complex levels are increased following G-CSF administration.

G-CSF induced mobilization of $CD34^+$ cells and improved survival of patients with acute-on-chronic liver failure (ACLF) [10]. This effect was attributed to the increased innate immune state, which potentially contributed to prevention from sepsis and multiorgan failure, and improved survival in the study group [11]. A recent experimental study has shown that the use of G-CSF recruited bone marrow-derived macrophages into the liver, which, on engraftment in the liver, did help in reducing the hepatic fibrosis and supported hepatic regeneration.

4. G-CSF Is Used for Treatment of Septic Patients

4.1. G-CSF Treatment Increased Immune Response of the Patients. According to a large number of clinical trials, G-CSF can decrease the incidence of infections and strengthen host defense in patients. G-CSF administration increased concentrations of IL-1ra, soluble TNF receptors (sTNFr), and IL-10 and reduced TNF-α, IFN-γ, and GM-CSF in healthy volunteers [12]. Application of a single dose of G-CSF resulted in the upregulation of neutrophils. These newly formed neutrophils were well equipped against bacterial infections in terms of Fcy RI expression, Fcy RI-dependent antibody-dependent cellular cytotoxicity, and strong-surface CD14 expression. In contrast the biological significance of the decreased surface-expression of Fcy RI11 and the high intracellular LAP content still needs to be elucidated.

4.2. G-CSF Has Beneficial Effects in Experimental Models of Sepsis. G-CSF has been used successfully in the past to prevent and to treat experimentally induced sepsis, for example, using the cecal ligation and perforation (CLP) model in rats and mice [13, 14]. It has been reported that the combination of G-CSF application and antibiotic prophylaxis was the most efficacious treatment in rats with polymicrobial peritonitis [15]. Prophylaxis with G-CSF increased the survival rate, decreased the bacterial load, and promoted the production of inflammatory cytokines directly, as demonstrated in experiments [16].

The protective effects were at least partially related to increased neutrophil function, as studies proved that similar phenomena were observed by increasing the neutrophil secretory proteins. Singer observed in a model of iNOs knock-out mice that the iNOs derived NO is a determinant of the proinflammatory phenotype acquired by the hepatic microvasculature during sepsis [5]. Luo et al. found that the neutrophil extracellular trap had a proinflammatory role in abdominal sepsis and regulated the pulmonary infiltration of neutrophils and tissue injury [17].

The results might provide insights and guidance for the application of G-CSF and antibiotic in the perioperative treatment of patients who are susceptible to infection following intra-abdominal surgery.

4.3. G-CSF Treatment of Patients with Sepsis Leads to Controversial Results. It has become well accepted that sepsis is composed of two, often concomitant, phases (Figure 1):

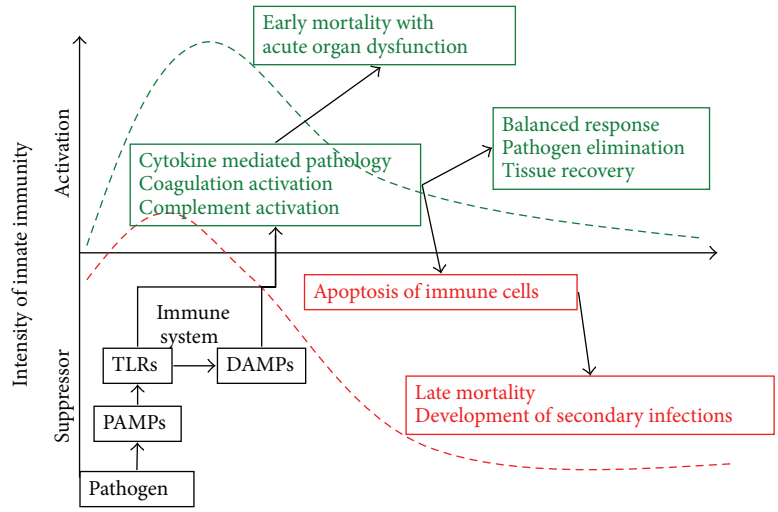

Figure 1: The development of sepsis has two phases, that is, activating and suppressing phase. The activation of the innate immune system can lead to a balanced response that can trigger the elimination of invading pathogens and the recovery of tissue but can also lead to an unbalanced response that can induce hyperinflammation or immune suppression.

an immune-activated phase and an immune-suppressed phase [1]. Identification of two distinct phases of sepsis calls for a stage dependent therapy. However, it remains difficult since the two stages can be hardly distinguished in experimental models but much less in patient with sepsis.

Therefore, treatment strategies targeting only one phase may fail and cause mortality.

G-CSF is frequently and successfully used in patients with severe neutropenia, who are at risk of sepsis [18]. However, when used in clinical trials to treat ongoing severe sepsis or when given as prophylactic treatment, G-CSF did not result in a clear benefit (Table 1). It is reported that perioperative G-CSF administration was effective in upregulating immune function in patients subjected to major surgery. G-CSF administration resulted in increased levels of natural circulating antagonists of TNF-α and IL-1, that is, TNF-R p55/p75 and IL-1ra in patients, thus increasing the threshold of triggering the inflammatory reaction. Perioperative prophylaxis with G-CSF in high-risk colorectal cancer patients resulted in improved recovery [19]. In acute-on-chronic liver failure (ACLF) patients, treatment of G-CSF significantly decreases the risk of sepsis [10]. Additionally, G-CSF is used in the treatment of neonates and adults with infection [20].

Although G-CSF administration is associated with a longer duration of survival in patients with severe sepsis, a meta-analysis from Bo et al. indicated that G-CSF therapy did not significantly reduce the overall mortality at 14 days or 28 days or in-hospital mortality in patients with sepsis [21]. Nelson et al. reported that G-CSF treatment did effectively increase neutrophil levels but did not affect the mortality rate of the patient with community-acquired pneumonia [22]. A multicenter clinical trial of G-CSF had shown similar results that the G-CSF treatment did not substantially reduce mortality and complication rate in patients with pneumonia and severe sepsis [23]. The clinical trials in nonneutropenic septic patients indicated that the use of G-CSF decreased the risk of sepsis in nosocomial pneumonia patients, although the effect did not reach statistical significance [24]. Similarly, a clinical observation from melioidosis patients indicated that G-CSF treatment was associated with longer survival duration but was not associated with an overall survival benefit [25]. Another study from the same group showed that the G-CSF treatment did not improve the outcome in patients with septic shock, [26]. Altogether, the G-CSF administration in infectious disease was not associated with a clear therapeutic benefit (Table 1).

5. The Therapeutic Effect of G-CSF Might Be Enhanced by Reducing the Inflammatory Response

5.1. G-CSF Treatment Induces LPS Sensitization. Our own results demonstrated that G-CSF pretreatment was not only mobilizing neutrophils but also inducing LPS sensitization to inflammatory response [27]. The inflammatory response contributed to bacterial clearance but did become deleterious if circulating LPS was abundant. Therefore, both components of the disease—the state of innate immunity and the inflammatory response—have to be addressed appropriately to maximize the therapeutic efficiency of GCSF in sepsis.

According to the sepsis treatment guidelines [28–30], therapy consists of elimination of the septic focus by early antibiotic treatment ("hit early, hit hard") as well as supportive therapy consisting of volume resuscitation and maintenance of organ function. Antibiotic treatment is very efficient to reduce the bacterial load. However, the antimicrobial, cytolytic properties of antibiotics may induce the release of LPS from the outer membrane of Gram-negative bacteria [31]. Antibiosis does not address the effect of high circulating

Table 1: Clinical studies investigating effect of G-CSF treatment on infectious disease.

Study	Design	Treatment groups	Disease	G-CSF treatment schedule	Conclusion	Effect of G-CSF
Garg et al., 2012 [10]	Effect of G-CSF on survival rate in patients with liver failure	Placebo G-CSF	Acute-on-chronic liver disease	5 μg/kg	G-CSF decreased risk of sepsis significantly	Positive
Stephens et al. [26]	Effect of G-CSF on patients with septic shock	Placebo G-CSF	Septic shock	263 μg/day, 3 days	G-CSF did not improve outcome in patients with septic shock	Indifferent
Cheng et al., 2007 [25]	Effect of G-CSF on severe septic patients with melioidosis infection	Placebo G-CSF	Melioidosis	263 μg/day, 3 days	G-CSF was associated with a longer duration of survival but was not associated with a higher survival rate	Indifferent
Hartmann et al., 2005 [24]	Effect of G-CSF on pneumonia patients	Placebo G-CSF	Nosocomial pneumonia	300–480 μg/day for 7 days	G-CSF decreased risk of sepsis but did reach significance	Indifferent
Hartung et al., 2003 [55]	Perioperative treatment of G-CSF before abdominal surgery	Placebo G-CSF	Mixture, operation	5 μg/kg for 3 times or continuous administration of 5 μg/kg for 5 days after an initial bolus of 5 μg/kg	G-CSF decreased the post-operative infection	Positive
Root et al., 2003 [23]	Effect of G-CSF on pneumonia and sepsis patients	Placebo G-CSF	Pneumonia and severe sepsis	300 μg/day for 5 days	G-CSF was not efficacious in reducing mortality and complications from infection	Indifferent
Tanaka et al., 2001 [56]	Lung injury was investigated after G-CSF treatment	Placebo G-CSF	Sepsis	2 μg/kg for 5 days	G-CSF attenuated inflammatory response	Positive
Ishikawa et al., 2000 [8]	G-CSF with relative neutropenia septic patients	Absolute neutrophil count: high, moderate, and low	Mixture	2 μg/kg for 5 days	G-CSF was effective in septic patient with a percentage of immature neutrophils, but less effect with high percentage of immature neutrophils and bone marrow was depressed.	Positive
Nelson et al., 1998 [22]	Effect of G-CSF on pneumonia patients	Placebo G-CSF	Community-acquired pneumonia	300 μg/day for 10 days	G-CSF did increase neutrophil levels but did not affect the mortality of patient with pneumonia	Indifferent

endotoxin levels. Current guidelines do not call for reducing the overwhelming inflammatory response to circulating free endotoxin. New evidence indicated that the controversial clinical observations might be related to the G-CSF modulated inflammatory response.

5.2. Strategies to Decrease the LPS Induced Inflammatory Response. A number of strategies exist to reduce circulating LPS levels or to minimize the response to LPS [32]. Elimination of LPS using polymyxin columns is troublesome because of the unproven benefit, significant costs, and potential risks [33]. Neutralization of LPS using antibodies reduced the LPS induced inflammatory response [32]. Currently, vaccination strategies to increase the elimination of pathogens are under investigation [34]. None of these antiendotoxin strategies reached wide clinical acceptance.

Minimizing the response to LPS was explored by blocking different pathways. Blockade of the interaction between LPS and TLR4 signal pathway decreased TNF-α levels in LPS induced SIRS model and *E. coli* induced sepsis model [35]. The intracellular signal transduction cascade can be blocked to prevent the excessive induction of proinflammatory cytokines. However, clinical trials using TNF-α antibodies were not promising [36].

Recently TLR4 blockade gained increasing attention [37]. The TLR4 signaling pathway leading to LPS-mediated NF-kappa B activation constitutes an important therapeutic target for sepsis therapy. Various molecules are involved in regulating TLR4-expression on the cell membrane and act as new adjuvant therapies that are able to weaken the deleterious effects of exaggerated host response to infection [38].

Eritoran tetrasodium is a nonpathogenic endotoxin analog that antagonizes inflammatory signaling via the immune receptor TLR4 [39]. Therefore, eritoran tetrasodium (E5564) was investigated as promising molecular candidate to treat sepsis [40]. Christ et al. proposed that E5531, a slightly different analogue, would antagonize LPS activity at its cell-surface receptor leading to inhibition of transmembrane signal transduction [41]. E5531 protected mice from lethal doses of LPS and from viable *E. coli* infections in combination with antibiotics. However, E5531 did not affect bacterial counts. In contrast, additional administration of antibiotic dramatically decreased blood bacterial counts, but plasma endotoxin levels were concomitantly increased in these animals.

5.3. LBP Blockade Is a Novel Strategy to Reduce LPS Induced Inflammatory Response. Our previous study indicated that G-CSF pretreatment induced upregulation of LPS binding protein (LBP) [27]. LPS binding protein (LBP) is an acute phase plasma protein with a molecular weight of 60 KD that can be detected in the acute phase serum of different species such as mice, rabbits, and human [42]. The serum LBP binds to the lipid A component of bacterial endotoxin and facilitates its transfer to the CD14 antigen, which is needed for triggering the inflammatory response via TLR4-NFκB signaling pathway [43]. Martin et al. reported that the binding between LBP and LPS increased the bioactivity of LPS by 100 to 1000 times [44], playing an important role in triggering the inflammatory response in adult respiratory distress syndrome patients and rabbit, respectively [45]. However, upregulation of LBP, in turn, did augment the inflammatory response [46, 47]. It has been shown in different models that upregulation of LBP prior to an LPS challenge potentiates the inflammatory response which may largely contribute to LPS toxicity in sepsis [46].

Minimizing the inflammatory response to LPS can be achieved by interfering with the interaction of LPS and LBP. The inflammatory response to endotoxin can be decreased by reducing circulating endotoxin levels or by reducing the response of the organism to circulating endotoxin. Experimental strategies include using LBP inhibitory peptide [35], or LPS analogues [48], or using LBP deficient mice [47, 49]. Knapp et al. reported [50] that LBP(−/−) mice were associated with diminished early tumor necrosis factor alpha, interleukin-6, cytokine-induced neutrophil chemoattractant, and macrophage inflammatory protein production and attenuated recruitment of polymorphonuclear leukocytes to the site of infection, indicating that acute inflammation was promoted by LBP. However, LBP(−/−) mice were highly susceptible to *E. coli* peritonitis, as indicated by increased mortality, earlier bacterial dissemination to the blood, impaired bacterial clearance in the peritoneal cavity, and more severe remote organ damage.

Le Roy et al. demonstrated that neutralization of LBP accomplished by blocking either the binding of LPS to LBP or the binding of LPS/LBP complexes to CD14 protected the host from LPS induced toxicity [51]. Araña et al. showed that application of the LBP inhibitory peptides blocked the LBP-LPS interaction efficiently and prevented death of animals in an endotoxin shock model by suppressing the TNF-α response to an LPS challenge [35]. This was confirmed in another publication using CLP-19, a synthetic peptide derived from *Limulus* (anti-LPS factor) [52].

6. Outcome of Sepsis Was Improved by Modulating Neutrophil Function and Inflammatory Response

The role of G-CSF pretreatment and subsequent LBP upregulation was investigated in a SIRS model and a sepsis model. Interestingly, In the SIRS model, G-CSF pretreatment enhanced and accelerated the uptake of LPS by the liver. Subsequently, G-CSF pretreatment caused an overwhelming inflammatory response to LPS leading to the death of all animals in response to an otherwise sublethal dose of LPS. This response could at least be partially attributed to the upregulation of LBP prior to the LPS challenge, as blocking of LBP using an inhibitory peptide abrogated the effects of G-CSF pretreatment [27].

In contrast, in the sepsis model, G-CSF pretreatment was associated with an increased survival rate when compared with an untreated control group [53]. This was paralleled by a reduced inflammatory response. Of note, this response could also be at least partially attributed to the upregulation of LBP prior to the septic insult, as blocking of LBP using an inhibitory peptide abrogated the effect of G-CSF pretreatment. Moreover, LBP upregulation during the infection

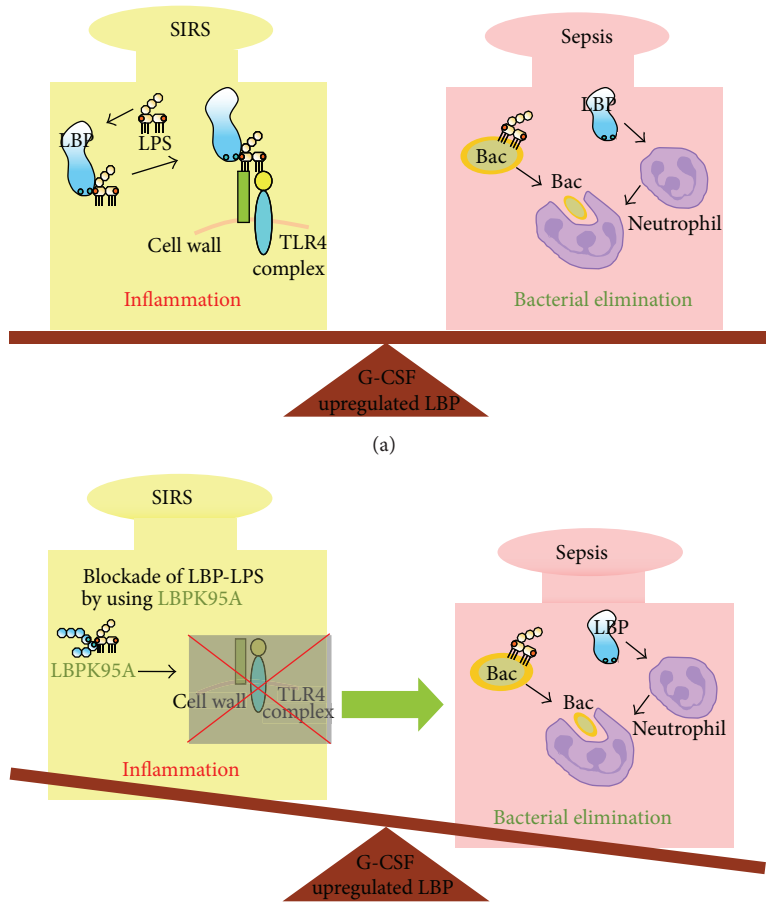

Figure 2: Balanced modulation of LBP-effect by LBP blockade and G-CSF pretreatment in sepsis. (a) on one hand, LBP promotes bacterial clearance, but on the other hand, it contributes to the sensitization of inflammatory response via activation of NF-κB signaling pathway. (b) Interfering with the LBP-mediated inflammatory response by LBP blockade reduces the inflammatory injury and improves outcome after septic insult.

has seemingly a dual function. Yang et al. found that LBP deficient mice showed delayed neutrophil influx in case of a peritoneal infection [54]. This led to the idea that LBP might have a dual role: augmenting the inflammatory response to bacterial toxin such as LPS and contributing to bacterial elimination via the associated and enhanced neutrophil infiltration (Figure 2). These two functions might help to explain that either LBP blockade or G-CSF treatment alone was not useful in the therapy of patients with severe sepsis.

7. Conclusion

The seeming contradictory results above support the idea that augmenting the neutrophil response via G-CSF treatment is a two-edged sword. Elimination of bacteria by increasing the innate immunity seemed improved via the mobilization of neutrophils, whereas LPS response was augmented via upregulation of LBP [47]. Therefore, these evidences suggest that the therapeutic strategies by combining the increased bacterial elimination via improving the innate immunity and decreasing the inflammatory injury could be a worthwhile therapeutic strategy for sepsis. Further G-CSF-based therapeutic strategies should be designed to potentially combine with effects of increasing bacterial elimination via mobilization of neutrophil and decreasing the inflammatory response by blocking the LPS response.

Conflict of Interests

The authors declare that there is no conflict of interests.

Acknowledgments

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Review Article

Neutrophil Leukocyte: Combustive Microbicidal Action and Chemiluminescence

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Neutrophil leukocytes protect against a varied and complex array of microbes by providing microbicidal action that is simple, potent, and focused. Neutrophils provide such action via redox reactions that change the frontier orbitals of oxygen (O_2) facilitating combustion. The spin conservation rules define the symmetry barrier that prevents direct reaction of diradical O_2 with nonradical molecules, explaining why combustion is not spontaneous. In burning, the spin barrier is overcome when energy causes homolytic bond cleavage producing radicals capable of reacting with diradical O_2 to yield oxygenated radical products that further participate in reactive propagation. Neutrophil mediated combustion is by a different pathway. Changing the spin quantum state of O_2 removes the symmetry restriction to reaction. Electronically excited singlet molecular oxygen ($^1O_2^*$) is a potent electrophilic reactant with a finite lifetime that restricts its radius of reactivity and focuses combustive action on the target microbe. The resulting exergonic dioxygenation reactions produce electronically excited carbonyls that relax by light emission, that is, chemiluminescence. This overview of neutrophil combustive microbicidal action takes the perspectives of spin conservation and bosonic-fermionic frontier orbital considerations. The necessary principles of particle physics and quantum mechanics are developed and integrated into a fundamental explanation of neutrophil microbicidal metabolism.

Respectfully dedicated to my deceased mentor Dr. Richard H. Steele and to my friend and colleague Dr. Randolph M. Howes

1. Introduction

Considered as an organ, the collective mass of hematopoietic bone marrow in a healthy adult is greater than that of the liver. The major proportion of this hematopoietic activity is directed to producing neutrophil leukocytes. Each day a healthy human adult releases about a hundred billion neutrophils into the circulating blood [1]. This baseline production is greatly expanded in inflammatory states and by treatment with a granulocyte-colony stimulating factor (G-CSF) such as filgrastim [2]. In addition to stimulating neutrophil hyperplasia, that is, increased production, G-CSF treatment also results in hypertrophic changes, that is, larger neutrophils with severalfold greater azurophilic granule content and proportionally increased myeloperoxidase (MPO) [3].

The neutrophil serves as the principal leukocyte of the acute inflammatory response and is the primary microbicidal phagocyte of the innate and acquired immune defense systems. Accomplishing such function starts in the circulation with intricate neutrophil-endothelial contact. When stimulated by microbial products, complement activation peptides, cytokines, *et cetera*, neutrophils fuse their specific (a.k.a., secondary) granules with their cytoplasmic membrane (i.e., specific degranulation), thus providing the increased surface-to-volume ratio necessary for locomotion and exposing the cytokine receptors and opsonin receptors required for close endothelial contact and diapedesis (i.e., transit) from the vascular space through the endothelial lining into the tissue interstitial space. Chemotactic locomotion to the site of infection is directed by concentration gradients of bacterial products, anaphylatoxin, cytokines, *et cetera*.

Contact and receptor-mediated recognition of an opsonified (e.g., complement-, immunoglobulin-labeled) microbe results in phagocytosis, formation of a phagosome, and

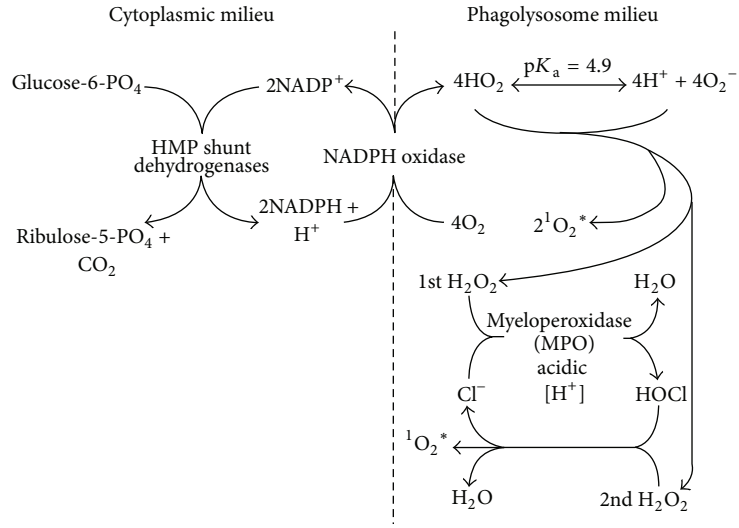

Figure 1: Schematic depiction of neutrophil HOCl and $^1O_2{}^*$ generation.

fusion with azurophilic (a.k.a. primary) granules containing myeloperoxidase to produce the phagolysosome [4].

2. Respiratory Burst Metabolism

The morphologic changes of phagocytosis are associated with magnitudinal increase in hexose monophosphate shunt metabolism of glucose and with proportionally increased molecular oxygen (O_2) consumption, that is, the respiratory burst. This mitochondria-independent metabolic activity is required for effective microbicidal action [5, 6]. The resulting microbicidal oxygenation reactions have exergonicities sufficient to produce electronic excited products yielding light emission in the visible spectrum, that is, chemiluminescence or luminescence [7].

Phagocytosis is linked to activation of NADPH oxidase. This complex flavocytochrome oxidase drives the respiratory burst [6, 8]. Two reducing equivalents (i.e., two electrons (e^-) plus two protons (H^+)) are transferred from NADPH to the oxidase where they distribute in a manner allowing for univalent (one equivalent) reduction of O_2, yielding hydrodioxylic acid (HO_2; a.k.a. hydroperoxyl radical) [9, 10]. HO_2 is an acid with a pK_a of 4.9 and, as such, dissociates yielding a proton and its conjugate base, the superoxide anion ($O_2{}^-$). Production of HO_2 within the neutrophil phagosome or phagolysosome dynamically acidifies the confined space. As the pH of the space approaches the pK_a (i.e., 4.9), the ratio of HO_2 to $O_2{}^-$ approaches unity, removing the anionic barrier to direct radical-radical disproportionation [10, 11]. In such acidic milieu, HO_2 reacts directly with $O_2{}^-$ to produce hydrogen peroxide (H_2O_2) and singlet molecular oxygen ($^1O_2{}^*$) [10–12].

NADPH oxidase dynamically acidifies the phagolysosome and produces H_2O_2. These activities provide the optimal milieu and H_2O_2 substrate for myeloperoxidase (MPO) oxidation of chloride (Cl^-) to the chloronium (Cl^+) state yielding hypochlorous acid (HOCl) [13]. Spontaneous reaction of HOCl with an additional H_2O_2 yields Cl^-, H_2O, and $^1O_2{}^*$ [14]. Both HOCl and $^1O_2{}^*$ are potent microbicidal reactants. The metabolic generation of these reactants is depicted in Figure 1.

3. Oxygen Reactivity

With regard to electronegativity, oxygen is second only to fluorine. The order of electronegativity values for fluorine (F), oxygen (O), and chorine (Cl) is 4.0, 3.5, and 3.0, respectively [15]. The energy difference separating O_2 from water (H_2O) is large, that is, a difference of 1.23 volts (V) or 96.5 kilocalories per mole (kcal mol^{-1}), and provides the driving force of life on earth.

The Merriam-Webster Dictionary defines combustion as a chemical reaction that occurs when oxygen combines with other substances to produce heat and usually light. As such, neutrophil microbicidal action is combustive. Dioxygenation reactions are among the most exergonic in biology. The heat of reaction calculations for the reaction of O_2 with ethylene liberates 93 kcal mol^{-1} (i.e., 389 kilojoules per mole (kJ mol^{-1})) or the energy equivalent to an einstein (i.e., one mole) of ultraviolet photons [16]. The electronegativity of oxygen predicts the high exergonicity of oxygenation reactions, but such reactions are not spontaneous. Placed together in a chamber, O_2 does not react with ethylene. For reaction to occur, a photon or spark with energy sufficient to initiate ethylene bond homolytic cleavage must be applied.

This presentation addresses the questions: why does not oxygen spontaneously react with organic material? Why is oxygen reduction, a process that decreases its thermodynamic potential, required as a first step for neutrophil combustive microbicidal action? And what is the best viewpoint for considering oxygen reactivity? The review provides the background physics and chemistry for a fundamental perspective with regard to oxygen reactivity in particular, neutrophil combustive microbicidal action with its associated

luminescence, and reaction chemistry in general. Hopefully, it will encourage interested researchers out of their comfort zone and broaden their scientific outlook.

4. Particle Physics, Quantum Mechanics, and Reaction Symmetry

Particles are of two symmetry types, each with unique statistical mechanical properties [17, 18]. They are either Bose-Einstein particles (bosons) or Fermi-Dirac particles (fermions). According to the exchange principle, a pair of particles, that is, a and b, can be described by a wave function, $\Psi(a, b)$, representing the space and spin coordinates of the particles. Exchanging the particles generates a new wave function, $\Psi(b, a)$. Even if the particles are indistinguishable (e.g., electrons), the particle sites are distinguishable. Each site is unique with regard to its spin-state, that is, spin-up ($\uparrow$) or spin-down ($\downarrow$). Each combination is distinct. For indistinguishable particles, the result of exchange can differ by no more than a quantum phase factor. There are only two symmetry possibilities. Exchange can be symmetric: $\Psi(a, b) = \Psi(b, a)$; or exchange can be antisymmetric: $\Psi(a, b) = -\Psi(b, a)$ [19].

4.1. Bosons. The wave functions of bosons are symmetric to exchange of a pair of particles; that is, $\Psi(a, b) = \Psi(b, a)$. Bosons obey ordinary commutation; $a \times b = b \times a$. Rotating a boson through 360 degrees, Ψ—360° $\rightarrow \Psi$, returns it to its original state. Bosons are symmetric particles with integral spin. Photons, the force carrier particles of electromagnetic energy, are bosons and are described by the Planck equation: $E = h\nu$, where E is energy, h is Planck's constant, and ν is frequency.

Two antisymmetric fermions can also couple to produce a symmetric bosonic product. Such products include large bosons with mass, such as alpha (α) particles. The character of atomic and molecular orbitals can likewise be considered as bosonic or fermionic [20]. As will be developed subsequently, the frontier orbital of an atom or molecule is bosonic if composed of paired antisymmetric electrons. As such, the highest fully occupied atomic or molecular orbital (HO(A)MO) has bosonic character. Such atoms and molecules are nonradical and diamagnetic.

4.2. Fermions. The wave functions of fermions are antisymmetric to exchange of particles; that is, $\Psi(a, b) = -\Psi(b, a)$. Spin is an intrinsic property of the particle and is quantized. Fermions are characterized as having half-integer spin and appear as multiples of the basic unit $(1/2)\hbar$, where $\hbar$ (h-bar) equals Planck's constant (h) divided by 2π. Fermions anticommute; that is, $a \times b \neq b \times a$. Rotating a fermion through 360 degrees, Ψ—360° $\rightarrow -\Psi$, changes the phase but does not return the fermion to its original state. An additional 360 degrees' rotation, $-\Psi$—360° $\rightarrow \Psi$, is required to return the antisymmetric particle to its original state.

Fermions compose the solid stuff of the universe and are subject to time. Electrons, protons, and neutrons are fermions. The frontier orbitals of atoms, such as hydrogen (H), nitrogen (N), and oxygen (O), and molecules, such as O_2 and nitrous oxide (NO), have singly occupied atomic or molecular orbitals (SO(A)MO). An orbital with a single electron has fermionic character. Atoms and molecules with fermionic orbitals are radical and paramagnetic.

4.3. Principal, Radial, and Angular Quantum Numbers. Atomic hydrogen (H) is composed of a positively charged nuclear proton (H^+) and a negatively charged electron (e^-). Both H^+ and e^- are fermions. H^+ is thousandfold more massive than e^-, and, as such, the kinetic and potential energies of e^- are described as its "orbit" relative to the massive H^+. The orbital wave function describes the energy possibilities. Using polar coordinates, the position of e^- can be described as its distance, r, from its nucleus and two angles, θ and φ. The total wave function is isolated into 3 separate contributions, $\Psi(r, \theta, \varphi) = R(r)\Theta(\theta)\Phi(\varphi)$, where $R(r)$ is the radial component and $\Theta(\theta)$ and $\Phi(\varphi)$ are the angular components. Solution of each component yields a quantum number. The radial component yields the principal quantum number, n. The angular components yield the azimuthal quantum number, l, and the magnetic quantum number, m_l.

The principal quantum number, n, describes the energy of the orbital. The degree of orbital degeneracy is the square of the principal quantum number, n^2. When $n = 1$, the degeneracy is $1^2 = 1$, yielding the 1s orbital. When $n = 2$, the degeneracy is $2^2 = 4$, yielding the 2s, $2p_x$, $2p_y$, and $2p_z$ orbitals. The azimuthal quantum number, l, describes the shape of the orbit and the orbital angular momentum of e^-. The magnetic quantum number, m_l, describes the number of orbitals with a given value of l. The value of the total orbital angular momentum, L, is $L = \sqrt{[l(l+1)]}\hbar$.

4.4. Spin Quantum Number. Electrons and other fermions possess intrinsic angular momentum that is independent of orbital motion. This intrinsic quantum mechanical property, that is, spin, is described by the spin quantum number, s. The magnitude of s is restricted to a value of 1/2. The total spin angular momentum, S, of a system is expressed by the equation $S = \sqrt{[s(s+1)]}\hbar$. The intrinsic spin with its value of $(1/2)\hbar$ (abbreviated to 1/2) is a quality of fermions without analogy in classical physics. Just as l gives rise to m_l, s gives rise to the spin quantum number m_s. Only two values are allowed for m_s. When $m_s = 1/2$, e^- is described as spin-up ($\uparrow$); when $m_s = -1/2$, e^- is described as spin-down ($\downarrow$).

4.5. Pauli Exclusion Principle. Each e^- of an atom or molecule is defined by its five quantum numbers: n, l, m_l, s, and m_s. The Pauli exclusion principle states that no two electrons of a given atom can have identical quantum numbers; that is, the total wave function for a system must be antisymmetric to the exchange of any pair of electrons. For an orbit to accommodate two electrons, the electrons must have opposite spins. If one orbital e^- has $m_s = 1/2$ ($\uparrow$), the other orbital e^- must have $m_s = -1/2$ ($\downarrow$). As such, the total spin quantum number, S, for an orbital electron-couple is $1/2 + -1/2 = 0$ ($\uparrow\downarrow$). Orbital coupling of the fermionic electrons results in spin-neutralization. In effect, the antisymmetric fermions combine

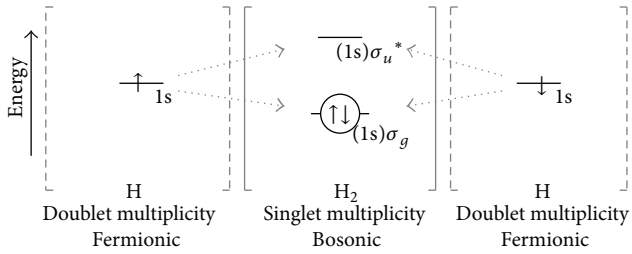

Figure 2: Combination of two doublet multiplicity hydrogen atoms (H, shown on the left and right of the graphic) yields singlet multiplicity molecular hydrogen (H_2, shown in the center of the graphic). Multiplicity is defined as $|2(S)| + 1$, where S is the total spin number. Constructive overlap of the two antisymmetric 1s SOAOs of the H atoms yields the HOMO σ of H_2. The (1s) notation before σ indicates the atomic source of the molecular orbital. σ^* is the LUMO antibonding orbital ($*$ indicates antibonding) of H_2. The energy differences in the figures are for illustration and not drawn to scale.

into a symmetric boson. This concept is illustrated in Figure 2 by the reaction of two H atoms to generate molecular H_2.

4.6. Multiplicity. Multiplicity is defined as $|2(S)| + 1$, where S is the total spin number. Multiplicity is a spectroscopic term and indicates the number of wave functions possible for the system; that is, singlet indicates 1, doublet indicates 2, triplet indicates 3, *et cetera*. In its ground (lowest energy) electronic state, atomic H has a single e^- in the 1s orbital and has an S value of 1/2 or −1/2; thus $|2(1/2 \text{ or } -1/2)| + 1 = 2$; the multiplicity is doublet. For molecular H_2 the value of S is 0; thus, $|2(0)| + 1 = 1$; the multiplicity is singlet. In Figure 2, the orbital possibilities are depicted by a horizontal bar (—). The spin quantum number of each e^- is represented as spin-up ($m_s = 1/2 = \uparrow$) or spin-down ($m_s = -1/2 = \downarrow$). The circle surrounding the σ orbital electron-couple of the HOMO of H_2 symbolizes the bosonic character of the filled orbital.

Orbital overlap of the two H atoms can be constructive or destructive. When the electrons are antisymmetric, that is, ↑ and ↓, overlap is constructive resulting in chemical bonding; that is, there is an increased probability of finding electrons in the internuclear region between the two H nuclei. Combining the two atomic 1s orbitals yields the bonding sigma molecular orbital, σ. Note that bonding lowers the energy of the system. When the electrons are ↑ and ↑ or ↓ and ↓, the overlap is destructive and no bonding occurs.

5. Frontier Orbital Theory

Chemical reaction involves frontier orbital interaction. Frontier orbital theory focuses on the initial orbital conditions of the reactants and on reactive transition with special emphasis on the highest occupied and lowest unoccupied orbitals [21, 22]. The frontier orbitals, that is, the highest occupied atomic or molecular orbital (HO(A)MO) and the lowest unoccupied molecular orbital (LUMO), define the reactive possibilities. As depicted in Figure 2, the electrons of the singly occupied atomic orbital (SOAO) of the two H atoms constructively overlap in a radical-radical (i.e., doublet-doublet) annihilation producing the σ bonding HOMO orbital of singlet multiplicity, diamagnetic molecular hydrogen (H_2).

For the H atoms, the electrons of the 1s orbitals have identical energies, but with covalent bonding to form H_2, the wave functions overlap and split producing two molecular orbitals, one with lower and one with higher energy than the original 1s atomic orbitals. The bonding orbital (σ) is lower in energy and stable, thus promoting bonding. The antibonding orbital (σ^*) is of higher energy. Populating the antibonding orbital promotes bond breaking [23]. Consequently, if a photon of sufficient frequency is captured by a σ electron, its electronic excitation to the σ^* orbital results in H_2 bond cleavage.

Table 1: Spin conservation rules. Multiplicity states from the perspective of the bosonic-fermionic orbital character of reactants and products.

Reactants	Product(s)
Singlet + singlet	Singlet
Bosonic + bosonic	*Bosonic*
Singlet + doublet	Doublet
Bosonic + fermionic	*Fermionic*
Singlet + triplet	Triplet
Bosonic + bifermionic	*Bifermionic*
Doublet + doublet	Singlet (triplet)*
Fermionic + fermionic	*Bosonic (bifermionic)**
Doublet + triplet	Doublet (quartet)*
Fermionic + bifermionic	*Fermionic* (*trifermionic*)*
Doublet + quartet	Triplet
Fermionic + trifermionic	*Bifermionic*
Triplet + triplet	Singlet
Bifermionic + bifermionic	*Bosonic*
Triplet + quartet	Doublet
Bifermionic + trifermionic	*Fermionic*
Quartet + quartet	Singlet
Trifermionic + trifermionic	*Bosonic*

*The products in parentheses are symmetrically possible but improbable.

6. Wigner-Witmer Spin Conservation and Boson-Fermion Orbital Symmetry

The Wigner-Witmer spin conservation rules describe reaction symmetry possibilities in terms of reactant and product multiplicities [24, 25]. The spin conservation rules state that the overall spin angular momentum of a system must be conserved. The symmetries of reactants and products must correlate. Reactant and product state possibilities can also be considered in terms of fermionic and bosonic frontier orbital character [20].

In Table 1 reactants and products are presented in terms of multiplicity and also in terms of bosonic or fermionic orbital character. As depicted in Figure 2, combining two doublet multiplicity H atoms produces singlet multiplicity

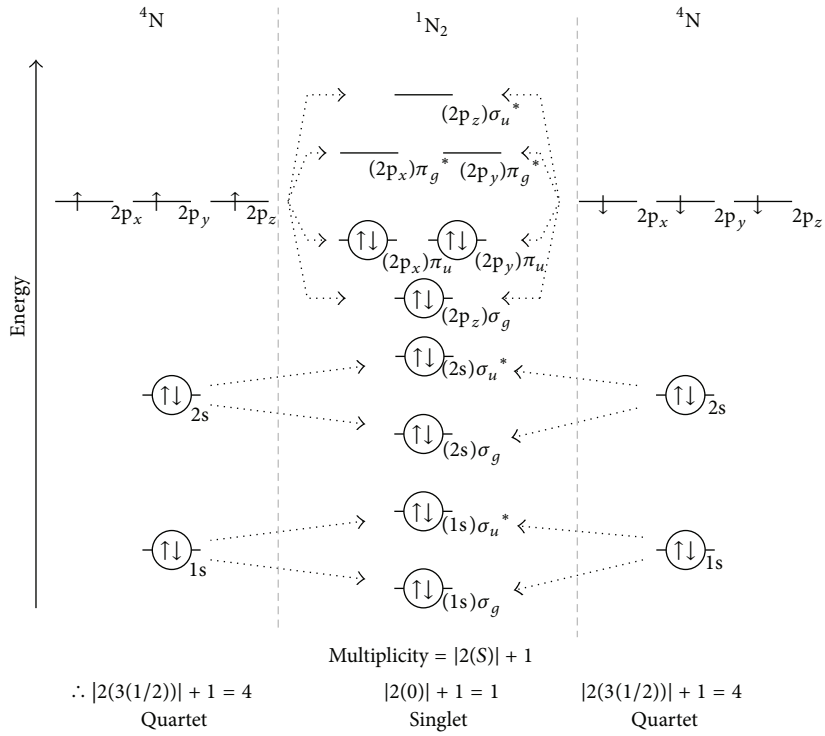

Figure 3: Combination of two quartet multiplicity nitrogen atoms (^{4}N; the superscript 4 indicates multiplicity) to yield singlet multiplicity molecular nitrogen ($^1N_2$).

H_2. With regard to orbital spin character, the two antisymmetric fermionic electrons of the atomic orbitals are coupled (condensed or combined) resulting in the bosonic electron pair of the σ orbital of H_2. Since the Pauli principle requires the total wave function for any system of electrons to be antisymmetric to exchange an orbital pair of electrons, only phase-opposite electrons can occupy a given orbital state. Orbital coupling of such fermionic electrons imposes bosonic character.

6.1. Hund's Maximum Multiplicity Rule. Hund's maximum multiplicity rule states that the electronic configuration with highest multiplicity has the lowest energy. The greater the number of wave functions possible for a system, the lower the energy. Figure 3 depicts the combination of two nitrogen atoms (N) to yield molecular nitrogen (N_2). Ground state atomic N is a paramagnetic triradical with one electron in each of its three 2p orbitals. Note that each electron has the same m_s value resulting in quartet multiplicity; that is, $|2(3(1/2 \text{ or } -1/2))|+1 = 4$. The orbitals of higher multiplicity states are more contracted than those of lower multiplicity. Higher multiplicity states produce greater nuclear-electron attraction and are of lower energy [26].

Combining the atomic orbitals of the two quartet multiplicity nitrogen atoms (N) generates the filled (bosonic) (1s and 2s) σ and σ^* orbitals; constructive overlap of the phase-opposite electrons of the three 2p orbitals of the two N atoms generates one σ and two π bonds of triple-bonded ground state singlet multiplicity N_2. The triradical N atoms combine to produce nonradical N_2. From the fermion-boson frontier orbital perspective, each N presents a complex of trifermionic SOAOs, that is, Ψ (i.e., $S = 3(1/2)$) or $-\Psi$ (i.e., $S = 3(-1/2)$). Consistent with Table 1, antisymmetric coupling produces triple-bonded bosonic (i.e., $S = 0$) N_2.

6.2. Oxygen Chemistry. Frontier orbital overlap of two oxygen atoms with antisymmetric SOMO is constructive producing $^1O_2{}^*$ as depicted in Figure 4. As per Table 1, combining two ground state triplet multiplicity paramagnetic, diradical oxygen atoms (O) produces an electronically excited singlet multiplicity, diamagnetic, nonradical $^1O_2{}^*$. Note the product $^1O_2{}^*$ obeys the spin symmetry rules but violates Hund's maximum multiplicity rule. As such, $^1O_2{}^*$ is electronically excited (indicated by $*$) with an energy of 22.5 kcal mol^{-1} greater than that of triplet multiplicity ground state O_2 [14].

The spin conservation rules predict that the change in spin multiplicity, that is, a singlet-to-triplet transition, is of low probability. As such, electronically excited $^1O_2{}^*$ is metastable with a relatively long half-life. The estimated four-microsecond lifetime of $^1O_2{}^*$ is sufficient to allow its participation as an electrophilic reactant in dioxygenation reactions. However, such reactions are restricted to within a radius of about 0.2 microns (μm) from its point of generation [27, 28]. As depicted in Figure 5, unreacted $^1O_2{}^*$ relaxes to its triplet multiplicity ground state ($^3O_2$) by emitting a 1270 nm (near infrared) photon.

As per the maximum multiplicity rule, ground state molecular oxygen is a triplet multiplicity, paramagnetic diradical with one e^- occupying each of its two π^* SOMOs;

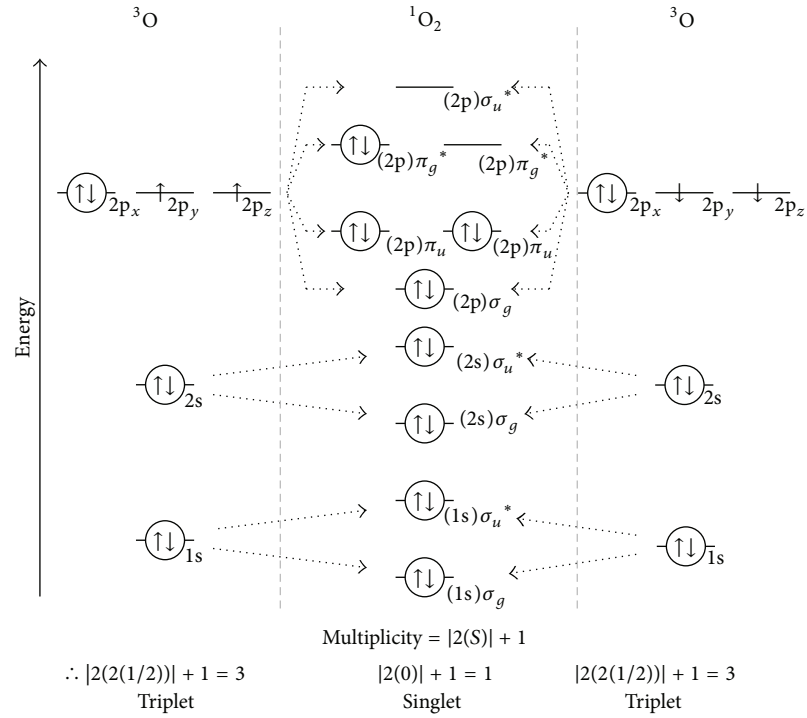

Figure 4: Combination of two triplet multiplicity oxygen atoms (^{3}O) to yield electronically excited singlet multiplicity molecular hydrogen ($^1O_2{}^*$).

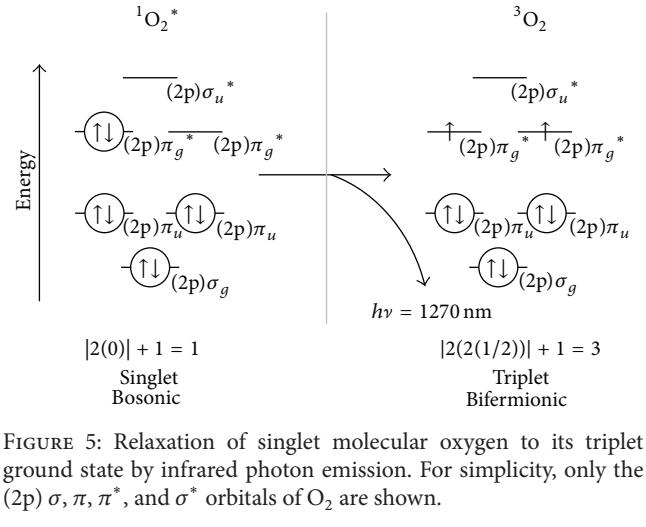

Figure 5: Relaxation of singlet molecular oxygen to its triplet ground state by infrared photon emission. For simplicity, only the (2p) σ, π, π^*, and σ^* orbitals of O_2 are shown.

it is bifermionic. Ground state O_2 can be in either the Ψ (i.e., $S = 1/2 + 1/2 = 2(1/2) = 1$) or the $-\Psi$ (i.e., $S = -1/2 - 1/2 = 2(-1/2) = -1$) state; thus, the multiplicity is $|2(1) + 1| = 3$, that is, triplet.

The vast majority of biological molecules are singlet multiplicity—that is, $S = 0$—and, as such, present bosonic frontier orbitals. For such molecules, chemistry is confined to bosonic HOMO-LUMO exchange of a composite orbital electron-couple. The fermionic orbital character of radicals is atypical and favors radicals-radical reaction. Antisymmetric fermionic frontier orbital overlap is constructive resulting in covalent bonding of the bosonic singlet product. As described in Table 1, constructive SOMO-SOMO overlap of a doublet reactant and a triplet reactant produces a doublet product; that is, the fermionic character is preserved.

Direct reaction of ground state triplet multiplicity O_2 with singlet multiplicity organic molecules violates the spin conservation rules, and, as such, combustion is not spontaneous. Overlap of the bifermionic π^* (the 2 SOMO) frontier orbitals of triplet multiplicity O_2 with the empty LUMO or bosonic HOMO of singlet multiplicity organic molecules is not constructive. Such reactions are symmetry-restricted, and the only product possible would also be a bifermionic triplet. O_2 with its triplet multiplicity ground state is relatively exotic. The triplet multiplicity states of most molecules are electronically excited. The relaxation of such excited triplet multiplicity molecules to their singlet ground state, that is, triplet-to-singlet transition, violates the conservation rules and is of low probability. The delayed relaxation of an excited triplet to its singlet ground state by photon emission is responsible for the phenomenon of phosphorescence [29].

6.3. Neutrophils Change the Spin Number of O_2. Considering the complexity and variety of potentially pathogenic organisms, neutrophil microbicidal action must be simple, potent, and focused so as to maximize microbicidal action and minimize collateral damage. Microbicidal combustion is realized by changing the frontier orbitals of oxygen from bifermionic to bosonic. Neutrophil combustive action is expected to produce light. Oxygenation reactions yield electronically excited products that relax to ground state by emission

of light in the visible spectral range. Bioluminescence and chemiluminescence reactions are essentially limited to such oxygenation reactions. Neutrophil luminescence is energetic proof of such combustive activity.

Respiratory burst metabolism mobilizes the reducing equivalents required for changing the frontier orbitals of O_2. One equivalent (radical) reduction is unusual in cytoplasmic metabolism. When it does occur, the semiquinone of a riboflavin prosthetic group is typically involved. Such flavoenzymes mark the departure from two equivalent cytoplasmic transfers to one equivalent cytochrome transfer. One equivalent reduction of O_2 changes its multiplicity from triplet to doublet, thus reducing its overall fermionic orbital character. The flavocytochrome enzyme NADPH oxidase catalyzes the reduction of bifermionic triplet multiplicity O_2 to fermionic doublet multiplicity HO_2 [9, 10]. Acidic dissociation of HO_2 yields its conjugate base, doublet multiplicity superoxide anion (O_2^-) [11]. As per Figure 1 and Table 1, direct SOMO-SOMO orbital overlap of HO_2 with O_2^- produces singlet multiplicity ground state H_2O_2 plus $^1O_2{}^*$ [10, 12].

The H_2O_2 produced serves as substrate for myeloperoxidase (MPO) oxidation of chloride producing hypochlorous acid; that is, $H_2O_2 + Cl^- \rightarrow H_2O + HOCl$. Note that all reactants and products are exclusively singlet multiplicity (bosonic). The hypochlorous acid (HOCl) produced reacts directly with additional H_2O_2. This reaction involves the bosonic HOMO of H_2O_2 and the bosonic LUMO of HOCl producing the intermediate singlet multiplicity chloroperoxy acid (HOOCl) and singlet multiplicity H_2O [14]. Disintegration of the chloroperoxy intermediate yields ground state singlet multiplicity chloride and electronically excited singlet multiplicity oxygen. The net reaction is $H_2O_2 + HOCl \rightarrow {}^1O_2{}^* + H_2O + Cl^- + H^+$. The bosonic orbital symmetry of $^1O_2{}^*$ allows direct constructive spin-allowed overlap with the bosonic frontier orbitals of biological substrates producing singlet multiplicity dioxygenated products of bosonic symmetry.

In conventional combustive burning, sufficient energy must be applied to a nonradical (singlet) substrate to produce homolytic bond cleavage yielding two radical (doublet) products. The fermionic SOMO of these doublet multiplicity radical products can now constructively overlap with one of the fermionic SOMOs of triplet multiplicity ground state O_2. As described in Table 1, the product of doublet-triplet reaction will have doublet multiplicity. Such radical-diradical reactions yield heat plus additional radical products that can further participate in the radical propagation process of burning.

Neutrophil microbicidal action is combustion by a different pathway. Instead of radicalizing a substrate to facilitate reaction with diradical O_2, the bifermionic orbitals of ground state triplet O_2 are converted to the bosonic orbitals of $^1O_2{}^*$. In its electronically excited singlet multiplicity state, the bosonic frontier orbitals of oxygen can participate in spin-allowed electrophilic oxygenation reactions with a broad spectrum of singlet multiplicity biological molecules. As per spin conservation, the products of these reactions are singlet multiplicity. Such products are nonradical, diamagnetic, and unable to participate in the radical propagation reactions that are characteristic of burning.

The potent electrophilic reactivity of $^1O_2{}^*$ is restricted to its metastable lifetime. The microsecond lifetime range of $^1O_2{}^*$ confines reactions to within about a 0.2 μm radius from its point of origin [27, 28]. Thus, phagolysosomal generation of $^1O_2{}^*$ in close proximity to the target microbe guarantees that combustive action is focused on the microbe with minimal collateral damage to the host. By changing the spin quantum number of oxygen, the neutrophil not only realizes its potent electrophilic microbicidal action, but also limits such combustive action to the target microbe.

6.4. Neutrophil Luminescence. The endoperoxide and dioxetane products of dioxygenation are of relatively high energy. These dioxygenated products are typically unstable and of singlet multiplicity. Disintegration of such products yields two carbonyl functions. One carbonyl is in the ground singlet multiplicity state, and the other carbonyl is in the electronically excited $n\pi^*$ singlet multiplicity state. As depicted in Figure 6, the $n\pi^*$ description indicates that an electron from the ground state nonbonding (n) orbital of the atomic oxygen (O) component occupies the π^* orbital of the electronically excited carbonyl function. When the spin of the excited π^* electron is antisymmetric to that of the ground state n electron of O, the electronically excited carbonyl is singlet multiplicity. Thus, π^*-to-n relaxation to ground state is spin-allowed and yields a photon with energy proportional to the difference between the two orbital states.

In bioluminescence and chemiluminescence processes, dioxygenated products disintegrate by oxygen-oxygen bond cleavage producing the singlet multiplicity $n\pi^*$ electronically excited carbonyl. This electronically excited state is chemically generated. In fluorescence, electronic excitation to the $n\pi^*$ state can occur when a photon of proper energy is captured by an electron in the nonbonding (n) orbital of the O component of the carbonyl.

As depicted in Figure 6, relaxation of the electron from π^* of the singlet excited carbonyl back to the n orbital of O results in photon ($h\nu$) emission and returns the carbonyl function to its ground singlet state. The frequency of the emitted photon is the energy difference that separates the n orbital from the π^* orbital. Photons are symmetric bosons; their absorbance or emission does not affect the electron spin.

In fluorescence, photon capture promotes an electron of an orbital to an appropriately higher energy orbital. Such photon excitation results in transient antibonding and fermionic character, but the overall spin symmetry is retained; that is, singlet character is retained. Relaxation of an $n\pi^*$ electronically excited singlet multiplicity carbonyl to its singlet ground state by fluorescence or chemiluminescence is spin-allowed, and, as such, the lifetime of the excited state is very short-lived, typically in the nanosecond range. Consequently the excited carbonyl is unable to participate in reaction chemistry.

6.5. Native and Substrate Specific Chemiluminescence. The chemiluminescence produced by neutrophils is an energy byproduct of microbicidal combustion and varies with the

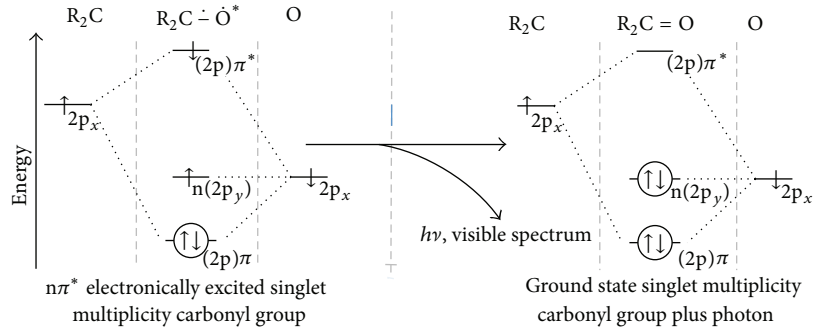

Figure 6: Relaxation (pi antibonding to nonbonding ($\pi^* \rightarrow$ n) electronic orbital transition) of a singlet electronically excited carbonyl product of dioxygenation to its singlet ground state by photon emission.

molecular composition of the microbe. Different microbes present substrates that vary with regard to dioxygenation susceptibility and combustion quantum efficiencies, that is, the photon yield per dioxygenation reaction. Native luminescence is easily measured using a luminometer, but the photon yield is insufficient for measurement of less than a hundred thousand neutrophils. In order to increase photon yield and impose reaction specificity, chemiluminigenic substrates can be introduced to the milieu. Chemiluminigenic substrates are susceptible to dioxygenation producing endoperoxides or dioxetanes ultimately yielding $n\pi^*$ electronically excited carbonyl products. Such substrates increase the sensitivity for detecting dioxygenation activity by several orders of magnitude [30]. In addition to increasing sensitivity, chemiluminigenic substrates can be selected for specificity with regard to the type of oxygenation activity measured [31].

The state of the circulating neutrophil reflects the state of inflammatory immune defense [32]. The sensitivity and specificity obtained using selective chemiluminigenic probe in combination with phagocytic and chemical stimuli allow simultaneous quantification of multiple neutrophil metrics using a microliter or less of unseparated whole blood. When subjected to discriminant function analysis, such neutrophil luminescence measurements allow assessment of host inflammatory state and hematopoietic marrow stress [32–35].

7. Neutrophil Myeloperoxidase and Lactic Acid Bacteria (LAB)

Neutrophil leukocytes respond to cytokines and other agents by fusing specific granules with the cytoplasmic membrane, that is, specific degranulation. Such fusion effectively increases the surface-to-volume ratio of the neutrophil, as is required for locomotion and phagocytosis, and for upregulated membrane expression of cytokine and opsonin receptors, as is required for effective recognition and phagocytosis. Microbicidal effectiveness is optimized by coordinating phagocytosis with activation of NADPH oxidase, resulting in dynamic acidification and H_2O_2 production. Fusion of the lysosomal azurophilic granules with the microbe-containing phagosome produces a phagolysosome with a relatively high MPO concentration in an acidic milieu replete with H_2O_2. The phagolysosomal milieu is optimal for combustive microbicidal action.

The neutrophils of patients with chronic granulomatous disease (CGD) have defective NADPH oxidase function and consequently have defective microbicidal combustion and do not chemiluminesce [36]. These CGD neutrophils phagocytose microbes and release MPO into the phagolysosomal space, but the neutrophil respiratory burst is defective and microbicidal action is poor. Consequently, CGD patients have an increased susceptibility to infection. Interestingly, the incidence of pneumococcal and other streptococcal infections is not increased in CGD patients; that is, infection rates for these microbes are about equivalent to those for healthy subjects [37]. Pneumococci and streptococci are cytochrome-deficient lactic acid bacteria (LAB) that produce lactic acid and H_2O_2 as metabolic products [38, 39]. Phagocytosis of live LAB by CGD neutrophils does result in microbicidal action. In the case of CGD neutrophils, the phagocytosed LAB provides the acid and H_2O_2 necessary for effective MPO-mediated microbicidal combustion and chemiluminescence [36].

Human neutrophils contain a relatively high concentration of MPO (about 5% of the dry weight of the neutrophil) in their azurophilic granules [40]. Following departure of the neutrophil leukocyte from the hematopoietic marrow and a relatively short transit time in the circulating blood, the neutrophil leaves the circulating and enters the tissue pool [1]. Neutrophils characteristically migrate to sites of injury and infection and accumulate in high concentrations. However, even in the absence of inflammation and infection, healthy humans have significant concentrations of neutrophils present in tissue fluids. The oral and vaginal cavities have neutrophil concentrations in proportion to blood neutrophil concentrations [41, 42].

When neutrophils transit from the blood to tissue and body spaces, they carry MPO into these spaces. Each neutrophil carries about four femtograms of MPO. As such, neutrophils deliver relatively large quantities of MPO into body spaces on a routine basis. The oral and vaginal cavities have acidic pH. The normal florae of these spaces are cytochrome-negative LAB that generate lactic acid and H_2O_2 as metabolic products. The transit of neutrophils into such spaces delivers MPO into milieux that are rich in LAB.

MPO selectively binds to Gram-negative and many Gram-positive bacteria. However, LAB show relatively poor MPO binding [43]. When small concentrations of neutrophil-free MPO are added to bacterial suspensions containing viridans streptococci with other competing bacteria, a potent synergistic microbicidal action is directed against MPO-binding Gram-positive, for example, *Staphylococcus aureus*, and Gram-negative, for example, *Pseudomonas aeruginosa* and *Escherichia coli*, microbes [44]. Damage to the LAB is only observed at significantly higher MPO concentrations. This LAB-MPO synergistic action may explain the dominance of streptococci and lactobacilli in healthy oral and vaginal cavities [45].

Selective binding of MPO to a microbe guarantees that $^1O_2{}^*$ is generated in close proximity to the target microbe, resulting in selective, focused combustive microbicidal action. The $^1O_2{}^*$ lifetime of a few microseconds limits damage to within about a 0.2 μm radius of its point of generation [27, 28]. Thus, combustion is focused on potential pathogens with minimal collateral damage to the host cells and the normal LAB flora [44].

8. Conclusions and Perspectives

There are no restrictions on biologic evolution other than those imposed by physical reality. The spin conservation rules describe fundamental symmetry restrictions that define reactive possibilities. These rules forbid the direct reaction of triplet oxygen with singlet organic molecules, and, as such, combustion is not spontaneous. Chemical reactions involve frontier orbitals. Changing the multiplicity of oxygen changes its frontier orbitals, eliminating the symmetry barrier to its electrophilic reactive potential.

Neutrophils kill microbes by a unique, focused combustive action. The redox reactions of neutrophil respiratory burst metabolism change triplet ground state (diradical) O_2 to singlet electronically excited (nonradical) $^1O_2{}^*$. In addition, other singlet multiplicity (nonradical) microbicidal reactants, for example, H_2O_2 and HOCl, are generated. These agents are uniformly singlet multiplicity (nonradical) reactants with bosonic frontier orbitals. Their reactions with microbes are consistent with the spin conservation rules. $^1O_2{}^*$ has one empty (LUMO) π^* orbital and one full (HOMO) π^* orbital with bosonic character. This highly electrophilic LUMO can participate in spin-allowed bosonic LUMO-HOMO reactions with biological molecules. The dioxygenated products of such combustions yield the electronically excited carbonyl functions that relax by photon emission, that is, luminescence or chemiluminescence.

Considering frontier orbital reactivity in the bosonic-fermionic terms of particle physics broadens the perspective for explaining oxygen chemistry in particular and for appreciating reaction chemistry in general. Biological reactions typically involve constructive frontier HOMO-LUMO overlap. Radical reactions involving singly occupied atomic or molecular orbitals (SO(A)MO) are uncommon in biology under normal conditions, that is, in the absence of high energy radiation or heat producing homolytic bond cleavage. Biological reactions involving one electron transfer are typically restricted to flavoenzymes and to highly ordered cytochrome electron transport systems such as those found in mitochondria. Cytoplasmic redox reactions typically involve two equivalent transfers and are nonradical. Orbital pairing of antisymmetric fermionic electrons imposes a bosonic (nonradical) character.

Chemical reactions can be generalized as constructive bosonic transfers (i.e., HOMO-LUMO) or constructive fermionic annihilations. Both reactions produce bosonic products. Radical-radical (i.e., SO(A)MO-SO(A)MO) orbital overlap is typically constructive; that is, fermionic radicals react with fermionic radicals. Overlap of a bosonic HOMO with a fermionic SO(A)MO is not constructive. As illustrated in Figure 1, the first steps of the hexose monophosphate metabolic pathway involve dehydrogenation of glucose-6-phosphate and reduction of $NADP^+$ yielding NADPH; these redox reactions involve balanced two equivalent bosonic transfers. Bosonic orbital pair transfers are the norm in biochemistry. The bosonic nature of the antisymmetric orbital electron-couple may be essential for such biologic redox transfer. Any fermionic electron transfer would open the possibility for reaction with bifermionic oxygen.

There are advantages to transferring electrons as a bosonic orbital-couple. As stated by Dirac, "If a state has zero total angular momentum, the dynamical system is equally likely to have any orientation, and hence spherical symmetry occurs" [19]. A bosonic orbital electron-couple has $S = 0$. This has significance with regard to the Heisenberg uncertainty principle which states that the uncertainty of momentum (Δp) multiplied by the uncertainty of position (Δx) is always equal to or greater than $(1/2)\hbar$; that is, $\Delta p \Delta x \geq (1/2)\hbar$ [46]. The spin momentum of the bosonic orbital electron-couple is known, that is, zero. Consequently, the positional uncertainty of the electron-couple must be proportionally large. Thus, the intermolecular redox transfer of a bosonic orbital electron-couple may proceed by a quantum tunneling mechanism analogous to the emission of a bosonic alpha particle from an atomic nucleus in alpha radiation decay [20]. Such a quantum tunneling mechanism explains the facility for electron-couple transfer in biologic redox reactions.

Conflict of Interests

The author declares no conflict of interests regarding the publication of this paper.

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Review Article

Reconciling the IPC and Two-Hit Models: Dissecting the Underlying Cellular and Molecular Mechanisms of Two Seemingly Opposing Frameworks

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Inflammatory cascades and mechanisms are ubiquitous during host responses to various types of insult. Biological models and interventional strategies have been devised as an effort to better understand and modulate inflammation-driven injuries. Amongst those the two-hit model stands as a plausible and intuitive framework that explains some of the most frequent clinical outcomes seen in injuries like trauma and sepsis. This model states that a first hit serves as a priming event upon which sequential insults can build on, culminating on maladaptive inflammatory responses. On a different front, ischemic preconditioning (IPC) has risen to light as a readily applicable tool for modulating the inflammatory response to ischemia and reperfusion. The idea is that mild ischemic insults, either remote or local, can cause organs and tissues to be more resilient to further ischemic insults. This seemingly contradictory role that the two models attribute to a first inflammatory hit, as priming in the former and protective in the latter, has set these two theories on opposing corners of the literature. The present review tries to reconcile both models by showing that, rather than debunking each other, each framework offers unique insights in understanding and modulating inflammation-related injuries.

1. Introduction

Many models have been put forward as an attempt to explain and counteract the real-life outcomes of several different inflammatory events in which neutrophil leukocytes play an outstanding role. Trauma, infection, hemorrhage, the response to both elective and emergency surgical interventions, and other pathological processes are incredibly prevalent in the human population. Such conditions are often complicated by nefarious immune responses that arise from these events that are, at least partially, mediated by neutrophils [1–4], since these are cells known for their central role in the mechanisms of inflammation in mammals [5, 6].

Logically, there is an ongoing effort to explain the inflammatory dynamics arising from these types of insults, as a first step in the direction of modulating and perhaps coordinating such responses in order to improve outcomes, reduce hospitalization times, and even prevent death.

The two-hit or multiple-hit hypothesis is a model that explains how sequential insults can synergically contribute to an inappropriate immune response [7] in which MODS/MOF (multiple organ dysfunction syndrome/multiple organ failure) is often the endpoint. As a broad definition, the two-hit model hypothesizes that an initial inflammation-triggering event, such as pancreatitis, trauma, burns, excessive bleeding, or elective surgery, can set in motion a priming condition for the immune system that can cause limited expression of SIRS (systemic inflammatory response syndrome) or other mild effects if left alone. Additional hits or insults (e.g., second-look laparotomy, infection, further blood loss, or ischemic

injury during the process of aneurysm repair) are capable of causing an extraordinary and exaggerated immune response [8] that can evolve to MODS/MOF and death.

Ischemic preconditioning or IPC, on the other hand, does not represent an actual attempt to explain the inflammatory processes involved in SIRS/sepsis and its continuum of MODS/MOF. Rather, it is a collection of techniques that make use of the dynamics of the inflammatory response to generate a modulatory effect over these events. IPC is a demonstrable, observable, reproducible phenomenon in which a nonlethal, mild, and often cyclic ischemic event has the capacity to protect organs and tissues from a secondary, prolonged, and otherwise deleterious ischemic event [9], mitigating the response to ischemia and the ischemia-reperfusion injury (IRI).

Incidentally, the predominance of information in the literature about both models has a marked timeline difference. Throughout the late 1980s and early 1990s the two-hit model was considered a good standard as an intuitive and empirical explanation for some real-life chronologically based events seen in trauma and septic patients. Conversely, while the manipulation of the inflammatory response through exposure to controlled ischemic scenarios was already underway in the late 1980s [10], it was not until recently that IPC was shown to have clinical and surgical applications that far surpass those initially conceived and has, not without merit, been increasingly present in the literature.

The shift in the literature and the seemingly obvious difference as to how the two models treat a first inflammatory event, on one hand as a first/priming hit, and on the other hand as a protective/beneficial insult, have led to the notion that one model is capable of debunking the previous theory. In the following pages, we shed some light on some of the paradoxes regarding the coexistence of both models and try to reconcile both theories as simultaneously valid answers to some very different questions.

2. Two-Hit Model: An Intuitive Explanation for Empirical, Readily Observable Conditions

Sometime by the late 1990s the two-hit model soared to a unique position as a theory that successfully explained and accounted for many of the bedside events that accompanied trauma patients, which by the nature of their injuries were often exposed to sequential insults [11]. Another relevant well-known real-life example of the application of the two-hit model is the correction of ruptured aortic aneurysms [12], which requires imposing a second long-duration dry ischemic event for the actual repair of the initial naturally occurring hemorrhagic injury [13]. A host of experimental models has also been developed to mimic the events of multiple or sequential hits in order to further understand the processes involved in the augmentation of the inflammatory response. One example of those models is from the researchers that in 1998 demonstrated that neutrophil recruitment to the lung was increased when hemorrhagic shock (first hit) was followed by inoculation of LPS (second hit) if compared to a single-hit process [14]. Another group, on the following year, demonstrated the same marked increase in PMN recruitment to the lung when subjects were exposed to a second hit with direct lung injury from LPS and immune complexes after a septic event that served as the first hit [15]. Nevertheless, examples of multiple-hit models that failed to induce the anticipated augmented immunological response can also be found in the literature. For instance, an American study from 2000 in which subjects were exposed to intratracheal injection of acid with or without previous induction of sepsis by CLP (cecal ligation and puncture) could not demonstrate a synergistic or even additive effect on the inflammatory response. Such study compared a two-hit insult versus a single-hit murine model, although their evaluation was limited to the number of PMN and the concentration of albumin present after BAL (bronchoalveolar lavage) [16]. This goes to show that the type and dynamics of the insult are to be considered when experimental models are designed to reproduce the inflammatory effects of multiple-hit insults.

As a rule, patients can be exposed to a variety of first-hit events, such as trauma itself or any number of hemorrhagic, ischemic, or infectious insults. During their hospital stay, patients are exposed to second, third, or further sequential events (e.g., laparotomy, fluid replacement therapy, blood transfusions, fracture repair surgeries, infection via catheter, or other sources). The mechanics behind it is that the first hit serves as a priming event that sets the patient towards the establishment of SIRS (systemic inflammatory syndrome). SIRS in itself is a fairly straightforward diagnosis, consisting in the identification of two or more of the following criteria [11]: (a) body temperature below 36°C or above 38°C; (b) heart rate higher than 90 bpm; (c) respiratory rate in excess of 20 mpm or $PaCO_2$ lower than 32 mmHg; and (d) total white blood cell count above 12.000 mm^3 or below 4.000 mm^3 or the presence of over 10% band forms. If the first insult is by any chance infectious in nature, SIRS is loosely termed sepsis [17].

After the establishment of SIRS, a secondary, seemingly trivial, insult can jumpstart a detrimental organic response that can culminate in potentially lethal conditions such as MODS/MOF [14]. Depending on the type of sequential hits, the path following SIRS (which in this text is generally considered being end result of the first hit) is somewhat dualistic in nature. A first, anti-inflammatory state can ensue, which is called CARS (compensatory anti-inflammatory response syndrome) that, in and of itself, can be dangerous since it predisposes the body to infection that can in turn serve as one of the following hits. During CARS, immunosuppression occurs via impairment of T-cell function that can deteriorate the pathophysiological cascade and lead to infection, sepsis, MODS/MOF, and death [18–24]. A second proinflammatory state is triggered depending on the nature of the sequential events (additional hits). Major surgery, IR-like injury by fluid reperfusion in a previously hypovolemic patient, and infection by loss of gut barrier are some of the events that can serve as second or sequential hits. Cytokines and other molecular markers for both the anti- and proinflammatory states can be simultaneously found in patients facing hospitalization from several causes of SIRS/sepsis/MODS/MOF. This observation

implies that these two biological conditions are not so clearly separated in real-life biological conditions but rather serve as a dynamic modulatory system that ideally keeps both the suppression and augmentation of the inflammatory response in check throughout the clinical evolution of the patient. Additional hits tend to throw this system out of balance, causing the inflammatory response and clinical presentation to escalate. Regardless of the type of insult, the two-hit model postulates that the inflammatory response to the additional challenges is generally exaggerated, since the body has already been primed by the first event, or hit. The complex molecular cascades and the serious remote injuries triggered by this proinflammatory state are responsible for the potentially fatal state of MODS/MOF [17]. The two-hit model clearly shows that severely injured or ill patients are commonly more easily susceptible to the sequential insults, which singly or cumulatively lead to their unfavorable outcomes, observation which indeed can be easily correlated to common clinical outcomes.

The molecular pathways that explain the two-hit model are incredibly complex and, to this date, there has not been a definitive flow of events identified. One can easily understand why this is the case, since the variable nature of the first and subsequent events imposes a humongous challenge to the definition of the underlying inflammatory biochemistry behind the two-hit model theory. For that reason, any discussion of the events occurring during the progression of the multiple inflammatory hits has to take into account the specifics of every single type of insult. Below we discuss some general molecular and physiological aspects underlying the two-hit or multiple-hit inflammatory events.

2.1. Cellular Responses. After an initial insult the immune system is affected at a cellular level as the inflammatory cells become easily susceptible and primed to any sequential stimulus/insult and are therefore further activated by a minor sequential exposure, allowing mildly injurious stimuli to synergistically set off the inflammatory machinery and cause tissue damage [25–28] both locally and remotely. The immune response induced by the first hit can be traumatic in nature and may be limited locally as in monotrauma or it may be a massive systemic immune activation as in polytrauma [18, 21, 29–31]. Different trauma-related inflammatory actors have been recently characterized among which the complement system stands out as a key mediator [20, 21, 24, 32–37]. When the complement system is activated by any of its three pathways, it plays a pivotal role in eliminating foreign pathogens by opsonization/phagocytosis (C3b, C4b) and chemotaxic attraction of leukocytes (C3a, C5a) and also directly lysing the pathogens through the membrane attack complex (MAC, C5b-9) [35, 38–40]. The anaphylatoxins like C3a and C5a recruit phagocytes and polymorphonuclear leukocytes (PMN) to the site of injury as these anaphylatoxins are strong chemoattractants for phagocytes [41] and also induce the degranulation of mast cells, basophils, and eosinophils [35, 39, 40]. It is clear from clinical and experimental studies that after trauma the complement system gets activated both locally and at the injury site, as well as systemically [42–47]. Tissue damage and cell injury cause the release of alarmins, which are non-pathogen-derived danger signals capable of activating the innate immune responses. These include annexins, heat-shock proteins (HSPs), defensins, and classical markers of tissue injury like S100 protein and high mobility group box-1 (HMGB1) nuclear protein [48, 49]. Alarmins correlate with the heterogenic innate immune inflammatory molecules and pathogen-associated molecular patterns (PAMPs), which are recognized by the immune system as foreign molecules because of their characteristic molecular pattern [50, 51]. Together, alarmins and PAMPs form a large family of damage-associated molecular patterns (DAMPs) and are recognized by immune cells that express multiligand receptors, such as Toll-like receptors (TLRs), on their surfaces. Therefore DAMPs are capable of activating innate immune responses after trauma either when the traumatic injury is a standalone event or when the traumatic event is further complicated by infection [24, 48]. It is reasonable to assume that similar triggers are present when the first and sequential hits are not traumatic in nature.

2.2. Molecular Responses. Whatever the cause of the insult, it is generally accepted that cytokinemia or cytokine storm is of major importance during the biological responses inside the two-hit model [12]. Cytokines are molecules of low molecular weight that are secreted by immune cells and serve as mediators for the communication between leukocytes, interlinking innate and adaptive immune responses. Traumatic tissue injuries induce the expression of proinflammatory cytokines, such as tumor necrosis factor (TNF) and interleukin- (IL-) 1β, IL-6, IL-8, IL-12, IL-15, and IL-18 [52–54]. In addition to other biological roles, cytokines also activate neutrophils which are key players in the early inflammatory response to trauma or other first-hit insults [55]. Neutrophils are pulled away from the circulation to the site of injury by chemotactic molecules, such as complement anaphylatoxins and chemotactic cytokines, called chemokines, most notably IL-8 [39, 56]. Several studies have tracked cytokine production during inflammatory conditions, demonstrating their primary role in tissue damage by cellular priming/activation and also in the pathophysiology of SIRS. In particular, tumor necrosis factor-α (TNF-α), IL-1β, IL-6, and IL-8 have been consistently present during these observations. Nevertheless inconsistent results in terms of levels of TNF-α, IL-1β, and IL-6 have prevented a definitive association between high concentration of these agents and the risk for development of MODS/MOF [57, 58]. In an ideally regulated immune response, neutrophils play an important role in the defense and repair of injured tissues. PMN priming for cytotoxicity covers a wide range of physiologic responses, like degranulation of enzymes, superoxide anion generation, lipid mediator (LTB4) and cytokine (IL-8) production, decreased selectin expression (L-selectin), enhanced integrin expression (CD11b/CD18), cellular elongation, reduced deformability, and delayed apoptosis [59–63] in addition to other cellular events such as adhesion, rolling, and ultimately diapedesis [20, 64, 65]. Neutrophil priming results from the preexposure of the cell to priming molecules like platelet activating factor (PAF), anaphylatoxin C5a, granulocyte macrophage-colony stimulating factor (GM-CSF), LTB4, substance P, IL-8, interferon, TNF-α, LPS, L-selectin cross-linking, and

CD18 cross-linking [20, 66–68], which could arise from exposure to first-hit events [66, 69]. Some investigators have suggested that circulating monocytes and tissue macrophages also become primed after severe injury [70–72]. Despite the beneficial effects of neutrophils in host defense, a dysfunction in priming and subsequent cellular activation may result in an overwhelming inflammatory response. Such response leads to tissue injury of previously healthy sites via the local release of toxic metabolites and enzymes that may lead to acute respiratory distress syndrome (ARDS), MODS/MOF, secondary blood-brain barrier dysfunction, and brain edema after traumatic brain injury [21, 68, 73–79].

2.3. Vascular Responses. Aside from circulating cytokines and signaling molecules, the response of the endothelium and its relationship with immune cells are central in understanding the process through which sequential insults can cause tissue injury. Endothelial cells are obviously present in virtually every single organ and are in constant contact with immune molecular and cellular mediators. Therefore, rather than simply being a passive pipe-like structure, the endothelium serves as an important and complex immune agent and its dysfunction is closely associated with increased morbidity in SIRS and its complications via an increase of uncontrolled vascular permeability [80]. In addition, microvascular changes caused by a first hit such as hemorrhagic shock have been implicated as one of the mechanisms through which a second hit such as infection is able to cause an exaggerated systemic inflammatory response [7] that is more likely to trigger MODS/MOF.

Central to the role of the endothelium in immune responses is the glycocalyx, a thin and complex structure of proteoglycans, glycosaminoglycans, glycoproteins, and other soluble molecules, which serves as a dynamic interface through which the vascular bed communicates with the flowing blood through continuous shedding and synthesis of this layer [81, 82]. Functions of the glycocalyx vary widely, from conferring the outer surface of the endothelium an overall negative charge to regulating vascular permeability and fluidic balance, all the way to preventing erroneous and inadvertent adhesion of leukocytes and platelets to the vascular wall by mechanically shielding molecules such as intercellular adhesion molecule 1, vascular cell adhesion molecule 1, and selectins [81, 83]. The binding of cytokines in the glycocalyx also plays an important role in enclosing and effectively hiding these molecules from circulating leukocytes cell surface receptors. Loss of glycocalyx function has been observed under many inflammatory processes such as diabetes, atherosclerosis, sepsis, and IR injury due to, amongst other things, changes in the interaction of the exposed vascular bed and circulating leukocytes and increased vascular permeability [81, 82].

Endothelial cells express innate immune receptors, such as Toll-like receptors, which can trigger intracellular inflammatory responses through mediators such as MAPK and NF-κB that can ultimately modulate vascular permeability and coagulation [84]. It is even conceivable that thrombi inside the microvasculature may have a role in mechanically preventing infectious agents from spreading. Albeit never actually dormant, the overt activation of the endothelium can be triggered as a natural and physiological response to the stimulation of the innate immune response via reactive oxygen signaling dominance due to an uncoupled state of the endothelial nitric oxide synthase (eNOS) or as a pivotal part of many different disease processes involved mainly in cardiovascular illnesses [85].

In healthy and undisturbed endothelium, cell junctions are constantly regulated to preserve overall vascular barrier integrity while allowing for the passage of small molecules and immune cells that are responsible for tissue surveillance [86, 87]. Activated neutrophils and their interaction with the endothelium, involving neutrophil adhesion and subsequent transendothelial migration, play a crucial role in SIRS pathophysiology and are closely related to endothelial dysfunction, associated with loss of functional intracellular contact sites. This loss of integrity of cell-to-cell contact results in tissue edema and impairment of microcirculation and ultimately leads to organ dysfunction [88–90]. Microvascular endothelium also has an integral role in postinjury priming of the innate inflammatory response. Neutrophil priming agents such as LPS and TNF cause endothelial activation by stimulating the expression of adhesion molecules (e.g., ICAM and VCAM) on the vascular bed that can account for tissue damage-driven cell migration [91–93].

The process of neutrophil activation involves complement dependent and complement independent mechanisms. When the blood comes in contact with the activated endothelium it strongly activates the complement and clotting cascades which in turn causes neutrophil activation via the anaphylatoxins C3a, C5a, and C5b9 [94], as previously discussed. Neutrophil adhesion to the endothelium is carried out by cytokines such as IL-1 and PAF but also by adenosine, prostacyclin, and cAMP. Endothelial activation causes the high expression of adhesion molecules or activation of constitutively expressed molecules like ICAM-1, leukocyte function-associated antigen-1 (LFA-1), or E-selectin [95, 96]. The endothelial activation by cytokines leads to the upregulation of adhesion molecules and can be accompanied by the expression of anaphylatoxins C3a and C5b, PAF, and LTB4. TNF-α and IL-1 result in neutrophil degranulation and further activation of endothelial cells. For example, neutrophil degranulation induced by TNF-α leads to vascular endothelium architecture destruction by proteolytic enzymes such as elastase, gelatinase, and collagenase and subsequently to the disruption of vascular wall integrity as elastase degrades the endothelial homodimeric cadherin-cadherin binding [97]. The overall result of this process is augmented permeability, adhesion, and migration of PMN and other leukocytes to locally or remotely affected tissues and organs, causing tissue damage, organ dysfunction, and ultimately MODS/MOF [91–93].

The combination of these cellular, molecular, and vascular phenomena needs to be constantly and ever so delicately balanced and regulated. The sheer simplicity of the reasoning behind the two-hit argument is that sequential and superimposed insults tend to challenge this fragile environment and are capable of synergistically rerouting the inflammatory response to a tissue injury-driven path that can be ultimately

recognized through the progression of the complex SIRS/sepsis/ARDS/MODS/MOF continuum, and possibly death.

3. IPC: An Elegant and Ingenious Solution for Modulating the Inflammatory Response to Ischemia

As early as 1986, researches were already noticing the protective effects that short-term nonlethal ischemic cycles could have over the heart if it were to be later exposed to a prolonged, potentially fatal ischemic event [10] and in 1990 a similar phenomenon had already been identified in the brain of gerbils [98]. This basic mechanism itself can be established in a variety of different organs and systems, and the initial protection-inducing ischemia can be of varying types and profiles. IPC or ischemic preconditioning is the general term that describes such phenomena. Some aspects of preconditioning through ischemia can even be naturally occurring as it is seen in patients that suffer from cerebral transient ischemic attacks (TIAs) and do not incur in structural damage to neurons but rather are actually protected from subsequent major episodes [99] demonstrating that IPC-like mechanisms can be adaptive in nature.

In general terms, IPC can be achieved by relatively gentle and often cyclic ischemic events. A tissue that undergoes an ischemic event is more likely to survive a subsequent prolonged deprivation of oxygen. Ideal duration and proportion of these initial insults of preconditioning are species- and tissue-specific. For example IPC can be achieved in rats during one to three cycles of IR [100, 101], while in rabbits a single 5 min cycle of IR is sufficient [102] and in dogs a 2.5 min single event of IR has been proven to be effective [103].

Processes involved during reperfusion of severe ischemic injuries are a consequence of a complex sequence of events leading to changes in capillary permeability, neutrophil recruitment, complement activation, and generation of reactive oxygen species [104], similar to other inflammation-driven insults. Preconditioned systems tend to attenuate these responses to IR and ultimately ameliorate IR injury. Two distinct phases of IPC can be observed [102, 105]. An early or classic stage of protection, independent of protein syntheses, begins almost immediately after the mild ischemic insult and can be sustained for up to 3 hours [106]. A second or delayed phase lasts for up to 24 hours after the first preconditioning hit and is based on synthesis of new proteins and altered gene expression [106].

Aside from local protection of tissues close to the preconditioned area, remote or distant protection of organs (RIPC, or remote ischemic preconditioning) can also occur. In broader terms, this notion means that exposing various tissues or organs (such as limbs) to mild preconditioning ischemic events can cause other distant organs and tissues to be more resilient to following ischemic insults, even though the latter were not directly preconditioned in the first place. This had already been demonstrated in 1993 when McClanahan and colleagues showed in a preliminary report that myocardial infarct size was reduced if rabbits were previously exposed to a brief transient occlusion of the renal artery [107]. In 1996 Gho et al. proved that brief occlusion of adjacent coronary arteries, left renal artery, and anterior mesenteric artery protected the myocardium from a subsequent prolonged ischemic event [108]. A murine model subject to two hours of ischemia of the hind limb showed significant protection against local (leg skeletal muscle) and distant (intestinal injury and lung infiltrates) organ injury by a subsequent severe ischemic event [109]. IPC also caused a marked mortality decrease up to one week after ischemia [109]. Due to its effect on decreasing neutrophil infiltrates in the lung [109–111] and because of the systemic attenuation of subsequent inflammatory responses, IPC could be a tool for modulating systemic inflammation and for preventing local and distant organ injury or even SIRS/sepsis or ARDS. Another clinical application of remote or distant IPC is the protection that it delivers against acute kidney injury following major cardiac surgery, demonstrated by the reduced rate in which patients subject to the procedure demand renal replacement therapy [112]. The possibility of remotely achieving IPC is pivotal in a number of conditions. During heart surgery, for instance, the repetitive clamping and declamping of major blood vessels to induce local IPC on the heart can cause the formation of emboli in addition to the risk that short repetitive ischemic episodes can be traumatic in nature to the organ itself [106]. Therefore, the possibility of triggering remote systemic protection against ischemia in organs such as the heart is invaluable [113, 114].

The molecular mechanisms through which IPC or RIPC protection occurs are still unclear and not a single definite pathway has been established. It seems that the protective state of IPC is achieved through a combination of humoral, neural, and systemic components [106, 115]. Principle mediators are adenosine, reactive oxygen species (ROS), NF-κB, bradykinin, opioids, angiotensin, endocannabinoids, and nitric oxide (NO) that alter cellular metabolism via ATP-sensitive K^+ ion channels and receptors that direct transcription of survival proteins and activation of intracellular kinases that ultimately protect against oxidative stress [105, 115–123]. The role of the endothelium seems to be central during the development of ischemic resistance through IPC. Local and systemic endothelial function was proven to be greatly enhanced when human subjects were exposed to daily short-term limb ischemia for a period of 7 days, with measurable improvements in resting skin microcirculation and brachial artery function assessed by FMD (flow-mediated dilation), effect which lingered after the late phase of ischemic protection was over [25]. Another study has shown that IPC is capable of protecting tight junctions both functionally and structurally on hearts that would otherwise suffer from cell-to-cell collapse and edema when exposed to severe IR injury [26]. Moreover, the role of endothelial nitric oxide synthase (eNOS) and its NO output has been implicated as relevant for the ischemic preconditioning both in early and late IPC [27, 124, 125].

The reasoning behind the triggering mechanisms of IPC is that short preconditioning insults generate enough tissue damage upon reperfusion as to cause the release of adenosine (from the breaking of ATP molecules), bradykinin, and ROS [105, 126]. These substances trigger a molecular cascade that

begins in the translocation and activation of protein kinase C and culminates in the phosphorylation of HSP27 via p38 MAPK and the opening of ATP-sensitive K^+ channels [126]. The role of organelles such as mitochondria and their function in regulating intracellular Ca^{2+}/K^+ exchange has also been implicated in the mechanisms of IPC protection in neuronal tissues [127], since the artificial opening of ATP-sensitive K^+ channels can mimic some of the effects of IPC [128]. Many molecular mechanisms of IPC have the inhibition of mPTP (mitochondrial permeability transition pore) channels as their endpoint, which, if opened, mediate cell death by ATP depletion and mitochondrial swelling [115, 129]. Activation of PKC measured by its intracellular translocation and the role of several kinases such as PI3, ERK/MAPK, and JAK/STAT are pivotal in conferring the state of protection during IPC [115, 121, 129].

Furthermore, IPC can attenuate or eliminate O_2^- production and its effects by suppressing endothelin-1 (ET-1) secretion via the opening of these mitochondrial KATP channels prior to subsequent ischemic events, since ET-1 generation is related to increased production of superoxide anion and endothelial dysfunction with increased P-selectin expression and neutrophil adhesion [130].

Nevertheless, due to the complexity of these mechanisms, results generally lack consistency when it comes to defining pathways through which IPC occurs. For example, in two separate knockout models, NO produced by an increased expression of NF-κB has been shown to be relevant in the ischemic protection of the heart [131], while the same role could not be demonstrated in the protection of the intestines and brain tissue [132].

The suppression of TNF-α and Bax (both involved in apoptotic stimulation) and the stimulation of cellular survival mechanisms such as phosphorylation of ERK-1/2 and Akt simultaneous to the upregulation of Bcl-2 have been identified in IPC of the myocardium [133] as mechanisms through which cell survival surpasses the rate of apoptosis after IR injury. Once again, results concerning the role of TNF-α in IPC are inconsistent. Some studies have suggested a protective self-regulatory effect that the secretion of TNF-α could have on the stimulation of NF-κB and on the suppression of proinflammatory proteins during subsequent ischemic injury [133].

Other recently unveiled novel molecular mediators of IPC have risen to the stage as possible targets in the investigation of the protective cascades of preconditioning. A study from 2013 showed that concentrations of HIF1-α and procaspase-3 were higher in patients receiving RIPC by upper limb ischemia before cardiopulmonary bypass. These same patients had higher right atrial tissue and systemic concentrations of IL-1β, IL-8, and TNF-α, indicating the direct influence of RIPC in the modulation of apoptosis and inflammation [134]. The role of Cx43 (connexin 43) has also been established in both local and remote IPC, which attenuates the ischemia-induced dephosphorylation of Cx43 that would otherwise cause the mechanical, chemical, and electrical instabilities in cardiomyocytes gap junctions by opening of the Cx43 hemichannels [135, 136]. This stabilization of Cx43 might be related to its association with PKC and p38MAPK [135]. A relevant role of microRNA 144 has also recently been revealed. Levels of mRNA 144 were found to be increased after RIPC and the exogenous intravenous administration of mRNA 144 was capable of mimicking the protective effect of RIPC in rescuing tissue from IR injury. Furthermore, increased activation of Akt and p44/42MAPK and decreased levels of mTOR were observed after the administration of mRNA 144, suggesting that the molecule could serve as a biomarker for the efficacy of a conditioning procedure [115, 137].

IPC as a technique has very practical applications. It can potentially be used as therapeutic preparation for surgical procedures that require some kind of ischemic episode, like in organ transplants. Another very important application of the model for real-life pathologies is the identification of novel molecular targets that are involved in IR injury and in the protection against said injuries. The modulation of some of these molecular targets and pathways can serve as treatment for naturally occurring ischemic insults seen in strokes, in thromboembolic injuries, and in acute myocardial infarction, to name a few.

4. Conclusion

As stated before, neither IPC nor the multiple-hit hypotheses are particularly new ideas, but it was not until the beginning of the century that more and more research has focused on one of the two models. During this paradigm shift, the idea that the two-hit model can satisfactorily account for many real-life pathological processes has been somewhat pushed aside.

It is logical to assume that, because of its nature, IPC is a mechanism that can be spontaneously found in the body and its artificial mimicking consists of a way to tap into the underlying responses of the immune system upon inflammatory insults and can serve as a tool for modulating these responses. Therefore, the two-hit model and IPC are not competing models in principle. While the former serves as a tool to explain the intricate processes that can be brought about via multiple sequential inflammatory insults which occur during a host of disease processes, such as trauma, hemorrhagic shock, and sepsis, the latter is an active investigation of techniques and strategies that can modulate the inflammatory response to a number of ischemic insults.

Nevertheless, there is an obvious paradox surrounding the subject: how can an inflammatory, ischemic insult drive the triggering of a protective mechanism to a subsequent ischemic insult, while the two-hit model states that a priming insult, including ischemia, should prepare the body to an exaggerated response to a second hit or insult, which incidentally can also be ischemic in nature. To our knowledge, there are no definitive answers to this conundrum. That the initial preconditioning ischemic event should not be harsh enough to cause serious tissue injury while maintaining enough potency to trigger the molecular mechanisms relevant to preconditioning seems reasonable and intuitive, but, other than that, no clear differentiation has been established concerning the nature of the first event in each model. Researchers have

already mapped protocols to ensure that certain ischemic events are limited in intensity and duration so that they would not serve as priming insults for the host while still holding their beneficial effects [100–103], but aside from this intuitive distinction little is known concerning what makes an insult behave in either a priming or protective manner.

During this review many pathways, examples, and applications of both the two-hit and IPC models were explored, and little juxtaposition was found. The goal behind this text was exactly that of reconciling both models and demonstrating that each of the seemingly contradictory perspectives has a lot to offer as platforms to better understand and intervene in some very real and relevant medical situations, while maintaining the fact that both theories are not mutually exclusive.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Carlos F. M. Morris and Muhammad Tahir contributed equally to this work.

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Research Article

fMLP-Induced IL-8 Release Is Dependent on NADPH Oxidase in Human Neutrophils

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N-Formyl-methionyl-leucyl-phenylalanine (fMLP) and platelet-activating factor (PAF) induce similar intracellular signalling profiles; but only fMLP induces interleukin-8 (IL-8) release and nicotinamide adenine dinucleotide phosphate reduced (NADPH) oxidase activity in neutrophils. Because the role of ROS on IL-8 release in neutrophils is until now controversial, we assessed if NADPH oxidase is involved in the IL-8 secretions and PI3K/Akt, MAPK, and NF-κB pathways activity induced by fMLP. Neutrophils were obtained from healthy volunteers. IL-8 was measured by ELISA, IL-8 mRNA by qPCR, and ROS production by luminol-amplified chemiluminescence, reduction of ferricytochrome c, and FACS. Intracellular pH changes were detected by spectrofluorescence. ERK1/2, p38 MAPK, and Akt phosphorylation were analysed by immunoblotting and NF-κB was analysed by immunocytochemistry. Hydroxy-3-methoxyaceto-phenone (HMAP), diphenyleneiodonium (DPI), and siRNA Nox2 reduced the ROS and IL-8 release in neutrophils treated with fMLP. HMAP, DPI, and amiloride (a Na^+/H^+ exchanger inhibitor) inhibited the Akt phosphorylation and did not affect the p38 MAPK and ERK1/2 activity. DPI and HMAP reduced NF-κB translocation induced by fMLP. We showed that IL-8 release induced by fMLP is dependent on NADPH oxidase, and ROS could play a redundant role in cell signalling, ultimately activating the PI3K/Akt and NF-κB pathways in neutrophils.

1. Introduction

Polymorphonuclear neutrophils (PMNs) are the first line of defence against microorganisms and are the main cellular component in the acute inflammatory response.

Neutrophils are primarily activated by chemotactic factors such as fMLP [1] and PAF [2].

Both compounds bind to the neutrophil cell surface via specific seven transmembrane domain G-protein coupled receptors [3, 4] and induce the activation of MAPK, PI3K, and NF-κB pathways in neutrophils [5, 6]. Notably, only fMLP has been described as a potent inducer of IL-8 in human neutrophils [7]. IL-8 is a member of the CXC chemokine family, relevant to the pathogenesis of several acute inflammatory processes and to the tissue damage associated with neutrophils [8]. Because PAF alone is not able to induce IL-8 production [9], the existence of differential mechanisms of cell signalling, essential for neutrophil IL-8 release induced by fMLP, seems likely. It is widely known that fMLP induces a significant increase in superoxide production via NADPH oxidase; in contrast, basal levels of superoxide are unaltered in PAF activated neutrophils [9, 10]. In fact, neutrophils produce a strong respiratory burst, resulting in the release of a diversity of radical oxygen species (ROS), during phagocytosis or following stimulation with a wide variety of agents [11]. ROS originate from the activation of NADPH oxidase, which is assembled at the plasma membrane. This reaction produces two superoxide anions (O_2^-) and $2H^+$. The H^+ accumulation induces a transient intracellular acidification that activates several compensatory mechanisms such as Na^+/H^+ exchanger (NHE), H^+ channels, and V-ATPase [12], which promote intracellular

alkalinisation [5]. ROS have been proposed as signalling molecules that regulate diverse responses in neutrophils, including cytokine expression [13–15]. It has been described that the superoxide anion induces NF-κB activation (IκBα degradation and p65 NF-κB translocation) and increases the expression of TNFα and macrophage inflammatory protein-2 in neutrophils [16]. However the role of ROS in cytokine expression is until now controversial in neutrophils. Human neutrophils from chronic granulomatous disease (CGD) that have genetic mutations in any of the components of the NADPH oxidase enzyme show an increase of IL-8 production induced by fMLP, suggesting that ROS reduce the IL-8 production in neutrophils [17]. Moreover, exposure of bone marrow-derived neutrophils to extracellular H_2O_2 diminished LPS induced activation of NF-B and expression of NF-B-dependent proinflammatory cytokines [18, 19].

In the present work, we present evidence that supports the role of NADPH oxidase in IL-8 release, the PI3K/Akt pathway, and NF-κB activity in human neutrophils treated with fMLP.

2. Materials and Methods

2.1. Reagents. Platelet-activating factor (C-16), fMLP, actinomycin D, SN50, UO126, LY294002, and SB203580 were obtained from Calbiochem (La Jolla, CA, USA). Histochoice, andrographolide, 4-hydroxy-3-methoxyacetophenone (HMAP), diphenyleneiodonium (DPI), and monoclonal antibody against β-actin were purchased from Sigma-Aldrich (St. Louis, MO, USA). The Akt inhibitor (sc-394003) 1L-6-hydroxymethyl-chiro-inositol-2-[(R)-2-O-methyl-3-O-octadecylcarbonate] was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Hank's balanced salt solution (HBSS), Iscove's Modified Dulbecco's medium (IMDM) Penicillin-streptomycin, certified foetal bovine serum, hydroethidine (HE), BCECF-AM, and nitrocellulose membrane were purchased from Invitrogen (Grand Island, NY, USA). Monoclonal antibodies against phospho-ERK1/2, phospho-p38, phospho-Akt (ser473), Akt, p38, rabbit IgG-HRP, and mouse IgG-HRP were purchased from Cell Signalling (Beverly, MA, USA). Polyclonal antibodies against ERK1 (sc-94), p65 NF-κB, Nox2 (sc-5827), gp91-phox siRNA, nonsilencing control siRNA, siRNA Transfection Reagent (sc-29528), and siRNA Transfection Medium (sc-36868) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Human IL-8 CytoSet Kit was purchased from Biosource International (Camarillo, CA, USA) and PE Mouse Anti-Human IL-8 (#554720) was purchased from BD Pharmingen. Proteases inhibitors were purchased from Roche Diagnostics (Indianapolis, IN, USA). Affinity Script Reverse Transcriptase and Brilliant II SYBR Green QPCR master mix were purchased from Stratagene (USA). SV Total RNA Isolation System was obtained from Promega (Madison, WI, USA). All other reagents and chemicals were purchased from Merck (Darmstadt, Germany).

2.2. Isolation of Neutrophils. Neutrophils were obtained from the fresh blood of healthy adult human volunteers in accordance with guidelines set by and with the approval of the Bioethical and Bio-Safety Committee of Universidad Austral de Chile. Blood was collected in ACD vacutainer tubes, and neutrophils were purified by discontinuous Percoll gradient centrifugation. Neutrophils were suspended in Hank's Balanced Salt Solution (HBSS) (5.33 mM KCl, 0.441 mM KH_2PO_4, 138 mM NaCl, 0.34 mM Na_2HPO_4, and 5.56 mM D-glucose). Purity and viability were greater than 95% as determined by May-Grünwald Giemsa staining and trypan blue exclusion, respectively.

2.3. Cell Viability. Neutrophils (5×10^4/well) suspended in 100 μL HBSS were incubated with 500 μM HMAP, 10 μM DPI, 100 and 500 μM amiloride, 1 μM UO126, 10 μM LY294002, 10 μM SB203580, 10 μM AKT inhibitor, or vehicle (0.2% DMSO) for 30 min and stimulated with fMLP 100 nM for 4 h at 37°C. After that, we used the CellTiter-Glo Luminescent Cell Viability Assay according the manufacturer instruction (Promega, Madison, WI, USA).

2.4. Determination of IL-8 Release by ELISA. Neutrophils (2×10^6) were incubated with HMAP, DPI, amiloride, UO126, LY294002, SB203580, or vehicle for 30 min and stimulated with PAF or fMLP for 4 h. Supernatants were collected, and IL-8 was determined according the manufacturer's instructions (IL-8 Kit, Biosource).

2.5. Determination of Intracellular IL-8 by Flow Cytometer. Neutrophils (1×10^6) in HBSS were incubated with 500 μM HMAP, 10 μM DPI, 500 μM amiloride, 1 μM UO126, 10 μM LY294002, 10 μM Akt inhibitor, 10 μM SB203580, or vehicle (0.2% DMSO) for 30 min and stimulated with fMLP for 4 h at 37°C. Afterward neutrophils were centrifuged (300 ×g) for 6 min. The cells were fixed using paraformaldehyde 4% in PBS by 10 minutes at room temperature. Then the cells were washed twice using 500 μL of PBS. Afterward, the cells were permeabilized using 0.5% triton X-100 in PBS for 15 min and afterward washed twice with PBS. Then, neutrophils were incubated overnight at 4°C in 1% BSA-PBST (PBS-Tween 0.1%) containing PE Mouse Anti-Human IL-8 (1 : 100) or 0.25 μg mouse isotype antibody (5415 from Cell Signaling). A sample lacking the primary antibody was included as a control. Cells incubated with isotype antibody were incubated with 1% BSA-PBST with 1 : 1000 PE goat anti-mouse Ig (#550589) from BD Pharmingen (CA, USA) for 2 h at room temperature in the dark. Finally, the cells were washed with PBS and suspended in 300 μL of PBS, and they were assessed by flow cytometry FACSCanto II (BD, CA, USA) flow cytometer and analysed using FlowJo 7.6 software (FlowJo, OR, USA).

2.6. Real Time PCR of IL-8. Neutrophils (4×10^6) were incubated with fMLP for 1 h, and then 10 μM actinomycin D, 500 μM HMAP, 10 μM DPI, or vehicle was added for 1 or 3 h and total RNA was isolated. The RNA was treated with DNase and cDNA synthesis was made using 200 ng of total RNA. Real time PCR was performed using SYBR Green and primers of IL-8 and β-actin in MX3000P QPCR (Stratagene, USA) according to the conditions described elsewhere [20].

2.7. ROS Production. Luminol-amplified chemiluminescence: neutrophils (1×10^6) were suspended in HBSS (250 μL/well) with 50 μM luminol in the presence or absence of inhibitors (HMAP, DPI, amiloride, UO126, LY294002, or SB203580) for 10 min. Cells were subsequently stimulated by the addition of different concentrations (1 nM–10 μM) of PAF or fMLP, or 100 nM PAF or fMLP, and the emission of light was recorded by a luminometer at 37°C for 30 min.

Superoxide production: O_2^- release was monitored spectrophotometrically at 37°C by measuring O_2^- dismutase-inhibitable reduction of ferricytochrome c at 550 nm. Assays were performed in 96-well microtiter plates [21]. Control wells contained all components of the assay mixture plus O_2^- dismutase (20 U/mL) to correct for ferricytochrome c reduction by agents other than O_2^-. Cells (3×10^5) were suspended in HBSS (200 μL/well), incubated with inhibitors for 10 min, and stimulated by the addition of 100 nM PAF or fMLP. Absorbance (optical density) at 550 nm was recorded by a microplate reader (Tecan, Sunrise). O_2^- release was measured under conditions of linearity with respect to time and cell number, and O_2^- release was expressed as nmol $O_2^-/3 \times 10^5$ PMNs [21]. Additionally, superoxide production was assessed by flow cytometry using the fluorescent probe hydroethidine (HE). HL-60/neutrophils cells were loaded with 10 μM HE for 5 min at 37°C; then vehicle or fMLP was added and the superoxide production was measured at 10 min in FACSCanto II (BD, CA, USA) flow cytometer, with excitation at 488 nm and emission using a 610 nm absorbance long pass filter.

2.8. RNA Interference Assay. Small interfering RNA (siRNA) targeting human gp91-phox (Nox2) and a nonsilencing control RNA were used. HL-60 cells were differentiated to neutrophils using 1.3% DMSO in IMDM medium for 5 days. Differentiated HL-60 cells were transiently transfected with each siRNA in siRNA Transfection Medium according to the manufacturer's protocol. Approximately 48 h posttransfection total proteins were isolated and gp91-phox levels were detected by immunoblot. Also, cells were stimulated with fMLP and assessed for superoxide production by flow cytometry and IL-8 production by ELISA, according to the protocols described above.

2.9. Neutrophil Intracellular pH. PMNs (2×10^7 cells/mL) were suspended in a pH 7.2 buffer (140 mM NaCl, 10 mM glucose, 1 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 20 mM HEPES) and incubated with BCECF-AM (2.5 μM; Molecular Probes, Oregon, USA) for 30 min at 37°C. The cells were then washed twice and suspended at 4×10^6 cells/mL. The 8×10^6 BCECF-loaded neutrophils were incubated with either vehicle, HMAP, DPI, amiloride, UO126, LY294002, or SB203580 for 10 min, followed by exposure to fMLP or PAF. Fluorescence was measured in a thermoregulated spectrofluorometer (LS55 Perkin-Elmer) at 490 and 440 nm of excitation and 535 nm of emission. The solution was continuously stirred. Fluorescence was converted to pH units using nigericin methods of calibration [5].

2.10. Immunoblotting. Neutrophils (5×10^6) were incubated with HMAP, DPI, amiloride, or vehicle for 30 min and then incubated with fMLP or PAF (100 nM) for 2 min. For ERK1/2, p38 MAPK, and Akt phosphorylation determinations, total protein extracts were prepared and resolved (50 μg) by 12% SDS/PAGE. Immunoblotting was performed using monoclonal antibodies against phospho-ERK1/2 and total ERK1/2, phospho-p38 and total p38, and phospho-Akt (ser473) and total Akt [5]. Blots were developed with ECL. The primary antibodies were stripped, and each membrane was reprobed with an antibody recognising total nonphosphorylated protein. Reprobed signal was detected as described above.

2.11. Immunocytochemistry. Neutrophils were incubated with UO126, LY294002, SB203580, HMAP, DPI, SN50, or vehicle for 30 min and stimulated with fMLP for 30 min. Cytospin was performed, and cells were fixed with Histochoice for 10 min and washed three times with PBS. Cells were subsequently permeabilized with 0.3% Triton X-100 in PBS for 15 min and washed three times with PBS. Each cytospin spot was then incubated with blocking buffer (1% BSA, 5% nonfat milk, and PBS) for 1 hour followed by incubation with an antibody directed against p65 NF-κB in blocking buffer overnight at room temperature. Cells were then washed three times with PBS and incubated with Alexa Fluor 488-conjugated goat anti-rabbit antibody (1 : 200) for 2 hours in the dark; nuclei were counterstained with propidium iodide. Cells were then washed with PBS, mounted with fluorescence medium, and examined by confocal microscopy (Fluoview 1000, Olympus). The Image ProPlus software 4.5.1 (Media Cybernetic, MD, USA) was used to measure nuclear or cytoplasm localization of p65 NF-κB.

2.12. Statistical Analysis. Results are expressed as fold increase compared to control, percentage, or area under the curve (AUC) and reported as mean ± SE. An ANOVA was performed and Dunnett's multiple comparison test was applied using GRAPH PAD V 2.0. The level of significance used was 5%.

3. Results

3.1. fMLP Produces High Levels of IL-8 and ROS and Increases the Intracellular pH. We determined the effects of fMLP and PAF on IL-8 release and ROS production as well as intracellular pH. The concentration of IL-8 was assessed by ELISA in supernatants of cells treated with each chemotactic factor for 4 hours. Only fMLP 1 induced an increase in IL-8 release compared to the basal control; IL-8 release induced by PAF was similar to the basal control (Figure 1(a)). Production of ROS was assessed by luminol-amplified chemiluminescence and reduction of ferricytochrome c, to measure total ROS and extracellular superoxide release, respectively. A rapid and significant increase in ROS and superoxide production was observed in fMLP treated neutrophils with maximum peak at 112 s (Figure 1(b)). Following this peak, ROS release decreased rapidly and a second peak of smaller intensity in some volunteers was observed. This minor peak was distinctive and unique for each individual volunteer.

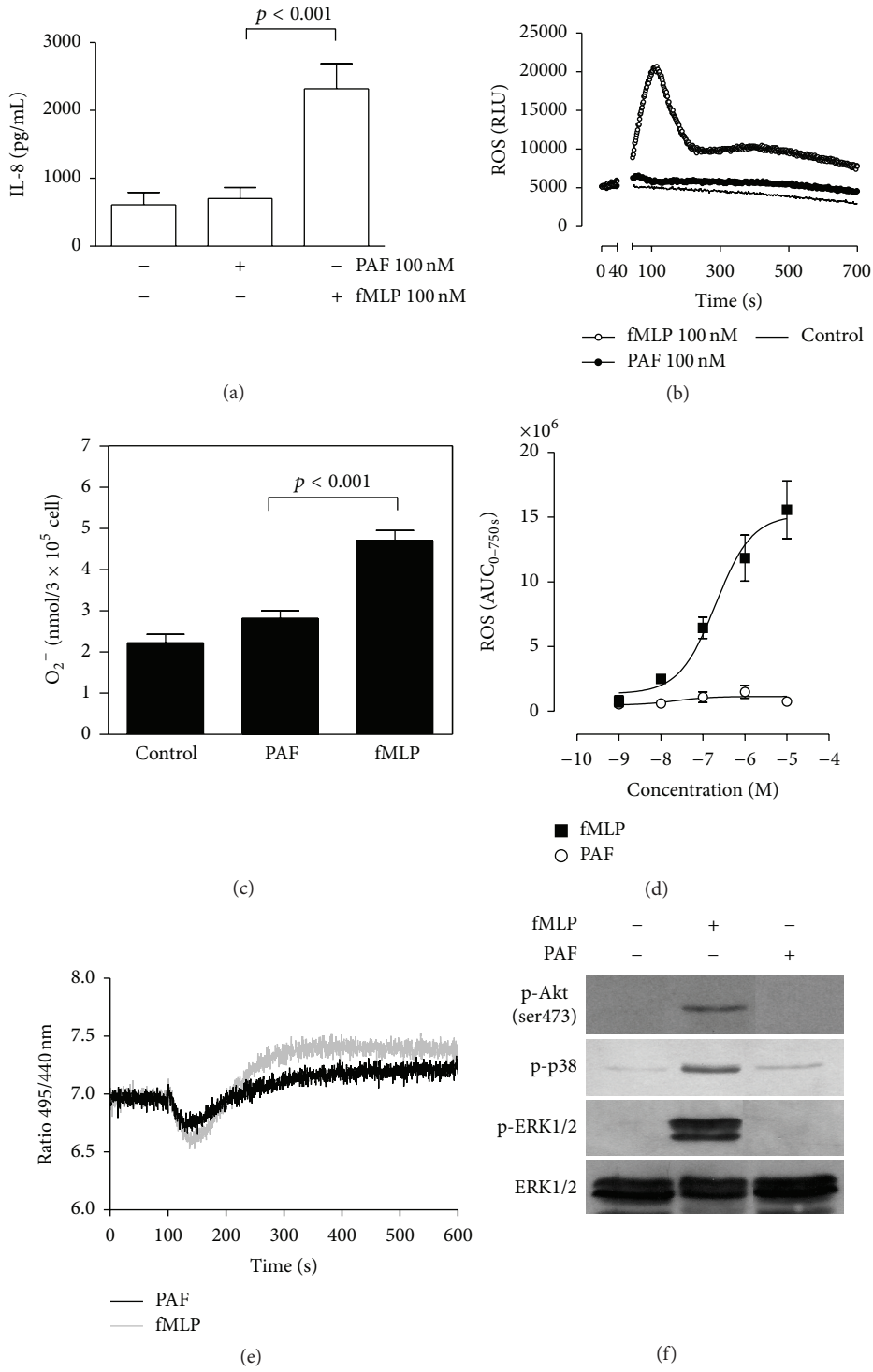

Figure 1: fMLP increases IL-8 release, ROS production, intracellular pH, and ERK1/2, p38 MAPK, and Akt phosphorylation. Human neutrophils were incubated with 100 nM fMLP or PAF for 4 h, and the IL-8 concentration in the supernatants was measured by ELISA (a). Neutrophils were incubated for 5 min at 37°C before fMLP or PAF was added. ROS production was monitored for 1200 s using a luminescence assay. RLU: relative luminescence unit (b), and superoxide production was measured following 30 min of incubation by a cytochrome c reduction assay (c). Curve dose response of ROS production in neutrophils stimulated with fMLP or PAF. AUC: area under curve for 700 s (AUC_{700}) (d). BCECF-AM-loaded neutrophils were incubated for 5 min at 37°C, fMLP or PAF was added, and the signal was measured for 600 s in a spectrofluorometer (e). Neutrophils were incubated with fMLP or PAF for 2 min, and total protein was analysed by immunoblot with specific antibodies against the phosphorylated form of Akt, p38 MAPK, and ERK1/2. In this case the same membrane was used after stripping procedure for reprobed and total ERK1/2 antibody was used as a charge control (f). Mean ± SE, $n = 3$.

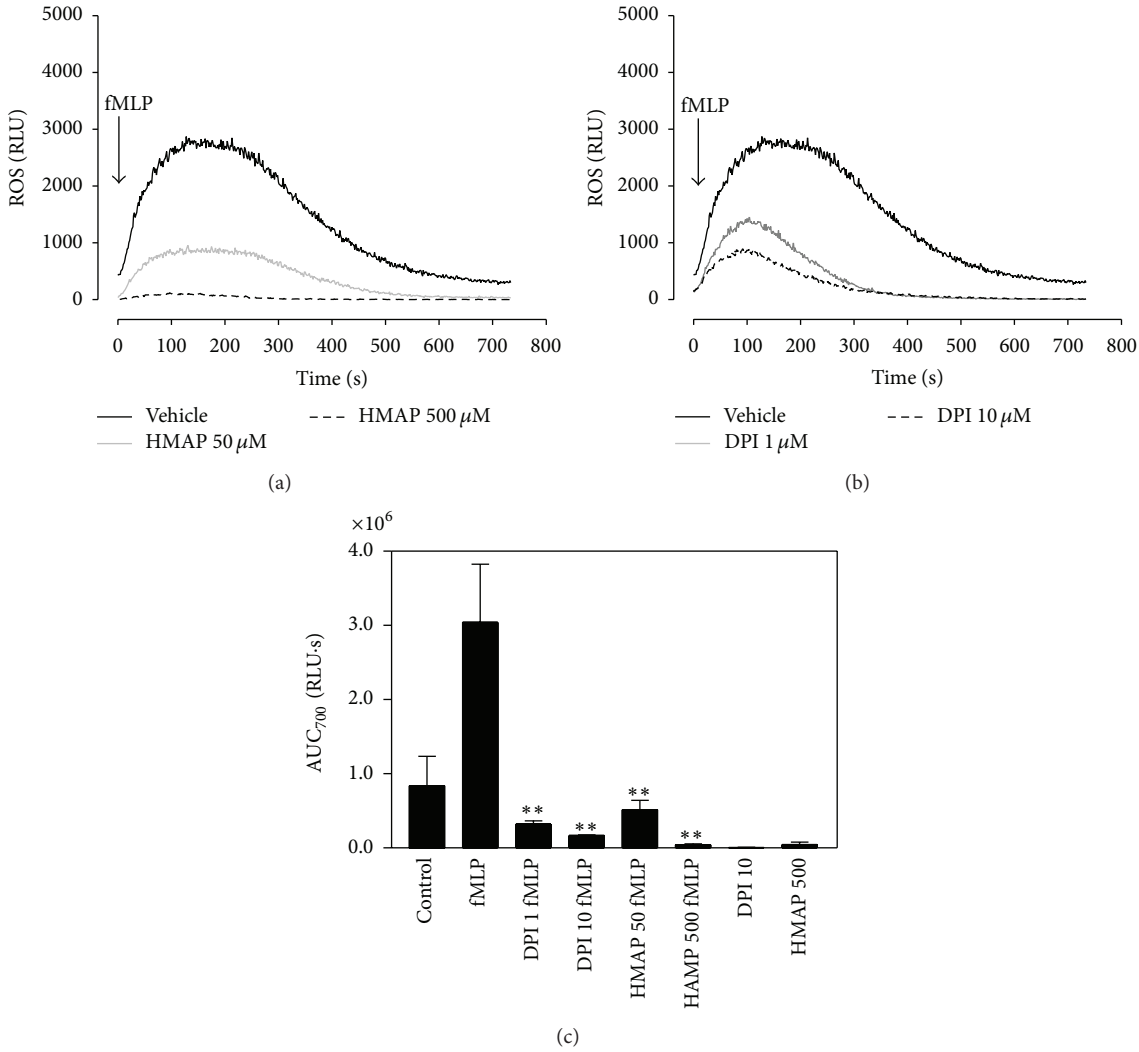

Figure 2: NADPH oxidase inhibition reduces ROS production. Neutrophils incubated with 50 or 500 μM HMAP (a), 1 or 10 μM DPI (b), or vehicle for 10 min were stimulated with 100 nM fMLP, and ROS production was detected by luminescence assay. In (c), the effect of NADPH oxidase inhibitors on AUC for 700 seconds (AUC_{700}) of ROS production is shown. Mean ± SE; $^{**}p < 0.01$ compared to fMLP; $n = 3$.

After 2 min of incubation, fMLP but not PAF stimulated an increase in superoxide release (Figure 1(c)). Only fMLP, in a dose-dependent manner, increased the ROS production (Figure 1(d)). NADPH oxidase activation also induces H^+ release in the intracellular space, contributing to slight and transient neutrophil acidification [12]. This lower pH is spontaneously restored to normal by activation of the Na^+/H^+ exchanger, producing an increase of intracellular pH [5]. We assessed the changes in intracellular pH in fMLP or PAF treated neutrophils using a fluorescent BCECF-AM probe. We found that fMLP and PAF induce a similar intracellular acidification in neutrophils; however, fMLP triggered a large rebound increase in intracellular pH following the period of acidification (Figure 1(e)).

Previously, it had been demonstrated that MAPK and Akt phosphorylation are induced by fMLP and PAF in neutrophils [6, 22, 23]. Here we show that ERK1/2, p38 MAPK, and Akt phosphorylation are more intense when induced by fMLP compared to PAF (Figure 1(f)).

3.2. NADPH Oxidase Inhibition Reduces IL-8 Release by Neutrophils Treated with fMLP. ROS have been described as second messengers for the induction of cytokines [13]; however in neutrophils the role of ROS in the IL-8 release induced by fMLP is until now controversial. We assessed the role of ROS in IL-8 release by using DPI and HMAP, two known NADPH oxidase inhibitors. We observed that 1 and 10 μM DPI as well as 50 and 500 μM HMAP reduce, in a dose-dependent manner, the ROS production induced by fMLP in neutrophils as measured by luminol-amplified chemiluminescence (Figures 2(a)–2(c)).

Subsequently, IL-8 release was measured in neutrophils treated with DPI or HMAP for 30 min and stimulated with fMLP for 4 hr. We observed that IL-8 release was reduced following treatment with 500 μM HMAP and 10 μM of DPI (Figure 3(a)). Additionally, we assessed the effect of HMAP or DPI on stability of IL-8 mRNA. Neutrophils were stimulated with fMLP for 1 h and then actinomycin D plus HMAP, DPI, or vehicle for 1 or 3 h was added. The total RNA was used

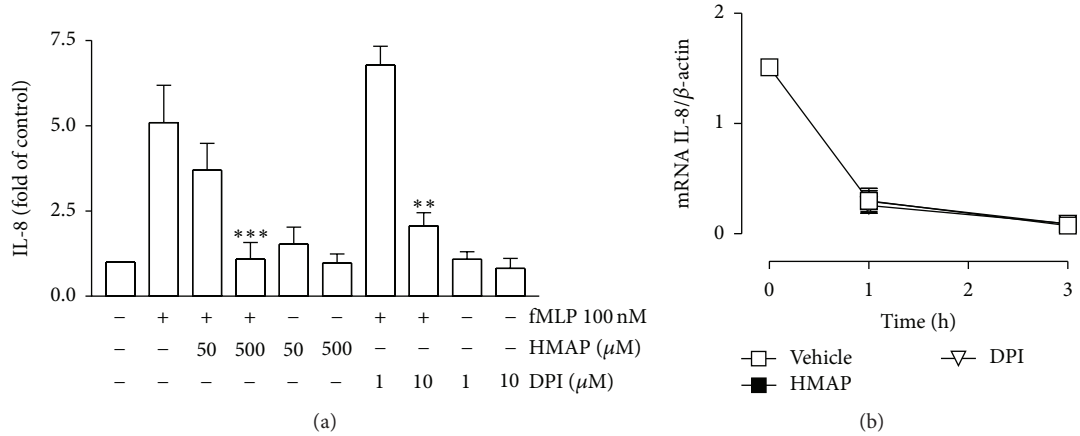

Figure 3: NADPH oxidase inhibition reduces IL-8 production. Neutrophils were treated with HMAP, DPI, or vehicle for 30 min and stimulated with fMLP for 4 h. IL-8 was measured in the supernatants by ELISA (a). Neutrophils were incubated with fMLP for 1 h, and then 10 μM actinomycin D and 500 μM HMAP, 10 μM DPI, or vehicle were added and incubated for 1 or 3 h. Total RNA was isolated and cDNA synthesis and qPCR of IL-8 and β-actin were done (b). Mean ± SE; $^{**}P < 0.01$; $^{***}P < 0.001$ compared to fMLP; $n = 3$.

for cDNA synthesis and real time PCR of IL-8 and β-actin. Figure 3(b) shows that the treatments with HMAP or DPI did not modify the slope of IL-8/β-actin compared to the vehicle, indicating that NADPHox inhibitors did not affect the mRNA stability, suggesting an effect on IL-8 at transcriptional level. siRNA assay targeting human Nox2 was used to verify the effect of NADPHox inhibition on IL-8 release. HL-60 cells differentiated to neutrophils were used for transfection assay.

Untransfected or transfected with siRNA Nox2 or siControl HL-60/neutrophils were used to determine Nox2 level, superoxide production, and IL-8 release. The transfection of siRNA Nox2 decreased the level of Nox2 compared to untransfected and siControl group (Figure 4(a)).

Also, a reduction of the superoxide production induced by fMLP in HL-60/neutrophils transfected with siRNA Nox2 compared to the untransfected or siControl group was observed (Figure 4(b)). Finally, we observed that the IL-8 release induced by fMLP was significantly reduced in HL-60/neutrophils transfected with siRNA Nox2 compared to untransfected or siControl transfected cells (Figure 4(c)).

3.3. HMAP and DPI Interfere with Intracellular pH Changes Induced by fMLP. Intracellular pH changes induced during fMLP activation could be associated with the respiratory burst [24]. The intracellular pH drop induced by chemoattractants is transient (Figure 5(a)); the recovery of intracellular pH is NHE dependent [5]. To assess the impact of NADPH oxidase inhibitors on intracellular pH changes, we assessed the effects of HMAP and DPI on intracellular pH changes induced by fMLP. We observed that HMAP partially and DPI completely inhibited intracellular acidification. In addition, HMAP, but not DPI, partially interfered with the intracellular alkalinisation induced by fMLP (Figures 5(b) and 5(c)). We observed that amiloride, a NHE inhibitor, strongly reduced the intracellular alkalinisation induced by fMLP (Figure 5(d)).

3.4. Amiloride Reduces Release of IL-8 and ROS Production in Neutrophils Treated with fMLP. It has been proposed that NHE is involved in IL-8 release [25]. To assess the role of NHE on ROS production and IL-8 release, we evaluated the effects of amiloride on human neutrophils treated with fMLP. Neutrophils were incubated with amiloride (an NHE inhibitor) for 30 min and stimulated with fMLP for 4 hr; IL-8 release was measured by ELISA. Amiloride (100 and 500 μM) reduced IL-8 release in neutrophils stimulated by fMLP, which suggests a role for NHE in the release of this chemokine (Figure 6(a)). However, it appears that amiloride interferes with ROS production, and the reduction of IL-8 release is secondary (Figure 6(b)). This was evident when we measured the AUC of ROS production over 25 min (Figure 6(c)), supporting that amiloride also affects the ROS production in neutrophils activated by fMLP.

3.5. fMLP Induces IL-8 Release via MAPK, PI3K/Akt, and NF-κB. We analysed the signalling pathways that control IL-8 release. Neutrophils were pretreated with UO126 (MEK1/2 inhibitor), SB203580 (p38 MAPK inhibitor), or LY294002 (PI3K inhibitor) or with a specific Akt inhibitor for 30 min and stimulated with fMLP for 4 hr. A reduction in fMLP-induced IL-8 release was observed with all inhibitors analysed (Figure 7(a)). Furthermore, we demonstrated that andrographolide, a well-known NF-κB inhibitor [26–28], reduces the IL-8 release induced by fMLP. Neutrophils were incubated with the vehicle or inhibitors (UO126, LY294002, or SB203580) for 30 min and stimulated with fMLP for 2 min before ERK1/2, p38 MAPK, or Akt phosphorylation were analysed by immunoblotting (Figure 7(b)). fMLP induced an increase in ERK1/2 phosphorylation, a response that was completely inhibited by UO126. The p38 MAPK phosphorylation induced by fMLP was inhibited by SB203580. Furthermore, the increase in Akt phosphorylation induced by fMLP was inhibited by LY294002. Notably, Akt phosphorylation was also reduced by SB203580, suggesting that p38 MAPK could be upstream of Akt; this observation is consistent with previous reports in other cells which have indicated a possible role for p38 MAPK in regulating Akt phosphorylation [29].

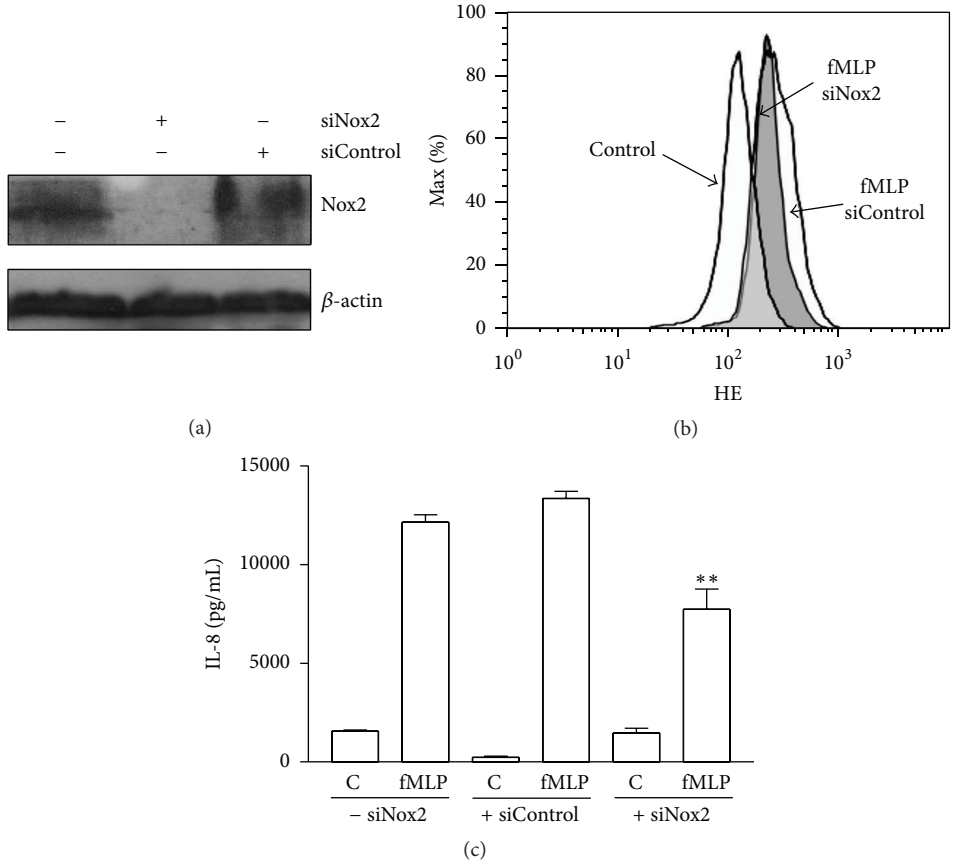

Figure 4: Nox2 siRNA interferes with ROS and IL-8 production in HL-60-derived neutrophilic cells. HL-60 cells were differentiated to neutrophils and transfected with Nox2 siRNA or control siRNA. (a) A representative immunoblot of Nox2 from cells untreated with siRNA or treated with unspecific siRNA (siControl) or specific siRNA (siNox2) is shown. As control β-actin was used. (b) HL-60/neutrophils transfected with siRNA siControl or Nox2 were loaded with HE and treated with vehicle (Control) or fMLP. The superoxide production was measured by flow cytometry. (c) HL-60/neutrophils untreated or treated with siRNA siControl or siNox2 were incubated with vehicle or fMLP for 4 h and IL-8 production in the supernatants by ELISA was analysed. Mean ± SE, $^{**}p < 0.01$ compared to the siControl cells treated with fMLP, $n = 3$.

3.6. NADPH Oxidase, NHE, MAPK, and PI3K/Akt Inhibitors Increase the Intracellular IL-8 Level in fMLP Treated Cells. Because an interference of fMLP-induced IL-8 release was observed with the use of NADPH oxidase, NHE, MAPK, and PI3K/Akt inhibitors, a possible increase at intracellular level could be involved. To test this assumption we performed FACS experiments to assess the intracellular content of IL-8. We observed that DPI and HMAP increased the intracellular content of IL-8 in neutrophils stimulated with 100 nM fMLP. In a lesser extent, the inhibition of NHE or interference of PI3K/Akt, p38 MAPK, and ERK1/2 pathway also increased the intracellular level of this chemokine (Figure 8). Moreover, we discard a cytotoxic effect because none of these inhibitors affect the cellular viability (Supplemental Figure in Supplementary Material available online at http://dx.doi.org/10.1155/2015/120348).

3.7. NADPH Oxidase Inhibition Reduces Akt Phosphorylation. Because MAPK and PI3K/Akt participate in IL-8 release, we investigated whether NADPH oxidase activity has a role in ERK1/2, p38 MAPK, and Akt phosphorylation. Neutrophils were pretreated with DPI or HMAP for 30 min and subsequently stimulated with fMLP for 2 min; ERK1/2, p38 MAPK, and Akt phosphorylation were detected by immunoblotting. It was demonstrated that DPI and HMAP, in a dose-dependent manner, reduced Akt phosphorylation but did not affect ERK1/2 and p38 MAPK phosphorylation (Figure 9(a)). Also, we investigated the role of NHE in regulating MAPK and Akt phosphorylation. Amiloride strongly inhibited Akt phosphorylation but did not affect ERK1/2 or p38 MAPK phosphorylation, a pattern similar to that observed with NADPH oxidase inhibitors (Figure 9(b)).

3.8. fMLP Induces NF-κB Activation via MAPK, PI3K/Akt, and NADPH Oxidase. We evaluated NF-κB activation using confocal microscopy to analyse the p65 NF-κB translocation, a strongly expressed isoform in neutrophils [30]. In control cells, p65 NF-κB preferentially showed a cytoplasmic distribution. However, when the cells were activated by fMLP, p65 NF-κB was mainly localised in the nucleus (arrows in Figure 10); this localization was also visualised with the nuclear stain propidium iodide in merged images (Figure 10). UO126 markedly inhibited the nuclear translocation of p65

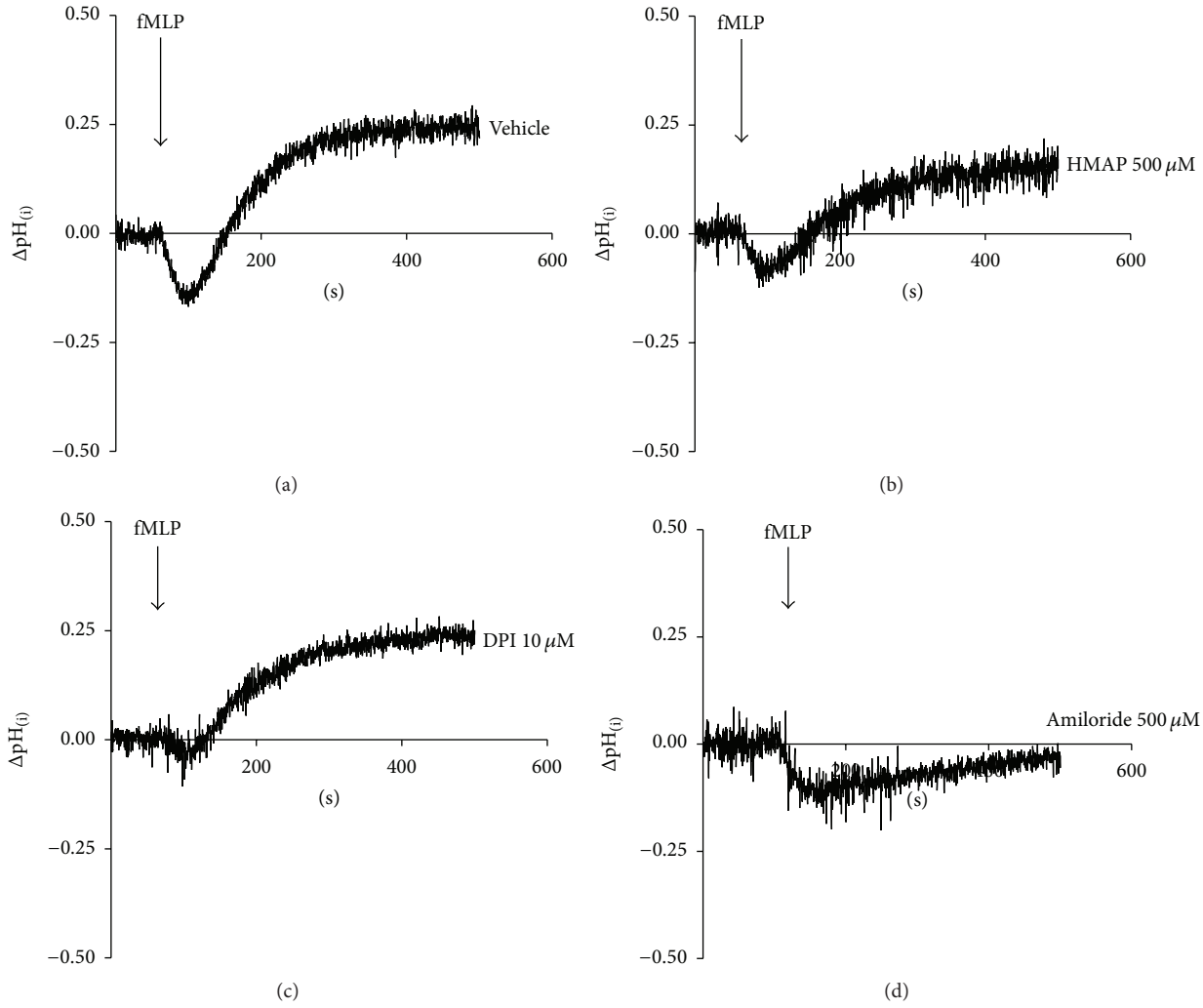

Figure 5: NADPH oxidase inhibition interferes with intracellular pH changes. BCECF-AM-loaded neutrophils were incubated with vehicle (a), 500 μM HMAP (b), 10 μM DPI (c), or 500 μM amiloride (d) for 10 min. A basal level was measured before 100 nM fMLP was added, and the intracellular pH was recorded for 500 s.

NF-κB in PAF activated cells, confirming participation of the ERK1/2 pathway in NF-κB activation. In cells activated with fMLP, preincubation with UO126, LY294002, and SB203580 significantly inhibited the p65 NF-κB translocation, resulting in a distribution similar to that of the control cells (Figure 10). These results demonstrate the importance of the ERK1/2, PI3K, and p38 MAPK pathways in NF-κB activation induced by fMLP. Additionally, we pretreated neutrophils with HMAP and DPI before stimulating the cells with fMLP. We observed that DPI and HMAP significantly interfered with NF-κB translocation, suggesting that NADPH oxidase is involved in the activation of this pathway. SN50, a cell-permeable peptide carrying a functional domain, nuclear localization sequence, that inhibits nuclear translocation of NF-κB/Rel complexes in intact cells, was used as control. SN50 significantly reduced the nuclear localization of p65 NF-κB induced by fMLP ($p < 0.01$). This result was in concordance with an inhibition of p65 NF-κB translocation from cytoplasmatic compartment.

4. Discussion

It has been reported that fMLP [7], but not PAF [9], is a potent inducer of IL-8 in human neutrophils. In fact, we demonstrated that fMLP, but not PAF, increases IL-8 release by neutrophils. Both chemoattractants induce a similar pattern of intracellular signaling pathways [5, 6, 31]. However, using two different approaches (luminol-chemiluminescence and reduction of cytochrome c) a clear difference between ROS production induced by fMLP and that induced by PAF in neutrophils was observed. It is widely known that PAF does not induce respiratory bursts and is considered mainly a priming stimulus in neutrophils [9]. There exist controversial antecedents in the role of ROS in neutrophils cytokine production. We hypothesised that fMLP induces IL-8 release via NADPH oxidase activity in neutrophils. We tested two NADPH oxidase inhibitors: HMAP, which reduces NADPH oxidase activity by competing with NADPH for the oxidase binding site [32], and DPI, which blocks flavin adenine dinucleotide binding to the oxidase [33]. HMAP and DPI,

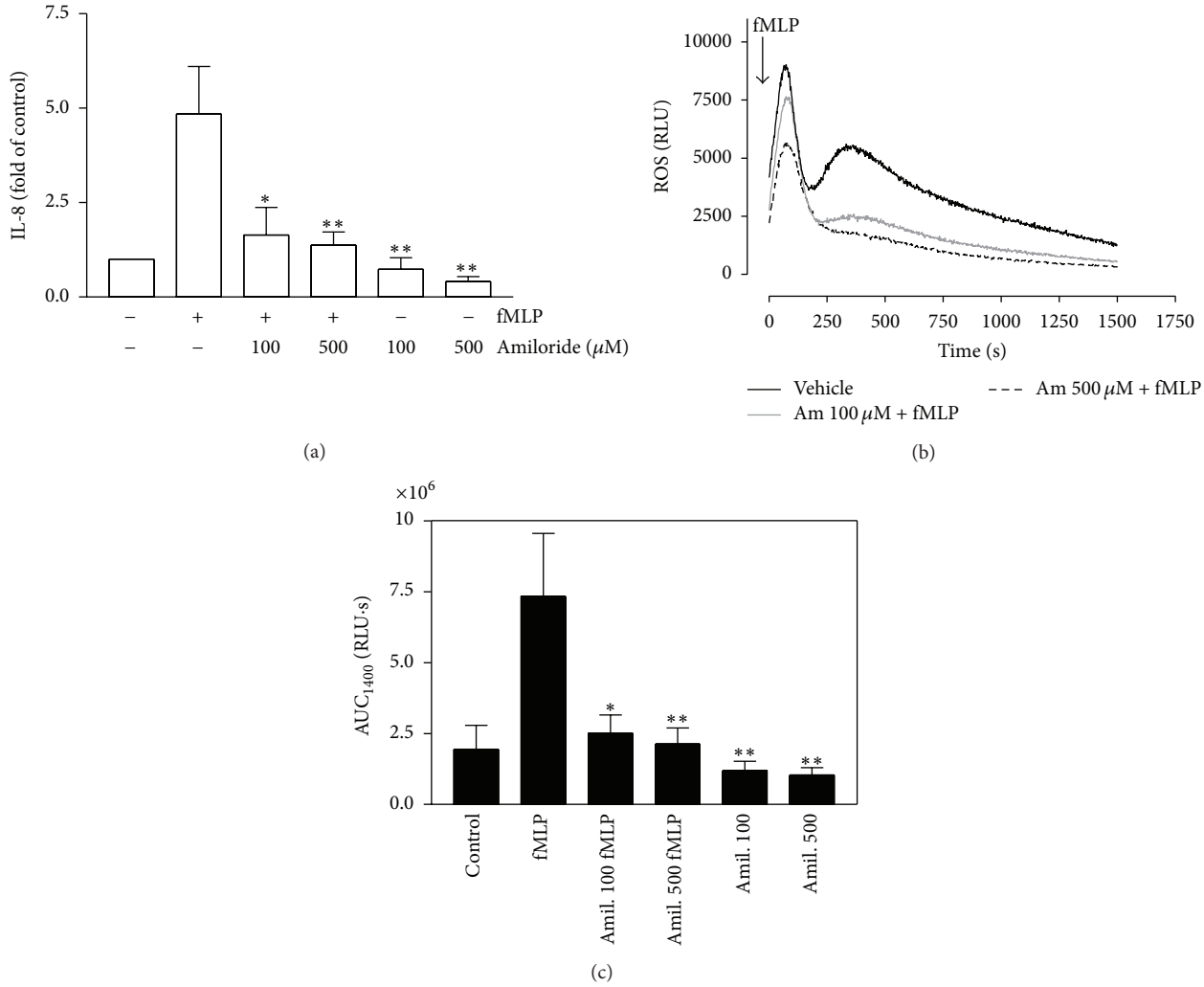

Figure 6: Amiloride reduces IL-8 and ROS production induced by fMLP. Neutrophils were incubated with vehicle or amiloride (100 or 500 μM) for 30 min and stimulated with fMLP for 4 h. IL-8 production was analysed in the supernatants by ELISA (a). Neutrophils were incubated with vehicle or amiloride (100 or 500 μM) for 10 min and then stimulated with 100 nM fMLP and the ROS production was detected by luminescence assay (b). In (c), the effect of amiloride on AUC for 1400 seconds (AUC_{1400}) of ROS production is shown. Mean ± SE; $^{*}p < 0.05$; $^{**}p < 0.01$ compared to fMLP; $n = 3$.

to a lesser extent, inhibited ROS production in neutrophils treated with fMLP. The observation that these compounds inhibit IL-8 release suggests that NADPH oxidase is involved in the secretion of this chemokine. We corroborate the role of NADPHox on IL-8 release induced by fMLP by using siRNA of Nox2 in HL-60/neutrophils. By the contrary, in neutrophils from chronic granulomatous disease that have genetic mutations in any of four components of the NADPH oxidase, fMLP increase the IL-8 neutrophil content [17]. These results could be explained by the different experimental conditions used. Because we measured the secretion of IL-8 but not total protein content, we propose that NADPH oxidase inhibition could be interfering with the release of IL-8, reducing the mobilization of a IL-8-containing organelle to the plasma membrane [34]. In fact, we observed that NADPH oxidase inhibitors increased the IL-8 at intracellular level in neutrophils treated with fMLP, suggesting interference in the release of this chemokine. In neutrophils, it has been observed that ROS are involved in IgE-induced IL-8 release [35]. Moreover, in neutrophils treated with LPS the IL-8 release was inhibited using OH radical scavenger [36].

Because NADPH oxidase activity is involved in intracellular acidification in neutrophils [24], we assessed the effects of the NADPH oxidase inhibitors on intracellular pH changes induced by fMLP. fMLP induced biphasic pH changes characterised by transient intracellular acidification followed by intracellular alkalinisation. HMAP partially affected the intracellular acidification and alkalinisation, and DPI only affected the intracellular acidification. Sustained intracellular acidification has been demonstrated to increase H_2O_2 but not O_2^-, which is explained by an increased rate of dismutation of O_2^- at acidic intracellular pH [24]. Our results suggest that transient acidification alone is insufficient to increase IL-8; furthermore, PAF produced a similar intracellular pH pattern but did not induce IL-8 release in neutrophils. To assess the impact of intracellular acidification on IL-8 product,

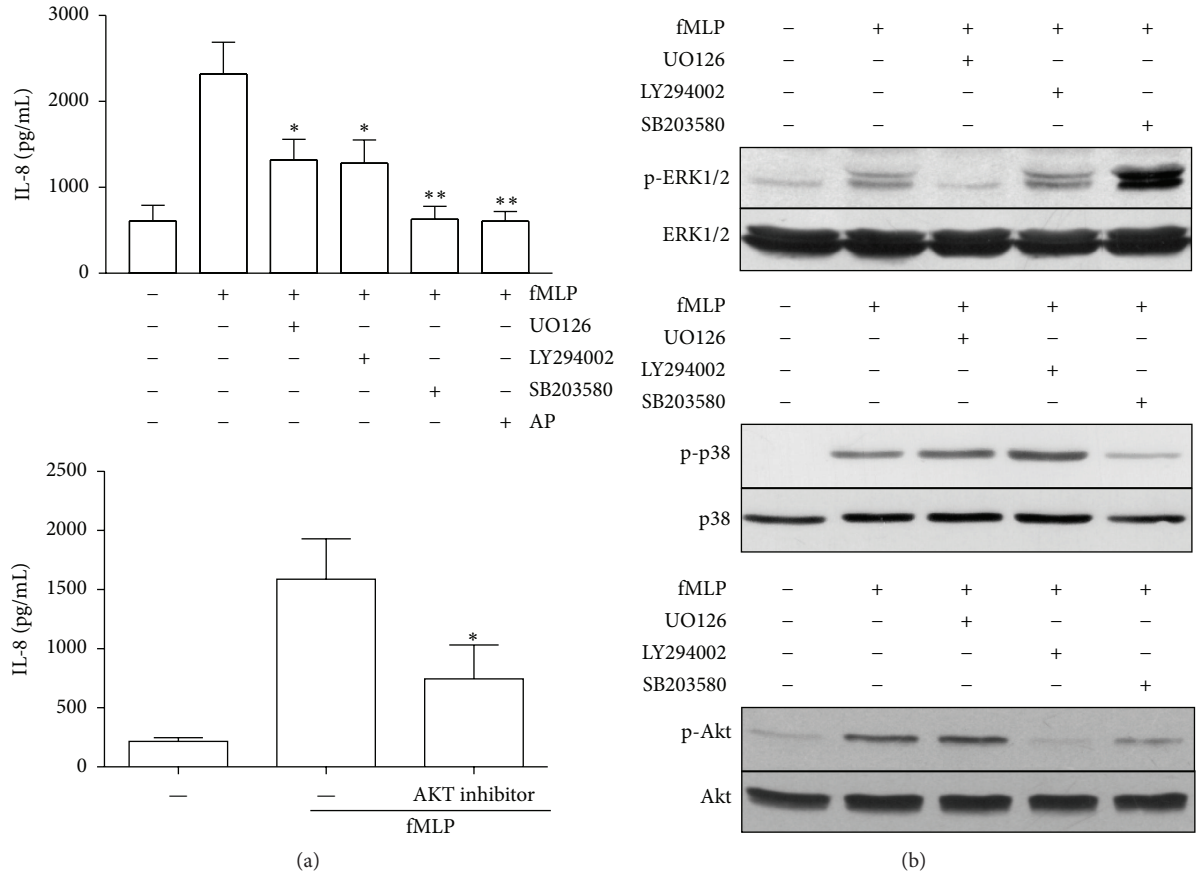

Figure 7: Effects of UO126, LY294002, Akt inhibitor, and SB203580 on IL-8 production and MAPK and Akt phosphorylation induced by fMLP. Neutrophils were incubated with vehicle, UO126 (1 μM), LY294002 (10 μM), SB203580 (10 μM), andrographolide (AP) (50 μM), or Akt inhibitor (10 μM) for 30 min and stimulated with fMLP for 4 h. IL-8 production was analysed in the supernatants by ELISA (a). Neutrophils were incubated with vehicle, UO126 (1 μM), LY294002 (10 μM), or SB203580 (10 μM) (b) for 30 min, stimulated with fMLP for 2 min, and analysed by immunoblot for ERK1/2, p38 MAPK, or Akt (Ser 473) phosphorylation. The blots were stripped and stained antibody specific to the unphosphorylated protein. Data presented are representative of three independent experiments. Mean ± SE; $^{*}p < 0.05$; $^{**}p < 0.01$ compared to fMLP; $n = 3$.

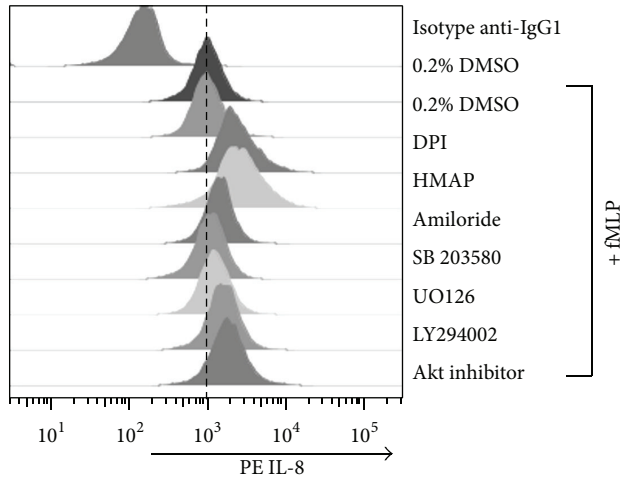

Figure 8: NAPDH oxidase, NHE, MAPK, and PI3K/Akt inhibitors increase the intracellular IL-8 level in fMLP treated cells. Neutrophils were incubated with vehicle, DPI (10 μM), HMAP (500 μM), amiloride (500 μM), SB203580 (10 μM), UO126 (1 μM), LY294002 (10 μM), or Akt inhibitor (10 μM) for 30 min and stimulated with fMLP (100 nM) for 4 h. IL-8 intracellular content was measured by FACS. Data presented are representative of three independent experiments.

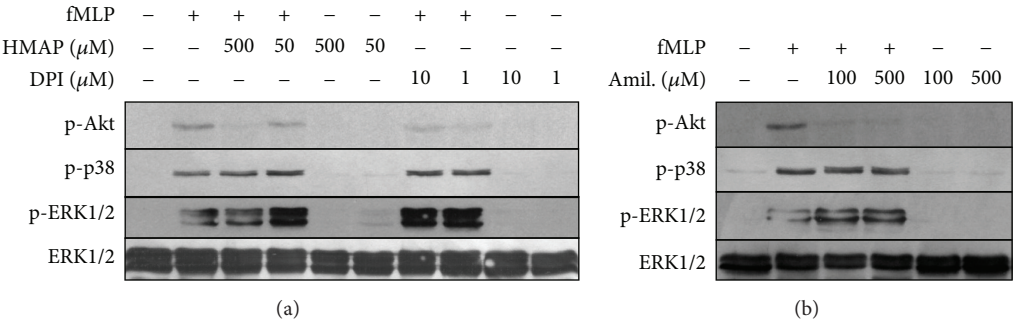

Figure 9: Effect of HMAP, DPI, and amiloride on MAPK and Akt phosphorylation induced by fMLP. Neutrophils were incubated with vehicle, HMAP, DPI (a), or amiloride (b) for 30 min, stimulated with fMLP for 2 min, and analysed by immunoblot for ERK1/2, p38 MAPK, or Akt (Ser 473) phosphorylation. The blots were stripped and stained antibody specific to the unphosphorylated protein. Data presented are representative of three independent experiments.

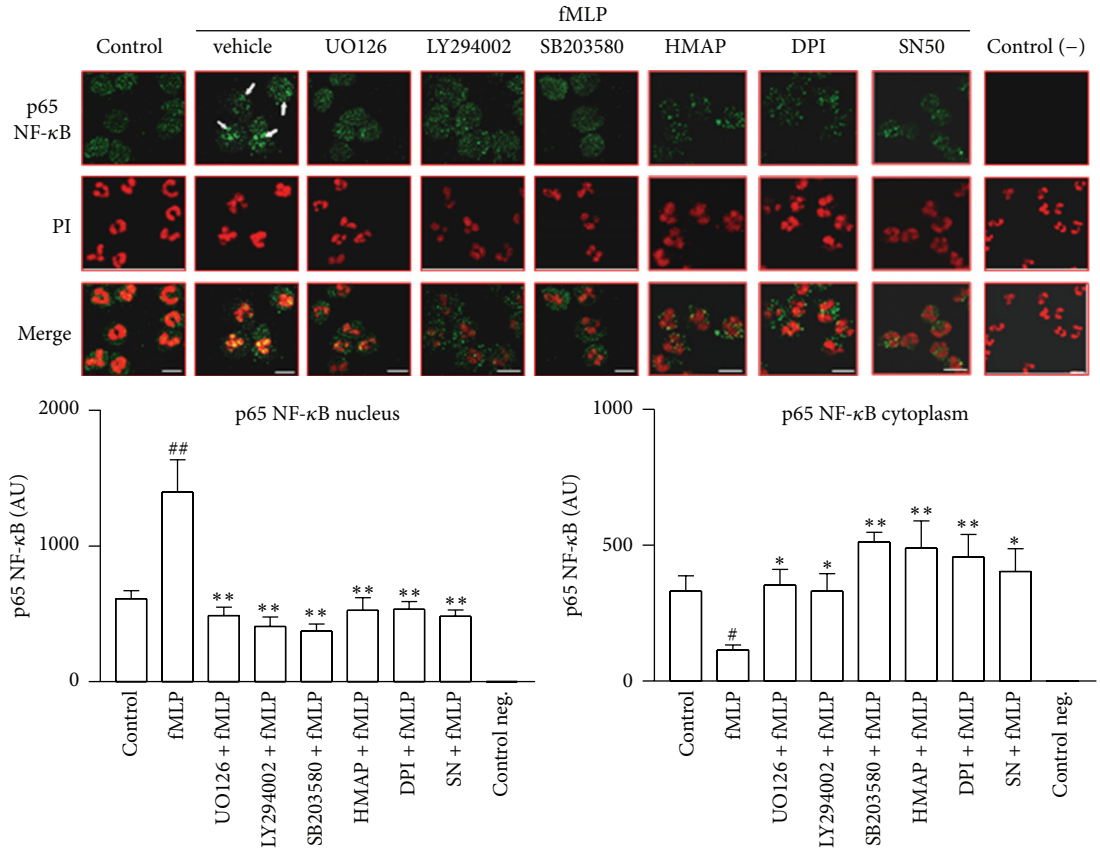

Figure 10: ERK1/2, PI3K, p38 MAPK, and NADPH inhibition reduce p65 NF-κB translocation induced by fMLP. Neutrophils were incubated with vehicle, UO126 (1 μM), LY294002 (10 μM), SB203580 (10 μM), HMAP (500 μM), DPI (10 μM), or SN50 (100 μg/mL) for 30 min and then stimulated with fMLP for 30 min. The cells were fixed and analysed by immunocytochemistry with an antibody targeted against p65 NF-κB. The nucleus was stained with propidium iodide (PI). The arrow shows the presence of p65 NF-κB in nucleus. The negative control was prepared without anti-p65 NF-κB. Bar: 10 μm. Figure is representative of three independent experiments. Each bar represents mean ± SE of arbitrary units (AU) of fluorescence intensity of p65 NF-κB, n = at least 5; $^{*}p < 0.05$; $^{**}p < 0.01$ compared to fMLP.

we subjected neutrophils to different concentrations of HCl. We found that intracellular acidification did not interfere with fMLP-induced chemokine secretion (data not shown). Additionally, we assess if intracellular alkalinisation via NHE could also be involved in IL-8 release. In monocytes and macrophages, LPS and IFN-γ via NHE promote the release of cytokines [25]. We found that amiloride inhibits fMLP-stimulated IL-8 secretion by neutrophils. However, amiloride also inhibited ROS production in neutrophils treated with fMLP, suggesting that NADPH oxidase could be involved in this IL-8 release. Moreover, PAF also activates NHE in neutrophils [5] but does not modify IL-8 basal levels

or ROS production. Recently, it has been described that NHE inhibitors directly reduce mitochondrial function, thus preventing ROS production in cat myocardium induced by angiotensin II and endothelin-1 [37]. Therefore, we suggest that the effects of NHE inhibitors on neutrophils could be in part attributed to inhibition of ROS production.

The ROS could contribute to chemokine expression by acting as an intracellular second messenger, either directly or indirectly influencing the signalling pathways activated by chemoattractants such as fMLP. Our results show that NADPH oxidase inhibitors and amiloride reduced only the phosphorylation of Akt in neutrophils treated with fMLP, which indicate that MAPK pathways are not directly regulated by NADPH oxidase, suggesting the existence of a redundant effect of NADPH oxidase on PI3K/Akt pathways in neutrophils treated with fMLP, effect that could be cell and ligand specific. In support of this, LPS in macrophages, via NADPH oxidase, contribute to the phosphorylation of Akt but not p38 MAPK or ERK1/2 [38, 39]. However, angiotensin II potently induces phosphorylation of p38 MAPK and ERK1/2 in neutrophils, which is inhibited by NADPH oxidase inhibitors (e.g., DPI) and ROS scavengers [40]. In addition, HMAP reduces phosphorylation of p38 MAPK and ERK1/2 induced by α-IgE in neutrophils from sensitised allergic patients [41]; moreover, nonlethal concentrations of H_2O_2 have been demonstrated to activate p38 MAPK and ERK1/2 [42]. We demonstrated that SB203580, but not UO126, reduced Akt phosphorylation induced by fMLP in neutrophils. Therefore, our result supports that NADPH oxidase could contribute directly to the activation of PI3K in neutrophils stimulated by fMLP and would be necessary for IL-8 release [43]. In support of this, the inhibition of PI3K or Akt reduced the release of IL-8 induced by fMLP and increased the intracellular concentration of this chemokine. Our proposal suggests that NADPH oxidase participates in the activation of the PI3K pathway in neutrophils treated with fMLP. In fact, it recently has been described that Akt phosphorylation can be induced by ROS. Elevation of ROS can activate the PI3K/Akt pathway in TonB.210 cells with Bcr-Abl activated [44].

MAPK and PI3K/Akt pathways simultaneously play crucial roles in NF-κB activation [45]; therefore, the contribution of NADPH oxidase on NF-κB could be relevant in the IL-8 production induced with fMLP. HMAP and DPI reduced the p65 NF-κB translocation in neutrophils. In addition, NF-κB inhibition using andrographolide [27, 28, 45] reduced the IL-8 release in neutrophils treated with fMLP. In concordance with our results other reports show that NF-κB controls the expression and release of IL-8 in LPS- or TNF-α-stimulated neutrophils [46].

In the present study, we additionally showed that fMLP induces p65 NF-κB translocation in neutrophils and is upstream mediated by ERK1/2, PI3K, and p38 MAPK pathways. It has been suggested that there is a link between ERK1/2 and NF-κB in neutrophils activated with group IB secretory phospholipase A2 [47]. Previously, other authors have indirectly suggested that ERK1/2 MAPK could participate in NF-κB activation in human neutrophils, mainly because ERK1/2 is activated by TNF-α, a potent NF-κB activator [48, 49]. A study showed that PI3K is upstream from NF-κB activation in neutrophils activated with type 1 IFN and exerts an antiapoptotic effect [50]. The role of p38 MAPK in the regulation of NF-κB activation in neutrophils is controversial. A report [46] concluded that p38 MAPK does not participate in NF-κB binding in LPS-stimulated neutrophils; however, in the present study we demonstrate a role for p38 MAPK in activating this transcription factor following fMLP stimulation. Our results agree with those of other authors who described the contribution of p38 MAPK to NF-κB activation in LPS-stimulated neutrophils [45]. The proteins downstream of p38 MAPK that control NF-κB are unknown in neutrophils; however, we did not exclude Akt as a possible candidate since inhibition of PI3K and p38 MAPK reduces Akt phosphorylation induced by fMLP. A similar cross talk between p38 and PI3K/Akt has been previously suggested in different cells [29].

Based on these results, we conclude that fMLP can increase NADPH oxidase activity and ROS production, favouring the activation of PI3K/Akt and NF-κB transduction pathways involved in the IL-8 release in neutrophils.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Research Article

CYP4F18-Deficient Neutrophils Exhibit Increased Chemotaxis to Complement Component C5a

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CYP4Fs were first identified as enzymes that catalyze hydroxylation of leukotriene B_4 (LTB_4). CYP4F18 has an unusual expression in neutrophils and was predicted to play a role in regulating LTB_4-dependent inflammation. We compared chemotaxis of wild-type and *Cyp4f18* knockout neutrophils using an *in vitro* assay. There was no significant difference in the chemotactic response to LTB_4, but the response to complement component C5a increased 1.9–2.25-fold in knockout cells compared to wild-type ($P < 0.01$). This increase was still observed when neutrophils were treated with inhibitors of eicosanoid synthesis. There were no changes in expression of other CYP4 enzymes in knockout neutrophils that might compensate for loss of CYP4F18 or lead to differences in activity. A mouse model of dextran sodium sulfate colitis was used to investigate the consequences of increased C5a-dependent chemotaxis *in vivo*, but there was no significant difference in weight loss, disease activity, or colonic tissue myeloperoxidase between wild-type and *Cyp4f18* knockout mice. This study demonstrates the limitations of inferring CYP4F function based on an ability to use LTB_4 as a substrate, points to expanding roles for CYP4F enzymes in immune regulation, and underscores the *in vivo* challenges of CYP knockout studies.

1. Introduction

CYP4Fs are a family of cytochrome P450 (CYP) enzymes that were first identified for their ability to catalyze end-chain hydroxylation and inactivation of leukotriene B_4 (LTB_4) [1]. The potency of LTB_4 as an inflammatory mediator in normal immune responses and pathologies is well established. LTB_4 is generated by the 5-lipoxygenase pathway of arachidonic acid metabolism and is implicated in the progression of diverse immune disorders such as inflammatory bowel disease, ischemia-reperfusion injury (IRI), arthritis, and asthma [2, 3]. Therefore, CYP4Fs are predicted to play a significant role in the regulation of inflammation and prevention of disease. There is growing evidence to support this possibility. CYP-dependent LTB_4 hydroxylase activity limits neuroinflammation in mouse models [4] and might contribute to the beneficial effects of retinoids in the treatment of inflammatory skin diseases [5, 6]. Neutrophils and colonic mucosa from patients with inflammatory bowel disease have reduced LTB_4 hydroxylase activity [7, 8], and genetic association studies link variants of the *CYP4F2* and *CYP4F3* genes with celiac disease and Crohn's disease [9, 10].

Human neutrophils have been used for detailed studies of CYP-dependent LTB_4 metabolism: hydroxylation at the terminal (ω) position generates 20-hydroxy LTB_4, which is subsequently converted to the inactive metabolite 20-carboxy LTB_4 [11]. This is the major pathway for the inactivation of LTB_4 in human neutrophils [12, 13]. The enzyme responsible for the initial ω-hydroxylation step was identified as CYP4F3 [14, 15], and subsequently we demonstrated that this enzyme is an alternative splice form of the *CYP4F3* gene designated as CYP4F3A [16]. A second splice form, CYP4F3B, has lower activity for LTB_4 and is expressed in different locations such as liver and kidney [17]. The unusual localization and high expression of CYP4F3A in human neutrophils, and its high activity for LTB_4 as a substrate, suggest that inactivation of LTB_4 is a specialized function of the enzyme. There is

evidence for temporal expression of CYP4Fs consistent with the resolution phase of inflammation in some experimental models [18], but expression of CYP4F3A in neutrophils does not fit this time frame. Neutrophils are short-lived cells associated with the early stages of inflammation, and CYP4F3A is expressed at a high constitutive level both before and during inflammatory recruitment of the cells [19]. It is possible that LTB_4 inactivation functions to restrain neutrophil infiltration and prevent excessive inflammation. An alternative possibility is that LTB_4 inactivation plays a role in neutrophil polarization, which is required to maintain normal chemotaxis [20].

We developed mouse models to better understand the role of CYP4Fs in neutrophil-dependent inflammation. We identified the CYP4F18 enzyme as the mouse homologue of CYP4F3A [21] and generated targeted deletions in the *Cyp4f18* gene. Neutrophils from *Cyp4f18* knockout mice exhibit a null phenotype for end-chain hydroxylation of LTB_4 [22]. However, there are significant differences between mice and humans. The *Cyp4f18* gene is not alternatively spliced and generates a single enzyme that is homologous to CYP4F3A in sequence, localization to neutrophils, and high activity for LTB_4. The products of end-chain hydroxylation by CYP4F18 are 19-hydroxy LTB_4, and to a lesser extent 18-hydroxy LTB_4, not 20-hydroxy LTB_4 [21, 22]. It is not known whether ω-1 and ω-2 hydroxylation of LTB_4 represents an efficient inactivation pathway. Furthermore, mouse neutrophils have an alternative pathway of LTB_4 metabolism that involves a 12-hydroxydehydrogenase. Knockout of *Cyp4f18* does not impact neutrophil infiltration into kidney tissue and disease pathology in a mouse model of renal IRI [22], although inhibition of LTB_4 synthesis does have observable effects in this model [23]. It appears that CYP4F18 is redundant for LTB_4 inactivation in mouse neutrophils, and we speculated that it might have an alternative function in these cells.

Since the discovery of CYP4Fs, numerous *in vitro* substrates have been identified [1]. There are 7 members of the human CYP4F family including the two splice forms of CYP4F3 (4F2, 4F3A, 4F3B, 4F8, 4F11, 4F12, and 4F22) and 9 members of the mouse family (4F13, 4F14, 4F15, 4F16, 4F17, 4F18, 4F37, 4F39, and 4F40). CYPs typically have broad and overlapping substrate specificity, and a single enzyme such as CYP4F3B might have the capacity to catalyze multiple reactions including inactivation of LTB_4, generation of 20-hydroxyeicosatetraenoic acid (20-HETE), and modification of fatty acid epoxides [1, 24]. This suggests potentially diverse and prominent roles for CYP4Fs in immune regulation but creates a challenge for the identification of physiologically relevant substrates [25]. It is possible that CYP4Fs have different functions in different tissue locations, and new experimental systems will be required to determine the significance of particular reactions and disentangle the effects of multiple CYP4Fs. CYP4F18 is the only CYP4F family member expressed at high levels in mouse neutrophils, so *Cyp4f18* knockout mice provide a novel system to dissect diversity of function. In this report we demonstrate that neutrophils from *Cyp4f18* knockout mice show increased chemotaxis to complement component C5a that is independent of LTB_4, an unexpected result that is not predicted by known CYP4F substrates.

2. Materials and Methods

2.1. Mice. *Cyp4f18* knockout (−/−) mice were generated in a C57BL/6 background as previously reported [22] and are available at the Mutant Mouse Regional Resource Center (MMRRC) with the designation B6.129S4(Cg)-Cyp4f18$^{tm1.1Pchr}$. *Cyp4f18* +/− heterozygous mice were maintained at the Massachusetts General Hospital and mated to generate *Cyp4f18* −/− homozygous knockouts and *Cyp4f18* +/+ wild-type littermates for experiments. Genotyping assays are as previously described [22]. Breeding and experimentation of mice were performed in accordance with the guidelines of the Massachusetts General Hospital/Partners Committee on Research Animal Care. Mice of 6–12 weeks of age were used for experiments.

2.2. Isolation of Bone Marrow Neutrophils. Mouse bone marrow cells were isolated from femurs and tibias by perfusion with phosphate buffered saline (PBS). The cells were filtered through a 40 μm cell strainer, washed in PBS, and layered on top of a discontinuous two-layer gradient of Histopaque 1077/1119 (Sigma). After centrifugation at 700 ×g for 30 min at RT, neutrophils were separated from other cells including erythrocytes and recovered at the interface of the 1077 and 1119 fractions. Purity of the isolated polymorphonuclear cells was confirmed (>90%) by staining nuclei with DAPI and examination under a Nikon Eclipse Ti microscope with confocal imaging. Viability of the cells (>95%) was assessed by trypan blue exclusion.

2.3. In Vitro Chemotaxis Assay. Bone marrow neutrophils were isolated as described above and resuspended in migration medium (RPMI + 0.5% FBS) to a concentration of 3×10^6 cells/mL. Chemotaxis was performed using a 12-well plate with Transwell 12 mm polycarbonate membrane inserts of 3.0 μM pore size (Corning 3402). The cells were placed in the insert (0.5 mL, 1.5×10^6 cells per insert), and 1.5 mL of migration medium containing different concentrations of chemoattractant was placed in the lower well. Migration medium containing vehicle but no chemoattractant was used as a control to measure background chemotaxis, and chemoattractant was added to both the insert and the lower well as a control for chemokinesis. The 12-well plates were incubated for 3 hours at 37°C in a 5% CO_2 incubator. The medium in the lower well was transferred to a 12 × 75 mm sterile culture tube (BD Falcon), and the well was washed with PBS. The tubes were then centrifuged for 5 min at 200 ×g, and the cell pellet was resuspended in 0.1 mL migration medium. The total number of cells was determined using a hemocytometer. To calculate the chemotactic index, the number of cells migrated in response to chemoattractant was divided by the number of spontaneously migrated cells (background).

The chemoattractants tested were LTB_4 (Cayman), mouse complement component C5a (R&D Systems), mouse

CXCL1/KC (R&D Systems), and WKYMVdM peptide (Sigma) as an agonist for the mouse formyl peptide receptor. In some experiments, bone marrow neutrophils were incubated with or without inhibitors of eicosanoid synthesis or BLT1 for 30 min at 37°C prior to adding the cells to the 12-well plate inserts. This included incubations with 0.5 μM of the FLAP inhibitor MK 886 (Cayman 10133), 1 μM of a cPLA2α inhibitor (Calbiochem 525143, PubChem CID 9833099), and 10 μM of the BLT1 antagonist LY223982 (Cayman 10010024). Each experimental condition was performed in duplicate or triplicate on a single 12-well plate, and each experiment was performed at least 4 times ($n \geq 4$).

2.4. Flow Cytometry. Bone marrow cell suspensions in PBS + 0.5% BSA were preincubated with Mouse Fc Block (BD Biosciences) for 5 min at 4°C (0.5 μg/10^6 cells/100 μL) and then incubated with fluorophore-conjugated anti-mouse monoclonal antibodies for 30 min at 4°C (antibodies diluted to 1 μg/mL). Anti-CD45-PerCP (clone 30-F11) and anti-Ly6G-FITC (clone 1A8) were from BD Biosciences. Anti-C5aR(CD88)-APC (clone 20/70) was from BioLegend. The cells were fixed in BD stabilizing fixative (BD Biosciences). Labeled cells were analyzed at the Flow Cytometry Core Facility, Massachusetts General Hospital, using a BD SORP 7 Laser LSRII and FlowJo software, as in previous studies [19, 22]. The cells were gated on forward versus side scatter, then for CD45 expression, prior to double plot analysis of Ly6G and C5aR (numbers were assigned to each quadrant to indicate percentage of total CD45+ cells). Bone marrow samples from 5 wild-type mice and 5 *Cyp4f18* knockout mice were analyzed.

2.5. RNA Isolation and Real Time PCR. Total RNA was isolated from cells and tissues using the RNeasy Plus Mini Kit with QIAshredder (Qiagen). Reverse transcription was performed with a High Capacity cDNA Reverse Transcription Kit (Life Technologies, Applied Biosystems). The cDNA was analyzed for target gene expression using TaqMan primer sets and a StepOnePlus real time PCR machine from Applied Biosystems. A standard reaction protocol was followed (50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 sec and 60°C for 1 min). Relative quantification of gene expression in knockout samples compared to wild-type was performed by the ΔΔCt method using mouse GAPDH as endogenous control. Each sample was run in triplicate to determine ΔCt values, $2^{-\Delta Ct}$ values, or fold-differences in expression ($2^{-\Delta\Delta Ct}$). Values from four experiments were expressed as mean ± SEM. The TaqMan primer-probe sets were purchased from Life Technologies (Applied Biosystems) as listed in Table 1.

2.6. Dextran Sodium Sulfate (DSS) Colitis. DSS (MP Biomedicals) of average molecular weight 42 kDa (35–50,000) was administered to 8-week-old mice ad libitum at a concentration of 4% in drinking water for 9 days. Control mice received the same drinking water without DSS ($n = 10$ mice in each group). Changes in body weight were calculated every day. A disease activity index (DAI) was determined by assigning a score of 1–4 for weight loss (1: 1–5%, 2: 5–10%, 3: 10–15%, and 4: >15%), rectal bleeding (ranging from 1: positive to 4: gross bleeding), stool consistency (ranging from 1: loose stools to 4: diarrhea with fecal material adherent to anal fur), and body posture/lethargy (ranging from 1: mild to 4: severe). The total scores were averaged to give a value between 0 (normal) and 4 (maximum). Mice were sacrificed on day 9, and colonic tissue samples were collected for further analysis. Myeloperoxidase (MPO) was measured using an ELISA kit from Hycult Biotech (HK210). The assay was performed on tissue samples that had been frozen and stored at −70°C. The samples were thawed, weighed, and homogenized in a lysis buffer containing 200 mM NaCl, 5 mM EDTA, 10 mM tris, 10% glycerin, 1 mM PMSF, 1 μg/mL leupeptin, and 28 μg/mL aprotinin, pH 7.4 (20 μL lysis buffer per mg tissue). The homogenate was transferred to a 1.5 mL microfuge tube and centrifuged two times at 1500 ×g for 15 min at 4°C. Supernatants were snap frozen in liquid nitrogen and stored at −70°C. MPO was assayed by ELISA according to the manufacturer's instructions.

Table 1: Summary of primers.

TaqMan primers for real time PCR (Applied Biosystems/Life Technologies)	
C5ar1	Mm00500292_s1
Cyp4a10	Mm01188913_g1
Cyp4a12a	Mm00514494_m1
Cyp4a12b	Mm00655431_gH
Cyp4a14	Mm00484135_m1
Cyp4a29	Mm01188902_g1
Cyp4a30b	Mm01181463_m1
Cyp4a31	Mm03047753_m1
Cyp4b1	Mm01193710_m1
Cyp4x1	Mm01181487_m1
Cyp4f13	Mm00504576_m1
Cyp4f14	Mm00491623_m1
Cyp4f15	Mm00506542_m1
Cyp4f16	Mm00775893_m1
Cyp4f17	Mm01345625_m1
Cyp4f39	Mm00624134_m1
Cyp4f40	Mm01342246_m1
Custom TaqMan primers	
Cyp4f18	
Forward	AGTGGACTTTCCTGGATCCTGTAC
Reverse	GGCAGCGCTCCTGGTATTC
Probe	ACCTGGCAAGACACC
Cyp4f37	
Forward	TCCCGCCTCAGATGTTTCC
Reverse	CCCAAGTGACCCAAAAACCA
Probe	TCAGCCTCCTAAAAGA
Mouse blt1	
Forward	CCGACTTGGCTGTGTTGCT
Reverse	GTGCCTCGAGCCAGAAAGTG
Probe	ACTGCTCCCTTTTTC

Sequences are shown in 5′ to 3′ direction.

2.7. Statistical Analysis. The data was analyzed using Graphpad Prism version 5 statistical software. The results are expressed as the mean ± SEM. A t-test was used for comparisons of paired data, and multigroup data were analyzed by ANOVA. A P value of less than 0.05 was considered significant.

3. Results

3.1. LTB_4-Dependent Neutrophil Chemotaxis. LTB_4-dependent chemotaxis of neutrophils from wild-type and *Cyp4f18* knockout C57BL/6 mice was compared using an *in vitro* assay. A dose-dependent increase in chemotaxis was observed (Figure 1). For wild-type (WT) neutrophils, the chemotactic index increased from 1.27 ± 0.12 at 1 nM LTB_4 to 2.73 ± 0.7 at 10 nM LTB_4 and 8.53 ± 0.64 at 100 nM LTB_4. For *Cyp4f18* knockout (KO) neutrophils, the chemotactic index increased from 1.4 ± 0.1 at 1 nM LTB_4 to 3.3 ± 0.77 at 10 nM LTB_4 and 9.27 ± 0.77 at 100 nM LTB_4. At each concentration, there was no significant difference between the chemotactic index for wild-type and *Cyp4f18* knockout neutrophils ($n = 10$, $P > 0.05$).

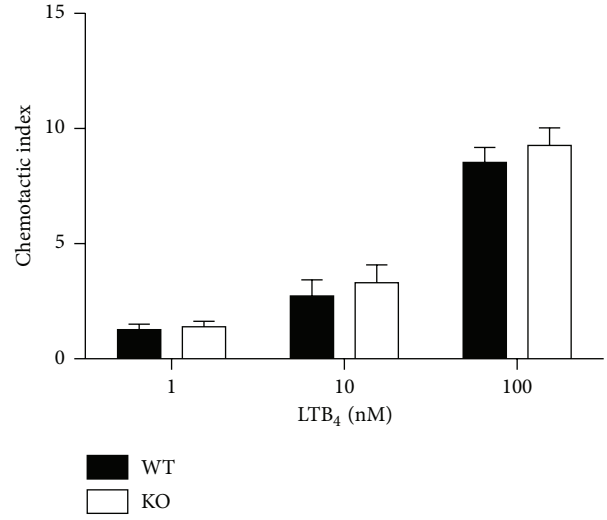

Figure 1: *Cyp4f18* knockout neutrophils show no change in LTB_4-dependent chemotaxis compared to wild-type. Chemotaxis of bone marrow neutrophils from wild-type (WT) and *Cyp4f18* knockout (KO) mice was measured using LTB_4 as a chemoattractant. The chemotactic index represents the number of cells migrated in response to LTB_4 divided by background (error bars represent SEM, $n = 10$).

3.2. C5a-Dependent Neutrophil Chemotaxis. The complement component C5a was originally used as a control in these experiments, because C5a-dependent chemotaxis was not expected to be affected by loss of *Cyp4f18*. Surprisingly, significant differences in chemotaxis were observed when comparing wild-type and knockout neutrophils (Figure 2). For wild-type neutrophils, the chemotactic index increased from 1.7 ± 0.46 at 1 ng/mL C5a to 4.79 ± 0.48 at 10 ng/mL C5a and 9.2 ± 0.79 at 100 ng/mL C5a. For *Cyp4f18* knockout (KO) neutrophils, the chemotactic index increased from 2.2 ± 0.38 at 1 ng/mL C5a to 11.2 ± 1.1 at 10 ng/mL C5a and 17.3 ± 1.7 at 100 ng/mL C5a. There was a significant difference between the chemotactic index for wild-type and *Cyp4f18* knockout neutrophils at 10 ng/mL C5a ($P < 0.01$) and 100 ng/mL C5a ($P < 0.05$), but not at 1 ng/mL C5a (Figure 2(a)). Overall, the relative difference in chemotactic index for knockout compared to wild-type was 2.3-fold at 10 ng/mL C5a and 1.9-fold at 100 ng/mL C5a (Figure 2(b)). No significant differences between *Cyp4f18* knockout and wild-type neutrophil chemotaxis were observed when CXCL1/KC or WKYMVdM peptide was used as chemoattractant (data not shown).

Previous studies demonstrated that LTB_4 is a signal relay molecule during neutrophil chemotaxis: LTB_4 synthesis and secretion are induced by primary chemoattractants such as C5a, and this amplifies neutrophil migration [20, 26]. The amplification is reduced by MK 886 [20], a FLAP inhibitor that is known to block 5-lipoxygenase activation and leukotriene synthesis [27]. As expected, we observed a decrease in C5a-dependent chemotaxis in neutrophils treated with MK 886 (Figure 2(a)), but the decrease was equivalent in wild-type and *Cyp4f18* knockout neutrophils (0.45–0.56-fold), such that the relative difference in chemotactic index remained unchanged: 2.21-fold higher in *Cyp4f18* knockout neutrophils compared to wild-type at 10 ng/mL C5a and 1.92-fold higher at 100 ng/mL C5a (Figure 2(b)). Similar results were obtained when neutrophils were treated with a cPLA2α inhibitor: there was an equivalent decrease in C5a-dependent chemotaxis in wild-type and *Cyp4f18* knockout neutrophils of 0.3–0.37-fold (Figure 2(a)), and the relative difference in chemotactic index remained unchanged: 2.3-fold higher in *Cyp4f18* knockout neutrophils at 10 ng/mL C5a and 1.75-fold higher at 100 ng/mL C5a (Figure 2(b)). The cPLA2α inhibitor is less specific than the FLAP inhibitor: it blocks eicosanoid production by preventing release of arachidonic acid from membrane phospholipids [28] and therefore inhibits synthesis of a wide range of lipid mediators in addition to leukotrienes. Overall, the data show that loss of CYP4F18 results in increased neutrophil chemotaxis to C5a and suggest that this is independent of LTB_4 and other eicosanoids.

LY223982 is a synthetic BLT1 (LTB_4 receptor) antagonist that has been used to study neutrophil function [29]. We incubated neutrophils with LY223982 prior to chemotaxis assays using 10 ng/mL C5a. This resulted in a 1.7-fold increase in C5a-dependent chemotaxis in wild-type neutrophils and a 2-fold increase in *Cyp4f18* knockout neutrophils, compared to cells that were not treated with LY223982 (Figure 2(a)). There is previous evidence for cross-desensitization between neutrophil chemoattractant receptors [30], and this might account for our data: less cross-desensitization of the C5a receptor following inhibition of BLT1 could result in an increased chemotactic response to C5a. However this does not account for the differences between *Cyp4f18* knockout and wild-type neutrophils, because the relative difference in chemotactic index remained undiminished following treatment with LY223982: 2.8-fold higher in *Cyp4f18* knockout neutrophils compared to wild-type (Figure 2(b)).

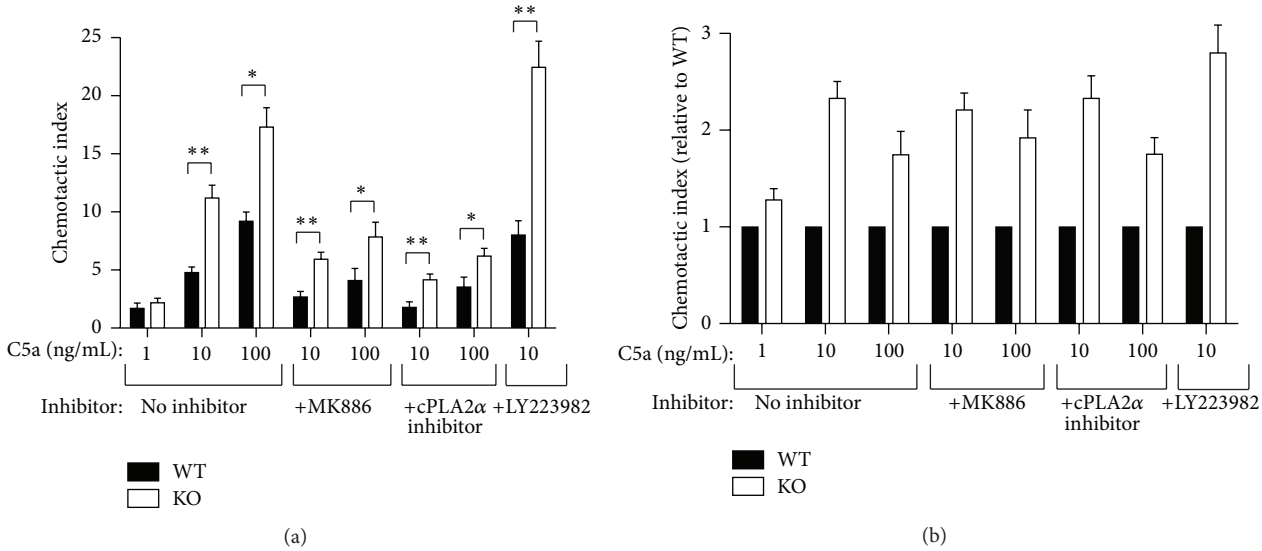

Figure 2: *Cyp4f18* knockout neutrophils show increased C5a-dependent chemotaxis compared to wild-type. Chemotaxis of bone marrow neutrophils from wild-type (WT) and *Cyp4f18* knockout (KO) mice was measured using C5a as a chemoattractant. Prior to chemotaxis, the neutrophils were incubated with or without the FLAP inhibitor MK 886 (0.5 μM), a cPLA2α inhibitor (CID 9833099, 1 μM), or a BLT1 antagonist (LY223982, 10 μM). (a) Chemotactic index represents the number of cells migrated in response to C5a divided by background (error bars represent SEM, $n = 5$, $^{*}P < 0.05$, $^{**}P < 0.01$). (b) Schematic plot for the relative difference in chemotactic index in *Cyp4f18* knockout neutrophils compared to wild-type for each experimental condition.

3.3. Comparison of Gene Expression in Cyp4f18 Knockout and Wild-Type Neutrophils. We have previously used multicolor flow cytometry analysis of cells from mouse bone marrow and other tissues to investigate protein expression in different cell lineages [19, 22]. Ly6G is a component of the myeloid marker Gr-1 (ly6G + Ly6C) that is preferentially expressed in neutrophils, and fluorophore-conjugated monoclonal antibodies to Ly6G are useful to identify neutrophils in flow cytometry studies [22]. Using this approach, mouse bone marrow cells were stained with anti-Ly6G-FITC and anti-C5aR-APC, and analysis of double plots shows an equivalent level of C5a receptor (C5aR) expression in neutrophils from *Cyp4f18* knockout and wild-type mice (Figure 3(a)). Real time PCR analysis of isolated bone marrow neutrophils (Figure 3(b)) determined that there is no significant difference in mRNA expression of C5aR or BLT1 in *Cyp4f18* knockout neutrophils compared to wild-type ($n = 4$, $P > 0.05$). A plot of $2^{-\Delta Ct}$ values shows that C5aR is expressed at approximately 10-fold higher levels than BLT1 in the cells (Figure 3(b)). The values for relative expression ($2^{-\Delta\Delta Ct}$) of C5aR in knockout neutrophils compared to wild-type ranged from 0.92 to 1.05.

A real time PCR assay designed to detect exons 8 and 9 of Cyp4f18 was used to confirm loss of expression of these exons in knockout mice, as previously described [22]. We routinely performed real time PCR analysis of isolated bone marrow neutrophils to determine if changes in expression of other *Cyp* genes might account for differences in the *Cyp4f18* knockout. We have previously shown that *Cyp4f18* is the predominant *Cyp4f* subfamily member in bone marrow neutrophils and that *Cyp4f13* and *Cyp4f16* are detected at lower levels [22]. In this study, we extended the analysis to include all *Cyp4* family members in mouse (Figure 3(c)). No other *Cyp4* transcripts were detected in wild-type or knockout neutrophils. Importantly, there were no changes in *Cyp4* expression in knockout neutrophils that might compensate for the loss of *Cyp4f18* or lead to differences in activity of the cells.

3.4. Mouse Model of DSS Colitis. A mouse model of DSS colitis was used to investigate the consequences of increased C5a-dependent chemotaxis *in vivo* (Figure 4). Inhibition of C5a activity has been shown to reduce disease pathology in this model in C57BL/6 mice [31]. Continuous administration of 4% DSS in drinking water to 8-week-old mice resulted in a rapid decline in body weight from 98.7 ± 1.35% of initial weight on day 6 to 78.5 ± 3.8% of initial weight on day 9 in wild-type mice. There was a corresponding increase in disease activity index (maximum = 4) from 1.3±0.29 on day 6 to 3.2 ± 0.24 on day 9. There were no significant differences in the values for loss of weight or increase in disease activity in *Cyp4f18* knockout mice ($n = 10$, $P > 0.05$). MPO, an enzyme produced mainly by neutrophils, was measured in colonic tissue to quantify inflammatory cell infiltration (Figure 5). On day 9, the MPO level was 57 ± 9.0 ng/mg tissue in wild-type mice and 55±7.1 ng/mg tissue in *Cyp4f18* knockout mice ($n = 5$, $P > 0.05$). Based on the similarity of these indicators, we did not proceed with further histological analysis of colon tissue.

4. Discussion

We previously generated *Cyp4f18* knockout mice [22], and predicted that the mice would exhibit altered LTB_4-dependent inflammation based on the ability of the CYP4F18

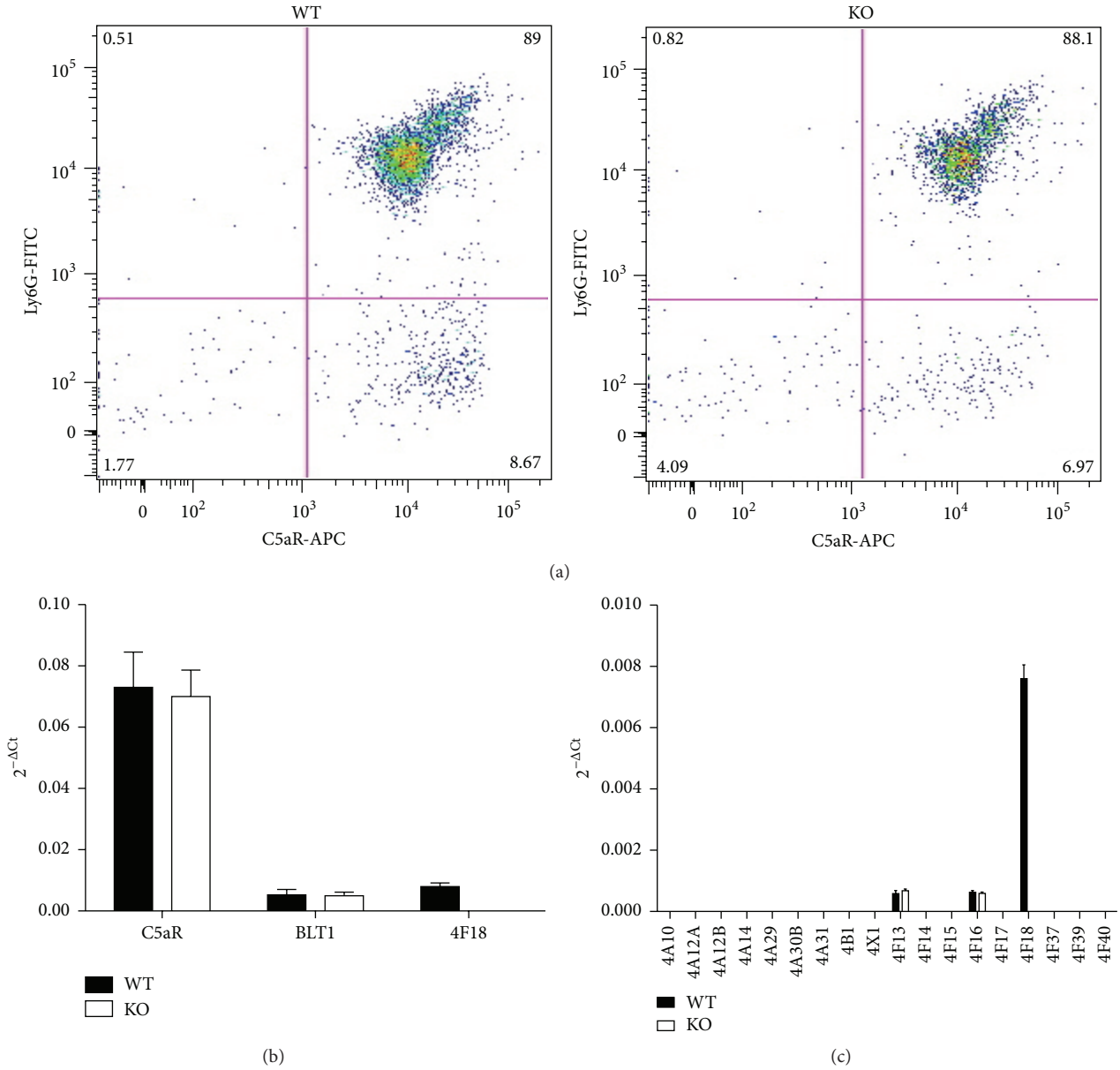

Figure 3: Gene expression in wild-type and *Cyp4f18* knockout neutrophils. Flow cytometry analysis of bone marrow cells from wild-type (WT) and *Cyp4f18* knockout (KO) mice using antibodies to Ly6G (neutrophil marker) and C5aR; representative double plots from individual mice are shown (a). Reverse transcription and real time PCR analysis of C5aR mRNA expression (b) and CYP4 mRNA expression (c), in isolated bone marrow neutrophils. ΔCt values were determined for each transcript using GAPDH as endogenous control, and $2^{-\Delta Ct}$ values were plotted for WT and KO samples ($n = 4$). Relative quantitation ($2^{-\Delta\Delta Ct}$) determined that there was no significant change in expression of any of the transcripts tested in knockout samples compared to wild-type, except for the loss of CYP4F18.

enzyme to metabolize LTB_4 in neutrophils. However, there were no significant differences in inflammation and injury in a mouse model of renal IRI compared to wild-type [22], although inhibition of LTB_4 synthesis does ameliorate pathology in this model [23]. The results of an *in vitro* chemotaxis assay are consistent with these previous observations *in vivo*. LTB_4 stimulated chemotaxis of neutrophils in a dose-dependent manner, but there were no significant differences in the response of *Cyp4f18* knockout and wild-type cells (Figure 1). In the renal IRI model, we measured infiltration of neutrophils into kidney tissue by traditional histological approaches and flow cytometry and demonstrated a comparable time course and magnitude of infiltration in *Cyp4f18* knockout and wild-type mice [22]. However, the loss of CYP4F18 might be compensated by other CYP4Fs in the complex tissue physiology of inflammation. The *in vitro* assay reported here confirms that the similarity between wild-type and knockout is inherent to neutrophils.

There are a number of possible explanations for the unaltered chemotactic response to LTB_4 in *Cyp4f18* knockout

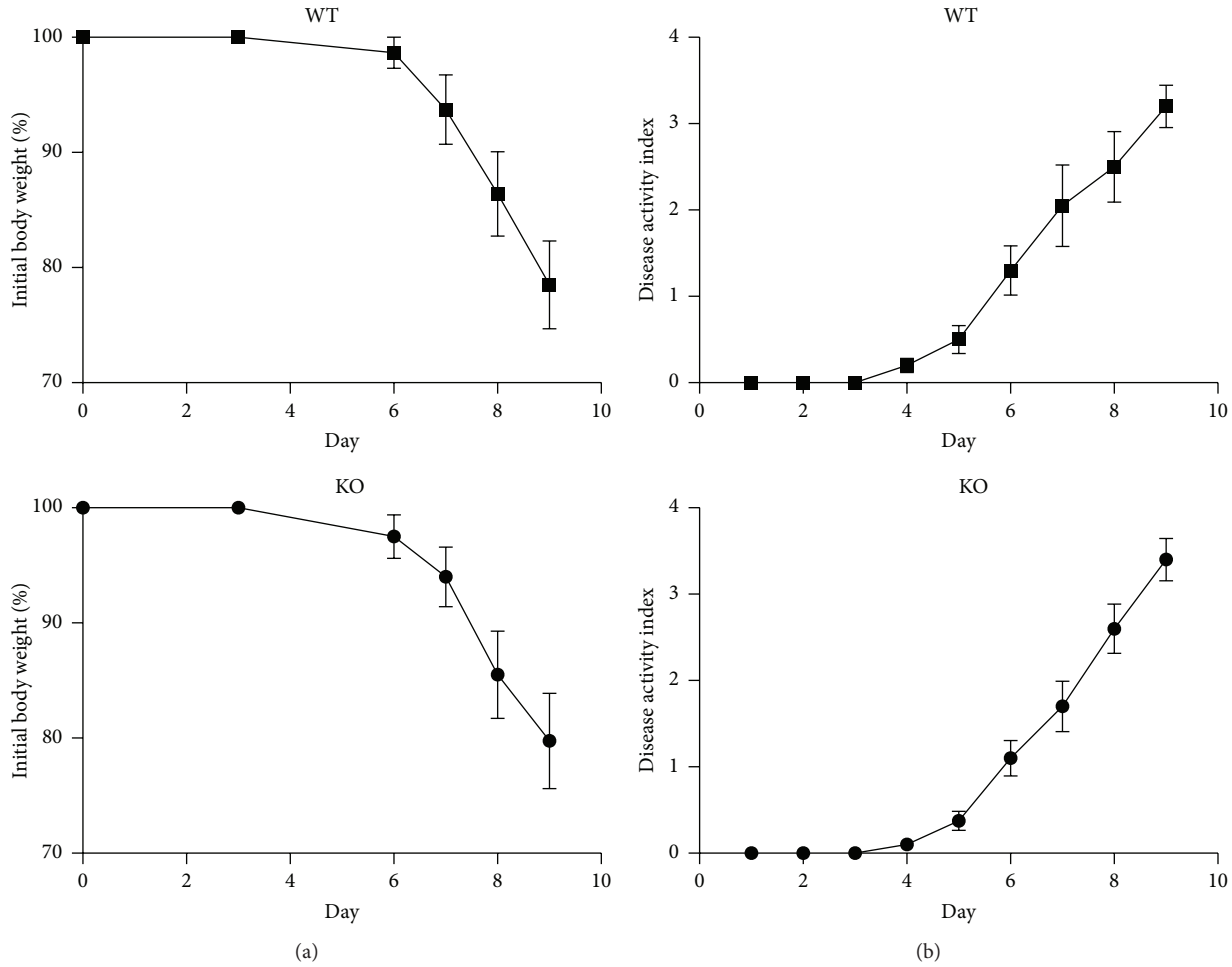

Figure 4: Mouse model of DSS colitis. Wild-type (WT) and *Cyp4f18* knockout (KO) mice were treated with 4% DSS in drinking water for 9 days, and the change in body weight (a) or disease activity index (b) was measured each day. There were no significant differences between WT and KO mice (data shows mean values ± SEM, $n = 10$, $P > 0.05$).

neutrophils. CYP4F18 converts LTB_4 to 19-hydroxy LTB_4, and to a lesser extent 18-hydroxy LTB_4, in mouse neutrophils, not 20-hydroxy LTB_4 as seen in humans. These products were not detected in *Cyp4f18* knockout neutrophils [22], but it is not known whether ω-1 and ω-2 hydroxylation of LTB_4 represents an efficient inactivation pathway. These studies underscore the importance of accurately identifying the metabolites produced by CYP4Fs. It is sometimes difficult to distinguish the ω, ω-1, and ω-2 metabolites of CYP hydroxylases, but this can have physiological and pharmacological importance. For example, 19-HETE is produced by a number of CYPs and is an antagonist of 20-HETE [32]. We have provided experimental details for the identification of 18- and 19-hydroxy LTB_4 [21, 22], but further studies are needed to clarify the roles of these metabolites. Another possibility is that CYP4F18 is redundant for LTB_4 metabolism, because mouse neutrophils have an alternative pathway for LTB_4 inactivation involving a 12-hydroxydehydrogenase that is not affected in *Cyp4f18* knockouts [22]. We speculated that CYP4F18 might have an alternative function in mouse neutrophils and that its homologue evolved to be the dominant LTB_4-metabolizing enzyme in humans coincident with its ability to generate 20-hydroxy LTB_4. This would predict that *Cyp4f18* knockout neutrophils exhibit LTB_4-independent phenotypes.

Unexpectedly, a difference in response to complement component C5a was identified using the *in vitro* chemotaxis assay. Compared to wild-type neutrophils, *Cyp4f18* knockout neutrophils show an increase in chemotaxis of 2.3-fold at 10 ng/mL C5a and 1.9-fold at 100 ng/mL C5a (Figure 2). Primary chemoattractants such as C5a stimulate LTB_4 synthesis and secretion, which amplifies neutrophil chemotaxis [20, 26]. Therefore, it is possible that increased C5a-dependent chemotaxis in *Cyp4f18* knockout neutrophils might be caused by increased levels of LTB_4 or other eicosanoids arising from endogenous synthesis. We used two different inhibitors to investigate this possibility: a FLAP inhibitor (MK 886) that blocks activation of 5-lipoxygenase and a cPLA2α inhibitor that blocks eicosanoid production by preventing release of arachidonic acid from membrane phospholipids. These inhibitors reduced C5a-dependent chemotaxis to an equivalent degree in both wild-type and *Cyp4f18* knockout neutrophils, such that the relative difference in knockout

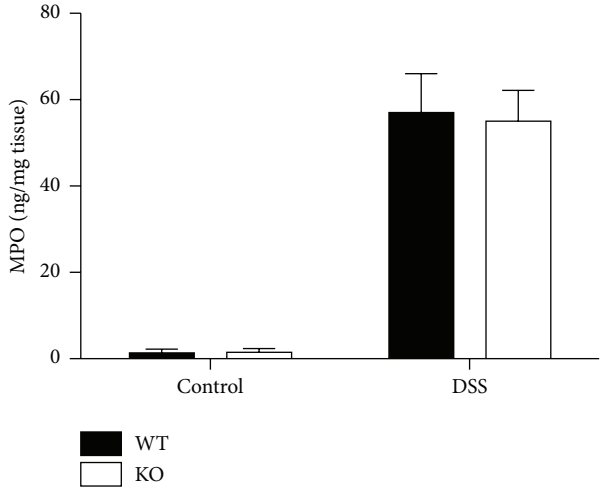

Figure 5: Myeloperoxidase (MPO) levels in colonic tissue in DSS colitis. MPO was measured by ELISA in colonic tissue homogenates from wild-type (WT) and Cyp4f18 knockout (KO) mice treated for 9 days with 4% DSS in drinking water (error bars represent SEM, $n = 5$). Control mice received drinking water without DSS.

cells compared to wild-type remained unchanged (Figure 2). A BLT1 antagonist increased C5a-dependent chemotaxis in both wild-type and *Cyp4f18* knockout neutrophils (Figure 2), possibly by blocking cross-desensitization between the chemoattractant receptors [30]. The relative difference in chemotaxis was undiminished following treatment with the BLT1 antagonist: 2.8-fold higher in *Cyp4f18* knockout neutrophils compared to wild-type (Figure 2). Overall, the data suggest that the increase in C5a-dependent chemotaxis in knockout neutrophils is unrelated to LTB_4, other eicosanoids, or receptor cross-desensitization. An alternative possibility is that CYP4F18 metabolizes a novel lipid substrate involved in the downstream signaling pathways and relay systems that mediate C5a activity (C5a is a polypeptide and therefore not a CYP substrate).

CYPs typically have broad and overlapping substrate specificity, and redundancy of function is a consistent problem with CYP knockout studies. It is often difficult to observe a phenotype for deletion of one CYP *in vivo*, because of the effects of multiple related CYPs in complex tissue environments. There are advantages to studying *Cyp4f18* knockout neutrophils *in vitro*, because CYP4F18 is the only CYP4 transcript expressed at high levels in these cells (Figure 3). Another problem with CYP knockout studies is that deletion of one CYP sometimes leads to up- or downregulation of a different CYP that generates an observed phenotype. For example, knockout of *Cyp4a14* led to male-specific hypertension that was caused by upregulation of *Cyp4a12* [33]. We compared expression of all members of the CYP4 family in wild-type and *Cyp4f18* knockout neutrophils and detected no differences other than loss of *Cyp4f18* in the knockout (Figure 3).

We used a mouse model of DSS colitis for a preliminary investigation of the consequences of altered C5a chemotaxis *in vivo*. A C5a receptor antagonist ameliorates pathology in this model [31], and genetic association studies link variants of the *CYP4F2 and CYP4F3* genes with celiac disease in humans [9]. Mice were treated continuously with 4% DSS for 9 days, but the profile of weight loss, disease activity, and colonic tissue MPO was not significantly different in wild-type and *Cyp4f18* knockout mice (Figures 4 and 5). Selection and design of future *in vivo* strategies will benefit from more *in vitro* information about *Cyp4f18* knockout neutrophils. A comparison of lipid metabolism in wild-type and knockout neutrophils will help to determine if novel CYP4F18 substrates are relevant to C5a chemotaxis and might point to other activities affected by loss of CYP4F18.

5. Conclusions

CYP4F enzymes have the ability to catalyze oxidation of a diverse range of lipid substrates related to inflammation. Therefore, these enzymes are emerging as potentially prominent players in immune regulation. A significant challenge is to identify physiologically relevant substrates among multiple possibilities and to assign functions to individual CYPs. Knockout studies are problematic because of the ability of related CYPs to compensate for function or to change expression and lead to phenotypes that are unrelated to the deleted CYP. Neutrophils provide a useful tool for our studies of *Cyp4f18* knockout mice: CYP4F18 is the only CYP4 enzyme expressed at high levels in these cells, and other CYP4 enzymes do not change expression in *Cyp4f18* knockout neutrophils. In this report we demonstrate that there is no difference in LTB_4-dependent chemotaxis of mouse neutrophils that lack CYP4F18, despite the high activity of CYP4F18 for LTB_4 as a substrate. This is significant, because many studies assume that CYP4Fs regulate LTB_4 function based on known activity as LTB_4 hydroxylases. We identified an unexpected role for CYP4F18 in regulating C5a-dependent neutrophil chemotaxis, and this was independent of LTB_4. Further studies of *Cyp4f18* knockout neutrophils *in vitro* will inform the design of *in vivo* strategies to investigate immune regulation.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Regulators and Effectors of Arf GTPases in Neutrophils

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Polymorphonuclear neutrophils (PMNs) are key innate immune cells that represent the first line of defence against infection. They are the first leukocytes to migrate from the blood to injured or infected sites. This process involves molecular mechanisms that coordinate cell polarization, delivery of receptors, and activation of integrins at the leading edge of migrating PMNs. These phagocytes actively engulf microorganisms or form neutrophil extracellular traps (NETs) to trap and kill pathogens with bactericidal compounds. Association of the NADPH oxidase complex at the phagosomal membrane for production of reactive oxygen species (ROS) and delivery of proteolytic enzymes into the phagosome initiate pathogen killing and removal. G protein-dependent signalling pathways tightly control PMN functions. In this review, we will focus on the small monomeric GTPases of the Arf family and their guanine exchange factors (GEFs) and GTPase activating proteins (GAPs) as components of signalling cascades regulating PMN responses. GEFs and GAPs are multidomain proteins that control cellular events in time and space through interaction with other proteins and lipids inside the cells. The number of Arf GAPs identified in PMNs is expanding, and dissecting their functions will provide important insights into the role of these proteins in PMN physiology.

1. Introduction

Rapid recruitment of innate immunity cells such as polymorphonuclear neutrophils (PMNs) is a critical component of pathogen killing and removal during infection. PMNs are generated from hematopoietic stem cells located in the bone marrow. A normal adult is estimated to produce about 100 billion of these PMNs daily. As they differentiate, the cells begin to move toward the venous sinusoids prior to migration across the sinusoidal endothelium to reach the vascular lumen. These terminally differentiated cells have a short life [1] but nevertheless represent the most abundant leukocyte species in the circulation. PMNs are the first leukocytes to migrate from the blood to inflammatory sites [2, 3]. Following their activation by various proinflammatory cytokines such as IL-8, TNFα, or IL-1β secreted by resident macrophages, PMNs start rolling along the vessel wall, followed by firm arrest and transmigration through the inflammatory vascular endothelium [4–6]. Once in the extravascular environment, PMNs interact with extracellular matrix proteins and migrate along a chemotactic gradient to reach the site of injury [7]. At the site of infection, PMNs begin phagocytosis and killing of pathogens through production of toxic reactive oxygen species (ROS), secretion of lysosomal enzymes, and formation of NETs (Figure 1(a)) [2, 8]. Activated PMNs also regulate the innate and the adaptive immune responses by secretion of various cytokines and chemokines, such as IL-1, IL-6, IL-8, TNFα [2, 9–11], and lipid mediators as well [12]. The interaction of PMNs with their environment is an indispensable determinant that tailors their functional responses, including correct timing of events leading to activation. The mechanisms that contribute to the maintenance of PMN homeostasis under normal and inflammatory conditions are tightly regulated through integration of external signals picked up by their transmembrane receptors. These receptors mediate intracellular signalling cascades through activation of two superfamilies of G proteins, the heterotrimeric G proteins, and the RAS superfamily of small monomeric GTPases [13, 14]. Whereas heterotrimeric G proteins directly interact with and are activated following stimulation of so-called

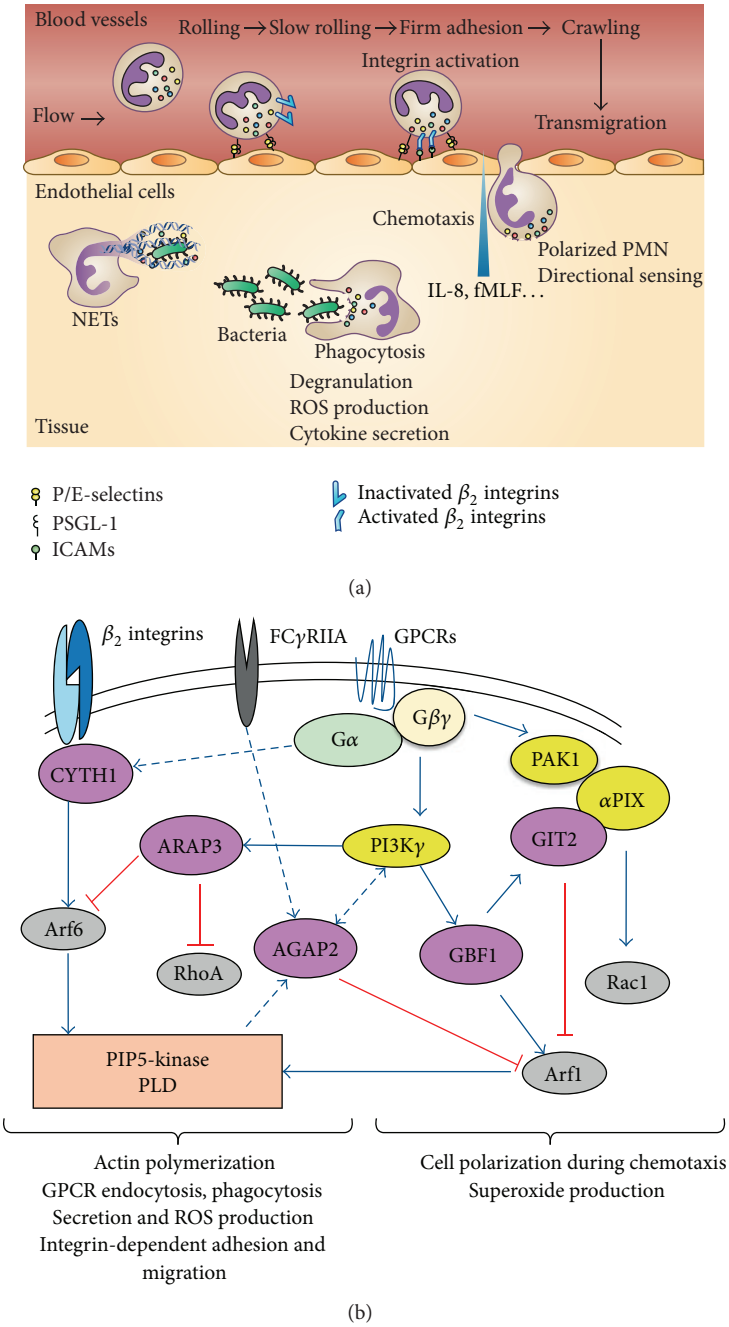

Figure 1: Main steps in PMN transmigration and regulation of PMN functional responses by Arfs and their regulators. (a) Schematic representation of PMN extravasation in infectious and noninfectious diseases. The first contact with endothelial cells is mediated by engagement of selectins with their counterreceptor P-selectin glycoprotein ligand-1 (PSGL-1) which results in capture and rolling of PMNs. Activation of PMNs by selectins and the different inflammatory signals like chemokines while rolling induces activation of the β_2 integrins (LFA-1 and Mac-1) and slow rolling. Binding of activated β_2 integrins to their counterreceptors ICAMs on endothelial cells induces PMN arrest due to firm adhesion and Mac-1-dependent crawling. Polarization of PMNs toward the chemoattractant source (i.e., cytoskeletal rearrangement, recruitment of regulators of Arfs, and activation of PI3Kγ, Arf1, and NADPH oxidase at the leading edge) initiates directional sensing and transmigration across the vascular endothelium. PMNs are guided by the gradient of chemoattractant factors and after arriving at the site of infection or tissue injury, the cells initiate phagocytosis or NETosis to kill pathogens and remove cellular debris. PMN granules are schematically represented by colored circles. (b) Signalling pathways downstream of GPCRs, Fcγ receptor IIA (FcγRIIA), and β_2 integrins by which Arfs and their regulators are thought to regulate PMN functional responses are presented schematically. Green arrows indicate direct activation either through lipid-protein or through protein-protein interactions, and negative feedback mechanisms are highlighted in red. Where direct interactions have not been established and/or the signalling mechanisms are unclear, lines are dotted. Cross talk between Arf and Rho family GTPases mediated by ARAP3 and the p21 protein- (Cdc42/Rac-) activated kinase 1 (PAK1)/PAK-interacting Exchange Factor alpha (αPIX) signalling complex is also shown.

G protein-coupled receptors (GPCRs) such as formyl-peptide receptors or CXCR1 chemokine receptor, small GTPases are generally activated by other regulatory proteins downstream of many transmembrane receptors (Figure 1(b)).

The RAS superfamily comprises more than 150 members divided into six subfamilies: Ras, Rho, Ran, Rab, Rheb, and Arf [15]. Small GTPases are molecular switches that exist in an active "on" form when bound to Guanosine-Triphosphate (GTP) and an inactive "off" conformation when bound to Guanosine-Diphosphate (GDP) [15, 16]. The activation-deactivation cycle is coordinated by three different factors. The Guanine Nucleotide Exchange Factors (GEFs) catalyze the removal of GDP and allow GTP binding to the conserved guanine nucleotide binding site of small GTPases. The binding of GTP rigidifies small GTPases in an active conformation that interacts with specific effector proteins and engages a limited number of downstream effects. Small GTPases have low intrinsic GTPase activity and need help from GTPase Activating Proteins (GAPs) to hydrolyse GTP and return to the inactive GDP-bound conformation [17, 18]. Some GTPases of the Rho and Rab family are also regulated by Guanine Nucleotide Dissociation Inhibitors (GDIs) that remove small GTPases from the membranes and sequester them in the inactive state as a cytosolic heterodimer.

Small GTPases are key elements of downstream signalling pathways regulating multiple effector proteins and functional responses of cells [21]. Numerous studies, including those from our laboratory, have shown that Arf proteins activate phospholipase D (PLD) and phosphatidylinositol 4-phosphate 5-kinase (PIP5-kinase) and regulate various PMN functions such as superoxide production, degranulation, and chemotaxis (Figure 1(b) and Table 1). In this review, we will focus on the Arf GTPases and their regulators in relation to PMN functional responses.

Table 1: Neutrophil functions modulated by Arfs and their regulators.

Detected in PMNs	Roles in PMNs (or PMN-like cells)	References
Arf1	PLD activation Secretion Golgi function	[32–35]
Arf6	PLD activation NADPH oxidase activity PIP5-kinase stimulation	[36–38]
CYTH-1	Arf6 activation PLD activation FPRL-1 internalization Regulation of β_2 integrins Adhesion Chemotaxis Phagocytosis NADPH oxidase activity	[37, 39–43]
CYTH-2/3	Unknown	[20, 39]
GBF1	Activation of Arf1 Cell polarisation Direction sensing Superoxide production	[44]
GIT2	NADPH oxidase activity Directional migration Arf1 inactivation	[44, 45]
ASAP1/2	Unknown	[46]
ACAP1	Unknown	[46]
ARAP1	Unknown	[46, 47]
ARAP3	Regulation of β_2 integrins Adhesion Chemotaxis ROS production	[46–49]
AGAP2	Phagocytosis	Unpublished
ADAP2	Unknown	[47]

2. Arfs

The Arf family was first identified and named according to its function as a cholera toxin cofactor stimulating the ADP-ribosylation of G protein Gα subunit [22]. Arf GTPases are ubiquitously expressed and their sequences are highly conserved among eukaryotes. They are divided into three classes based on sequence homology. Class I includes Arf1, Arf2 (absent in humans), and Arf3, class II comprises Arf4 and Arf5, and Arf6 is the only representative of class III [23, 24]. We cannot overlook the fact that small G proteins sharing structural features with Arfs also include Arf-like (Arl) proteins, Ras-related protein 1 (Sar1), and Arf-related protein 1 (Arfrp1) [25]. Like all small GTPases, Arfs are on-off molecular switches regulated by specific GAPs and GEFs. In contrast to other small G proteins that undergo posttranslational modification (prenylation or isoprenylation) at their C-terminus, Arfs share N-terminal amphipathic helix with a myristoylated N-terminal glycine residue. GTP binding to Arfs induces conformational changes in switch 1 and switch 2 regions that bind effector proteins [26], as well as a reorientation of the amphipathic helix that favours interaction with the membrane and insertion of the lipid tail into the phospholipid bilayer [27]. Arf1 and Arf3 are 3–10-fold more highly expressed than other Arfs in cells [28]. Arfs also have different distribution in the cells, which is thought to stem from the individual protein environment. Although classes I and II Arfs are mainly localized to the ER-Golgi, Arf1 and Arf3 are released in the cytosol, whereas Arf4 and Arf5 can associate with the Golgi and the trans-Golgi network (TGN) in their GDP-bound state [29]. Studies have determined that the GDP-GTP cycle of Arf6 takes place at the plasma membrane and that some GTP-binding defective mutants of Arf6 are trapped in endosomes [30, 31]. The two that have been the most studied are Arf1 and Arf6 (Table 1).

2.1. Arf Class I. Arf1 was first reported to regulate intracellular vesicular traffic from the Golgi to the endoplasmic reticulum (ER) and between Golgi cisternae, through the recruitment of clathrin and nonclathrin coats to membrane, a first step in the budding of transport vesicles [50–52]. In addition to stimulating PLD, Golgi-associated Arfs recruit phosphatidylinositol 4-kinase and PIP5-kinase to maintain the structure and the dynamics of the Golgi apparatus

through local synthesis of phosphatidylinositol-4-phosphate and phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P_2) [53]. Arf1 and Arf3 are required for the integrity of recycling endosomes but seem dispensable for the retrograde transport from endosomal compartments to the TGN [54]. Arf3 regulates trafficking of Toll-like receptor 9 (TLR9) [55].

In PMNs, Arf1 was shown to mediate formyl-methionyl-leucyl-phenylalanine (fMLF) dependent activation of PLD [56]. Studies from our laboratory have shown that the particulate agonist monosodium urate crystals [32], fMLF [33], and leukotriene B_4 induce Arf1 recruitment to membranes and PLD activation in PMNs [34]. A study using cytosol-depleted HL-60 cells suggested that Arf1 and phosphatidylinositol transfer protein (PITP) restore secretory function in cytosol-depleted cells by promoting PtdIns(4,5)P_2 synthesis [57]. PtdIns(4,5)P_2 synthesis could also be dependent on Arf1-mediated activation of PIP5-kinase in HL-60 cells [36]. In PMNs, Arf1 was shown to bind to complement receptor type 1 (CR1) storage vesicles and was suggested to play a role in regulation of their transport [58]. Though activated Arf1 has been shown to recruit arfaptin-1 and arfaptin-2 to Golgi membranes and has been suggested to regulate Golgi function in HL-60 cells [35], more recent studies showed that Arl1 but not other Arf proteins determine the association of arfaptins with and the biogenesis of secretory granules at the trans-Golgi in cells [59, 60].

2.2. Arf Class II. Less is known about the function of Arf class II. Arf4 and Arf5 play roles in transport mechanisms between the endoplasmic reticulum Golgi intermediate compartment (ERGIC) and the Golgi [61]. Arf4 was reported to regulate transport of ciliary cargoes [62], and the CREB3-Arf4 signalling cascade was suggested to be part of a Golgi stress response to pathogens [63]. In HeLa cells, Arf5 was reported to regulate internalization of the $\alpha_5\beta_1$ integrin and clathrin-mediated endocytosis of specific cargoes [64]. Though quantitative proteomics studies have identified Arf4 and Arf5 in PMNs [47, 65], the functions of Arf class II in PMNs have not been investigated.

2.3. Arf Class III. Arf6, the Arf GTPase most distantly related to Arf1, has been localized to the plasma membrane and endosomal compartments [31]. This GTPase has been implicated in different signalling pathways and in a wide diversity of cellular functions such as actin cytoskeleton remodelling, phagocytosis, endocytosis, membrane receptor recycling, and intracellular transport [52, 66–70]. Overexpression of Arf6 and of its regulators in metastatic cancers suggests important roles in regulating adhesion, migration, and invasive behaviour of cancer cells [71–73]. For example, in epithelial cells, E-cadherin is targeted by Arf6 to adherens junctions for maintaining barrier permeability, epithelial cell morphology, and polarity [74, 75]. In immune cells such as macrophages, a spatiotemporal recruitment of Arf6 to phagosomes regulates Fcγ receptor-dependent phagocytosis [76–79]. This GTPase is also involved in endocytosis and recycling of various GPCRs [80–82] such as mu-opioid [83] and β_2-adrenergic [84] receptors, or growth factor receptors as well [85]. Moreover, it is important to mention that Arf6 plays a major role in the signalling pathway of Toll-like receptor 4 (TLR4) and TLR9 [86, 87].

The expression of Arf6 in PMNs and neutrophil-like cells such as differentiated HL-60 or PLB-985 myeloid leukemia cells has been previously reported [36–38]. Arf6 protein is four to five times more abundant in these myeloid leukemia cells when compared to human PMNs [37]. In PMNs or differentiated PLB-985 cells, Arf6 plays roles in the signalling pathways elicited by the chemotactic peptide fMLF [37, 38]. PLB-985 cells, overexpressing the Arf6 (Q67L) mutant defective in GTP hydrolysis or the Arf6 (T27N) mutant defective in GTP binding, show increased and decreased NADPH oxidase activity, respectively [38]. Silencing of Arf6 in PLB-985 cells also reduces fMLF-mediated production of superoxide and PLD activation as well [37]. Inhibition of superoxide production by Arf6 mutants could be due to reduced PLD activity since overexpression of the Arf6 (N48R) mutant defective in PLD activation also reduced fMLF-induced NADPH oxidase activity in PLB-985 cells [38]. In addition to PLD, PIP5-kinase was reported to be a downstream effector of Arf6 [88, 89]. In this context, it is important to highlight that Arf proteins can be depleted from HL-60 cells by permeabilization and that addition of Arf6 to permeabilized cells contributes to the regulation of PdtIns(4,5)P_2 synthesis at the plasma membrane by directly activating PIP5-kinase [36]. Altogether, these studies suggest that remodelling of membrane phospholipids by Arf6-mediated activation of PLD enzymes and PIP5-kinase would regulate PMN functional responses such as NADPH oxidase activity, phagocytosis, and degranulation [90–92]. Direct demonstration of a role for Arf6 in PMN phagocytosis and degranulation awaits characterization of Arf6 knockout PMNs.

3. Arf GEFs

GEFs can signal from plasma membrane receptors directly to small GTPases, and in some cases GEFs serve as GTPase effectors or adaptor proteins that facilitate activation of other small GTPase family members [93]. The human genome contains GEFs that are family specific but some GEFs are highly specific toward one GTPase. Mammalian Arf GEFs comprise a family of 16 proteins [25]. The first characterized Arf GEFs comprise the yeast Gea1p and in mammals cytohesin-2 and BIG1 [94–96]. Although Arf GEFs show different substrate specificities, they share a conserved 200-amino acid region called the Sec7 domain that catalyzes the exchange of nucleotides. Some but not all Sec7 domains are the target of the drug Brefeldin A (BFA) [97]. Arf GEFs contain other motifs, like the Pleckstrin Homology (PH) domain involved in protein targeting to membranes through binding to polyphosphoinositides, homology downstream of Sec7 (HDS) lipid-binding domains, or SH2 and SH3 domains related to protein-protein interactions [25]. Arf GEFs are classified into six evolutionarily conserved families as follows: GBF1, BIG, PSD, IQSEC, cytohesins, and FBXO8.

The Golgi-specific BFA resistance factor 1 (GBF1) and the two yeast GBF1 orthologs, Gea1 and Gea2, localize in the Golgi. Lipid binding to the HDS1 domain immediately

downstream of the Sec7 domain is sufficient for targeting GBF1 to lipid droplets and Golgi membranes [98]. GBF1 recruits the COPI coat to the cis-Golgi [99–101]. *In vitro*, GBF1 acts preferentially on Arf5 [102]. In differentiated HL-60 cells, GBF1 has been reported to activate Arf1 in response to stimulation with fMLF [44]. Upon stimulation, Arf1 and GBF1 are relocalized from the Golgi to the leading edge of migrating cells in a phosphatidylinositol 3-kinase- (PI3K-) dependent manner. The silencing of GBF1 in HL-60 cells abrogates cell polarisation, direction sensing, and superoxide production induced by fMLF [44].

The BIG (BFA inhibited GEF) family of Arf GEFs comprises BIG1 and BIG2 in mammals. BIG2 has been shown to activate class I Arfs (Arf1 and Arf3) and to localize to the TGN and recycling endosomes [103–106]. BIG1 and BIG2 have redundant functions and are important determinants of Arf-based membrane traffic between the TGN and late endosomes [106]. In addition to lipid binding, the HDS1 domain of Sec7, the yeast orthologue of BIG2/BIG2, is important to localize this Arf GEF to Golgi membrane compartments on which Arf1 has already been activated [107]. A cascade in which GBF1-activated Arf4 and Arf5 regulate the recruitment of BIG1 and BIG2 to the TGN has been reported [108]. This study provides a mechanistic basis for the effects of a combination of Arf class I and class II knockdowns on Golgi morphology [61]. Although BFA has been reported to inhibit fMLF-mediated production of superoxide in PMNs, the BFA sensitive Arf GEF involved in this effect, if any, has not been characterized [109].

The PSD family of Arf GEFs, also known as EFA6 (Exchange Factor for Arf6), comprises four paralogs in vertebrates (EFA6A, EFA6B, EFA6C, and EFA6D) [110]. EFA6 is plasma membrane-targeted through interaction with PtdIns(4,5)P_2 and F-actin [111, 112]. EFA6 is involved in cytoskeletal rearrangement and clathrin-mediated endocytosis [112, 113]. There is no report on expression of EFA6 by PMNs.

The BFA resistant Arf GEFs (BRAGs) or IQSEC family contains three members in vertebrates [25]. BRAG1/2 were found associated to endosomes and were found to localize with Arf6 at the cell periphery [114–116]. In addition to Arf6, BRAG2 was reported to activate Arf4 and Arf5 and to regulate Arf5-dependent internalization of β_1 integrins in the clathrin-coated pits [64]. Other studies have shown that BRAG2 plays a role in cell adhesion and phagocytosis through regulation of β integrin trafficking in epithelial cells and monocytes, respectively [117, 118]. There is no information on BRAG proteins in PMNs.

Other Arf family GEFs include Sec12, a type II ER membrane protein that is a specific Sar1 GEF [119], and the F-box protein 8 gene family (FBXO8) [120]. FBXO8 might not function as a GEF but as a factor that controls the intracellular levels of Arf6 protein through ubiquitinylation-mediated proteasomal degradation [121].

The cytohesin (CYTH) family is represented by four BFA-insensitive Arf GEFs in vertebrates. The structural organization of CYTHs includes N-terminal coiled-coil domain involved in CYTH dimerization or protein-protein interaction, the Sec7 domain, and a C-terminal PH domain [122, 123]. The affinity of their PH domain for PtdIns(4,5)P_2 and phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P_3) is an important determinant for CYTH localization and/or recruitment to the plasma membrane [123]. Studies from our laboratory have reported the expression of cytohesin-2/ARNO (CYTH-2) and cytohesin-3 (CYTH-3) in undifferentiated HL-60 or PLB-985 cells and a strong induction of cytohesin-1 (CYTH-1) expression during granulocytic differentiation [20, 39]. In differentiated HL-60 cells, stimulation with fMLF induced a PI3K-independent and PI3K-dependent membrane recruitment of CYTH-1 and CYTH-2, respectively [20]. PMNs express mainly CYTH-1. We have previously shown that pharmacological inhibition of CYTH-1 with SecinH3 inhibited fMLF-mediated membrane translocation of Arf6 and Arf1 and activation of Arf6 in PMNs [37]. Studies using SecinH3 in PMNs and PLB-985 cells overexpressing CYTH-1 or silenced for Arf6 have highlighted a role for the CYTH-1-Arf6 signalling axis in PLD activation and two major bactericidal functions, degranulation and NADPH oxidase activity [37]. Furthermore, pharmacological inhibition or silencing of CYTH-1 was shown to reduce the internalization of FPRL-1 (formyl-peptide-like receptor 1) in fMLF activated granulocytes [39].

CYTH-1 was initially characterized as a positive regulator of the β_2 integrin LFA-1 (lymphocyte function antigen-1) functions in lymphocytes [40–42]. In our laboratory, we showed that overexpression of CYTH-1 in PLB-985 cells increases LFA-1-dependent adhesion to endothelial cells [43]. In contrast, PLB-985 cells silenced for CYTH-1 and PMNs treated with SecinH3 show decreased LFA-1-dependent adhesion to endothelial cells [43]. Further studies from our laboratory also documented that CYTH-1 associates with and restrains the activation of the β_2 integrin Mac-1 (macrophage antigen-1), thereby having a negative impact on PMN adhesion to fibrinogen, chemotaxis, and phagocytosis [39]. Altogether, these studies suggest that CYTH-1 in PMNs differentially regulates the activation of the β_2 integrins LFA-1 and Mac-1 [42].

4. Arf GAPs

GTPase Activating Proteins (GAPs) are proteins that accelerate the intrinsic GTP hydrolysis activity of small G proteins. The Arf GAPs contain a characteristic domain of about 130 amino acids, which has been shown to be the minimum unit with GAP activity [124]. The GAP domain has a zinc-finger structure that is unique to Arf GAPs, but similar to other GAPs for the Ras and Rho families of small GTPases, there is a conserved arginine that is essential for the catalytic activity of the so-called "arginine finger" [125, 126]. The human genome is predicted to encode thirty-one proteins containing the Arf GAP domain [127]. Mammalian Arf GAPs are selective for one or more Arfs [128] but are not active on Sar1 or Arl proteins. However, GAP selectivity *in vivo* is likely to depend on the localization of Arf GAPs and Arfs in cells, as well as on composition and shape of lipid bilayer membranes. In addition to the GAP domain, these proteins have a variety of other domains involved in intramolecular, protein-protein, and protein-lipid interactions [129]. The Arf GAPs have been

classified into two major groups according to the domain structure [130]. The ArfGAP1-type with N-terminal GAP domain includes the ArfGAP, SMAP, ADAP, and GIT protein subtypes. The AZAP-type with a GAP domain in a sandwich between a PH domain and the ankyrin (ANK) repeat motif comprises ASAP, ACAP, AGAP, and ARAP subtypes.

ArfGAP1 was the first Arf GAP identified in mammals [124]. Its GAP activity is stimulated by diacylglycerol [131]. ArfGAP1 contains two motifs termed amphipathic lipid packing sensor (ALPS) that allow binding to liposomes [132]. The ALPS motifs for Golgi localization make ArfGAP1 activity extremely sensitive to membrane lipid curvature [132, 133]. The primary function attributed to ArfGAP1 is regulation of COPI vesicle biogenesis by stimulating the hydrolysis of GTP bound to Golgi Arfs [124, 134]. ArfGAP2 and ArfGAP3 lack the ALPS motif of ArfGAP1 but instead possess dilysine retrieval motifs that confer Golgi localization through direct interaction with the COPI coat [135–137]. ArfGAP2 and ArfGAP3 are key components of the COPI coat lattice and coatomer-induced GAP activity may be required for proper vesicle formation [135–137]. There are no reports on ArfGAP1/2/3 in PMNs.

The SMAP subfamily comprises two members, SMAP1 and SMAP2 [138, 139]. SMAP protein structure includes a clathrin box that binds clathrin heavy chains and regulates the trafficking of clathrin-coated vesicles [138, 139]. SMAP1 was reported to be an Arf6 GAP regulating Arf6-dependent endocytosis of transferrin and E-cadherin receptors [140], whereas SMAP2 was involved in Arf1-dependent membrane trafficking between early endosomes and the TGN [141]. Although SMAP1 and SMAP2 were initially shown to have distinct functions, the proteins were also reported to interact which each other and to regulate transferrin receptor endocytosis [142]. SMAP1 deficient mice are more prone to develop myelodysplasia [143]. There are no reports, as of yet, on the expression of SMAP proteins in human PMNs and in human tumor-derived myeloid cell lines.

The GIT subfamily includes the two structurally related proteins GIT1 and GIT2 [127]. They possess the zinc-finger motif required for their GAP activity on Arf6 [144]. Though GIT proteins have no PH domain, their GAP activity was reported to be stimulated by PtdIns(3,4,5)P_3 [144]. GIT1 interacts with various GPCRs to regulate their endocytosis via the clathrin pathway in a G protein-coupled receptor kinase, β-arrestin, and dynamin-dependent manner [145, 146]. It is worth highlighting that GIT proteins can form complexes with PIX, a GEF specific for Rho GTPases Rac2 and Cdc42 [147]. GIT/PIX complexes regulate Cdc42/Rac-dependent activation of p21-activated kinase 1 (PAK1), a protein involved in microtubule-mediated focal adhesion disassembly [148]. GIT2 and a splice variant named GIT2-short have been characterized, with the expression of the latter being restricted to immune cells [149]. Overexpression of GIT2-short was reported to cause redistribution of Golgi protein β-COP, to affect the subcellular localization of paxillin, and to reduce the levels of actin-based fibers [150]. GIT2 is expressed in human lymphocytes and/or monocytes, mature PMNs, HL-60 promyelocytic leukemia cells, and the rat macrophage cell line RAW264 (Figure 2). In PMNs

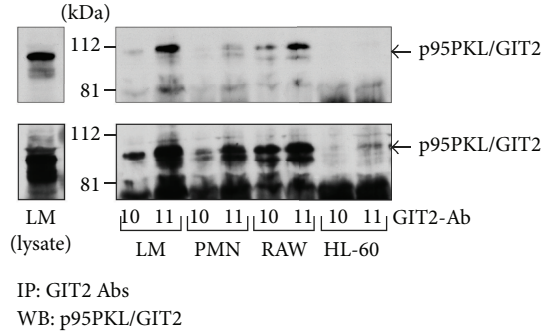

Figure 2: Expression of GIT2 in immune cells. RAW264 macrophages (1.5×10^7 cells), human PMNs (3×10^7), human lymphocytes/monocytes (LM, 3×10^7), and dimethyl sulfoxide-differentiated HL-60 cells (3×10^7) were mixed with an equal volume of boiling denaturing buffer and cell lysates were processed essentially as described by Marcil et al. [19]. The supernatants were then filtered through Sephadex G-10 columns to remove the denaturing agents and 0.1% Nonidet P-40, 20 μg/mL aprotinin, 20 μg/mL leupeptin, and 5 μL of bovine serum albumin (0.01% w/v) were added to the eluates. Samples were precleared with protein A-Sepharose and subsequently used for overnight immunoprecipitation with the polyclonal GIT2 antibodies 10 and 11 (5 μL). The beads were washed three times with ice-cold nondenaturing lysis buffer containing 1% Nonidet P-40 and boiled for 7 min at 100°C in 2x Laemmli's sample buffer as described previously [19]. Immunoprecipitated proteins were electrophoresed on 10% SDS-PAGE and proteins were transferred to Immobilon PVDF membrane (Millipore Corp., Bedford, MA, USA). Membranes were incubated with the p95PKL/GIT2 antibody (P94020; 1:1500) from Bection Dickinson (Mississauga, ON, Canada) and exposed to peroxidase-conjugated anti-rabbit IgG (1:20,000) for 1 h at 37°C. The membranes were covered with ECL+ detection reagents. Images were obtained by exposing Kodak X-Omat film to membranes for 20 sec (upper panel) and 5 min (lower panel).

obtained from GIT2-deficient mice, Arf1 was reported to be hyperactivated in response to stimulation with fMLF [45]. GIT2 deficiency was associated with reduced directional migration to fMLF and enhanced production of superoxide even if NADPH oxidase polarization at the leading edge of migrating PMNs was lost [45]. Interestingly, the Arf GEF GBF1 has been suggested to control the activation of Arf1 and to target p22phox and GIT2 to the leading edge of chemotaxing PMNs [44].

The ASAP subtype includes ASAP1, ASAP2, and ASAP3 [127]. This family possesses BAR, PH, and Arf GAP domains in tandem. More information on the domain structure of ASAPs can be found elsewhere [25, 127]. ASAP1 and ASAP2 have PIP_2-dependent GAP activity and both act on Arf1 and Arf5 and only weakly on Arf6 *in vitro* [151]. ASAP1 was involved in the regulation of cytoskeletal remodeling [152]. It was shown that ASAP1 is in an autoinhibited conformation in its native state. This is possibly due to intramolecular interaction between the BAR and PH domains, which affects GAP activity independently of the property of BAR domain in mediating association of ASAP1 with membranes [153]. Although ASAP1 and ASAP2 were detected in PMNs using

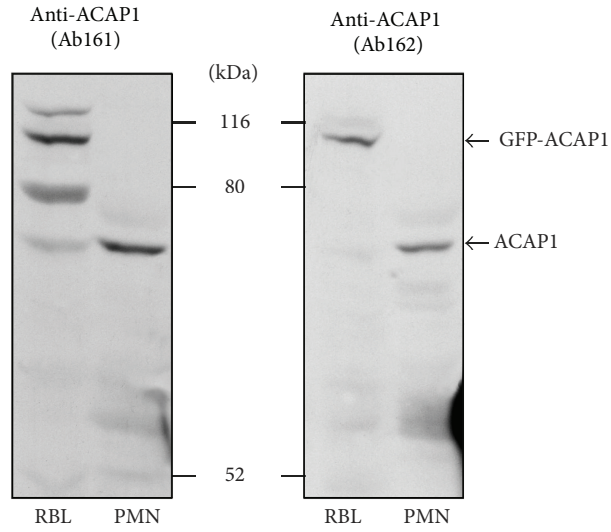

Figure 3: Expression of ACAP1 by human PMNs. Lysates from RBL-2H3 cells (0.5×10^6) overexpressing ACAP1-GFP and human PMNs (2×10^6) were subjected to 8% SDS-PAGE and proteins were transferred to Immobilon PVDF membrane. Membranes were incubated with our homemade polyclonal antibodies (serums 161 and 162) against ACAP1 (1 : 1000) and exposed to peroxidase-conjugated anti-rabbit IgG (1 : 20,000) for 1 h at 37°C. The membranes were covered with ECL+ detection reagents. Images were obtained by exposing Kodak X-Omat film to membranes.

proteomic analyses [46], their subcellular distribution and biological functions remain open questions.

The ACAP family comprises three members, with ACAP1 and ACAP2 being the best characterized. ACAP1 and ACAP2 are activated by PtdIns(4,5)P_2 and PtdIns(3,5)P_2 [154]. ACAP homologs in *Dictyostelium* were shown to affect the actin cytoskeleton and to regulate cytokinesis [155, 156]. However, their role in chemotaxis is still unclear [154–156]. A recent study suggested a role for ACAP2 in FcγR-dependent phagocytosis in macrophages [157]. Proteomic analysis has detected ACAP1 in PMNs [46]. Polyclonal ACAP1 antibodies generated in our laboratory detected a protein of about 75 kDa in PMNs and ACAP1-GFP overexpressed in RBL-2H3 cells (Figure 3). But to our knowledge, no one has yet explored the function of this Arf GAP in PMNs.

The three members of the ARAP subtype have Rho GAP domain in addition to an Arf GAP domain with multiple PH domains that recognize PtdIns(3,4,5)P_3 [25, 127, 158]. ARAP1 regulates endocytosis of epidermal growth factor receptor (EGFR) [159, 160]. Receptor internalization requires the interaction of ARAP1 with multiple proteins such as CIN85 and its phosphorylation by Src kinase [160, 161]. Further investigation is required to assess the impact of phosphorylation and protein-protein interaction on ARAP1 GAP activities. ARAP1 also regulated the filamentous-actin ring structure size of circular dorsal ruffles in NIH 3T3 cells through an Arf1/5-dependent mechanism [162]. ARAP2 was shown to regulate focal adhesion dynamics using Arf6 [163]. *In vitro* and *in vivo* ARAP3 has been reported to be a specific Arf6 GAP [158, 164]. ARAP1 and ARAP3 were detected in neutrophils using proteomics methods [46, 47]. Recent studies using an inducible ARAP3 KO mouse model suggest that this GAP affects β_2 integrin functions and several biological responses dependent on integrin activation such as adhesion-dependent ROS formation, granule release, and chemotaxis through modulation of RhoA but not of Arf6 activation [48, 49].

In humans, 11 genes are predicted to encode for AGAP-type Arf GAPs [127], with AGAP1 and AGAP2 being the most studied. AGAP1/2 have high GAP activity toward Arf1 and Arf5 and weak activity towards Arf6 [165, 166]. GAP activity is stimulated by PtdIns(4,5)P_2 and phosphatidic acid as well [165, 166]. The AGAP2 gene encodes for three protein isoforms; PIKE-L and PIKE-S, which are restricted to brain, whereas PIKE-A (AGAP2) is more ubiquitously expressed [167, 168]. As shown in Figure 4(a), purified recombinant AGAP2 is a very potent Arf1 GAP. GAP activity is strongly stimulated by PtdIns(3)P and PtdIns(3,5)P_2, the products of PI3Ks (Figure 4(b)). AGAP2 was reported to colocalize with AP-1 and transferrin receptors on recycling endosomes, and, together with Arf1, to regulate retrograde trafficking between early endosomes and the TGN [166, 169]. Moreover, AGAP2 plays a role in the signalling pathways and regulates the recycling of β_2-adrenergic receptors [170]. During cell migration, AGAP2 was shown to promote focal adhesion disassembly through binding to and stimulation of focal adhesion kinase [171]. We generated polyclonal AGAP2 antibodies that detect a protein of about 90 kDa in PMNs (Figure 5(a)). The 90 kDa protein recovered in 1% NP-40 PMN lysates was immunoprecipitated by the AGAP2 antibody but not by the preimmune serum (Figure 5(b)). The band was analysed by mass spectrometry. Overall, 32 peptides covering 44% of the AGAP2 amino acid sequence were identified. Among these peptides, two were unique to AGAP2 and there were no signature peptides for AGAP1 or PIKE-L (Figure 5(c)). Taken together, the data indicate that AGAP2, but not AGAP1 or PIKE-L, was expressed in PMNs. This work is still in progress, but preliminary observations

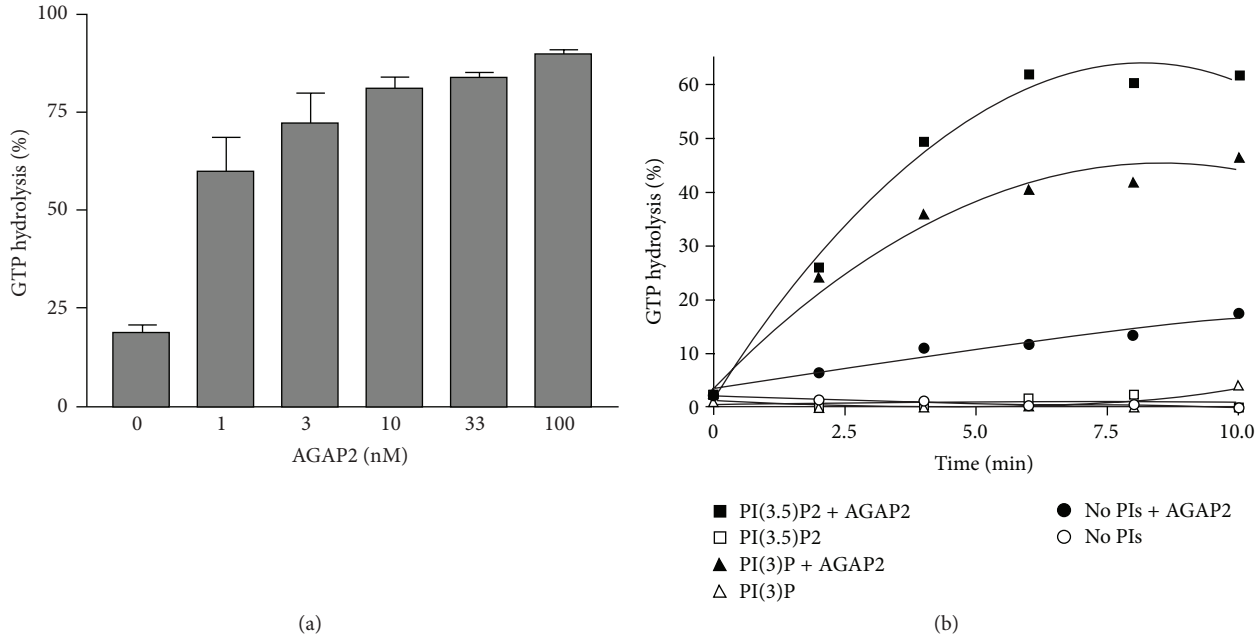

Figure 4: AGAP2 efficiently stimulates GTP hydrolysis on Arf1 and GAP activity is stimulated by products of PI3K, PtdIns(3)P, and PtdIns(3,5)P_2. Recombinant myristoylated Arf1 was purified from *E. coli* as previously described [20]. AGAP2 cDNA was inserted into the pACHLT-A baculovirus shuttle vector and cotransfected with linearized BaculoGold viral DNA into sf9 cells. Culture supernatants were used to infect sf9 cells with an MOI of 10. Insect cells were collected 48 h after infection and His6-AGAP2 was purified from sf9 lysates by chromatography on Ni-trap columns. (a) GTPα^{32}P was loaded onto Arf1 in the presence of 1 mg/mL of liposomes composed of phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine (molar ratio 40.55 : 31 : 28.45) for 30 min at 30°C. AGAP2 at the indicated concentrations was mixed with 0.3 μM GTPα^{32}P-loaded Arf1 and incubated for 30 min at 30°C in GAP buffer (20 mM Tris pH 8.0, 2 mM DTT, 100 mM NaCl, 1 mM $MgCl_2$, and 100 μg/mL liposomes). (b) GTPα^{32}P was loaded onto Arf1 in the presence of 1 mg/mL of liposomes composed of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine (molar ratio 40.55 : 31 : 28.45), and liposome-supplemented PtdIns(3)P or PtdIns(3,5)P_2 (molar ratio 37.4 : 28.5 : 26.2 : 7.9) for 30 min at 30°C. AGAP2 (10 nM) was mixed with 0.3 μM GTPα^{32}P-loaded Arf1 and incubated at 30°C in GAP buffer for indicated time points. Reactions were stopped by dilution in ice-cold stop buffer (20 mM Tris pH 8.0, 1 mM DTT, and 10 mM $MgCl_2$). Samples were filtered on Gelman GN-6 membranes and bound nucleotides were eluted with 2 M LiCl. GTP was separated from GDP by chromatography using polyethylenimine cellulose TLC plates developed in 1 M LiCl/1 M formic acid. The GTPα^{32}P/GDPα^{32}P ratios were calculated after exposure of TLC plates to a phosphorimager.

suggest that AGAP2 regulates phagocytosis independently of its GAP activity.

The ADAP subfamily includes two structurally related proteins with an Arf GAP and two PH domains in tandem [127]. ADAP1 is a brain specific PtdIns(3,4,5)P_3-binding protein that functions as an Arf6 GAP *in vivo* [172, 173]. ADAP1 also serves as a scaffold in several signalling pathways through interaction with proteins such as F-actin, the kinesin family protein K1F13B, Ran binding protein in microtubule organizing center, α-tubulin, and PKC family members to name a few (reviewed in [174]). Through interaction with components of the cytoskeleton, ADAP1 has been suggested to regulate neuronal actin and vesicle transport along microtubules [173, 175]. ADAP1 has been shown to be involved in dendritic cell differentiation and development [176]. ADAP2 is a GAP selective for Arf6 that regulates cortical actin formation at the plasma membrane [177]. ADAP2 is abundantly expressed in fat, heart, and skeletal muscles [178] and was suggested to play a role in heart development [179]. A recent proteomic analysis of PMN subcellular fractions has identified ADAP2 in cell membranes [47].

5. Concluding Remarks

The presence of Arf proteins including Arf1, Arf3, Arf5, and Arf6 has been reported in PMNs and/or neutrophil-like cells. Arf1 and Arf6 regulate various biological responses through stimulation of the lipid remodelling enzymes PLD and PIP5-kinase (Table 1). Pharmacological approaches and the use of neutrophil-like cells have permitted the investigation of the role of Arf6 in PMN functional responses such as NADPH oxidase activity, phagocytosis, and degranulation. Several regulators of Arfs have already been characterized. CYTH-1 and GBF1 are amongst the first Arf GEFs identified in PMNs. CYTH-1 was involved in PLD and NADPH oxidase activation, degranulation, and regulation of PMN adhesion through the β_2 integrins Mac-1 and LFA-1. GBF1 is part of a signalling pathway coordinating cell polarisation, direction sensing, and superoxide production in response to stimulation with chemoattractants. There is still fragmentary information available on the biological functions of the various Arf GAPs expressed by PMNs. The best characterized include GIT2, a negative regulator of Arf1, and ARAP3, a dual

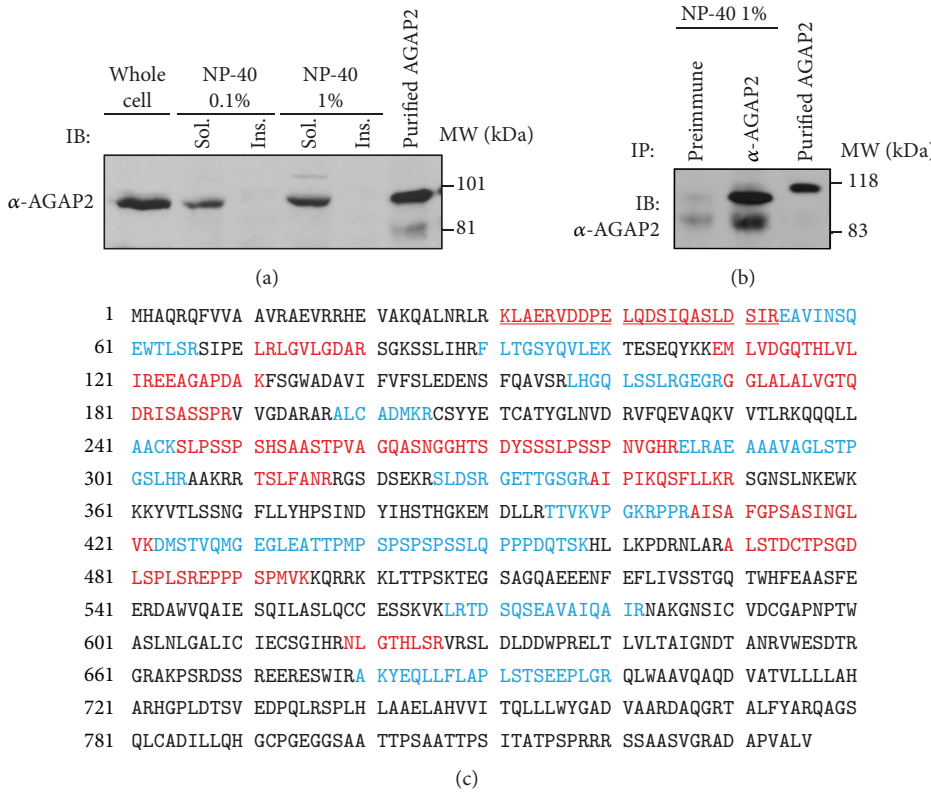

Figure 5: Expression of AGAP2 protein in PMNs. (a) Cells were lysed in nondenaturing isotonic (1% NP-40) or hypotonic (0.1% NP-40) lysis buffer. (b) PMNs were lysed in nondenaturing isotonic lysis buffer and AGAP2 was immunoprecipitated with preimmune serum or immune serum raised against AGAP2. Cell lysates derived from 1.5×10^6 PMNs (a) and immunoprecipitates (b) were resolved by SDS-PAGE. Purified His6-tagged AGAP2 was used as a control. (c) Amino acid sequence of human AGAP2 (NCBI Reference Sequence: NP_055585.1). AGAP2 was immunoprecipitated from PMNs (4×10^7/mL) as described in (b). The samples were resolved using 7.5–20% SDS-PAGE and the gel was stained with SYPRO Ruby. Bands of interest were analysed by mass spectrometry. The peptides identified by mass spectrometry are in red or blue. Peptides unique to AGAP2 are underlined.

Arf and Rho GAP. Whereas GIT2 is involved in PMN direction sensing and superoxide production, ARAP3 modulates β_2 integrin functions and adhesion-dependent formation of ROS, granule content release, and chemotaxis (Table 1). Further studies on the Arf GAPs recently identified in human PMNs (ASAP1, ASAP2, ACAP1, ACAP2, ARAP1, ADAP2, and AGAP2) are required to understand the significance of these proteins in PMN biology.

Conflict of Interests

The authors have declared no conflict of interests.

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Review Article

The Novel Functions of the PLC/PKC/PKD Signaling Axis in G Protein-Coupled Receptor-Mediated Chemotaxis of Neutrophils

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Chemotaxis, a directional cell migration guided by extracellular chemoattractant gradients, plays an essential role in the recruitment of neutrophils to sites of inflammation. Chemotaxis is mediated by the G protein-coupled receptor (GPCR) signaling pathway. Extracellular stimuli trigger activation of the PLC/PKC/PKD signaling axis, which controls several signaling pathways. Here, we concentrate on the novel functions of PLC/PKC/PKD signaling in GPCR-mediated chemotaxis of neutrophils.

1. Introduction

Neutrophils, also known as polymorphonuclear leukocytes (PMNs), are short-lived and highly specialized immune cells that form the first line of defense against bacterial and fungal infections [1]. Not only are neutrophils an essential part of the innate immune system, but activated neutrophils also secrete a number of cytokines and chemokines to help to shape lymphocyte-oriented adaptive immunity [2, 3]. The recruitment of neutrophils to inflammatory sites is through a cellular process known as chemotaxis, directional cell migration guided by extracellular chemoattractant gradients [4]. Rapid recruitment of neutrophils is crucial for host defense; however, excessive recruitment of neutrophils into healthy tissues causes damage and inflammatory diseases such as asthma and arthritis [5, 6]. Thus, neutrophil chemotaxis is tightly controlled *in vivo* through chemoattractants and their receptors.

Chemotaxing neutrophils display a polarized morphology in a chemoattractant gradient (Figure 1(a)). They extend their leading edges by assembling a force-generating actin network beneath the plasma membrane [7, 8]. Actin also collaborates with myosin to retract the rear of migrating cells and to prevent errant pseudopod extension [7]. Neutrophils detect and move toward chemoattractant gradients by G protein-coupled receptor (GPCR) signaling pathways [4]. The most important GPCRs expressed in neutrophils include formyl-peptide receptors (FPR1/2/3), classical chemoattractant receptors (BLT1/2, PAFR, and C5aR), and chemokine receptors (CXCR1/2 and CCR1/2) [9]. The engagement of chemoattractants with their GPCRs triggers dissociation/activation of the GPCR-specific Gα subunit from the G$\beta\gamma$ dimer [10, 11]. Both Gα and G$\beta\gamma$ activate downstream effectors, such as phospholipase C (PLC) [12]. It has been shown that Gαi and Gα12/13 are involved in neutrophil chemotaxis [11, 13] although the coupling mechanism of GPCRs and their specific Gα/$\beta\gamma$ remains unclear. Over the last decade, multiple signaling pathways have been revealed to control GPCR-mediated organization of actin cytoskeleton in directional cell migration [8]. At the leading edge, signaling pathways control the activity of Arp2/3 complexes that initiate the formation of new branches of actin filaments. In neutrophils, GPCRs/G protein activation triggers multiple signaling pathways to activate the Rho family of small GTPases (cdc42 and Rac1/2) to promote the growth of actin filaments (F-actin) [12, 14–19]. GPCR activation also regulates the activity of cofilin, the F-actin depolymerization factor, to facilitate the rapid growth of F-actin in the leading edge

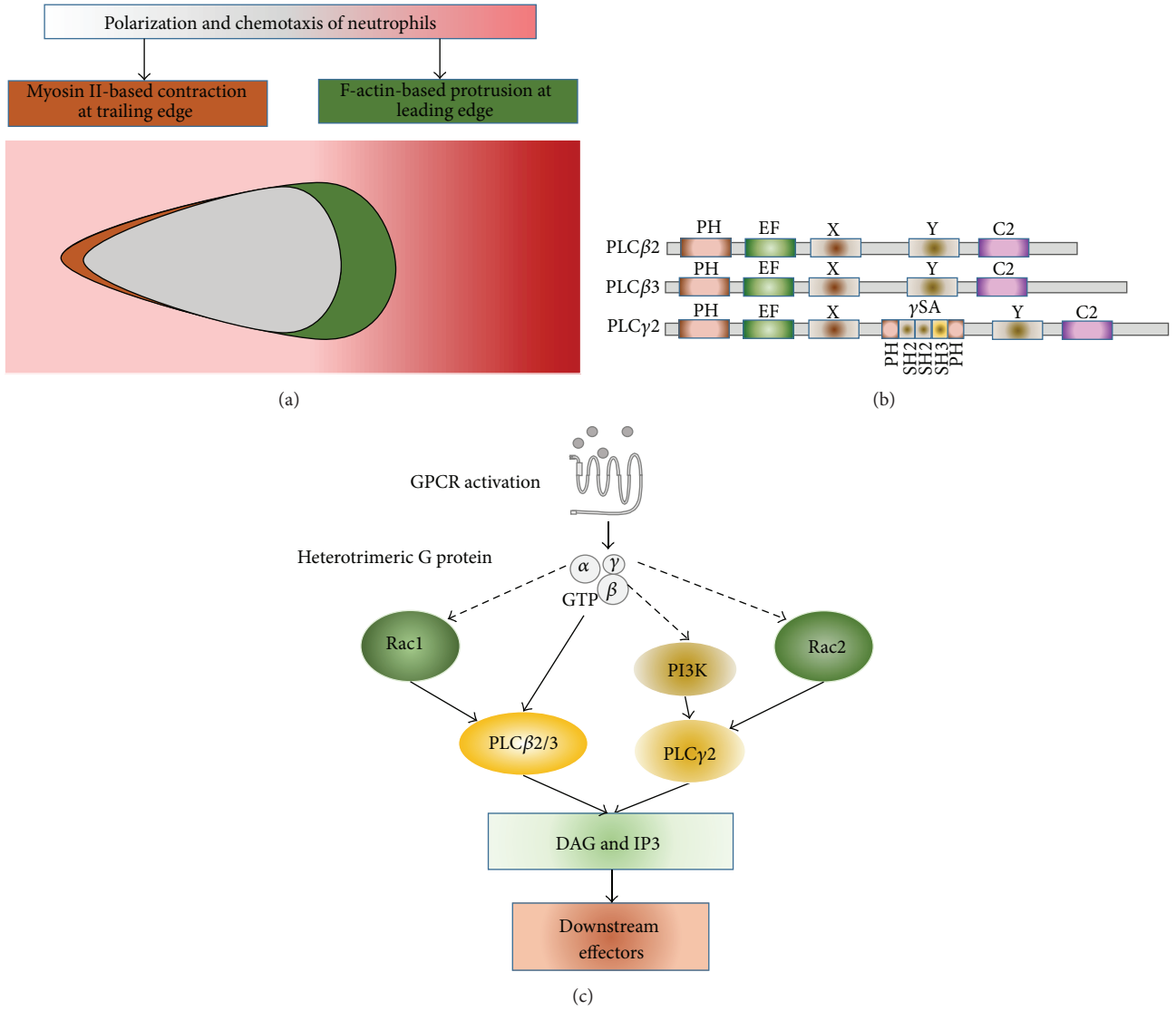

Figure 1: PLC isoforms and their signaling pathways in neutrophils. (a) F-actin-based protrusion in the leading edge and myosin-based contraction in the trailing edge of chemotaxing neutrophil cell. (b) Scheme shows the domain compositions of PLC isoforms expressed in neutrophils. Scheme shows the PH domain, EF-hand motifs, catalytic X and Y domains, and C2 domain in all PLC isoforms. In addition to the domains indicated above, PLCγ2 is characterized by the insertion of a highly structured region (PLCγ-specific array, γSA), which is comprised of a split PH domain flanking two tandem SH2 domains and an SH3 domain between the two halves of a TIM-barrel catalytic domain. (c) Signaling pathways which activate PLC isoforms in neutrophils.

[20–23]. Spatial-temporal activation of different signaling pathways for precisely controlled cell migration has just begun to be revealed.

PLC activation is an early event in response to numerous extracellular stimuli [24]. Upon activation, PLC produces two important second messengers: diacylglycerol (DAG) and inositol trisphosphate (IP_3). Both DAG and IP_3 play important roles in many signaling pathways, including the activation of protein kinase C (PKC) and protein kinase D (PKD) and the induction of calcium influx [12, 21, 23, 25]. It was shown that the signaling axis of PLC/PKC/PKD plays important roles in many signaling pathways [26]. In this review, we summarize the novel functions of the PLC/PKC/PKD signaling axis in GPCR-mediated chemotaxis of neutrophils.

2. PLC Signaling Is Required for GPCR-Mediated Neutrophil Chemotaxis

In response to various extracellular stimuli, PLC produces DAG and IP_3, which mediate multiple downstream pathways. In mammals, there are 13 phosphatidylinositide-specific PLCs that are divided into 6 subgroups: PLCβ, PLCγ, PLCδ, PLCε, PLCξ, and PLCη [24, 27–32]. Several excellent reviews have summarized the structures and activation mechanisms

of PLC isoforms [24, 29, 33]. Mammalian neutrophils express PLCβ2, PLCβ3, PLCγ2, and PLCε (Figure 1(b)). In murine neutrophils, chemoattractant stimulation robustly activates both PLCβ2 and PLCβ3 [12]. However, the evidence of PLC signaling function in neutrophil chemotaxis is contradictory. Early studies reported that murine neutrophils lacking both PLCβ2 and PLCβ3 are still able to chemotax [12]. Surprisingly, some leukocytes with a single PLCβ2 deficiency actually have enhanced chemotaxis [34], indicating that PLC signaling might not play essential role in neutrophil chemotaxis. However, a PLCβ/PI3Kγ/GSK3 signaling pathway has been shown to regulate the activity of cofilin phosphatase slingshot 2 (SSH2) and control neutrophil polarization and chemotaxis [21]. The authors further investigated *in vitro* chemotaxis behavior of murine neutrophils with PLCβ2 deficiency and suggested that the normal chemotaxis behavior of these murine neutrophils was rather context- and assay-dependent. A recent study has shown that when PLC activity is inhibited with the PLC inhibitor U73122, chemotaxis of human neutrophils is reduced, suggesting an essential role of PLC signaling in neutrophil chemotaxis [23]. Thus, PLC signaling appears to play a complicated role in neutrophil chemotaxis that is still not well understood.

In a chemoattractant gradient, PLC is recruited and activated at the leading edges of chemotaxing cells, suggesting its likely role in the remodeling of the actin cytoskeleton [23]. In neutrophils, GPCRs activate PLCβ through several mechanisms (Figure 1(c)). First, chemoattractant stimulation may trigger PLCβ activation through direct interaction with and activation by released heterotrimeric G proteins [12, 34]. Structural insights into GPCR-mediated PLCβ activation have been summarized in a recent review [33]. Although there are currently no reported structures of a G$\beta\gamma$-PLCβ complex that could shed light on the molecular basis for their interaction and activation, many studies have sought to map the interface of their interaction. GDP-bound Gαi can inhibit PLCβ activation, suggesting a common protein interaction interface on G$\beta\gamma$ [35]. Beside direct activation by heterotrimeric G protein, PLCβ2/3 might be activated by another mechanism. For example, in insect cells, PLCβ2/3 is activated by small GTPase Rac1 [36]. The crystal structure of the Rac1-PLCβ2 catalytic core complex has shown that the PH domain is the sole Rac1 binding site on PLCβ2 and that the PH domain-mediated Rac1 interaction is sufficient to activate PLCβ2/3 [37]. In neutrophils, chemoattractant stimuli trigger robust activation of Rac1 [38]. Moreover, in a chemoattractant gradient, the activated Rac1 localizes at the leading edge where PLCβ2 is highly activated in chemotaxing neutrophils [39]. Rac1-mediated PLCβ activation might provide an activation mechanism that is independent of GPCR or heterotrimeric G protein. It is intriguing to understand Rac1-mediated spatiotemporal activation of PLCβ and its possible function in neutrophil chemotaxis.

PLCγ2 also plays critical roles in integrin- and Fc receptor-mediated neutrophil functions, such as respiratory burst, degranulation, and cell spreading *in vitro* [22]. PLCγ1 is ubiquitously expressed and is mainly activated downstream of growth factor stimulation, including stimulation by platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), and fibroblast growth factor (FGF). PLCγ2 is predominantly expressed in hematopoietic cells and is activated by immune cell receptors such as B cell and Fc receptors. PLCγ1 and PLCγ2 share similar domain composition and molecular structure. The activation mechanisms and functions of PLCγ1/2 have been well summarized [29]. Recently, it has been shown that chemokine GPCRs also mediate the membrane targeting and subsequent activation of PLCγ2 in a phosphoinositide 3-kinase- (PI3K-) dependent manner [23]. This result is consistent with the finding that PLCγ1 activation is a consequence of the binding between the PH domain of PLCγ1 and PIP_3 produced on the membrane [40]. In addition, PLCγ2 might also be activated through another mechanism. It has been reported that PLCγ2 is specifically activated by Rac2 [41, 42]. In neutrophils, the engagement of chemoattractants with their GPCRs triggers the activation of Rac2 [18, 38]. The Rac1/2-mediated PLCβ/γ activation adds another layer of complexity to the existing signaling networks of PLC signaling. GPCR-mediated PLCγ2 activation in neutrophils might provide an explanation for the normal chemotaxis behavior in murine neutrophils with single or double PLCβ2/3 deficiency. However, the chemotaxis behavior of mammalian neutrophils lacking PLCγ2 remains unknown. Thus, it is difficult to evaluate the PLC isoform, and its activation is more important for neutrophil chemotaxis. Also, as a scaffold protein with numerous interacting partners, it is unlikely that GPCR- or Rac2-mediated PLCγ2 activation serves solely as the backup for PLCβ2/3 in neutrophils. Further investigation is urgently needed to understand the role of PLCγ2 in neutrophil chemotaxis.

Neutrophils also express a low level of PLCε, which is activated by GPCR and GPCR-regulated small GTPases, including Ras and Rap [24, 28, 43]. Recently, it was shown that PLCε plays a crucial role in the neutrophil-associated inflammatory response [44]. In PLC$\varepsilon^{-/-}$ mice, neutrophil infiltration is remarkably suppressed. Future work is needed to elucidate the temporal and spatial activation profiles of each PLC isoform and their molecular mechanisms and subsequent effects on neutrophil chemotaxis.

3. PKC Isoforms Play Different Roles in the Regulation of Neutrophil Chemotaxis

PKC isoforms share a similar overall structure consisting of an NH_2-terminal regulatory domain joined through a flexible linker to a conserved COOH-terminal catalytic domain that binds ATP and substrates [45]. The regulatory domain of PKC contains a pseudosubstrate domain that maintains the enzyme in an inactive conformation and membrane-targeting modules that control the subcellular localization of the enzyme. PKC isoforms are subclassified based on these membrane-targeting modules. Neutrophils express PKCα, PKCβI, PKCβII, and PKCδ [46]. PKCα, PKCβ1, and PKCβII are conventional PKCs and contain tandem C1A/C1B motifs that bind diacylglycerol (DAG) or phorbol esters (such as PMA), a C2 domain that binds anionic phospholipids in a Ca^{2+}-dependent manner,

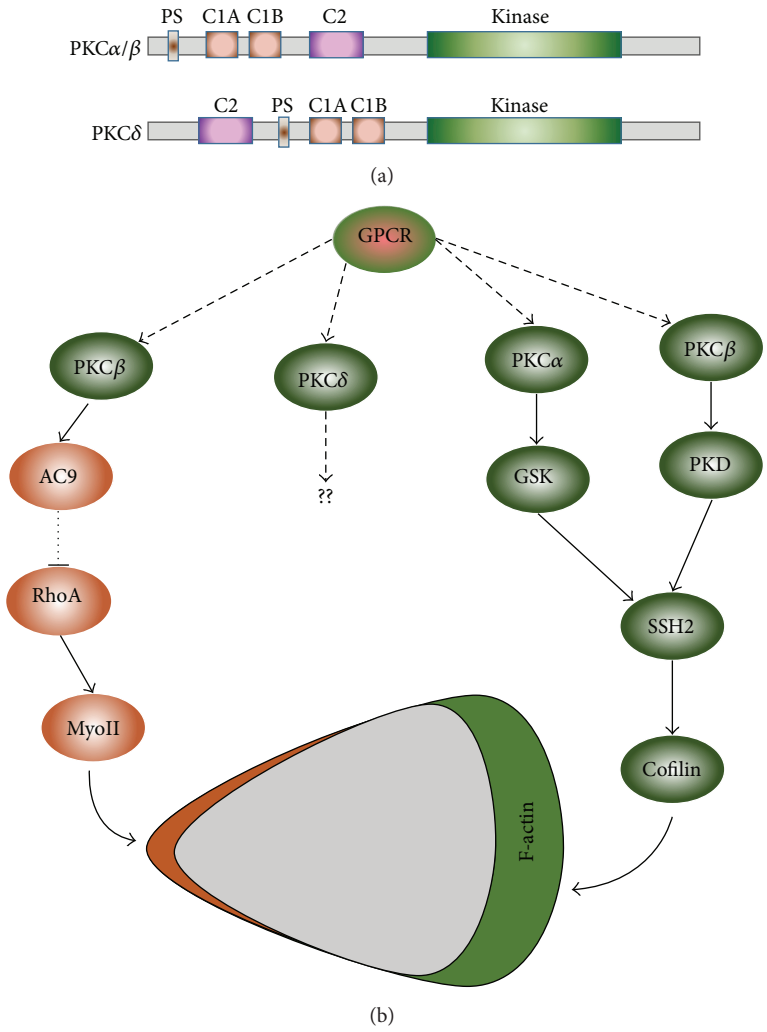

Figure 2: PKCα and PKCβ play different roles in neutrophil polarization and chemotaxis. (a) Scheme shows the domain compositions of PKCα, PKCβ, and PKCδ, which are expressed in neutrophils. All three PKC isoforms have an NH_2-terminal regulatory domain that is joined through a flexible linker to a conserved COOH-terminal kinase domain that binds ATP and substrates. PKC regulatory domains contain a pseudosubstrate domain (PS) that maintains the enzyme in an inactive conformation. Membrane targeting modules (C1A-C1B-C2 domains) control the subcellular localization of the enzyme. (b) Scheme shows the signaling pathways in which PKCα and PKCβ play essential roles in maintaining the polarization and chemotaxis of neutrophils.

and a Ser/Thr kinase domain (Figure 2(a)). PKCδ is a novel PKC that contains a nonfunctional C2 domain and therefore is insensitive to Ca^{2+}. Various stimuli activate all four PKC isoforms, and the activation of PKC is required for the oxidative burst of neutrophils [12, 47, 48]. PKCα, PKCβ, and PKCδ phosphorylate all phosphorylation sites on p47phox [47, 49]. However, it is PKCα and PKCδ, but not PKCβ, that play essential roles in fMLP- and PMA-induced superoxide generation in neutrophils or HL60 cells [48, 50], indicating that different PKC isoforms perform specific functions in neutrophils. The isoform-specific functions of PKCs have long been missing in the signaling pathways of neutrophil chemotaxis.

PKCα and PKCβ share remarkable similarities in molecular composition, structure, and activation mechanism (Figure 2(b)). In resting neutrophils, both of them localize in the cytosol. Uniformly applied chemoattractant induces membrane translocation and subsequent activation of PKCα and PKCβ in a PLC-dependent manner, indicating that the binding of DAG to their C1A domain serves as the major determinant for membrane translocation and activation [21, 23]. However, PKCα and PKCβ interact with and activate different effectors to regulate SSH2 activity. GSK3, a substrate of PKCα, is active in resting neutrophils and phosphorylates SSH2 to decrease its cofilin phosphatase activity, in turn, leaving cofilin in an inactive phosphorylated state [21]. Upon fMLP stimulation, PKCα phosphorylates GSK3 and inhibits its activity, consequently increasing SSH2 activity and the activity of its target cofilin. Recently, it has been shown that PKCβ interacts with and activates PKD1, and PKD1 phosphorylates SSH2 and inhibits its cofilin phosphatase activity [23]. By interacting with different effectors, both

PKCα and PKCβ regulate cofilin activity in order to regulate actin-based protrusion in the leading edge of chemotaxing cells. It was also reported that an mTORC2-specific activation of PKCβII regulates myosin II activity in the trailing edge of cells [51]. The authors revealed mTORC2-specific phosphorylation sites of PKCβII on its C-terminus. Point mutation of these sites resulted in impaired membrane translocation of PKCβII upon fMLP stimulation, providing an alternative membrane-targeting mechanism in addition to PLC signaling. The authors identified adenylyl cyclase 9 (AC9) as a downstream effector of PKCβII activation. Adenylyl cyclases (ACs) are activated and produce cAMP upon chemoattractant stimulation in both *D. discoideum* and neutrophils [52, 53]. In chemotaxing cells, cAMP is spatially restricted to the back of the cells to specifically regulate trail retraction and contraction in a MyoII-dependent manner [51–54]. This finding might also provide an example of how neutrophils utilize one common upstream activation pathway to precisely coordinate actin-based protrusion in the leading front and myosin II-based contraction in the trailing edge.

PKCδ translocates to the plasma membrane through the binding of its C1a domain with DAG or phorbol esters [8] and is involved in the oxidative burst in neutrophils [7, 55]. Recently, it has been reported that PKCδ is required for neutrophil transmigration mediated by IL-1β and fMLP (integrin-dependent), but not IL-8 (integrin-independent), by regulating adherence of neutrophils [4]. However, the molecular mechanism of PKCδ's function in neutrophil chemotaxis remains unclear. In corneal epithelial cells, PKCδ mediates CAP37 (neutrophil-derived granular protein) induced chemotaxis [56]. In fibroblast migration and pulmonary fibrosis development, mTORC2-mediated PKCδ phosphorylation and cell migration downstream of Gα12 have been reported [14]. Neutrophils express Gα12, which localizes and reinforces signaling networks at the trailing edge of cells [13]. It would be interesting to know whether similar signaling pathways exist in neutrophils.

4. PKD1 Is an Effector of the PLC/PKC Signaling Axis in Neutrophil Chemotaxis

Protein kinase D (PKD) belongs to a family of serine/threonine kinases that play critical roles in many physiological processes, including cell growth, protein trafficking, and lymphocyte biology [26]. All three PKD isoforms are highly expressed in neutrophils [46]. The essential role of PKDs in neutrophil chemotaxis has only recently been revealed [23].

PKD isoforms share a conserved structural motif, N-C1A-C1B-PH-KD-C, and display a high sequence homology, particularly in the catalytic domain and C1A and C1B domains (Figure 3(a)). The C1A domain binds to DAG for membrane targeting, while the C1B domain has a higher affinity for phorbol ester [26]. This explains the fact that chemoattractant stimuli trigger very similar dynamics of membrane translocation and cellular localization for all three PKDs in neutrophils [23]. However, the differences in the N-terminal region and in the regions flanked by the C1 and PH domains may confer isoform-specific functions [26]. PKD1 contains an alanine-proline-rich region (AP domain) at the N-terminus, while PKD2 has a proline-rich region (P domain). Interestingly, there was a distinct expression profile of PKD isoforms in a panel of leukocyte cell lines [23]. The expression pattern of these three isoforms is not affected by the knockdown of the other isoforms, excluding the possibility of functional compensation among the three isoforms. Accumulating evidence demonstrates the involvement of PKDs in a variety of cellular processes that contribute to cancer development [57]. It has also been shown that specific PKD isoforms are misregulated in several cancer types, including leukemia [57]. It is important to understand PKD isoform-specific functions in neutrophils.

PKD is activated by several mechanisms [57]. In one mechanism, PKD is activated by direct phosphorylation of two conserved serine residues in its activation loop by DAG-binding PKC isoforms. Subsequent autophosphorylation allows its full and sustained activation. In neutrophils, chemoattractant stimulation induces robust phosphorylation of PKD1 at Ser744/Ser748 (activation loop) [23]. Phosphorylation at both sites is severely inhibited by PKC inhibitor GÖ6983, indicating that activation of PKD is directly through phosphorylation at the activation loop by PKC. The author identified that PKCβII, a DAG-binding PKC isoform, interacts with PKD1 and is essential for neutrophil chemotaxis [23]. Phosphorylation at Ser916, an autophosphorylation site of PKD1, is also detected in response to chemoattractant stimuli, indicating that autophosphorylation of PKD1 also occurs in neutrophils following chemoattractant stimulation.

Membrane translocation of PKD is required for its activation in neutrophils. Membrane translocation of PKD1 is mediated through several mechanisms in response to various kinds of stimuli, such as growth factor, phorbol esters, and GPCR agonists [26]. In resting, neutrophil-like HL60 cells, all three PKD isoforms localize in the cytoplasm and nucleus. In contrast to its behavior in other mammalian cell lines, the kinase-inactive mutant PKD1 (K612W) also localizes in the cytoplasm of HL60 cells, indicating that kinase activity is dispensable for PKD's cellular localization. Uniformly applied chemoattractant stimulation triggers a robust membrane translocation of all three PKDs. In a chemoattractant gradient, PKD localizes at the rear of the leading edge of chemotaxing cells (Figure 3(b)). The kinase-inactive mutant of PKD is recruited to the leading edge of chemotaxing cells, indicating that kinase activity is not required for membrane targeting of PKD. Instead, membrane targeting is completely abolished by either treatment with PLC inhibitors or a mutation of the C1A domain (DAG-binding domain), which results in a decreased affinity toward DAG. This result indicates that the binding of C1A domain and DAG is the major determinant for membrane targeting of PKDs in neutrophils. DAG also recruits PKCβ to the membrane, where PKCβ phosphorylates and consequently activates PKDs [23]. Thus, translocation to the plasma membrane allows PKD1 to interact with its upstream activator, such as PKCβ, to be phosphorylated and subsequently activated. After being activated, this membrane localization might also provide close proximity for interaction with its substrates.

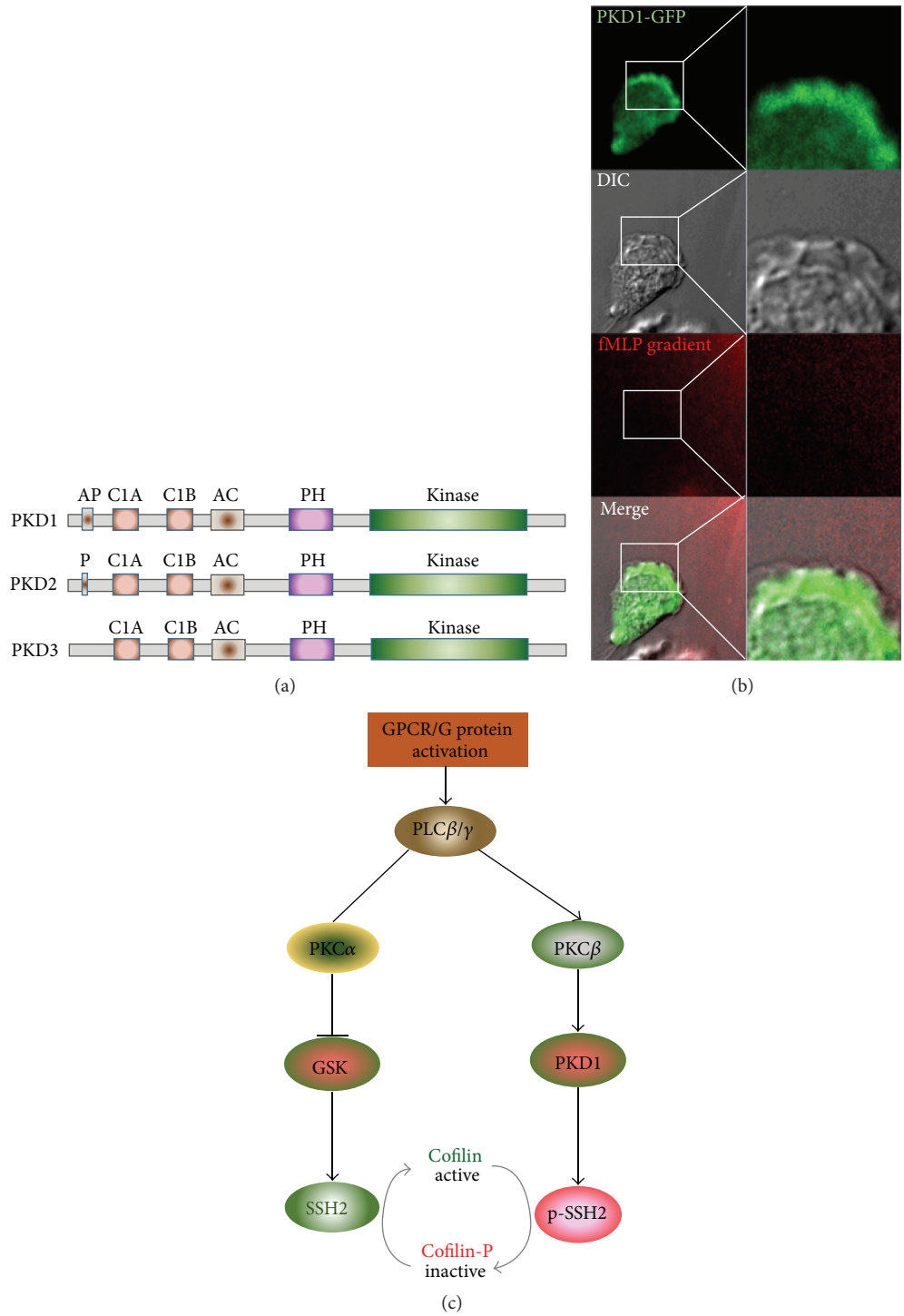

Figure 3: PKD, a direct effector of the PLC/PKC axis, is required for neutrophil chemotaxis. (a) Scheme shows the domain compositions of the three isoforms of the PKD family, PKD1–3. All three PKD isoforms have a conserved N-terminal C1A-C1B-AC-PH domain connected to a serine/threonine kinase domain at the C-terminal. The C1A domain binds to DAG for membrane targeting, while the C1B domain has a higher affinity for phorbol ester. C1A and C1B domains are separated by a long spacer, an acidic amino-acid-rich region (AC domain). The PH domain seals the kinase domain of PKD1 and inhibits its kinase activity. PKD1 also contains an alanine- and proline-rich region (AP domain) in its N-terminus while PKD2 has a proline-rich region (P domain) in its N-terminus. (b) PKD localizes at the backside leading edge of chemotaxis cells. HL60 cells expressing PKD1-GFP (Green) chemotax in 100 nM fMLP gradient (Red). In order to visualize the gradient, 100 nM fMLP was mixed with fluorescent dye Alexa 594. A differential interference contrast (DIC) image is also shown in order to portray the protrusion area of the leading edge. (c) Scheme shows the signaling pathways in which PKD1 phosphorylates cofilin phosphatase SSH2, ultimately regulating cofilin activity in GPCR-mediated neutrophil chemotaxis.

5. Downstream Effectors of the PLC/PKC/PKD Signaling Axis Regulate Neutrophil Chemotaxis

Each isoform of PLC, PKC, and PKD might have its own interacting partners in a separated signaling pathway in diverse functions of neutrophils. In the following two paragraphs, we are going to focus on the downstream effectors of the PLC/PKC/PKD axis that are involved in the remodeling of F-actin-based cytoskeleton and the regulation of other crucial signaling components.

The family of actin-depolymerizing factor (ADF)/cofilin proteins is comprised of cofilin-1 (a nonmuscle type of cofilin), cofilin-2 (a muscle type of cofilin), and ADF (also known as destrin) in mammals [58]. Active cofilin severs actin filaments and creates new barbed ends for actin polymerization [59]. Cofilin also contributes to F-actin assembly by increasing the actin monomer concentration for polymerization and consequently increasing the turnover rate of actin filaments in cells [60]. Cofilin might also increase new barbed ends by its intrinsic nucleation activity [61]. However, phosphorylation is the best-studied mechanism of regulating cofilin activity. LIM kinases (LIMKs) and testicular protein kinases (TESKs) phosphorylate cofilin to deactivate it while slingshot proteins (SSHs) and chronophin dephosphorylate p-cofilin to activate it [58]. In neutrophils, chemoattractants mediate the rapid dephosphorylation of cofilin [62]. A chemoattractant-mediated PLCβ/PI3Kγ/GSK3 signaling pathway has been found to increase the activity of SSH2, which dephosphorylates and activates cofilin (Figure 3(c)) [21]. The activation cycle of cofilin is especially important at the leading front, where rapid polymerization and depolymerization of F-actin cytoskeleton are required. Hirayama and his coworkers used HL60 cells to study the cofilin activation cycle and demonstrated a clear activation cycle [20]. A recent study has identified SSH2 as the direct target of the PLC/PKCβ/PKD signaling axis to regulate cofilin activity [23]. Taken together, GPCR activation triggers two pathways to control the cycle of cofilin activity. The cofilin activation cycle is essential for a rapid and coordinated cycling of F-actin polymerization and depolymerization at the leading edge of chemotaxing cells (Figure 3(c)). However, the signaling pathways and kinases that phosphorylate cofilin are still not fully understood in neutrophils. Future work is necessary to fully understand the regulation of cofilin activity upon chemoattractant stimulation. In the future, it will be particularly important to understand how spatiotemporally distinct signaling pathways control the rapid and precisely coordinated regulation of cofilin activity in the leading front of chemotaxing neutrophils. A live probe to visualize cofilin activity in migrating cells is urgently needed.

The PLC/PKC/PKD1 signaling pathway might also regulate the localization and functions of other key components involved in neutrophil chemotaxis. PI3Ks phosphorylate phosphatidylinositol(4,5)-biphosphate (PtdIns(4,5)P_2 or PIP_2) into phosphatidylinositol(3,4,5)-triphosphate (PtdIns(3,4,5)P_3 or PIP_3) and the phosphatase and tensin homolog (PTEN) converts PIP_3 back to PIP_2. Leading-edge localization of PI3K and trailing-edge localization of PTEN are key features of gradient sensing and polarization and are essential requirements for chemotaxis in neutrophils and *Dictyostelium discoideum* [15, 63–65]. It has recently been shown that PKD1 directly phosphorylates the p85α subunit of PI3K to enhance its interaction with PTEN, leading to polarized PTEN activity and thereby regulating neutrophil migration [66]. Moreover, PKD1 might also play a role in PTEN membrane localization. Membrane localization of PTEN is required for its function in both *D. discoideum* and neutrophils. The C2 domain of PTEN is required but not sufficient to recruit *D. discoideum* PTEN to the plasma membrane [67]. Li et al. have shown that small GTPase RhoA/Rock mediates PTEN membrane targeting in murine neutrophils [15]. Recently, Nguyen et al. generated a library that contains green fluorescent protein (GFP) fused to randomly mutated human PTEN and expressed the library in *D. discoideum* cells [68]. One cluster of mutations with an enhanced membrane association is located in the C-terminal tail phosphorylation sites. These results indicate that phosphorylation plays essential roles in PTEN membrane targeting [15, 68]. It is not clear whether PKD1 is responsible for the phosphorylation of these sites in PTEN. However, trailing-edge localization of PKDs has been reported [23]. It is of great importance to understand whether PKD is the kinase responsible for the phosphorylation of these sites in PTEN, because both PTEN and PKD have substantial functions in various types of cancer.

6. Concluding Remarks

In this review, we strove to summarize recent findings regarding novel functions of the classic PLC/PKC/PKD signaling axis in neutrophil chemotaxis. Future research should focus on revealing isoform-specific functions of PLCβ, PLCγ, and PLCε in GPCR-mediated neutrophil chemotaxis, specifically PLC isoform-specific activation and the function of downstream effectors such as PKCs.

Disclaimer

The contents are solely the responsibilities of the authors and do not necessarily represent the official views of the NIH.

Conflict of Interests

The authors declare no conflict of interests.

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Research Article

Regulation of Neutrophil Degranulation and Cytokine Secretion: A Novel Model Approach Based on Linear Fitting

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Neutrophils participate in the maintenance of host integrity by releasing various cytotoxic proteins during degranulation. Due to recent advances, a major role has been attributed to neutrophil-derived cytokine secretion in the initiation, exacerbation, and resolution of inflammatory responses. Because the release of neutrophil-derived products orchestrates the action of other immune cells at the infection site and, thus, can contribute to the development of chronic inflammatory diseases, we aimed to investigate in more detail the spatiotemporal regulation of neutrophil-mediated release mechanisms of proinflammatory mediators. Purified human neutrophils were stimulated for different time points with lipopolysaccharide. Cells and supernatants were analyzed by flow cytometry techniques and used to establish secretion profiles of granules and cytokines. To analyze the link between cytokine release and degranulation time series, we propose an original strategy based on linear fitting, which may be used as a guideline, to (i) define the relationship of granule proteins and cytokines secreted to the inflammatory site and (ii) investigate the spatial regulation of neutrophil cytokine release. The model approach presented here aims to predict the correlation between neutrophil-derived cytokine secretion and degranulation and may easily be extrapolated to investigate the relationship between other types of time series of functional processes.

1. Introduction

Historically, neutrophils were described as simple professional killers of invading pathogens to the human organism [1]. In this regard, it was considered that only the release of various antimicrobial and cytotoxic proteins synthesized and distributed into different types of granules participated to the innate immune response mediated by neutrophils. Granule types have been characterized to be readily mobilized upon an inflammatory stimulus at the plasma membrane in reverse order to their formation according to the *formed-first-released-last model* [2]. Indeed, in the different stages of neutrophil development, azurophil granules are formed first followed by specific granules, gelatinase granules, and, lastly, secretory vesicles, which are the most easily mobilized organelles in the mature neutrophils.

Due to recent progress, this classical view has been expanded by the acknowledgment that appropriately activated neutrophils constitute a substantial source of a variety of secreted cytokines supporting a direct contribution of these cells in the regulation framework of the adaptive immune response [3–5]. Neutrophils not only are a source of *de novo* synthesized cytokines dependent on gene induction but also have the capacity to express cytokines at a basal level from preformed stores [2]. However, precise intracellular localization of these packaged cytokines and mechanisms underlining their secretion remain largely elusive. The widely accepted assumption is that multiple secretory pathways coexist in neutrophils allowing the regulated release of diverse proinflammatory mediators [6]. Preformed cytokines are instantly released upon ligand-receptor signaling during the so-called "regulated exocytosis" process [7] whereas *de*

novo synthesized cytokines may be released after trafficking via recycling endosomes during the mechanism referred to as "constitutive exocytosis" [8, 9]. Additionally, variations of these two main classical secretion pathways have also been reported [10]. These distinct processes selectively control the combination of granule proteins and cytokines released into the local microenvironment from neutrophils over a temporal and spatial range and are thus regulatory mechanisms important for the onset and resolution of inflammation enabling the development of an appropriate inflammatory response [11].

It is now largely recognized that neutrophil-derived granule proteins and cytokines contribute to the maintenance of the inflammatory response and, when excessively secreted, to the ongoing process of tissue damage leading to the development of many chronic inflammatory disorders such as inflammatory bowel diseases [12], rheumatoid arthritis [13], chronic obstructive pulmonary disease [14], and atherosclerosis [15]. Determination of the regulatory mechanisms mediating the different patterns of cytokine trafficking and release may create opportunities to define new targets or strategies to selectively reduce cytokine secretion in clinical diseases.

Therefore, we selected relevant cytokines secreted by neutrophils, described to contribute to the development of chronic inflammatory diseases, in order to investigate their release in combination to degranulation upon stimulation with bacterial lipopolysaccharide (LPS).

Here, we propose an appealing model based on a linear fitting approach of cytokine secretion and degranulation giving a first basis for deeper understanding of the relationship between these two processes. It also provides a predictive view on the distribution of cytokines in neutrophils and offers an outstanding starting point to target future research on release mechanisms involved in inflammatory processes.

2. Materials and Methods

2.1. Purification of Human Neutrophils. Peripheral blood of healthy volunteers was collected in EDTA-containing tubes (BD Vacutainer, BD Biosciences, Erembodegem, Belgium). Samples were collected in accordance with the good clinical and ethical practices, which have been approved by the Ethics Review Panel (ERP) of the University of Luxembourg according to the "Comité National d'Ethique de Recherche" (CNER) from Luxembourg.

Neutrophils were isolated from blood samples by Polymorphprep separation procedure (Axis-Shield, Dundee, Scotland) according to manufacturer's instructions. Remaining erythrocytes in the neutrophil cell suspension were lysed for 10 min with red blood cell lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, and pH 7.4 [16]).

Neutrophils were washed and resuspended in PBS 1x (pH 7.4). Purity of isolated neutrophils was analyzed by the BD FACSCanto II flow cytometer (BD Biosciences) using two mixtures of selection markers CD66b-FITC/CD11b-PE/CD14-APC and CD15-FITC/CD16-PE/CD45-APC (Immunotools, Friesoythe, Germany) on 10,000 events in the gated population of homogenous (FSC-A versus SSC-A), single (SSC-A versus SSC-H), and living cells (negative cells for Sytox Blue staining (Invitrogen, Gent, Belgium)). Purified neutrophils are positive for all the selection markers used by flow cytometry. Human neutrophils were cultured in X-VIVO 15 medium with L-glutamine and gentamicin (Lonza) at 37°C and 5% CO_2 up to 24 h after purification.

2.2. Cell Stimulation. Purified neutrophils were stimulated with bacterial LPS from *E. coli* serotype O111:B4 (Sigma, Bornem, Belgium) for simulating proinflammatory conditions. For kinetic studies of cytokine secretion and degranulation, neutrophils were stimulated with 100 ng/mL LPS for 2, 4, 6, 12, and 24 h under serum-free conditions to avoid any serum component contamination, which could interfere with specific LPS-induced cell responses.

2.3. Cell Analysis by Flow Cytometry. In accordance with the literature, the most relevant markers have been selected for degranulation analysis [17]. Degranulation was determined by measuring the expression of CD markers characteristic for azurophil granules (CD63-PE), specific granules (CD15-FITC, CD66b-FITC), gelatinase granules (CD11b-PE), and secretory vesicles (CD13-APC, CD14-APC, CD18-FITC, and CD45-APC) at the plasma membrane by flow cytometry (all antibodies are from BD Biosciences except CD14-APC from Immunotools).

IgG1-FITC, IgG2a-PE (BD Biosciences), and IgG1-APC antibodies (Immunotools) were used as negative isotype controls to place the cells in the first decade of any plot, whereas CD45-FITC, CD45-PE, or CD45-APC (BD Biosciences) single dye staining was used to set compensations. Data analysis was performed by measuring the mean fluorescence intensity (MFI) for each CD marker with BD FACSDiva software (BD Biosciences) on the gated population of granulocytes (FSC-A versus SSC-A), single (SSC-A versus SSC-H), and living cells (negative cells for Sytox Blue staining (Invitrogen)). In total, 10,000 events were recorded *per* staining. The relative translocation of CD markers to the plasma membrane for each granule was determined by calculating the ratio between MFI of LPS-stimulated cells and nonstimulated control from the same time point.

2.4. Measurement of Cytokine Secretion by Cytometric Bead Array (CBA). Density of human neutrophils was adjusted to 10×10^6 cells *per* condition for subsequent quantitative measurement of cytokine secretion by LPS-stimulated cells, respectively. Fresh supernatants were collected and used directly for cytometric bead array (CBA, BD Biosciences) analysis. The multiplex standard curve composed of mixed cytokine standards was set up by serial dilutions according to the manufacturer's instructions. Selected capture beads were prepared and added to supernatants. The following beads were used: CCL2 (MCP1, bead D8), CCL3 (MIP1α, bead B9), CCL4 (MIP1β, bead E4), CCL5 (RANTES, bead D4), IL1a (bead D6), IL1b (bead B4), IL6 (bead A7), IL8 (CXCL8, bead A9), IL12b (bead E5), and TNFa (bead C4). After 1 h of incubation, detection reagent was added to each sample.

After 2 h of incubation, samples were rinsed with wash buffer and centrifuged. Samples were washed again prior to flow cytometry analysis (BD FACSCanto II, BD Biosciences). Results were quantified using the standard curves and the Flow Cytometric Analysis Program (FCAP) Array software (Soft Flow, Minneapolis, USA).

2.5. Linear Fitting Approach via R Statistical Software. Kinetic profiles of cytokine secretion and degranulation were imported into R statistical software (https://www.r-project.org/) and a linear regression approach was applied. This approach was used to find the optimal proportionality factor, namely, the slope of the model, and provide methods to evaluate the significance of our models. All ratio values between LPS-stimulated and nonstimulated control conditions (stimulation points 0, 2, 4, 6, 12, and 24 h) from the time series of cytokine secretion and degranulation were $\log_{10}$ normalized to minimize scale effect. For each granule-specific CD marker, a linear model has been fitted with the secreted cytokines. For each model, ANOVA analysis has been performed and the adjusted R-squared (RSQ) value and the slope of the model were retained. Models with a significant difference to the null model (p value ≤ 0.05) and with a high adjusted RSQ value between degranulation marker and secreted cytokine (RSQ $\geq$ r, with r being determined by simulations; see Section 2) were considered as underlying a strong similarity of pattern between a secreted cytokine and a mobilized degranulation marker. Then, cytokines linked to the same degranulation marker were clustered. To visualize these clusters, time series of the degranulation marker and its relative cytokines of the cluster were plotted. To permit comparison between time series from different scales, values from the secreted cytokines were divided by the slope of their linear model.

2.6. Simulations to Determine the Optimal RSQ Threshold. To define the optimal RSQ threshold, we simulated the fitting between pairs of granule marker and cytokine with controlled perturbations between them and choose the threshold by determining at which level of perturbation the RSQ was drastically dropping. The simulations were designed as follows: time series of each granule-specific marker and cytokines were taken individually (19 time series in total) and used to simulate matching time series with more or less perturbations. For the six points of each time series (0, 2, 4, 6, 12, and 24 h), a random number between $V_i - e$ and $V_i + e$ was drawn. V_i is the ith element of the time series and e is a predefined constant. e controls the intensity of the perturbation: the highest e is the more different both profiles are expected to be. All values from 0 to 1 with a step of 0.1 were tested for e (0, 0.1, 0.2, etc., to 1). Then, the RSQ of the original profile versus simulated profile was computed as mentioned in *linear fitting approach via R statistical software*. This process was repeated 1000 times and, for each of the 19 original profiles, the average of the RSQs was computed and plotted against the e value. To define the optimal RSQ threshold that defines a cut-off between linear fitting models with "high RSQ" and "low RSQ," we clustered the distribution of averaged RSQ into two groups, using a k-means approach (a silhouette analysis of all clustering solution with 2 to 10 clusters confirmed that using 2 clusters was the best solution, *data not shown*). The last element of the cluster with the highest center, namely, 0.796, was taken as RSQ threshold for our analysis.

2.7. Statistical Analysis. Statistical analyses were performed using the PRISM6 software (Graph Pad Software, La Jolla, CA, USA). When normality and homogeneity of variances were ascertained, as determined by the F-test, Student's t-test analyses were performed to establish two group comparisons. Otherwise, Mann-Whitney tests were used for two group comparisons. p values ≤ 0.05 were considered statistically significant.

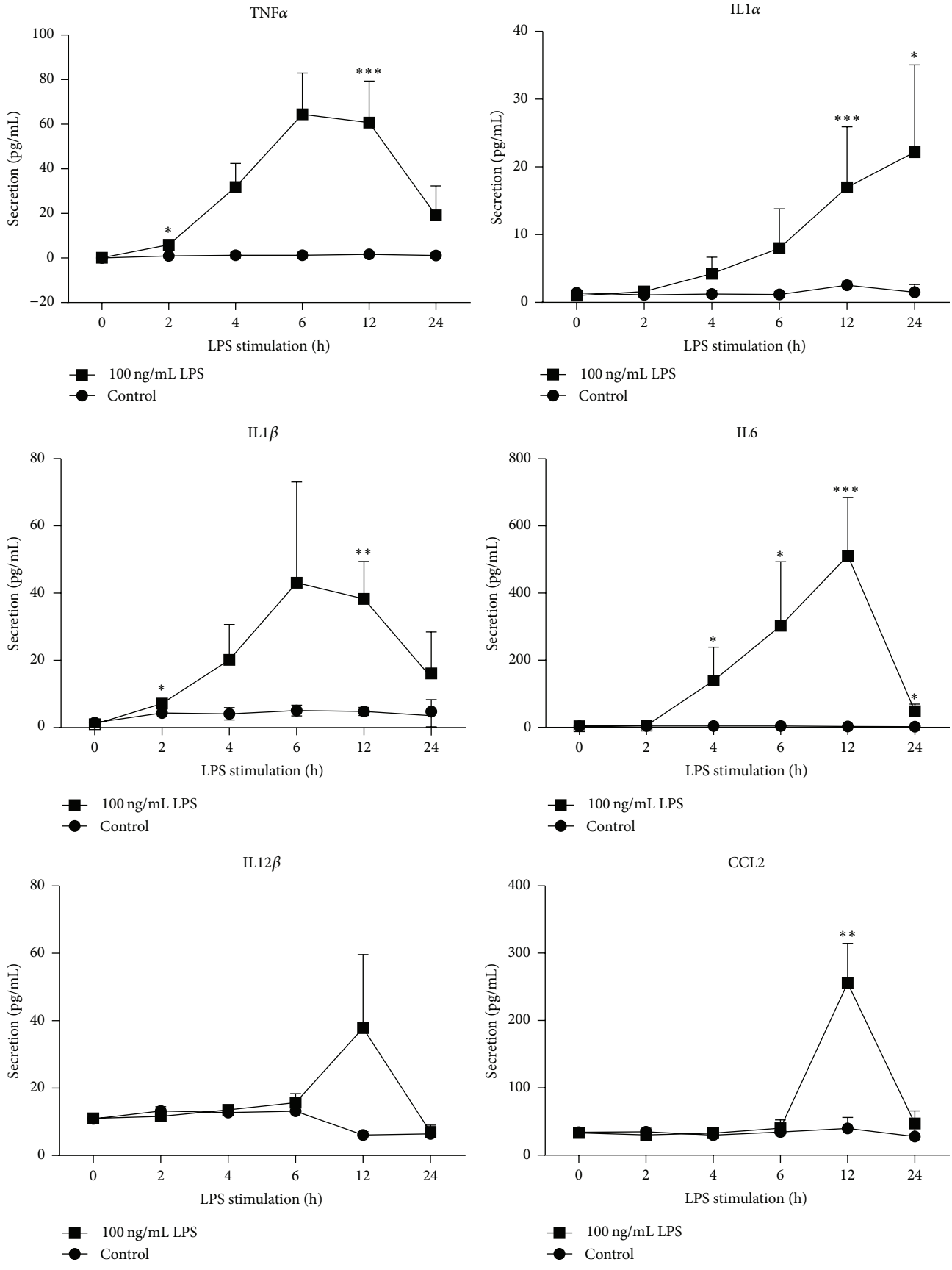
3. Results

3.1. Cytokine Levels Secreted by Human Neutrophils upon LPS Stimulation. Since recent reports have implicated neutrophils in the development of chronic inflammatory disorders, we wanted to characterize the regulatory mechanisms in the release of neutrophil-derived products. In a first step, cytokine candidates found secreted by highly purified (≥98%) neutrophils upon LPS stimulation [17] were selected for integration into our mathematical model. These cytokines have a particular relevance since they have been reported to contribute to the development of different chronic inflammatory diseases through the recruitment of diverse immune cells to the inflammatory site (Table 1).

To develop a reliable model that investigates the relationship between degranulation and cytokine secretion, different experimental data sets were generated. Time series of cytokine secretion were determined to serve as input to our model. Neutrophils were treated for 0 h, 2 h, 4 h, 6 h, 12 h, and 24 h with LPS 100 ng/mL since maximal peak of secretion for cytokines was reached at this concentration (data not shown). Subsequent quantitative measurement of cytokine secretion was performed by the CBA technique. A basal secretion level of all cytokines was detected in supernatants from neutrophils under nonstimulated conditions. Secretion of most of the cytokines released into the extracellular medium was augmented with increasing time of LPS stimulation, except for IL12β and CCL5 whose release was not significantly affected by LPS treatment (Figure 1).

The secretion pattern was different for each cytokine in the way that different profiles could be identified. Except for IL1α, secretion levels of cytokines were maximal 6 h or 12 h after treatment with LPS and decreased after 24 h. Maximal cytokine secretion was observed at (i) 6 h LPS for CCL3 and (ii) 12 h LPS for TNFα, IL6, IL8, CCL2, IL1β, and CCL4. Moreover, quantities of released cytokines were highly variable. TNFα, IL1α, IL1β, and CCL3 were only discretely secreted (≤150 pg/mL) whereas IL6, CCL2, and CCL4 were secreted at an intermediate level (~250–600 pg/mL) and IL8 was highly secreted (≥30000 pg/mL).

3.2. Effect of LPS on Degranulation in Neutrophils. To collect data for the implementation of the model, the second series



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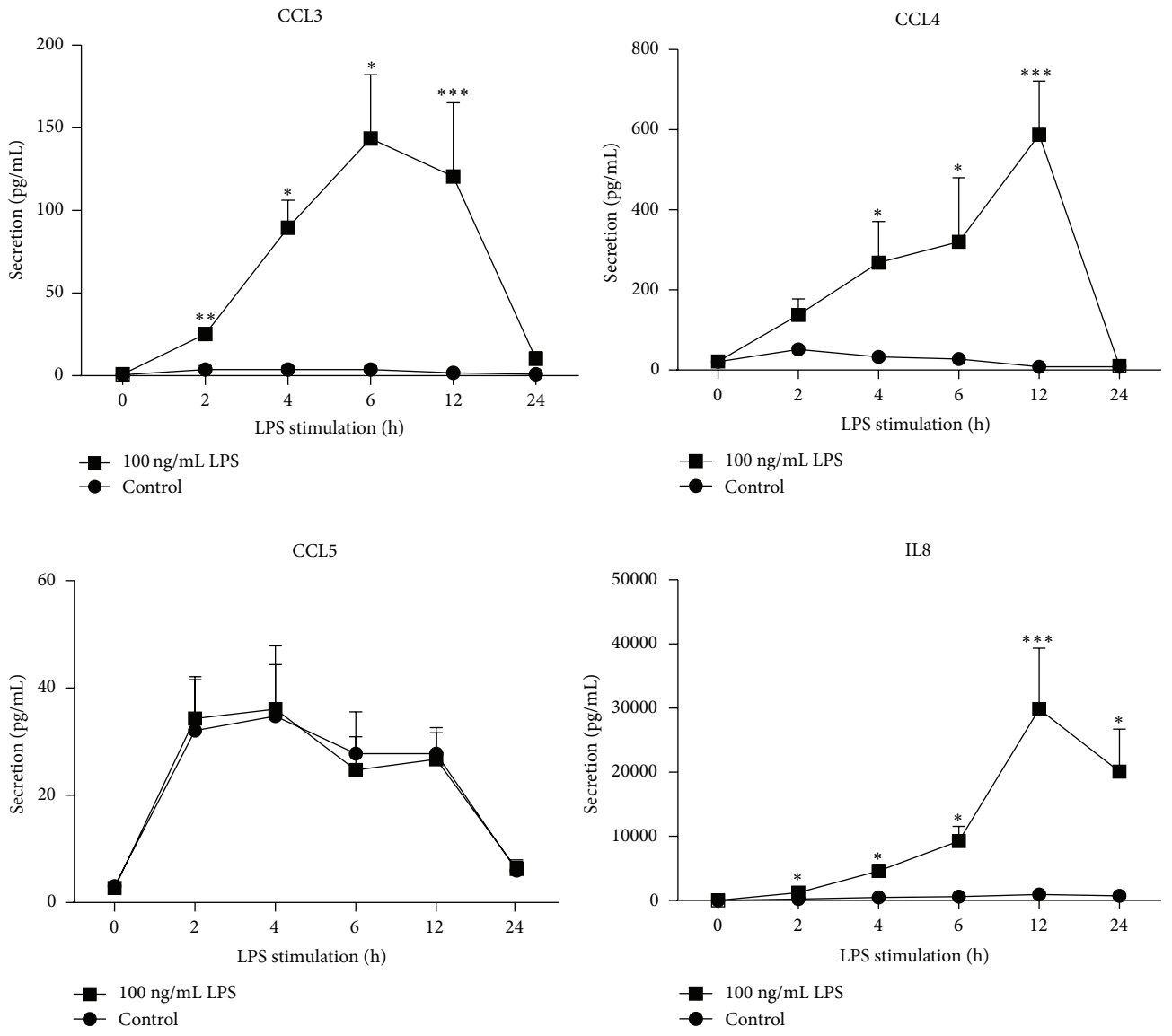
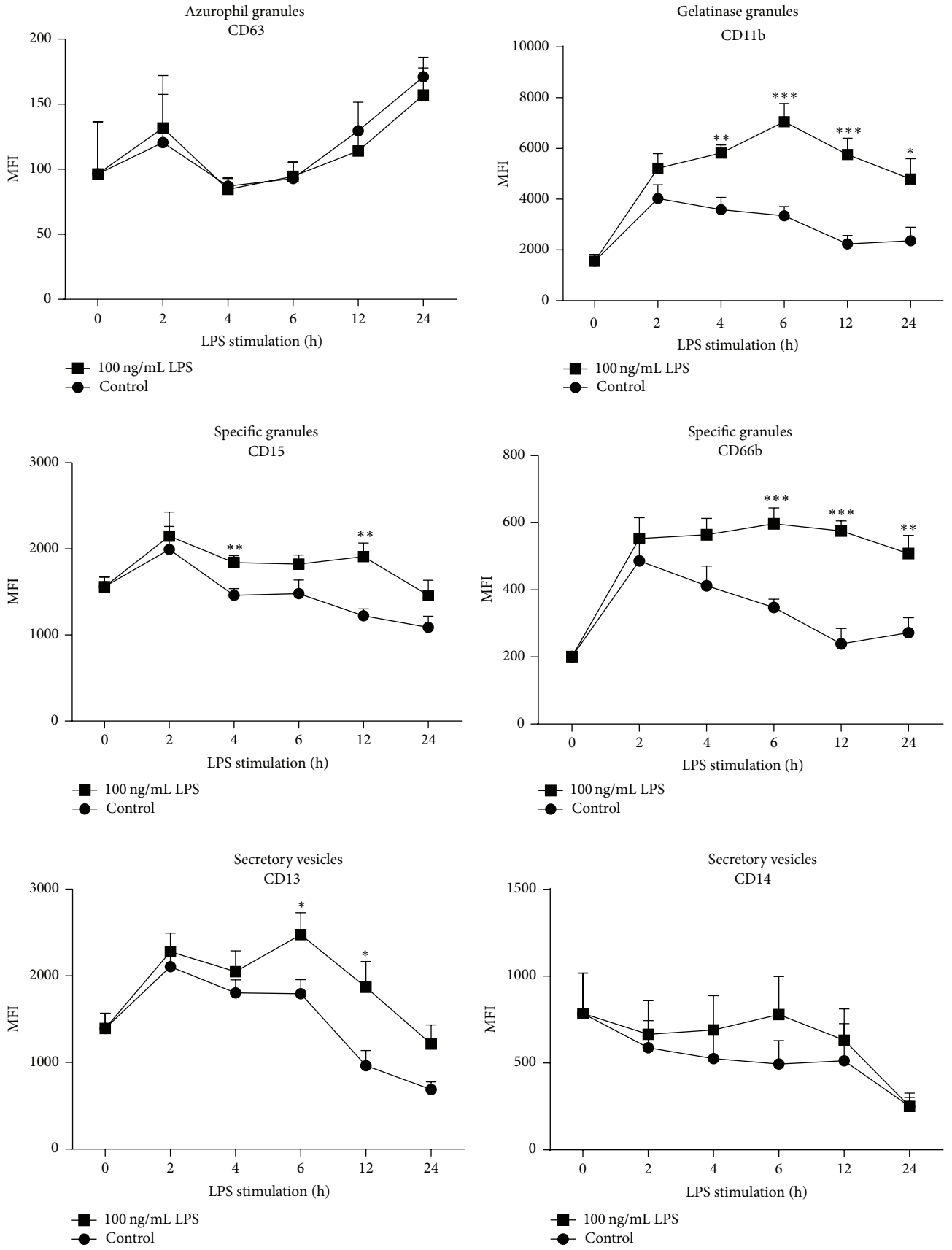


Figure 1: Time-dependent effect of LPS on cytokine secretion in human neutrophils. Cytokine secretion was measured by CBA upon stimulation with 100 ng/mL LPS for 0 h, 2 h, 4 h, 6 h, 12 h, and 24 h. Results are mean secretion (pg/mL) ± SEM of at least 3 independent experiments, significantly different from cytokine secretion in nonstimulated control at the corresponding time: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

Table 1: List of selected proinflammatory cytokines contributing to the development of chronic inflammatory disorders.

Cytokine	Cell recruitment	Chronic inflammatory disorders
TNFα	Monocytes, neutrophils, and dendritic cells	RA [13], IBD [18], A [19], and COPD [20]
IL1α	Neutrophils, T cells	RA [21], IBD [22]
IL1β	Neutrophils, thrombocytes, and T cells	RA [13]
IL6	Neutrophils, B cells	RA [13], A [23], and COPD [24]
IL12β	T cells	RA [25], IBD [26], and A [27]
CCL2	Monocytes, dendritic cells, and memory T cells	RA [21], A [28]
CCL3	Neutrophils, eosinophils, and basophils	RA [29], A [30]
CCL4	Monocytes, dendritic cells, NK cells, and T cells	RA [31], IBD [32], and A [30]
CCL5	Eosinophils, basophils, and T cells	RA [33], A [30]
IL8	Neutrophils, macrophages, and mast cells	RA [13], A [28], and IBD [34]

RA = rheumatoid arthritis, IBD = inflammatory bowel disease, A = atherosclerosis, and COPD = chronic obstructive pulmonary disease.



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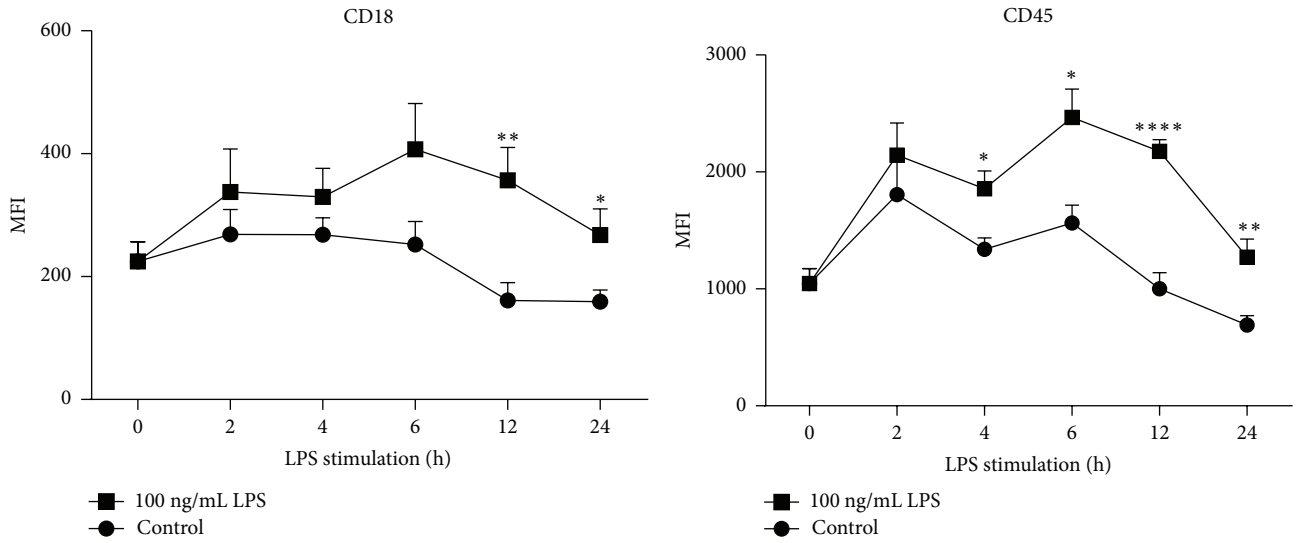


Figure 2: Temporal pattern of degranulation upon LPS stimulation in human neutrophils. Translocation of degranulation markers to the plasma membrane was assessed using flow cytometry after cell treatment with 100 ng/mL LPS for 0 h, 2 h, 4 h, 6 h, 12 h, and 24 h. Sytox Blue negative cells were gated to exclude dead cells from the analysis. Results are expressed in mean fluorescence intensity (MFI) of LPS-stimulated cells and nonstimulated control ± SEM of at least 3 independent experiments, significantly different from nonstimulated control at the same time point: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

of experiments consisted in identifying the kinetic degranulation profile of the different granule types upon time-dependent LPS stimulation. Degranulation can be determined by the upregulation of granule membrane molecules as a consequence of membrane fusion from granules with the plasma membrane [35]. Therefore, LPS-treated cells were analyzed for cell surface expression of several CD molecules known as degranulation markers.

Results showed that LPS stimulation affected the ease of mobilization of intracellular granule types in neutrophils in a time-dependent manner, as reflected by increased translocation of CD markers to the cell surface (Figure 2). In a temporal pattern, LPS stimulation induced the release of specific granules as demonstrated by the redistribution of CD15 and CD66b to the plasma membrane. Translocation of these CD markers towards the plasma membrane was scattered over a time interval of 4 h and 24 h.

In a similar way, LPS stimulation increased the presence of CD11b as well as CD13, CD18, and CD45 at the plasma membrane reflecting an increased release of gelatinase granules and secretory vesicles, respectively. As observed with CD15 and CD66b for the specific granules, increase of these CD markers was detected between 4 h and 24 h of LPS stimulation.

Maximal expression at the plasma membrane for all the CD markers was detected after 6 h or 12 h of LPS stimulation (Figure 2).

It must be noted that LPS was unable to trigger the mobilization of azurophil granules since CD63 expression was not changed at the plasma membrane.

3.3. Linear Fitting of Cytokine Secretion and Degranulation Kinetics. Many approaches exist for the examination of time series of expression data (e.g., [36]) but none of them could be applied to analyze short-time series of secretion. For this reason, we used a novel model approach to explore the relationship between cytokine secretion and degranulation by their kinetic profiles (Figure 3).

We hypothesized that time series of secreted cytokines with similar pattern to time series of degranulation markers present at the plasma membrane should have proportional values at each time point of LPS stimulation, so that a proportionality factor between the two profile curves can be defined. To address this question, we choose to use the linear regression approach, which fits best to our needs: it captures proportionality well, can be used with only one pair of profiles (in our case, cytokine versus granule-specific marker), and includes measures to evaluate the results (significance of the model and R-squared value, Section 2). All ratio values between LPS-stimulated and nonstimulated control conditions from the time series of cytokine secretion and degranulation (Table 2) were $\log_{10}$ normalized.

While the ANOVA analyses the efficiency of this model (i.e., proportional kinetic profile curves are significantly different from the null model), the adjusted RSQ value measures the correlation between the kinetic profiles (i.e., proximity to the linear fitting). The optimal RSQ value was determined by simulations, in which predefined perturbations were introduced to our kinetic profiles (Section 2).

How augmenting perturbations (e from 0 to 1) influenced the linear fitting of two time series, in our example, the linear fitting between the granule marker CD11b and the cytokine IL8, is presented in Figure 4(a). The original kinetic profile is depicted by the black line whereas the one with perturbations is represented by the red line. By plotting the average RSQ values (derived from 1000 repetitions of simulations) against

Table 2: Time-dependent (a) secretion of cytokines and (b) granules expressed in ratio between LPS-stimulated and nonstimulated cells ± SEM of at least 3 independent experiments, significantly different from nonstimulated control at the same time point: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

(a)

Relative secretion	0 h LPS	2 h LPS	4 h LPS	6 h LPS	12 h LPS	24 h LPS
TNFα	1.28 ± 0.28	6.30 ± 1.81*	24.82 ± 8.26	51.54 ± 14.79	36.70 ± 11.20***	16.48 ± 11.35
IL1α	0.73 ± 0.38	1.43 ± 0.21	3.36 ± 1.92	6.84 ± 4.92	6.67 ± 3.51***	14.70 ± 8.51*
IL1β	0.69 ± 0.69	1.64 ± 0.2*	4.87 ± 2.55	8.45 ± 5.89	7.89 ± 2.3*	4.20 ± 3.46
IL6	0.93 ± 0.18	1.30 ± 0.37	34.35 ± 23.38*	69.92 ± 50.91*	185.53 ± 62.99***	23.64 ± 10.51*
IL12β	1.02 ± 0.06	0.88 ± 0.04	1.06 ± 0.09	1.16 ± 0.19	6.20 ± 3.57	1.08 ± 0.25
CCL2	0.97 ± 0.13	0.86 ± 0.17	1.11 ± 0.18	1.17 ± 0.36	6.43 ± 1.48**	1.69 ± 0.66
CCL3	1.43 ± 1.43	6.72 ± 0.98**	24.35 ± 4.51*	66.06 ± 17.86*	65.98 ± 24.41***	11.05 ± 4.62
CCL4	1.06 ± 0.07	2.65 ± 0.76	8.10 ± 3.09*	11.69 ± 5.84*	69.14 ± 15.76***	1.22 ± 0.72
CCL5	0.88 ± 0.05	1.07 ± 0.24	1.04 ± 0.34	0.89 ± 0.22	0.96 ± 0.18	1.05 ± 0.29
IL8	1.02 ± 0.12	4.89 ± 1.47*	9.85 ± 2.31*	15.84 ± 3.93*	31.83 ± 10.15***	27.00 ± 8.89*

(b)

Relative degranulation	0 h LPS	2 h LPS	4 h LPS	6 h LPS	12 h LPS	24 h LPS
CD63	1.00 ± 0.42	1.09 ± 0.33	0.97 ± 0.10	1.02 ± 0.12	0.88 ± 0.10	0.92 ± 0.12
CD15	1.00 ± 0.07	1.08 ± 0.14	1.26 ± 0.06**	1.23 ± 0.07	1.56 ± 0.13**	1.36 ± 0.16
CD66b	1.00 ± 0.06	1.14 ± 0.13	1.37 ± 0.12	1.72 ± 0.14***	2.41 ± 0.13***	1.87 ± 0.20**
CD11b	1.00 ± 0.17	1.29 ± 0.14	1.62 ± 0.09**	2.11 ± 0.21***	2.57 ± 0.29***	2.03 ± 0.34*
CD13	1.00 ± 0.13	1.08 ± 0.10	1.14 ± 0.13	1.38 ± 0.14*	1.94 ± 0.31*	1.76 ± 0.32
CD14	1.00 ± 0.30	1.13 ± 0.33	1.31 ± 0.37	1.58 ± 0.44	1.23 ± 0.35	1.00 ± 0.31
CD18	1.00 ± 0.14	1.26 ± 0.26	1.23 ± 0.17	1.61 ± 0.3	2.22 ± 0.33**	1.68 ± 0.27*
CD45	1.00 ± 0.12	1.19 ± 0.15	1.39 ± 0.11*	1.58 ± 0.16*	2.18 ± 0.10****	1.84 ± 0.22**

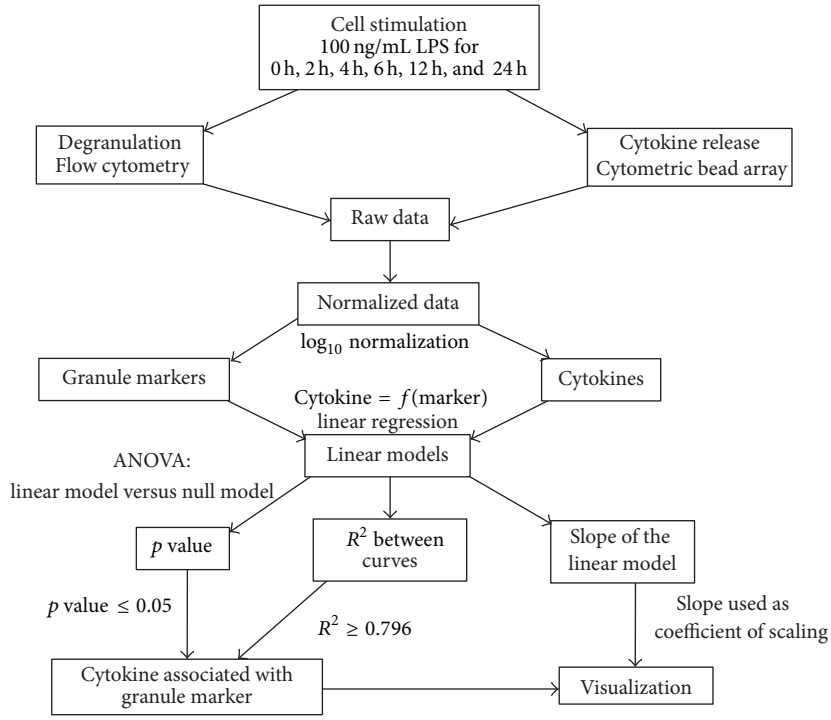
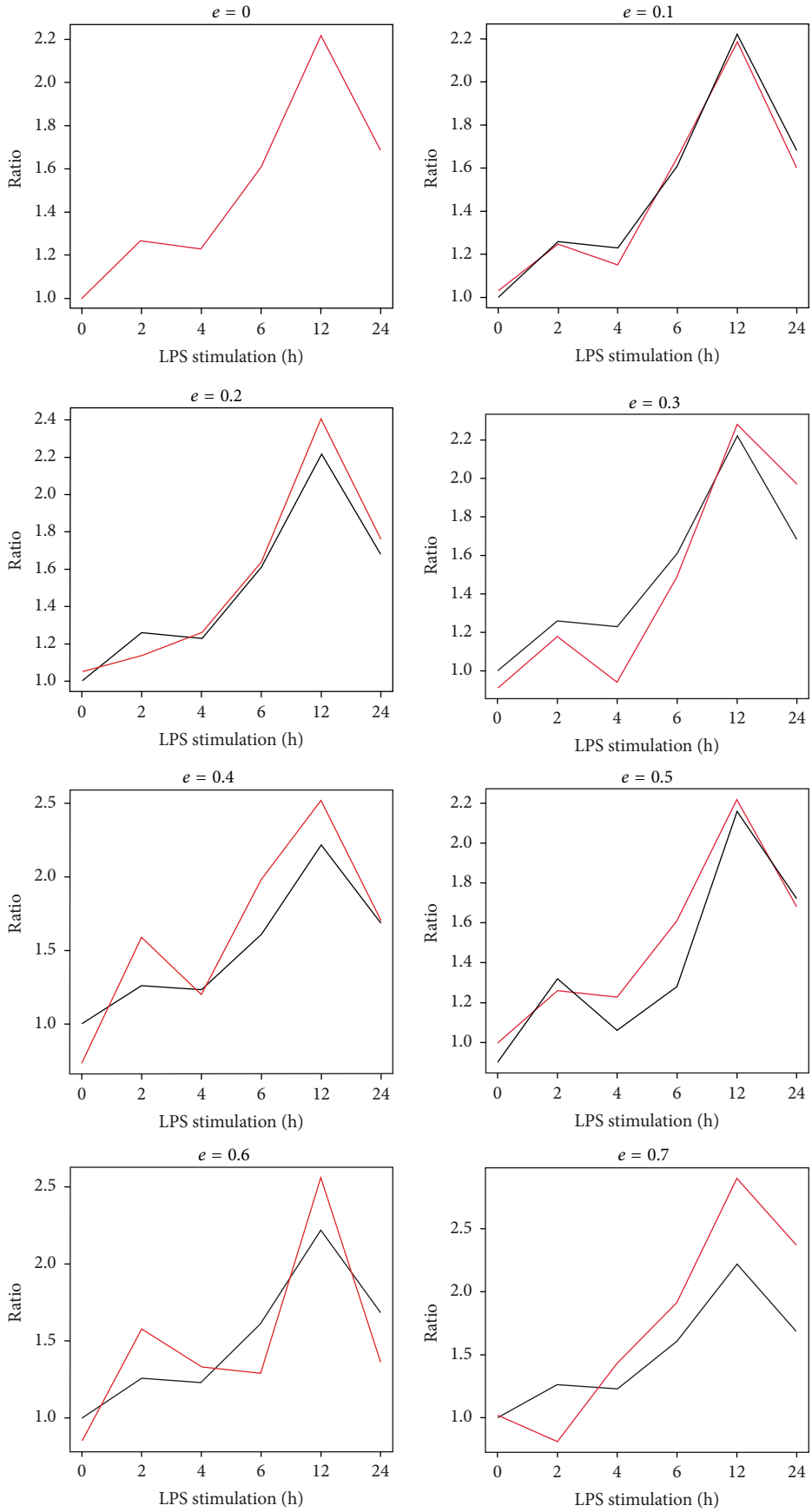


Figure 3: Workflow of linear fitting of LPS-mediated cytokine secretion and degranulation. A novel approach based on linear fitting was used to find linear relationship between short-time series of secreted cytokines and similar pattern to degranulation markers present at the plasma membrane. After importing data into R statistical software, all ratio values between LPS-stimulated and nonstimulated control conditions were $\log_{10}$ normalized and treated as mentioned in Section 2.



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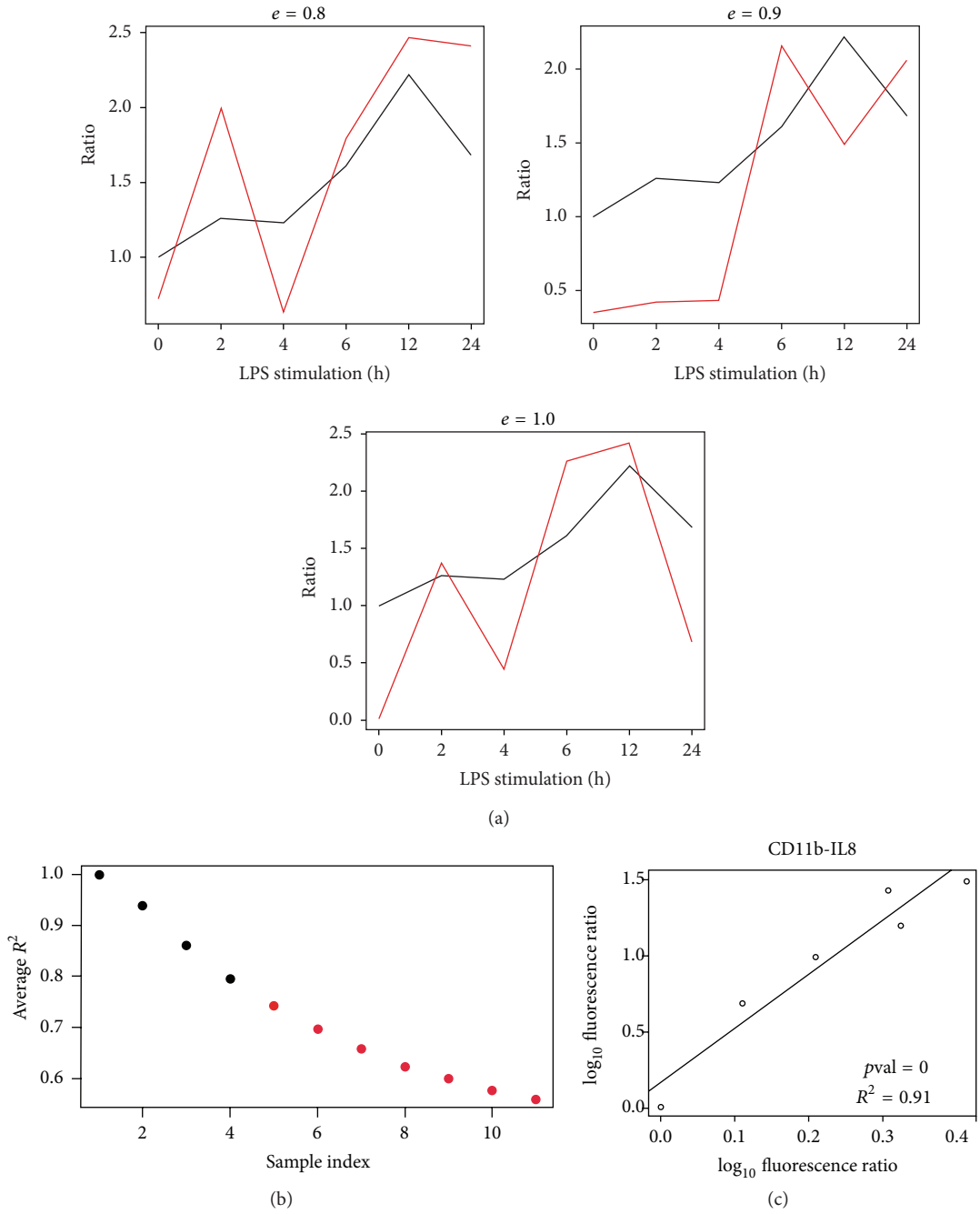

FIGURE 4: Linear fitting approach to investigate time series of degranulation and cytokine secretion. (a) Simulations were performed to find the optimal threshold for RSQ value. After introducing perturbations (e value) to the kinetic profiles, the linear fitting decreases in significance. (b) Plot of average RSQ deriving from simulations against augmenting e values. (c) Perfectly linear fitting model in which the behaviour of IL8 is correlated to CD11b. Due to its p value of 0 and RSQ of 0.91, the correlation between IL8 and CD11b corresponds to a perfect linear fitting model.

e values, k-means clustering can differentiate between "high" (black) and "low" (red) RSQ (Figure 4(b)).

By setting these parameters nonsignificant outcomes with "low" RSQ were eliminated, and the threshold for RSQ was set to RSQ ≥ 0.796.

An example of a significant linear fitting model is shown (Figure 4(c)), in which the behaviour of IL8 is correlated to CD11b. Due to its p value of 0 and RSQ of 0.91, the correlation between IL8 and CD11b fits to the model.

3.4. Relationship between Degranulation and Cytokine Secretion. After filtering only highly significant correlation data, results from human neutrophils were plotted. The granule membrane molecules (CD63, CD66b, CD11b, and CD45),

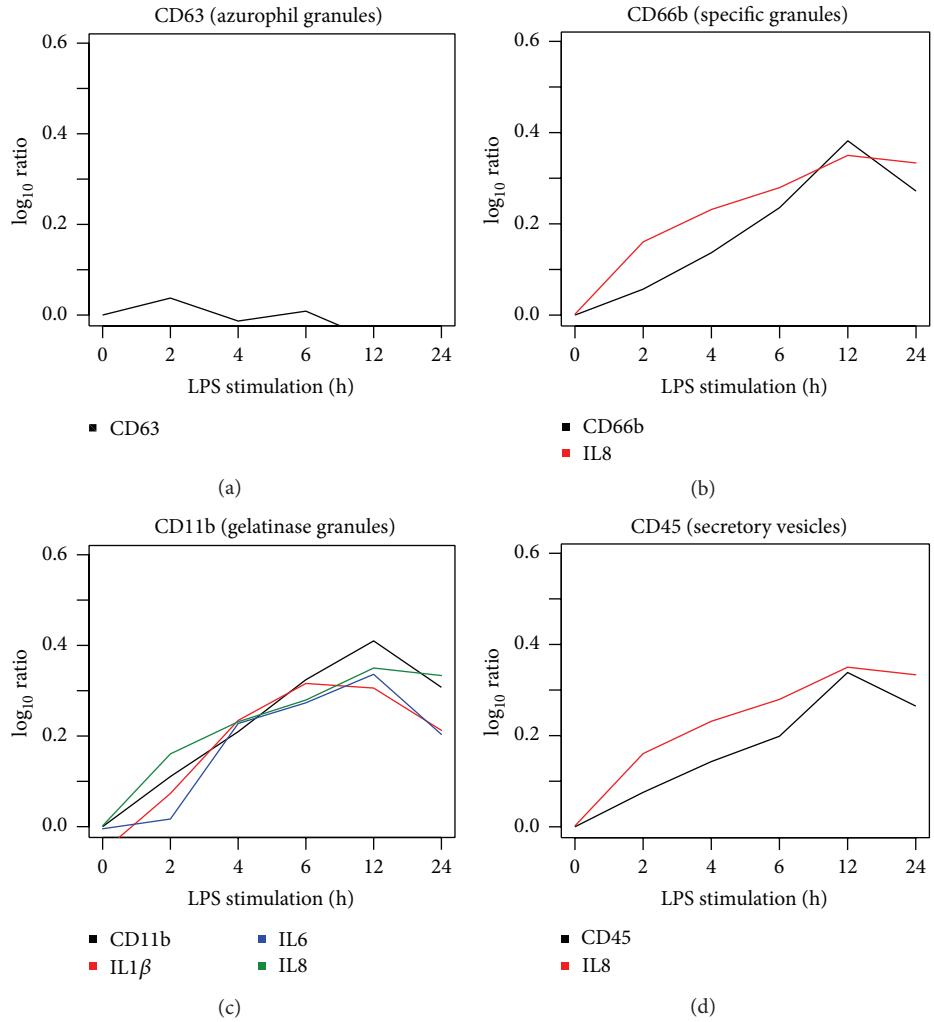

Figure 5: Linear fitting of LPS-mediated kinetics of cytokine release and degranulation in human neutrophils. Enlisted cytokines on the histograms fitted to the kinetics of the degranulation markers characteristic for (a) azurophil granules (CD63), (b) specific granules (CD66b), (c) gelatinase granules (CD11b), and (d) secretory vesicles (CD45), according to the selection of p value ≤ 0.05 and RSQ ≥ 0.82.

specific of each type of granule, that are most highly upregulated have been targeted to show the linear fitting approach (Figure 5).

This method applied to the time series data showed that the secretion of three selected proinflammatory cytokines (IL8, IL6, and IL1β) strongly correlated with the release of secretory vesicles, gelatinase granules, and specific granules (Figure 5).

The release of the cytokine IL8 fitted to CD66b suggesting that secretion of this cytokine correlated to specific granules (Figure 5(b)). Moreover, time series of IL1β, IL8, and IL6 release were strongly correlated to the degranulation marker CD11b, showing a relationship between gelatinase granules and these cytokines (Figure 5(c)).

Furthermore, secretory vesicles represented by the marker CD45 were fitted to IL8 (Figure 5(d)).

Since no significant cytokine correlation has been observed for CD63, azurophil granules are probably not associated with cytokine secretion (Figure 5(a)).

4. Discussion

For many years, the contributory impact of neutrophils to the development of chronic inflammation was not seriously taken into account since they have been considered as terminally differentiated cells synthesizing low amount of RNA and protein [37]. However, the vast number of neutrophils found at the site of infection cannot be neglected due to the fact that their secreted amounts of granule proteins and cytokines exert a cumulative and synergistic effect on the inflammatory tissue environment [15]. These proinflammatory soluble mediators are highly decisive for the onset of inflammatory processes and the activation and the recruitment of various immune cells to the infection site [1]. However, little is known about the combination in which cytokines and granule proteins are secreted by neutrophils. In the present report, we therefore aimed to predict the spatiotemporal regulation of proinflammatory mediator release in neutrophils. For this purpose, LPS has been used as stimulus agent since it has been

well described to induce the secretion of granule contents and cytokines [17, 38]. Once the model is established, it could constitute an important tool to investigate other stimulation conditions (fMLF, TNFα, IL8, or combination of stimuli) in order to mimic different microenvironmental conditions (e.g., healthy and pathological diseases) and help to improve our knowledge of inflammatory processes.

Our study is the first to propose an original approach allowing the establishment of a relationship between cytokine secretion and degranulation in neutrophils. We choose to use the linear fitting approach to integrate data generated from own experiments and obtained from LPS-mediated short-time series of degranulation and cytokine secretion. Other approaches, such as Pearson correlation, are based only on the average of all values correlated. In contrast, our model is able to reliably predict time-specific associations between the two dynamic functions in neutrophils, respecting each time point of stimulation. According to our results, a number of cytokines could be fitted to the different types of neutrophil granules. These granules have been characterized to be mobilized towards the plasma membrane in a hierarchically and more precisely reverse order to their formation according to the *formed-first-released-last model* [2]. Our model illustrates the fact that neutrophil-derived cytokines and granules are released in a hierarchical sequence in accordance with their roles during the microbial elimination processes and inflammatory response (Figure 6(a)).

In this study, the linear fitting approach (i) gives us information about the concurrent behavior of cytokine secretion and degranulation upon inflammation, thus underlining the key role of both functions in the regulation of inflammatory responses and (ii) can represent an attractive method to investigate the possible mobilization or localization of cytokines in the different types of granules.

Given that cytokines can exert pleiotropic functions, some of them are probably localized in different types of granules as suggested by our model. In this sense, we found that IL8 correlated to secretory vesicles (Figure 6(a)), which are the most easily mobilized organelles in mature neutrophils [35]. Furthermore, IL8 was correlated to gelatinase and specific granules. These data support the observations of Pellmé et al. [2], who reported that IL8 is stored in cytoplasmic granules in resting peripheral blood neutrophils and, thus, can be rapidly mobilized and released by the cells.

IL8 can be secreted into the extracellular milieu from intracellular stores or by *de novo* synthesis via the classical secretory pathway. Two phases of secretion have been described: an early secretory phase which is directly induced by LPS and a late secretory phase which results from LPS-stimulated release of other proinflammatory mediators such as TNFα and IL1β [39]. The fact that IL8 is stored in different types of granules could allow its secretion over a large time interval.

Large amount of IL8 released could be explained by a positive feedback loop generated by MMP-9 on the IL8-induced neutrophil activity. Indeed, it has been previously reported that MMP-9 released from gelatinase granules is able to process IL8 [40] which is stored in the same granules as MMP-9 before secretion as shown by our linear fitting approach. IL8 cleaved by MMP-9 can enhance neutrophil degranulation [40], in comparison to nondegraded IL8, and thus increase the quantity of MMP-9 and IL8 released leading to an amplification of this system.

Our results also show a significant fitting between kinetics of IL6 secretion and the release of gelatinase granules. In line with this observation, Terebuh et al. [41] showed by immunohistochemical staining of neutrophils that IL6 might be localized in gelatinase granules and secretory vesicles.

Finally, in our model, IL1β could be fitted to gelatinase degranulation as previously postulated [17]. IL1β is secreted by a nonclassical secretory pathway (independent of endoplasmic reticulum and Golgi apparatus). Different release models have been suggested for IL1β [42] but the mechanisms associated are poorly understood and still controversially discussed. Our data indicate that the process of IL1β secretion involves a trafficking via granules which could be related to gelatinase granules.

In contrast to other granules, degranulation of azurophil granules seems unchanged by LPS suggesting that either (i) this granule type may require further cell activation to induce its mobilization towards the plasma membrane [43] or (ii) the upregulation of CD63 at the plasma membrane might not be significant enough to detect since azurophil granules are rather poor in receptors in contrast to secretory vesicles [35]. For this reason, no cytokine could probably be fitted to CD63 in human neutrophils.

In this view, late release of azurophil granule contents can be explained by the involvement of these proteins in neutrophil extracellular trap formation [43]. In this regard, the role of these granules during cytokine secretion appears very restricted. This assumption is supported by our results showing that azurophil granules are not able to translocate to the plasma membrane upon LPS stimulation in neutrophils.

5. Conclusion

Intracellular localization of cytokines in neutrophils remains largely elusive due to the fact that reliable staining for electron microscopy is facing challenges as the low intracellular amount of cytokines and other techniques used to document subcellular organelle location of cytokines have limited resolution (e.g., subcellular fractionation) [44].

In comparison to the modelling approach proposed by Rørvig et al. [45], which is based on proteomic and mRNA array data to predict localization of proteins in granules, our approach is complementary by including functional data analysis. Hence, the linear fitting between degranulation and cytokine release in LPS-treated neutrophils represents an attractive method to investigate the possible localization of cytokines in the different types of granules even if additional experiments are required to confirm the intracellular localization of cytokines. Furthermore, since our linear fitting approach has been adapted to investigate secretion kinetics, it can easily be extrapolated for the analysis of other short-time series deriving from other cell types, disease, or developmental states, for example, protein arrays or proteomics data.

Our linear fitting approach primarily constitutes a tool aiming to investigate regulatory mechanisms during

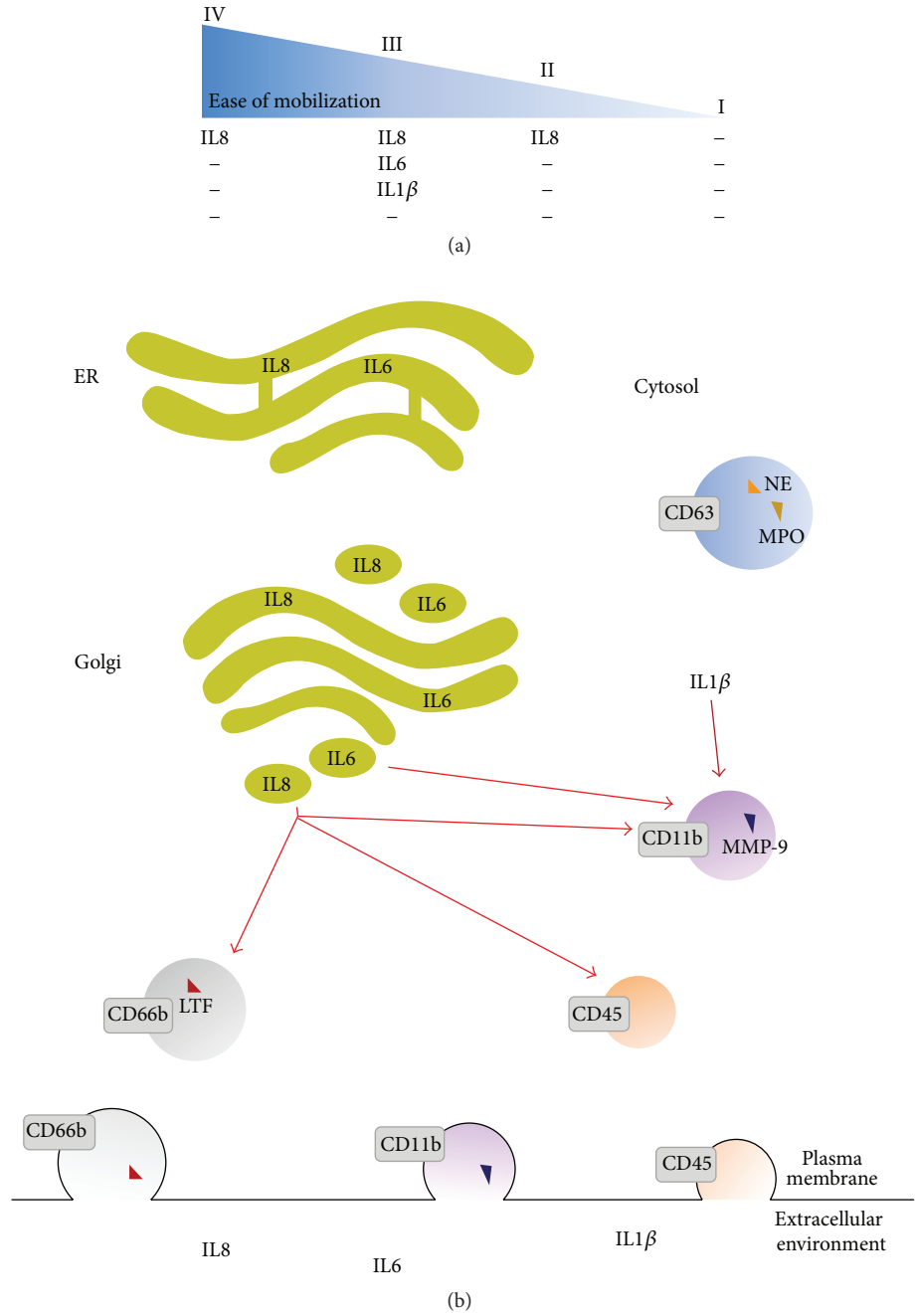

Figure 6: Hypothetical models of fitted cytokines and granules in human neutrophils. (a) Neutrophil-derived cytokines are released in a hierarchical sequence coincident with the roles of granules (IV secretory vesicles, III gelatinase granules, II specific granules, and I azurophil granules) during the microbial elimination processes and inflammatory response. (b) Cytokines and granules are released in a concurrent fashion but could additionally be localized in or mobilized to the different granule types. Classical secretory pathways are mediated through the endoplasmic reticulum (ER) and Golgi complex (IL6 and IL8). IL1β is secreted on a nonconventional pathway. Possible routes for cytokine trafficking (after ER-Golgi or after cleavage) and mobilization to the plasma membrane relative to degranulation are shown in red. Different types of granules are characterized by their CD markers and proteolytic enzymes (triangles): CD63, myeloperoxidase (MPO), and neutrophil elastase (NE) for azurophil granules; CD66b and lactoferrin (LTF) for specific granules; CD11b and matrix metallopeptidase-9 (MMP-9) for gelatinase granules; and CD45 for secretory vesicles. Upon mobilization of the granules to the plasma membrane, granule docking and fusion lead to the translocation of the CD markers to the plasma membrane and the release of proteolytic enzymes.

neutrophil exocytosis but can also serve as basis to identify regulatory proteins by the supplementary analysis of proteins involved in exocytosis (e.g., SNARE and Rab proteins) and the construction of a dynamic regulatory network [46].

Abbreviations

CBA: Cytometric bead array
LPS: Bacterial lipopolysaccharide.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Isabelle Naegelen and Nicolas Beaume contributed equally to this paper.

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Research Article

Adaptation to Resistance Training Is Associated with Higher Phagocytic (but Not Oxidative) Activity in Neutrophils of Older Women

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Failure in antimicrobial activity contributes to high morbidity and mortality in the geriatric population. Little is known about the potential effect of resistance training (RT) on the functional properties of the innate immunity. This study aimed to investigate the influence of long-term RT on the endocytic and oxidative activities of neutrophils and monocytes in healthy older women. Our results indicate that the phagocytosis index (PhI) of neutrophils (but not of monocytes) in the RT-adapted group was significantly higher ($P < 0.001$; effect size, (d) = 0.90, 95% CI: [0.75–1.04]) compared to that in sedentary subjects. In contrast, the oxidative activity of either neutrophils or monocytes was not significantly influenced by RT. Also, total energy and carbohydrate intake as well as serum IL6 levels had a significant influence on the phagocytic activity of neutrophils ($P = 0.04$), being considered in the model. Multivariate regression identified the physical condition of the subject ($\beta = 0.425$; $P = 0.01$) as a significant predictor of PhI. In conclusion, circulating neutrophils of older women adapted to a long-term RT program expressed higher phagocytic activity.

1. Introduction

The cascade of biological events that makes up the innate defense against infectious agents is a vital part of the immune system. Typically, this process is characterized by acute response triggered by the rapid increase in circulating inflammatory mediators [1]. However, during immunosenescence, there is complex remodeling of the immune system, characterized by exacerbation of the basal, possibly nonimmunologically derived profile of proinflammatory mediators and of reactive oxygen species, a phenomenon known as *inflammaging* [2]. This scenario is more evident after the fourth decade of life, when increased susceptibility to cancer, infections, and metabolic disorders is observed [3, 4].

Although the effects of aging on the innate immune system are not fully elucidated [5–7], it is assumed that this senescence-associated, subclinical inflammatory, and oxidative processes constitute a compensatory mechanism due to the decline of the endocytic capacity and reduced production of superoxides by phagocytic elements, among other age-triggered flaws of the immune function. Failure in neutrophil properties has been postulated as an important predictor of morbidity and mortality in the geriatric population [5].

Further studies aimed at exploring nonpharmacological interventions with potential to reverse the detrimental aspects of immunosenescence should be carried out [8, 9]. Although results already in the literature disclose benefits of resistance training (RT) on the systemic proinflammatory

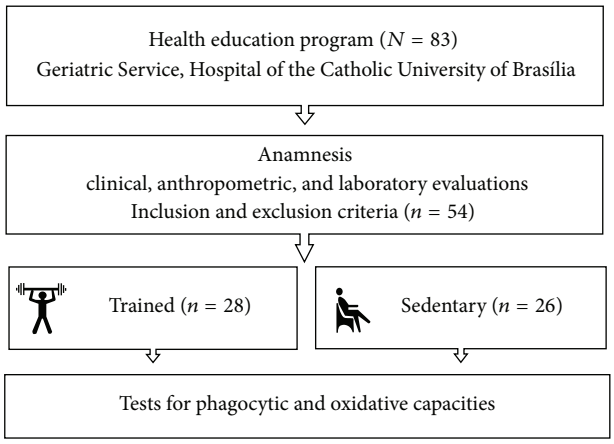

Figure 1: Procedures for sample selection and data collection.

milieu of elderly subjects [10–12], little is known about the influence of RT on the functional response of cells that form the first line of defense of the immune system, particularly in this age stratum. This study aimed to investigate the influence of long-term RT on the phagocytic and oxidative activities of neutrophils and monocytes in apparently healthy older women. The intake of the main macronutrients and the circulating levels of important serum mediators of immunosenescence and endocrinosenescence were taken as potential confounding factors and considered herein. Our hypothesis is that chronic physiological adaptations induced by RT can modulate the endocytic and oxidative capacities of peripheral phagocytes in older women.

2. Materials and Methods

2.1. Participants. The participants of this study were elderly women living in the community regularly followed by health education programs for prevention of chronic disorders developed by the Geriatric Service of the Catholic University of Brasilia Hospital, Brazil, known as the Prognosis and Therapeutics in Geriatrics (ProTeGer) study [13, 14]. After inspection of medical records, patients were excluded due to the following: uncontrolled type 2 diabetes, obesity, having smoked > 100 cigarettes over a lifetime, and/or having regularly consumed one dose (12 g) of alcohol per week or more over the last 12 months. Other exclusion criteria were use of immunomodulatory drugs or presence of neoplastic processes, acute infectious, and/or inflammatory signs at the time of clinical evaluations. Figure 1 presents the chart of procedures adopted during this study.

The Lipschitz equation was used to determine the body mass index (BMI) [15]. The waist-to-hip ratio (WHR) was calculated based on waist circumference measurements at the midpoint between the last rib and the iliac crest and hip circumference in the greater trochanter. Body mass was measured with participants barefoot and wearing light clothing on a calibrated scale with 100 g accuracy (Filizola, São Paulo, SP, Brazil). Height was measured with stadiometer with accuracy of 1 cm. Body composition measurements were performed at the Laboratory of Image of the Institution using dual energy X-ray absorptiometry (DXA; Lunar DPX-IQ model, software version 4.7e, Lunar Radiation Corp., Madison, WI, USA), with individuals in the supine position on a horizontal platform, with slightly apart and relaxed legs, arms at body sides, and palms down. The software provided fat and fat-free masses. In addition to absolute masses, relative fat and fat-free measures were obtained with adjustment to height (kg/m^2). The equipment was calibrated as recommended by the manufacturer. All examinations were performed by the same trained researcher.

2.2. Nutritional Assessment. Dietary data were determined based on food records of 3 days (2 working days and 1 day in the weekend), which is as accurate as records of 4 or 7 days [16, 17]. The food record was completed at home with patients being instructed to record food consumption in terms of number and size of portions. Values for each individual patient were expressed as the average intake of the 3 days reported. To ensure completion of food records, the staff of nutritionists provided personal or telephone assistance. The forms were returned during a clinical interview in which the amounts and types of foods were reviewed. Dietary contents were calculated using the Diet-Pro software, version 4.0 (A.S. Sistemas, Viçosa, MG, Brazil), configured for international food tables and complemented with a table for local food products [18]. Dietary intake of total protein, carbohydrates, and lipids was expressed as percentage of total energy and included in the analysis as continuous variables.

2.3. Resistance Training Prescription. For the RT prescription, the 1-maximum-repetition (MR) percentage was obtained according to methodology described by Kraemer et al. [19]. The group under RT was composed of volunteer women who have attended a moderate-intensity physical training (70% of 1 MR) consisting of exercise sessions three times a week on alternate days for 8.6 ± 0.3 months, with three sets of 12 repetitions per exercise at moderate speed and 1 minute of rest between sets. Each 50-minute session included nine exercises: horizontal leg press, knee extension, knee flexion, bench press, triceps extension in the pulley, biceps curling, seated rowing, plantar flexion, and abdominals. Whenever one participant was able to perform 13 repetitions or more in the third series of a given movement in the last weekday of training, loads were increased [20]. All equipment was manufactured by Righetto (Campinas, SP, Brazil), and warm-up exercises for 10 min preceded each training session.

All subjects were sedentary at baseline. The control group consisted of women who remained sedentary and participated during nine months in occupational activities not related to physical activity offered by the health education program. Attendance to either physical or occupational activities was entirely voluntary and freely chosen by each older-woman, as long as the three-session-per-week regimen was fulfilled when inscribed in RT-training. The study protocol was approved by the Research Ethics Committee of the institution and conducted in accordance with the Declaration of Helsinki.

2.4. Total and Differential Count of Leukocytes. Blood samples were diluted at 1 : 20 in Turk's solution. Leukocytes were counted in a Neubauer chamber (Labex, Aparecida de Goiânia, GO, Brazil) with concentration (cells/mm^3) determined under optical microscopy by the equation $[((Q1 + Q2 + Q3 + Q4)/4) \times 200]$, with Qn as the number of cells in the quadrant n of the chamber and 200 as the product of 10 (conversion factor to 1 mm^3, count area depth) and 20 (dilution factor). For differential leukocyte count, 100 cells in randomly distributed fields were considered.

2.5. Analysis of Serum Mediators. For measurement of immune mediators, blood samples were collected in the morning (8:00 to 9:30 a.m.) in tubes without endotoxin. For RT-adapted individuals, samples were collected at the end of the intervention period, along with samples from control subjects. Serum was separated within 1 hour after collection and stored at −80°C until processing day. Samples were analyzed using the enzyme-linked immunosorbent assay method (ELISA) with kits specific for interleukin 6 (IL6) and for tumor necrosis factor-alpha (TNFα) (eBioscience, San Diego, CA). Serum IGF1 levels were determined using automated Immulite 2000 Siemens system (Los Angeles, CA, USA). All samples were analyzed in duplicate.

2.6. Phagocytic Capacity Test. The endocytic capacity of circulating monocytes and neutrophils was tested *in vitro* by a *Saccharomyces cerevisiae* phagocytosis assay adapted from Muniz-Junqueira et al. [21]. Briefly, blood samples (40 μL) were added in duplicate onto glass slides with 7 mm wide excavations and incubated in humid chamber for 45 min at 37°C. Then, slides were washed with PBS solution, pH 7.2 at 37°C. Adherent cells were incubated with 2.5×10^5 yeasts in 20 μL of Hanks-Tris solution (Sigma, St. Louis, MO, USA), pH 7.2, with 10% fetal bovine serum (FBS) (Cultilab, Campinas, Brazil). After 30 min of incubation in humid chamber at 37°C, slides were rinsed with PBS solution at 37°C, washed with Hanks-Tris solution with 30% FBS at 37°C, fixed with methanol, and stained with Giemsa solution. The number of *S. cerevisiae* phagocytosed by 200 monocytes or 200 neutrophils in individual preparations was evaluated by optical microscopy. The fields used for counting were randomly selected and all monocytes or neutrophils found were examined. The phagocytosis index (PhI) was calculated as the product of the following factors: (a) average number of *S. cerevisiae* phagocytosed by neutrophils or monocytes and (b) proportion of neutrophils or monocytes involved in the phagocytosis.

Yeast suspension was prepared following the method described by Lachmann and Hobart [22]. Briefly, 50 g tablet of fresh yeast (Fleischmann) was dissolved in 220 mL of a 0.05 M isotonic, phosphate buffered saline solution (PBS), pH 7.2, autoclaved at 120°C for 30 minutes, and then washed for 3 times in PBS by centrifugation at 4,000 rpm for 5 min. This procedure was repeated to obtain a clear supernatant. The sediment was resuspended in 28 mL of PBS containing 2-mercaptoethanol at 0.1 M. After two hours of stirring at 37°C, the suspension was washed again and the sediment was resuspended in 55 mL in PBS solution containing 0.02 M iodoacetamide. After further stirring at 37°C for two hours, the suspension was washed three times, resuspended in 220 mL of PBS, autoclaved once more, and washed with PBS by centrifugation to obtain a clear supernatant, being finally resuspended in 110 mL of Veronal buffer of pH 7.2 containing sodium azide (200 mg/L). The yeast suspension was stored at 4°C for use for a period not exceeding one week after its production. Prior to each assay, the yeast suspension was washed in PBS, suspended in equal volume of Hanks-Tris solution, quantified, and used immediately.

2.7. Oxidative Capacity Test. The oxidative capacity test was adapted from technique described by Park et al. [23] to evaluate the production of reactive oxygen species based on the principle of reduction of nitroblue tetrazolium (NBT) up to its insoluble form (formazan). Briefly, phagocytes adhered to excavation, as previously described, were incubated with Hanks-Tris solution containing 0.05% of NBT for 20 min at 37°C in humid chamber. The slides were rinsed, fixed with methanol, and stained with aqueous solution containing 1.4% safranin and 28.6% glycerol. The proportion of phagocytes that reduced NBT in their cytoplasm was determined by counting 200 neutrophils and 200 monocytes found. Positive, NBT-reducing phagocytes were those that presented the typical blackish-blue cytoplasmic precipitate that is compatible with formazan formation.

The phagocytic and oxidative capacities were intended to be assessed simultaneously and for 2 or 3 subjects at once. After assaying all subjects, our first-round success rate scored at 90%, with minimum slide quality achieved whenever distinction of leucocytes and identification of yeasts were possible by visual inspection. Blanks were filled by new invitations for blood draw until data was completed for the whole sample. All tests were performed by the same trained biologist.

2.8. Statistical Analysis. Data analysis was performed using the Statistical Package for Social Sciences (SPSS) for Windows (version 8.0). The Kolmogorov-Smirnov test was used to test the data normality. To obtain a close-to-normal distribution, data were logarithmically transformed ($\log_{10}$), whenever necessary. Student's t-test for independent samples was used to compare groups in terms of body composition, calorie intake, age, white blood cell count, and levels of proinflammatory mediators. Analysis of covariance (ANCOVA) was performed using both groups (RT versus sedentary) as independent variables to investigate possible differences induced by exercise on the phagocytic activity, with IL6, BMI, and caloric intake measures as covariant. Associations of body composition, total protein, carbohydrates, lipids, IL6, TNFα, and IGF1 measurements with phagocytosis of monocytes and neutrophils were analyzed using Pearson's product-moment correlation coefficient. Where appropriate, the effect size (d) and confidence interval (95% CI = $d \pm Z_{0.025}S_d$) were estimated. Multivariate linear regression with a stepwise variable selection was used in order to test the relationship between phagocytic activity and potential predictors of the regression model. Assumptions of normality, homogeneity, and independence of wastes were investigated, the former two by visual inspection and the latter by the Durbin-Watson statistics ($d = 1.7$).

Table 1: Characteristics of the sample.

	Sedentary ($n = 26$)	Trained ($n = 28$)	P^*
Age, years	72.0 ± 6.9	70.6 ± 5.7	0.406
BMI, kg/m^2	27.9 ± 4.4	29.5 ± 5.5	0.223
Total fat-free mass, kg	36.5 ± 2.4	39.1 ± 5.9	0.040
Relative fat-free mass, kg/height^2	15.8 ± 0.9	16.4 ± 1.8	0.090
Total fat mass, kg	24.9 ± 7.2	25.8 ± 8.1	0.121
Relative fat mass, kg/height^2	11.8 ± 3.1	12.9 ± 3.8	0.208
Waist circumference, cm	97.4 ± 9.2	99.8 ± 12.2	0.406
Calorie intake, 10^3 kcal*	2.2 ± 0.6	1.7 ± 0.5	0.001
Total carbohydrate intake, %	50.1 ± 4.9	47.4 ± 8.3	0.050
Total lipid intake, %	34.1 ± 4.9	36.0 ± 7.6	0.129
Total protein intake, %	14.7 ± 3.8	15.5 ± 3.5	0.424
Systolic blood pressure, mmHg	134.3 ± 13.0	125.8 ± 14.1	0.022
Diastolic blood pressure, mm Hg	81.9 ± 9.9	80.8 ± 9.5	0.670
IGF1, ng/mL	0.60 ± 0.17	0.56 ± 0.19	0.410
Leukocyte numbers, mm^3	5.2 ± 2.1	5.7 ± 2.2	0.401
Neutrophils, %	55.4 ± 11.7	53.2 ± 9.7	0.449
Segmented	53.4 ± 11.3	52.1 ± 9.5	—
Banded	2.0 ± 1.5	1.1 ± 1.4	—
Monocytes, %	3.9 ± 2.2	3.8 ± 2.8	0.910
Lymphocytes, %	37.9 ± 10.4	39.8 ± 10.1	0.494
Eosinophils, %	2.7 ± 2.6	3.1 ± 2.7	0.608
TNFα pg/mL	0.73 ± 0.29	0.57 ± 0.20	0.036
IL6 pg/mL*	0.63 ± 0.12	0.44 ± 0.36	0.002

Values are expressed as mean ± standard deviation. *Statistical difference $P < 0.050$.

Variance inflation factor was used for the diagnosis of possible collinearity among variables (VIF's < 1.3). $P < 0.05$ was considered statistically significant in bilateral tests.

3. Results

After applying the exclusion criteria, 54 older women (71.3 ± 6.3 years) composed two groups, 28 in the RT-adapted subset and 26 as a sedentary counterpart. Participants' characteristics are described in Table 1. When compared to the sedentary group, women adapted to RT exhibited reduced average values for total caloric intake (~23%) and systolic blood pressure (~6%). Similarly, reduced circulating levels of proinflammatory biomarkers $\log_{10}$ IL6 (~30%) and $\log_{10}$ TNFα (~22%) were observed in the RT-adapted group.

Concerning phagocytosis by monocytes and neutrophils, comparisons between groups are shown in Table 2. Increased phagocytic capacity of neutrophils was observed among RT-adapted participants compared to the sedentary group, represented by both greater proportion of *S. cerevisiae* captured (~31%; $P < 0.001$) and a higher frequency of phagocyting cells (~18%, $P = 0.033$). Thus, the PhI of neutrophils was significantly higher in the RT-adapted group (~57%; $P < 0.001$, $d = 0.90$, 95% CI: [0.75–1.04]) compared to the group without exercise. Among covariates, total energy intake and the intake of carbohydrates had a significant influence on the phagocytic activity of neutrophils ($F(1.53) = 3.67$; $P = 0.04$), being necessarily considered to be covariables in our models. In contrast, no statistically significant differences in the phagocytic ability of monocytes between groups were observed. The production of reactive oxygen species in neutrophils and monocytes, as assessed by the proportion of cells that promoted NBT reduction, was not significantly influenced by RT.

Table 2: Phagocytic and oxidative activities in the samples.

	Sedentary ($n = 26$)	Trained ($n = 28$)	P^*
S. cerevisiae phagocyte/neutrophils, n	2.2 ± 0.5	2.9 ± 0.9	<0.001
S. cerevisiae phagocyte/monocytes, n	1.4 ± 0.2	1.5 ± 0.3	0.416
Neutrophils in phagocytosis, %	60.5 ± 19.6	71.2 ± 19.1	0.033
Monocytes in phagocytosis, %	40.4 ± 14.3	40.5 ± 10.6	0.868
Index of neutrophils phagocytosis	140.4 ± 69.5	221.2 ± 105.8	<0.001
Index of monocytes phagocytosis	53.8 ± 22.4	60.5 ± 18.2	0.160
Neutrophils reduced NBT, %	51.3 ± 23.0	45.3 ± 26.4	0.328
Monocytes reduced NBT, %	49.0 ± 19.6	46.8 ± 21.1	0.294

Values are expressed as mean ± standard deviation. ANCOVA adjusted for IL6, BMI, and calorie intake. *Statistical difference set at $P < 0.05$.

Results of correlation tests with inclusion of all participants showed a significant negative association of the neutrophils' PhI with circulating levels of IL6 ($r = -0.29$; $P = 0.03$) and the intake of total carbohydrates ($r = -0.32$; $P = 0.01$) (Figure 2). There were no significant associations between PhI and circulating levels of IGF1 and TNFα. Multivariate regression analysis identified the physical condition of subjects (RT-adapted or sedentary) as the most significant variable to predict PhI ($\beta = 0.425$; $P = 0.01$). Log_{10} IL6 and caloric intake were excluded from the model.

4. Discussion

In the field of immunology, it is generally recognized that rapid activation and recruitment of neutrophils reflect the integrity of important signaling pathways involved with the initiation of immune responses, to result in protective inflammation [24, 25]. The main findings of this study are consistent with a higher phagocytic activity of circulating neutrophils in older women adapted to a long-term RT program in relation to the sedentary group. Since the disparity in PhI between groups was not mitigated with statistical control for potentially confounding factors (as nutritional profile, anthropometry, and cytokine levels), it is suggested that the benefits of RT may be the result of independent physiological mechanisms (e.g., hormonal), as advocated by evidence presented elsewhere [26].

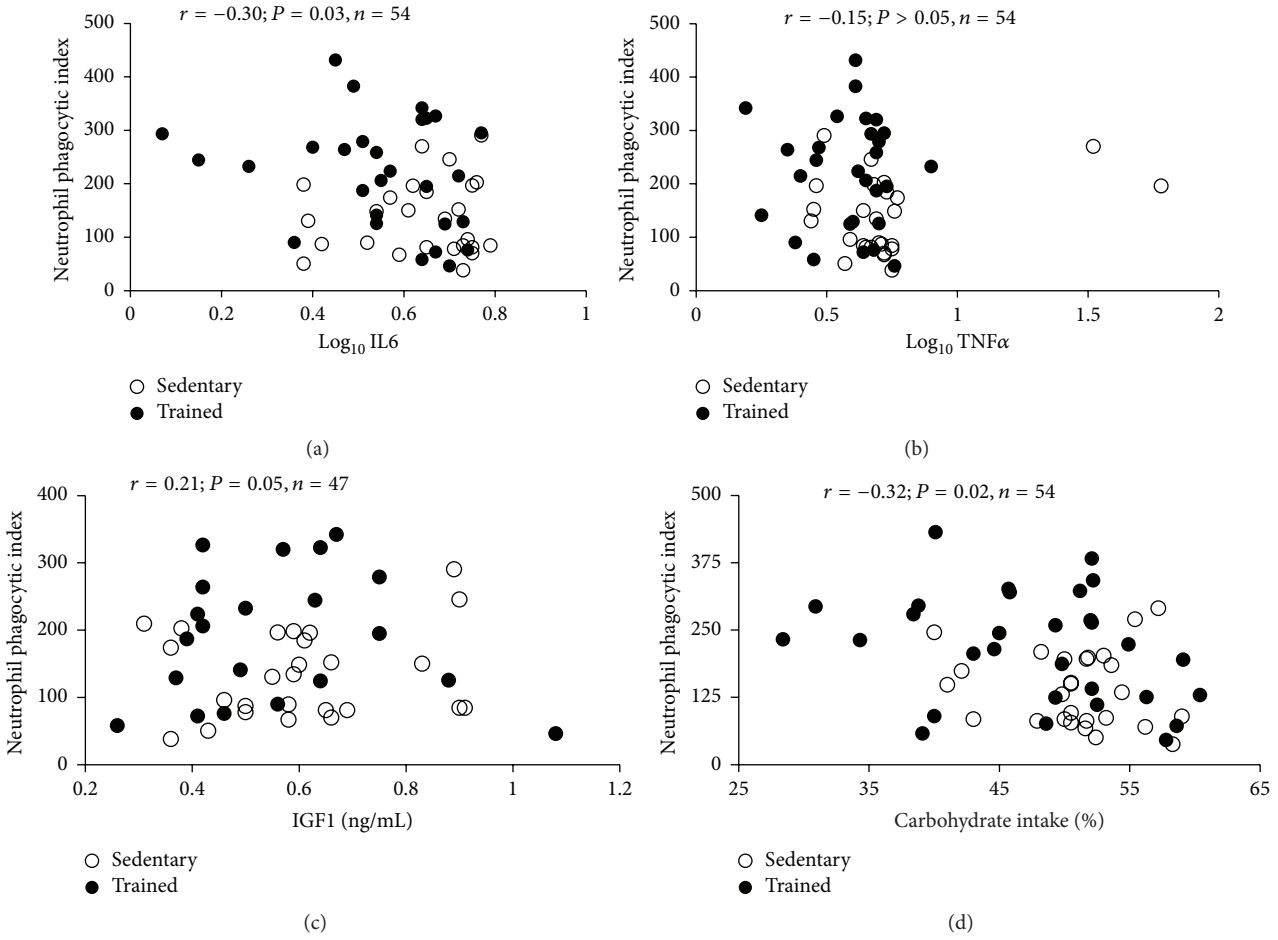

Figure 2: Correlations of $\log_{10}$ IL6 (a), $\log_{10}$ TNFα (b), IGF1 (c), and total carbohydrate intake (d) with neutrophil phagocytic index. r = Pearson's correlation coefficient.

Although little is known about the influence of exercise on the function of neutrophilic polymorphonuclear leukocytes in the geriatric populations [27], our results corroborate study that found lower phagocytic activity of neutrophils in sedentary elderly subjects compared to those physically active [28]. Study including middle-aged recreational soccer players showed higher phagocytic activity compared to sedentary subjects [29]. Although the cross-sectional nature of our study does not allow the establishment of causal relationship, these results suggest that the expected decline of the phagocytic response with aging [3, 5] may be at least partially reversed by adaptations induced by RT in populations under high risk of functional capacity decay.

On what concerns production of reactive oxygen species, our results did not show significant differences between groups, corroborating findings in different population strata and sportive modalities [29–31]. It has been well established that neutrophils may exhibit impaired capacity to produce superoxides in geriatric subjects [32, 33]. We do not rule out that this result can, at least in part, be influenced by the expected age-related reduction of dehydroepiandrosterone (DHEA) to levels that may restrict potential benefits from physical exercise, since this circulating steroid has proven to be important to the synthesis of superoxides in neutrophils [34].

Regarding the fact that the monocyte functions have not been influenced by RT, it is likely that the monocytes/macrophages system may depend on its developmental state to benefit from endocrine mediators promoted by physical exercise [35]. Monocytes are a class of relatively immature circulating phagocytes that demand migration to tissues as a requirement for full cell specialization. It is plausible that different phagocytic indexes between monocytes and neutrophils arise from this relative immaturity of monocytic types or that the adaptations induced by our intervention have been insufficient to promote important endocrine changes to the point of favoring direct activation of monocytes. In line with this, we do not rule out the possibility that the sample size was insufficient to obtain a significant effect on monocytes' indexes.

The analysis of the inflammatory profile of the subjects in this study corroborates evidence from literature that associates increased IL6 levels with an age-dependent dysregulation of the innate immune system [34]. Regarding the circulating TNFα level, the results showed no significant associations with the phagocytic activity. This finding is in

line with studies in human models that support the concept that TNFα is not a robust biomarker that reflects systemic inflammation in sedentary populations when compared to abnormal endocrine [36] and tissue [37] levels of IL6. Therefore, it is likely that the serum IL6 is a more robust predictor of physical condition in apparently healthy yet sarcopenia-prone geriatric populations, due to evidence that IL6 (but not TNFα) tends to be actively produced by the exercising muscle [38, 39].

Factors extrinsic to the immune system are implicated in the imbalance of the innate immune system with aging. Phagocytes are exposed to a variety of extraimmune agents (hormonal, mainly), which may affect their functional phenotype [7] by changes in intracellular signaling cascades [33], for instance. In our conditions, the phagocytic activity of neutrophils was not influenced by the circulating levels of insulin-like growth factor-1 (IGF1). Despite evidence that RT produces acute increases in serum IGF1 and in IGF1R on circulating monocytes [40], few studies have investigated the influence of RT on levels of this potent anabolic factor [41–43], with inconsistent results [44]. It is likely that the different protocols analyzed and the small frequency of postexercise assessments could explain these results or that neuroendocrine (e.g., catecholamines) signals are more effective modulators of phagocytic functions than the GH axis [26].

Despite the fact that total energy and carbohydrate intakes produced significant impact on the phagocytic response of neutrophils, it is important to observe that statistical control for such covariable did not suppress the association found between PhI and the physical status (RT-adapted or sedentary) of the older women investigated. The women in the RT group exhibited lower total caloric intake compared to sedentary subjects probably due to the reduced carbohydrate intake. Even though the authors consider this result to be important since the amount of nutrients ingested has been associated with changes in both cellular and humoral responses [10, 45], no explanation can be provided at this point for such disparity in the pattern of energy consumption displayed by the subjects. But we suggest that calorie intake should be controlled in studies investigating the relationship between exercise/physical activity and phagocytic response.

The cross-sectional nature of this study is a limitation, so the results should be interpreted with care. Prevalence bias cannot be ruled out, since an uneven distribution of preexisting health conditions not considered in the setting up of the groups may have resulted in more elderly women with an exceptional immune status to be included in the RT-trained group, for instance. On their behalf, the authors sustain that their analyses were designed with the purpose of controlling potential confounding variables associated with the effects of acute infectious processes, use of immunomodulatory drugs, systemic levels of relevant immune mediators, and nutritional factors prior to the phagocytic and oxidative assays.

5. Conclusion

The main findings of this study suggest that circulating neutrophils (but not monocytes) in older women adapted to long-term RT program bear higher phagocytic capability in relation to those of sedentary counterparts. There was no influence of RT on the oxidative activity of either neutrophils or monocytes. We suggest that the nutritional parameters and circulating immunomodulators also correlate with the phagocytic index of neutrophils and therefore must be controlled in studies addressing the relationship between exercise/physical activity and the phagocytic response in populations susceptible to significant weaknesses.

Disclosure

The authors declare minor self-plagiarism by reusing elements from published work of their own to help them describe the sample and methods, providing appropriate reference.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

João Bartholomeu-Neto and Vinícius Carolino Sousa executed the phagocytic assays and laboratory assessments of the immune mediators. Aparecido Pimentel Ferreira and David Junger Fonseca Alves executed the clinical anthropometric assessments. Juliana Oliveira Toledo, Roberta Silva Paula, and Clayton Franco Moraes executed the pharmacological assessments, dietary registries, and medical component of the study, respectively. Ciro José Brito advised on the statistical analysis and interpretation of results. Otávio Toledo Nóbrega and Cláudio Córdova designed and coordinated the study and analyzed and interpreted the results. João Bartholomeu-Neto, Ciro José Brito, Roberta Silva Paula, Otávio Toledo Nóbrega, and Cláudio Córdova participated in the preparation of the original paper as well as the revision process.

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