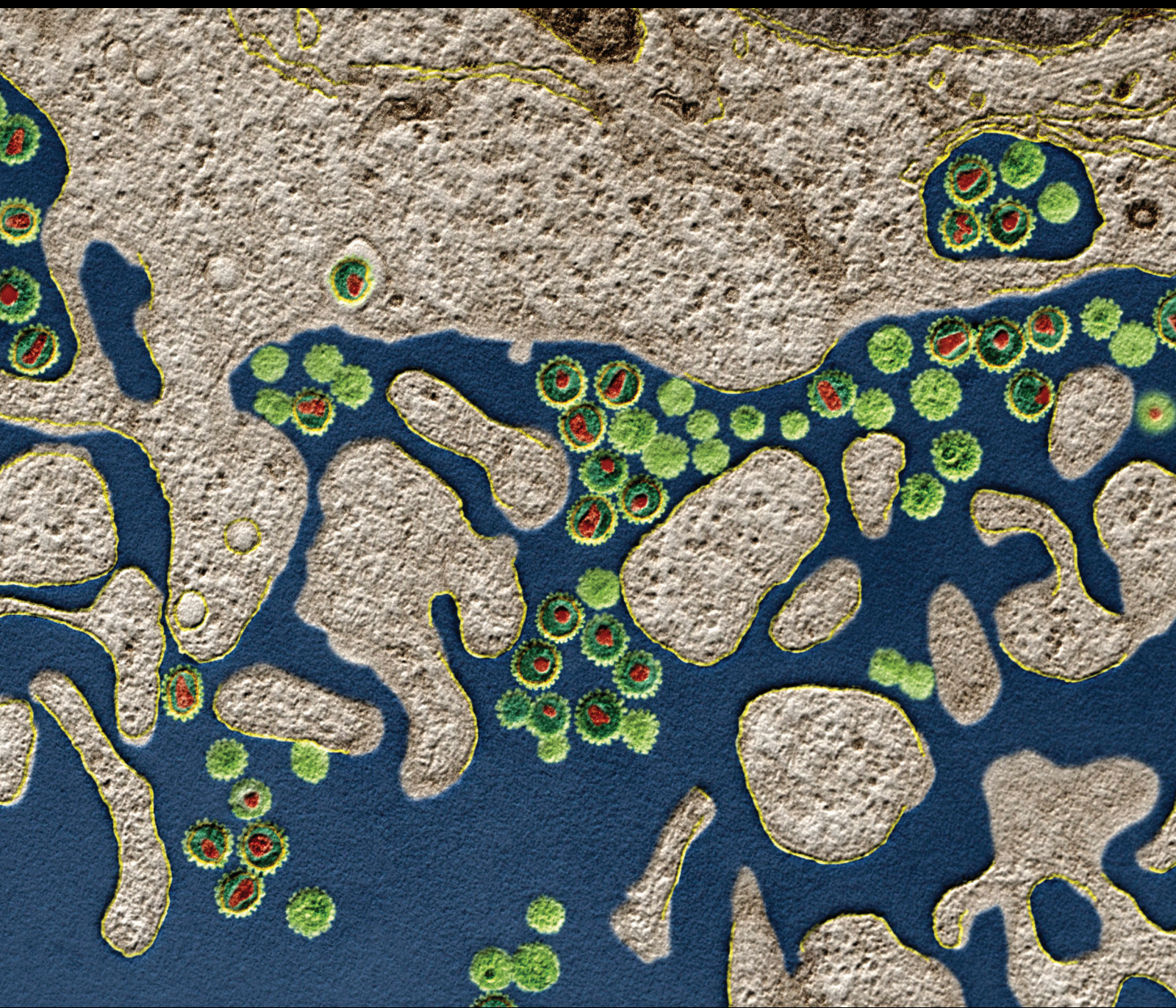
Journal of Immunology Research

Neutrophils: Their Role in Innate and Adaptive Immunity 2017

Lead Guest Editor: Carlos Rosales
Guest Editors: Michael Schnoor, Clifford Lowell, and Eileen Uribe-Querol



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Editorial Board

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Editorial

Neutrophils: Their Role in Innate and Adaptive Immunity 2017

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Neutrophils have long been regarded as the first line of defense against infection and one of the main cell types involved in initiation of the inflammatory response. It is generally accepted that the innate immunity functions of neutrophils are mainly mediated by phagocytosis, release of granules, and formation of neutrophil extracellular traps (NETs). In recent years, however, accumulating evidence has shown that neutrophils possess greater functional diversity than previously appreciated. Thus, the classical view of neutrophils as simple cytotoxic leukocytes against pathogens is currently being revisited. Neutrophils display an array of biological functions important for both innate and adaptive immune responses. Neutrophils can produce many cytokines and chemokines upon stimulation, and in this way, they can interact with endothelial cells, dendritic cells, macrophages, natural killer cells, T lymphocytes, and B lymphocytes. Through all these interactions, neutrophils can either activate or downregulate both innate and adaptive immunity. The novel functions of neutrophils reveal that these cells have unanticipated roles in homeostasis, as well as in several diseases such as atherosclerosis, stroke, chronic obstructive pulmonary diseases, and cancer.

To continue elucidating the complex role of neutrophils in infection, inflammation, and immunity, this second special issue has brought together original and review articles that will help us to better understand the complex and fascinating neutrophil biology.

As mentioned before, the primary role of neutrophils is the clearance of extracellular pathogens, through phagocytosis, release of a broad array of effector molecules, and the production of extracellular traps. However, some pathogens have also the capacity to overcome neutrophil-mediated host defense mechanisms and establish infections leading to disease. One such pathogen is the bacteria *Staphylococcus aureus*, which can block chemotaxis and phagocytosis, thus evading killing by neutrophils. In addition, *S. aureus can* survive within neutrophils and promote neutrophil cytolysis, causing the release of molecules that promote local inflammation. The article by T.-S. Teng and colleagues describes the mechanisms by which neutrophils kill extracellular pathogens and how pathogens evade neutrophil defense mechanisms. They also discuss ideas that might be useful for the development of novel therapies against infections caused by antibiotic-resistant pathogens.

Similarly, the role of NETs is primarily to entrap extracellular microbes and, in this way, to keep an early infection localized. Yet, besides microorganisms, other stimuli can also activate neutrophils to produce NETs. The article by B. Rada summarizes the recently described ability of different microcrystals to induce NET formation. Microcrystals are insoluble crystals with a size of 1–100 micrometers that can irritate phagocytes including neutrophils and typically trigger an inflammatory response. The effect on neutrophils by microcrystals in adjuvant and by microcrystals associated with diseases such as gout, atherosclerosis, and silicosis is discussed.

Neutrophils play an essential role during an inflammatory response. They are rapidly mobilized from the circulation into damaged tissues. The blood supply of neutrophils is at the same time replenished by a rapid recruitment of

neutrophils from the bone marrow to the vasculature. A great deal is known about the mechanism for neutrophil migration into tissues. However, there is very little information about the molecular signals that regulate the entry of neutrophils into the circulation. In an attempt to learn more about this process, the article by C. Zuñiga-Traslaviña and colleagues describes a zebrafish model, to assess the role of CXC-chemokines and CXC-receptor 2, in neutrophil migration into the blood circulation after injury. They found that the CXCL8b/CXCR2 axis is an important regulator of neutrophil entry into the bloodstream. On the other hand, when neutrophils arrive at sites of inflammation, they release various cytokines. The signaling machinery required for production of inflammatory and immunomodulatory cytokines and for extending the life of neutrophils at inflamed sites is poorly known. Thus, this is another area of intense study in the neutrophil field. The article by T. Ear and colleagues tells us about the constitutive expression of various Src family kinase isoforms and of spleen tyrosine kinase (Syk) in neutrophils and how the inhibition of these tyrosine kinases selectively blocks inflammatory cytokine production by acting posttranscriptionally. In contrast, delayed apoptosis seems to be independent of these kinases. These findings have implications for the future identification of potential molecular targets that could be useful in therapeutic intervention of chronic inflammatory conditions.

The involvement of neutrophils in several inflammatory pathologies is being recognized more and more, with the growing understanding of the proinflammatory and immunomodulatory properties of these cells. In some conditions, such as stroke and cancer, the numbers of immune cells can significantly predict the clinical outcome of the disease. In particular, the neutrophil-to-lymphocyte ratio was shown to predict hemorrhagic transformation and the clinical outcome of stroke. However, the immunological mechanisms underlying these effects are poorly understood. In the article by J. Ruhnau and colleagues, the role of neutrophils in brain ischemia is discussed. Neutrophils are the first cells to invade injured tissue after focal brain ischemia. In these conditions, they can enhance tissue damage and even promote more ischemic injury by inducing thrombus formation. Yet, neutrophils are also beneficial because they are involved in triggering the removal of cell debris and are essential for defense against bacterial infections. Thus, therapeutic interventions that target neutrophils to prevent stroke should preserve their functions outside the central nervous system.

In other pathologies, neutrophils also play an essential role. For example, respiratory diseases such as asthma and chronic obstructive pulmonary disease (COPD) are characterized by an excessive infiltration of neutrophils. It is generally accepted that subsequent activation of these neutrophils promotes production of reactive oxygen species and release of proteases resulting in tissue damage and alveolar airspace enlargement. The article by J. Liu and colleagues reviews the role of neutrophils in respiratory diseases, describing recent studies on mechanisms for neutrophil trafficking, activation, and cell death. The studies on neutrophil function with isolated cells do not provide a complete picture because cells are taken from the inflammatory environment of the disease. Animal models play an important role in studying the underlying mechanisms of respiratory diseases such as COPD as they address questions involving integrated whole body responses. The article by G. Huang and colleagues presents a review of the current animal models of COPD, focusing on their advantages and disadvantages on immune responses and neutrophilic inflammation. Finally, the article by C. K. Mårdh and colleagues discusses novel therapeutic approaches for respiratory diseases that target neutrophil function. They describe how targeting the chemokine receptors CXCR2 and CXCR1 could be regulated during neutrophil trafficking and how targeting the enzyme PI3K could modulate neutrophil function. Also, they explain how protease inhibitors that target matrix metalloproteinases and neutrophil serine proteases could prevent excessive tissue damage.

Together, these articles provide a sample of the multiple and complex functions of neutrophils for fighting infections and for controlling immunity. They also underline the relevant role of neutrophils in pathological conditions and provide guidance for future research on the cell biology of these fascinating leukocytes.

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Research Article

Cxcl8b and Cxcr2 Regulate Neutrophil Migration through Bloodstream in Zebrafish

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Neutrophils play an essential role during an inflammatory response, which is dependent on their rapid recruitment from the bone marrow to the vasculature. However, there is no information about the molecular signals that regulate neutrophil entry to circulation during an inflammatory process in humans. This is mainly due to the lack of a suitable model of study that contains similar set of molecules and that allows in vivo analyses. In this study, we used the zebrafish to assess the role of Cxcl8a, Cxcl8b, and Cxcr2 in neutrophil migration to blood circulation after injury. Using Tg(BACmpx:GFP)^{i114} transgenic embryos and two damage models (severe and mild), we developed in vivo lack of function assays. We found that the transcription levels of *cxcl8a*, *cxcl8b*, and *cxcr2* were upregulated in the severe damage model. In contrast, only *cxcr2* and *cxcl8a* mRNA levels were increased during mild damage. After knocking down Cxcl8a, neutrophil quantity decreased at the injury site, while Cxcl8b decreased neutrophils in circulation. When inhibiting Cxcr2, we observed a decrease in neutrophil entry to the bloodstream. In conclusion, we identified different functions for both Cxcl8 paralogues, being the Cxcl8b/Cxcr2 axis that regulates neutrophil entry to the bloodstream, while Cxcl8a/Cxcr2 regulates the migration to the affected area.

1. Introduction

Neutrophils are the most abundant types of leukocytes and neutrophil migration represents the hallmark of inflammation. Under homeostatic conditions, in humans as well as in other mammals, the great majority of neutrophils are retained in the bone marrow and only a small fraction is present in peripheral blood [1]. Under a stress condition, when an inflammatory process is triggered, this fraction rapidly increase ensuring proper response [2]. On the other hand, in several human inflammatory diseases, such as chronic obstructive pulmonary disease, cystic fibrosis syndrome, rheumatoid arthritis, and atherosclerosis, the excessive accumulation of neutrophils in the blood vessels can have deleterious effects. Therefore, it is crucial to precisely control neutrophil levels in the blood to ensure efficiency during wound or infection but at the same time prevent an enhanced response that could damage tissue which would worsen the situation. Although neutrophil migration by circulation is a critical step during an inflammatory process, there is no detailed information about the molecular signals that regulate this process in humans.

In mice, during homeostatic conditions, bone marrow neutrophil retention signals are favored because the CXCL12/CXCR4 pathway is dominant to the promigratory pathway mediated by CXCL1-CXCL2/CXCR2 [3–7]. On the other hand, when an aggression is produced, the levels of promigratory cytokines CXCL1 and CXCL2 increase, displacing the balance towards the migration, thereby increasing the amount of neutrophils that travel from the hematopoietic tissue to the bloodstream. In humans, the primary ligand of CXCR2 is CXCL8, which gene is not present in the mouse genome. Also, humans have a second CXCL8 receptor, CXCR1, absent in mice neutrophils [7, 8]. Therefore, the difference between humans and rodents regarding CXCL8 represents a considerable obstacle, especially when

considering that CXCL8 greatly contributes to several chronic diseases in which neutrophils are involved [9–12]. Consequently, it is of utmost importance to identify a suitable biological model that contains the CXCL8/CXCR2 axis and that allows in vivo analyses at the cellular and molecular levels to better understand the molecular signals that regulate inflammation in humans.

In the last decade, zebrafish (*Danio rerio*) have been increasingly used to study innate immunity, particularly in regard to neutrophil functions. As in humans, this teleost fish contains Cxcr1, Cxcr2, and Cxcl8 (found as paralogues Cxcl8a and Cxcl8b) [13, 14]. Also, under normal conditions, the majority of neutrophils are present at the hematopoietic tissue; they are immobile and retained there by the action of the Cxcl12a-Cxcr4 signaling pathway [15]. Therefore, zebrafish may represent a suitable model for understanding which chemokines regulate neutrophil migration by the bloodstream, a process likely to overlap with that present in humans. Previously, we determined that the inflammatory process triggered by severe damage, such a caudal fin transection, differs in several aspects from mild damage, such as a fin cut [16]. For example, in a severe damage model, first-responding neutrophils migrate across the interstitial tissue to reach the wound. Later, neutrophils start to migrate to the damaged tissue by circulation [16]. On the contrary, in a mild damage model, neutrophils only migrate to the affected area through the interstitial tissue. Likewise, the Gcsf-Chr19 cytokine is only upregulated in the severe damage model, acting as an important promoter of neutrophil entry to blood vessels [16], which is similar to the role played by its mammalian orthologue, GCSF [1].

Considering the unique tools available in zebrafish that permit coupling live images of specific, fluorescently labeled cell types with molecular strategies to manipulate gene functions [17–20], the aim of this study was to understand the roles of Cxcr2, Cxcl8a, and Cxcl8b during neutrophil migration by the bloodstream. To achieve this, a series of molecular and pharmacological approaches were used to analyze their participation in vivo. Severe and mild damage models were compared to differentiate the signals that control neutrophil entrance into blood circulation from those governing other steps of the inflammation process, such as final migration to the damaged area. The results obtained indicate that Cxcl8b and Cxcr2 are key regulators of neutrophil migration by the bloodstream in zebrafish.

2. Materials and Methods

2.1. Zebrafish Strains and Maintenance. Zebrafish were maintained and raised according to standard protocols [20]. The following strains of fish were used in this study: Tg(BACmpx:GFP)^{i114} [21] and Tg(fli1a:EGFP)^{y1} [22]. All embryos were collected through natural spawning, staged according to Kimmel et al. [23], and raised at 28°C in Petri dishes containing the E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, with methylene blue 0.01%, and equilibrated to pH 7.0), as previously described in Westerfield et al. [20]. Embryonic and larval ages were expressed as hpf or dpf. All damage experiments were performed at 48 hpf. All maintenance and experimental protocols were reviewed and approved by the Animal Ethics Committee of the Universidad Andrés Bello to ensure animal welfare.

2.2. Damage Models. Previous to receiving any injury, embryos were anesthetized with 0.017% tricaine [24]. For the mild damage model, the caudal fin, excluding muscle, was transected. For the severe damage model, the protocol described by Elks et al. [24] for caudal fin transection was followed. This damage model included a small section of muscle from the most caudal end of the embryonic body (Figure 1(a)). All injuries were performed on 56–58 hpf Tg(BACmpx:GFP)^{i114} or Tg(BACmpx:GFP)^{i114} X Tg(fli1a:EGFP)^{y1} transgenic embryos. In the latter case, at this stage, no more green myeloid cells were seen in the Tg(fli1a:EGFP)^{y1}. For Tg(BACmpx:GFP)^{i114} X Tg(fli1a:EGFP)^{y1} transgenic fish, embryos with 2-3 disruptions in the intersegmental vessels, but with no defect in the dorsal artery or caudal vein, were selected to ensure that neutrophils could travel through the main vessels of the embryo to reach the target destination (Supplementary Figure 1 available online at https://doi.org/10.1155/2017/6530531).

2.3. Neutrophil Quantification. Neutrophils in the dorsal and damaged area were quantified according to the computational method described by Ellet and Lieschke [25]. Following this method, Tg(BACmpx:GFP)^{i114} or Tg(BACmpx:GFP)^{i114} X Tg(fli1a:EGFP)^{y1} transgenic larvae were photographed, and every picture was analyzed using the ImageJ software. Quantification was measured in leukocytes units (LEU) or the percentage of neutrophils present in the damaged tissue in relation to the total amount of neutrophils in the larval tail. Neutrophils in blood circulation were quantified in the posterior cardinal vein of each embryo using a 5 min movie with 4 s intervals in the Cxcr2 inhibition experiments. For double transgenic experiments microinjected with Cxcl8a and Cxcl8b morpholino (MO), the neutrophils in circulation were quantified in the caudal vein using a 5 min movie with 10.5 s intervals in each embryo.

2.4. Knockdown Experiments. Both morpholino MO5-cxcl8a (from now on Cxcl8a MO) and MO1-cxcl8b.1 (from now on Cxcl8b MO) used in the present study were previously used and proved to be effective and efficient in inhibiting the splicing of their corresponding gene [26]. The corresponding sequences are shown in Table 1. Each embryo was injected with 8 ng of Cxcl8a MO or 20 ng of Cxcl8b MO at the 1-cell stage. The knockdown of *cxcl8a* and *cxcl8b* was confirmed through RT-PCR (Supplementary Figure 2).

2.5. RT-qPCR. Total RNA was extracted from 40 embryos at 0, 0.5, 1, 2, and 3 hours post performing mild or severe damage. Total RNA was extracted using the TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. The cDNAs were synthesized from RNA samples with a reverse transcription reaction that used oligo-dt primers and SuperScript II RT (Invitrogen) according to the manufacturer's instructions. Real-time PCR conditions were as follows: 40 cycles at 94°C for 30 s, 59°C for 25 s, and 72°C

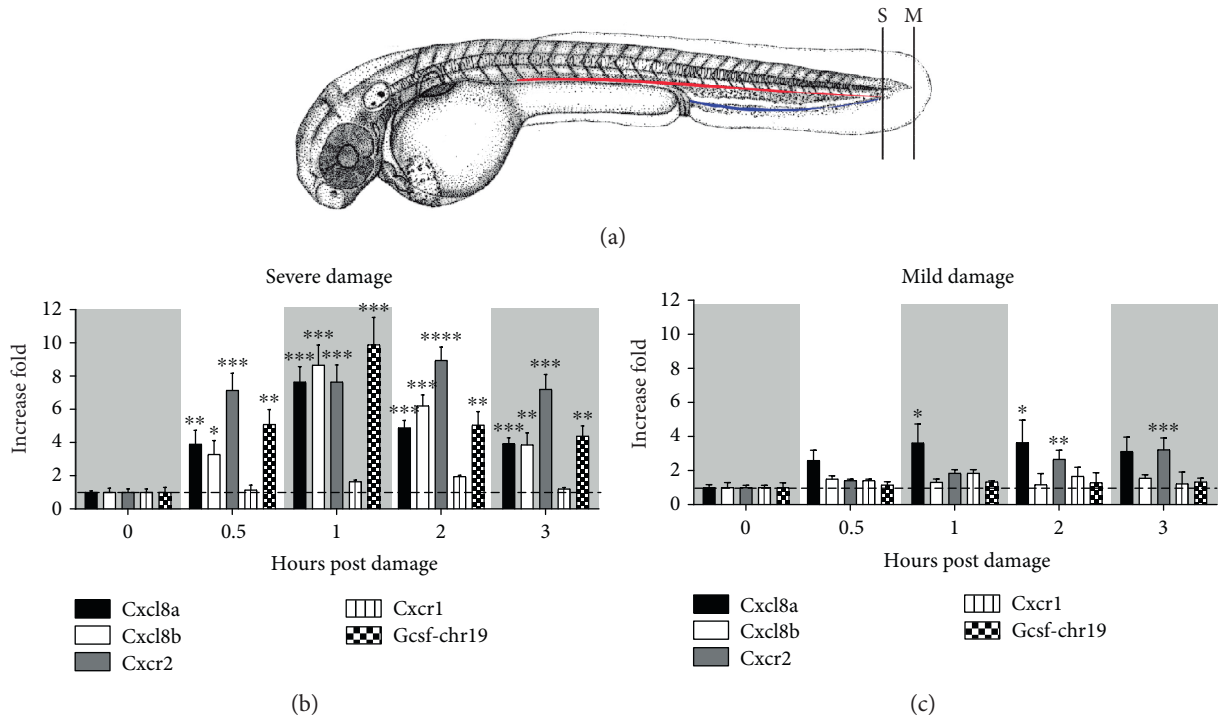

Figure 1: Severe and mild damage differentially regulate the transcription of cxcl8 paralogues in zebrafish. (a) Diagram showing the location of severe (S) and mild (M) damage on the caudal region and caudal fin of the embryo, respectively. The red line corresponds to the caudal artery, and the blue line to the caudal vein. (b, c) Transcription levels of *cxcl8a*, *cxcl8b*, *cxcr2*, *cxcr1*, and *gcsf-chr19* were quantified by qPCR after (b) severe or (c) mild damage. Data are presented as fold of change over each level at 0 hours post damage and normalized to *b-actin1*. $^{*}p$ value < 0.05; $^{**}p$ value < 0.01; $^{***}p$ value < 0.005.

Table 1: Morpholino sequences.

Gene	Sequence $5' \rightarrow 3'$	Concentration
Cxcl8a	GGTTTTGCATGTTCACTTACCTTCA	10 ng/embryo
Cxcl8b	TTAGTATCTGCTTACCCTCATTGGC	20 ng/embryo

Table 2: Primers sequences.

Gene	Primer	Sequence $5' \rightarrow 3'$
β-Actin1	Forward	TTCTGGTCGTACTACTGGTATTGTG
	Reverse	ATCTTCATCAGGTAGTCTGTCAGGT
Gcsf-chr19	Forward	GTGAGTTCCAGATCCCGACG
	Reverse	TGTGATGAAGCTCCAGACCG
Cxcl8a	Forward	TGTGTTATTGTTTTCCTGGCATTT
	Reverse	GCGACAGCGTGGATCTACAG
Cxcl8b	Forward	CTACCGAGACGTGGGTGATT
	Reverse	GCTCGGTGAATGGTCATTTT
Cxcr2	Forward	TGACCTGCTTTTTTCCCTCACT
	Reverse	TGACCGGCGTGGAGGTA
Cxcr1	Forward	TTCAGTTCGGCTGCACTATG
	Reverse	GGAGCAACTGCAGAAACCTC

for 30 s. Each gene was tested, and the melting curves were verified. The mean Ct values from each sample were normalized against the mean Ct value of a reference gene (β-*actin1*, housekeeping gene). The relative quantification of each gene was obtained with the Pfaffl method [27]. The primers used are shown in Table 2.

2.6. Cxcr2 Inhibition Experiments. Experiments with SB225002 (Cxcr2 inhibitor) were performed as previously described by Deng et al. [28]. Zebrafish embryos were preincubated 30 min before caudal fin transection with 5 μM of SB225002 (Calbiochem, EMD Millipore) in the E3 medium with 1% dimethyl sulfoxide. The embryos were maintained in this solution after fin transection over the entire course of the experiment.

2.7. Statistics and Imaging. In the case of qPCR (Figure 1), 40 individual were used for RNA extraction and RT-qPCR; the data showed is from one representative experiment from at least three biological replicates. Likewise, for the in vivo experiments (Figures 2, 3, 4, and 5), at least 20 individuals were included in each assay and three biological replicates were performed. In Figures 1, 2, and 3, data were analyzed using nonparametric Kruskal-Wallis, two-way ANOVA, and Dunn's multiple comparison tests. The data were normally distributed (analyzed by the D'Agostino-Pearson normality test), but variance was not homogenous (analyzed by the Brown-Forsythe test). For Figures 4 and 5, data were analyzed with the nonparametric test Mann–Whitney. All analyses were performed using Prism 6 (GraphPad Software), and the significance level was set at $P < 0.05$. Photographs were taken in an Olympus SZX16 stereoscope with

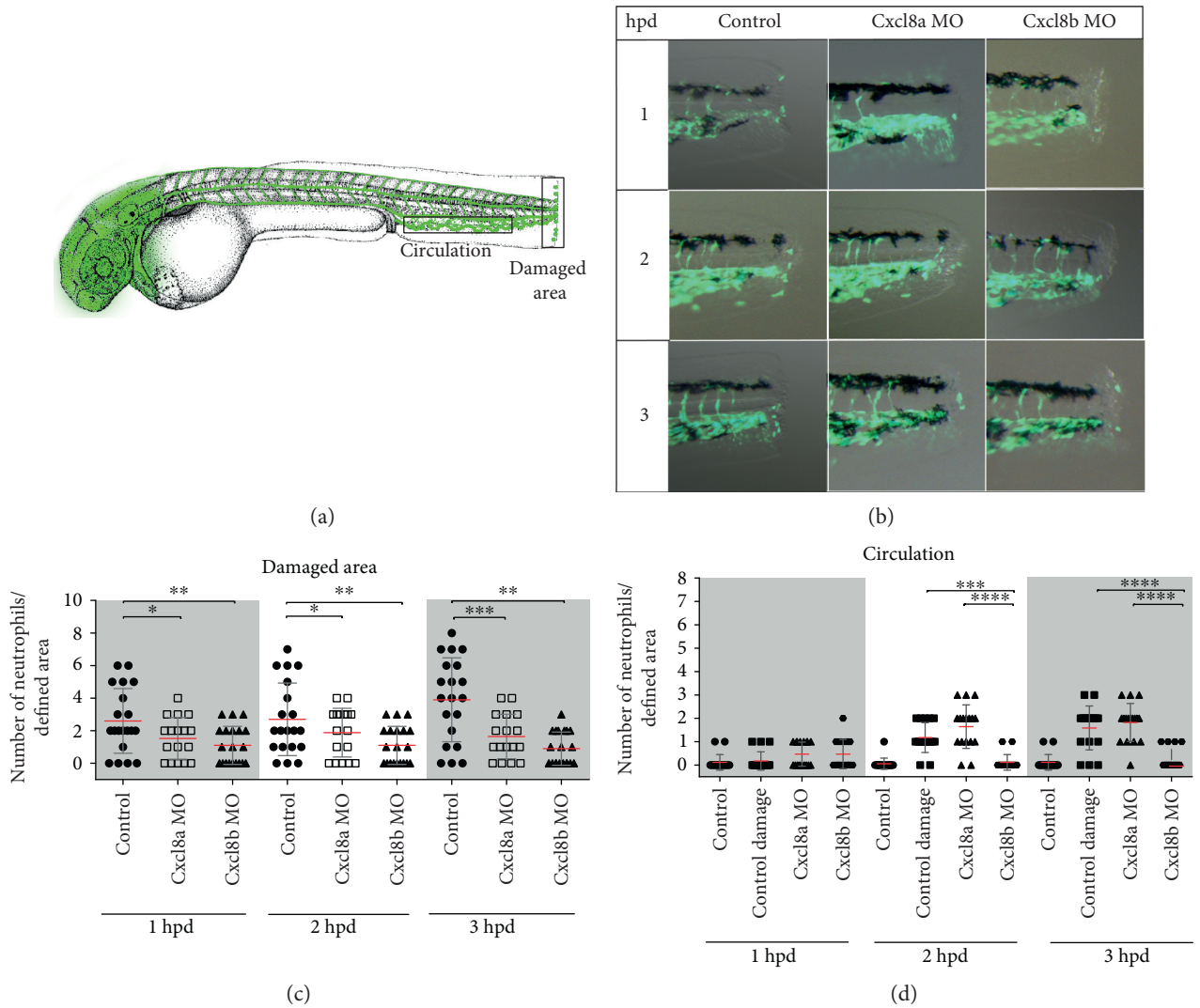

Figure 2: Neutrophil migration decreases when Cxcl8a or Cxcl8b are inhibited during severe damage. (a) Diagram showing the quantified neutrophils in two areas. (b) Lateral view of the caudal section of the embryo tail at 1, 2, and 3 hpd in control and morphant embryos. (c) Quantified neutrophils at the damaged area at 1, 2, and 3 hpd. (d) Quantified neutrophils in circulation during homeostasis, after severe damage and in the absence of Cxcl8a or Cxcl8b function. The neutrophils were quantified at 1, 2, and 3 hpd. $^{*}p$ value < 0.05; $^{**}p$ value < 0.01; $^{***}p$ value < 0.001; $^{****}p$ value < 0.0001.

the QImaging MicroPublisher 5.0 RVT camera. Images were processed with Photoshop CS5 or ImageJ 1.44o [29]. All of the described experiments were performed at least three times, and the images shown are representative of the effects observed in at least 70% of the individuals.

3. Results

3.1. Severe Damage Upregulates cxcl8a, cxcl8b, gcsf-chr19, and cxcr2, While Mild Damage Only Upregulates cxcl8a and cxcr2. To determine the roles of Cxcl8a (previously named Cxcl8l1 [14] and zCxcl8 [30]), Cxcl8b (previously named Cxcl8l2 [14]), and Cxcr2 in neutrophil migration through the bloodstream during mechanical damage, the transcriptional levels of these genes were determined in vivo using severe and mild damage models, taking into consideration the differences in the inflammatory processes generated by each type of injury [16]. As a control of the type of damage generated, the mRNA levels of *gcsf-chr19*, a critical cytokine for neutrophil blood vessel entry, were assessed [16]. Severe damage increased the mRNA levels of *cxcl8a*, *cxcl8b*, *gcsf-chr19*, and *cxcr2*. Specifically, as early as 30 minutes after severe damage, all of these molecules were upregulated, with peak expression occurring 1 hpd before slowly declining, reaching normal levels at 3 hpd (Figure 1(b)). On the other hand, mild damage only increased the transcription of *cxcl8a* and *cxcr2* (Figures 1(b) and 1(c)). Furthermore, *cxcl8a* and *cxcr2* upregulation was delayed in comparison with the severe damage model, starting at 1 hpd for *cxl8a* and at 2 hpd for *cxcr2* (Figure 1(c)). There was no increase in the mRNA levels of *cxcr1* during the entire time course for either severe or mild damage.

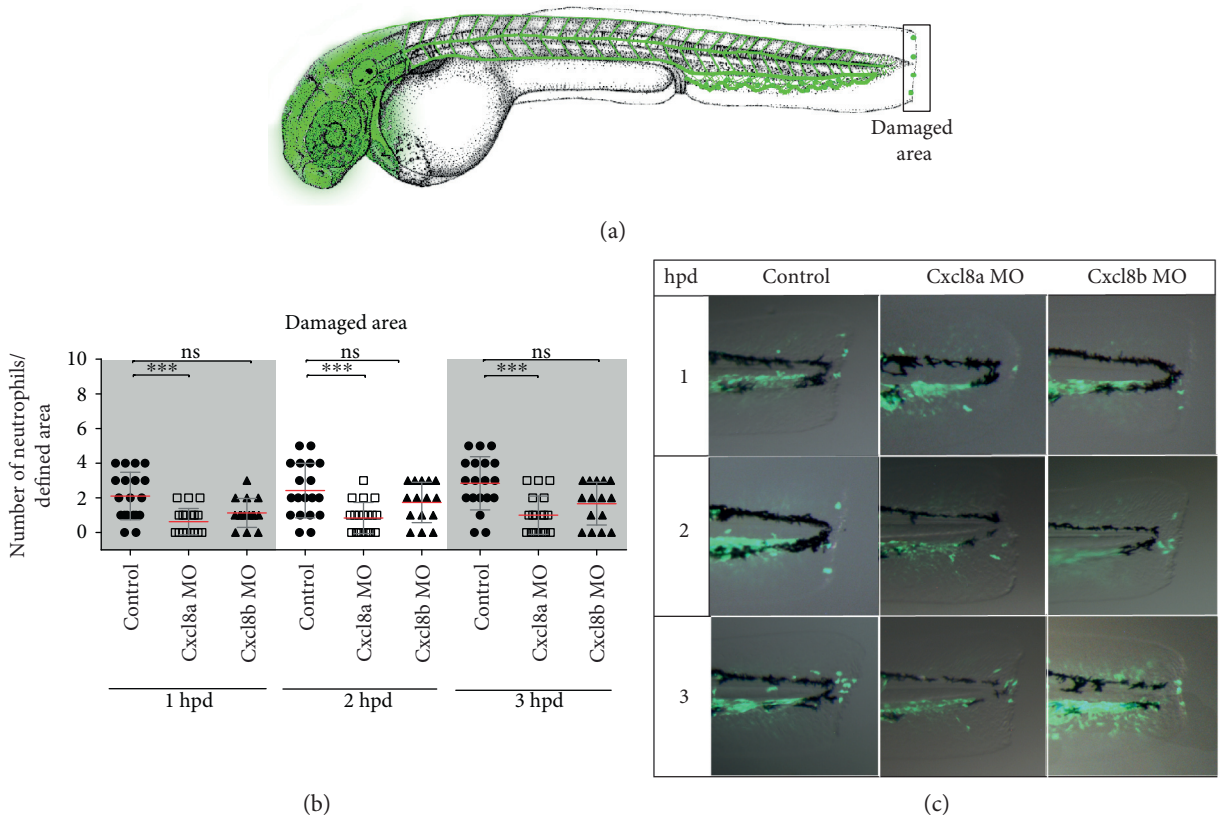

Figure 3: Neutrophil migration decreases when Cxcl8a, but not Cxcl8b, is inhibited during mild damage. (a) Diagram showing the quantified neutrophils at the wound area. (b) Quantified neutrophils at damaged area at 1, 2, and 3 hpd. (c) Lateral view of the caudal section of the embryo tail at 1, 2, and 3 hpd in the control and morphant embryos. $^{***}p$ value < 0.005.

3.2. Cxcl8a Knockdown Decreases Neutrophil Quantity at the Injury, While Cxcl8b Decreases Neutrophils in Circulation. Considering the transcriptional differences observed in the qPCR analysis for *cxcl8a* and *cxcl8b* between the severe and mild damage models, the functions of both genes were inhibited by MO and the effects of this on neutrophil migration to the wound were determined in vivo. Since Cxcl8 plays an important role in angiogenesis, particularly in intersegmental vessel formation [31], Cxcl8a MO or Cxcl8b MO was microinjected in double transgenic embryos, Tg(BACmpx:GFP)^{i114} X Tg(fli1a:EGFP)^{y1}, to correctly identify morphant embryos (Supplementary Figure 1). Both neutrophils and blood vessels of double transgenic fish are fluorescently labeled.

In the severe damage model, the absence of either Cxcl8a or Cxcl8b significantly decreased the amount of neutrophils present at the wound in comparison with that of control-damage embryos, in which the amount of neutrophils present at the damaged area continuously increased over the time course trial (Figures 2(b) and 2(c)). In addition, neutrophils in blood circulation were quantified (Figure 2(d)). In control-damage embryos, neutrophils were still high in circulation at 3 hpd (Video 1), in contrast to the noninjured control embryos that lack neutrophils in the bloodstream (Videos 2 and 3). No differences were observed between MO-injected Cxcl8a and control-damage fish. Remarkably, severely damaged morphant embryos for Cxcl8b showed no neutrophils in circulation, just as observed in the noninjured control embryos. On the other hand, in the mild damage model, only the absence of Cxcl8a affected the quantity of neutrophils at the wound. The amount of neutrophils that reached the wound in Cxcl8b morphant embryos presented no significant difference with control-damage embryos (Figure 3). Thus, the results obtained through in vivo analysis using MOs to block the functioning of each Cxcl8 paralogue were consistent with qPCR analyses and suggest different functions for Cxcl8a and Cxcl8b.

3.3. Pharmacological Inhibition of Cxcr2 Decreases the Amount of Neutrophil in the Bloodstream. To analyze Cxcr2 participation in neutrophil migration trough circulation, its function was pharmacologically inhibited, using the specific inhibitor SB225002. White and collaborators [32] demonstrated that SB225002 is a potent and selective nonpeptide inhibitor of Cxcr2, both in vitro and in vivo. Thus, in the severe damage model, we quantified neutrophil number in circulation and in damaged area and included a third area (dorsal area) as a nonspecific region (Figure 4(a)). The results obtained showed that the number of neutrophils present at the dorsal area was not different from that observed in control-damage embryos (Figure 4(d)). In contrast, the amount of neutrophils detected in the bloodstream of inhibitor-treated embryos was significantly lower than that of control-damage embryos at least until 3 hpd (Figure 4(c), Videos 4 and 5). Likewise, the number of neutrophils that

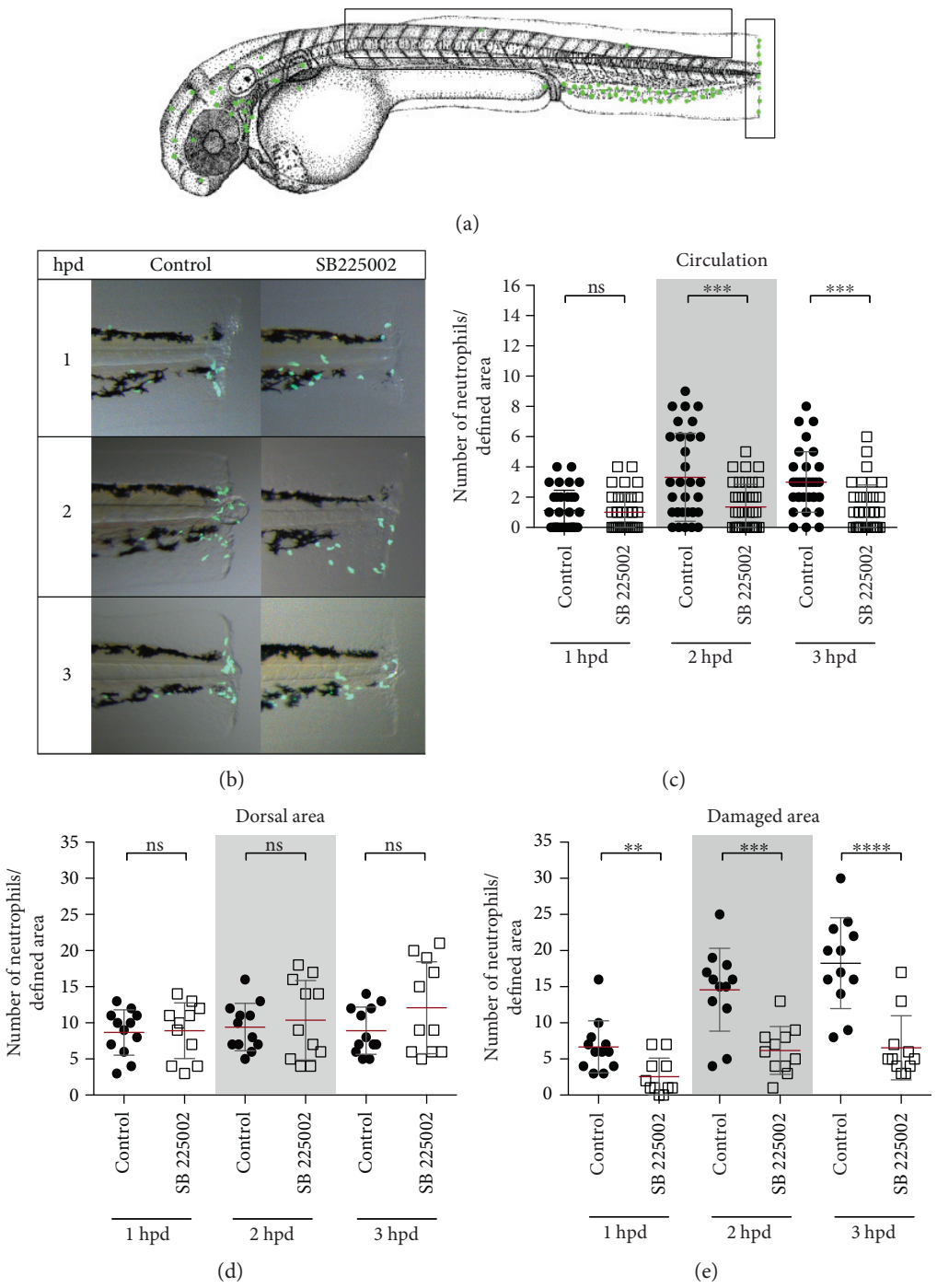

Figure 4: Cxcr2 inhibition decreases neutrophil entrance to the bloodstream and tissue infiltration in severe damage. (a) Diagram showing the quantified neutrophils, in circulation and at the dorsal and damaged area. (b) Neutrophils in circulation at 1, 2, and 3 hpd in control embryos and treated with the inhibitor SB225002. (c) Lateral view of the caudal section of the embryo tail at 1, 2, and 3 hpd in the control and treated embryos. (d, e) Quantified neutrophils at the dorsal and damaged areas at 1, 2, and 3 hpd. $^{**}p$ value < 0.01; $^{***}p$ value < 0.005; $^{****}p$ value < 0.0001.

reached the injury site was lower in inhibitor-treated embryos than that in controls during the entire time course trial (Figures 4(b) and 4(e)). In the mild damage model (Figure 5), the number of neutrophils present at the damaged area in inhibitor-treated embryos was drastically lower at each of the analyzed time points (Figures 5(a) and 5(c)). In contrast, the number of neutrophils detected in the dorsal area of inhibitor-treated embryos showed no difference compared with that of controls (Figure 5(b)).

4. Discussion

Neutrophils are the first cells to be recruited to a site of infection or damage, and neutrophil migration is regulated

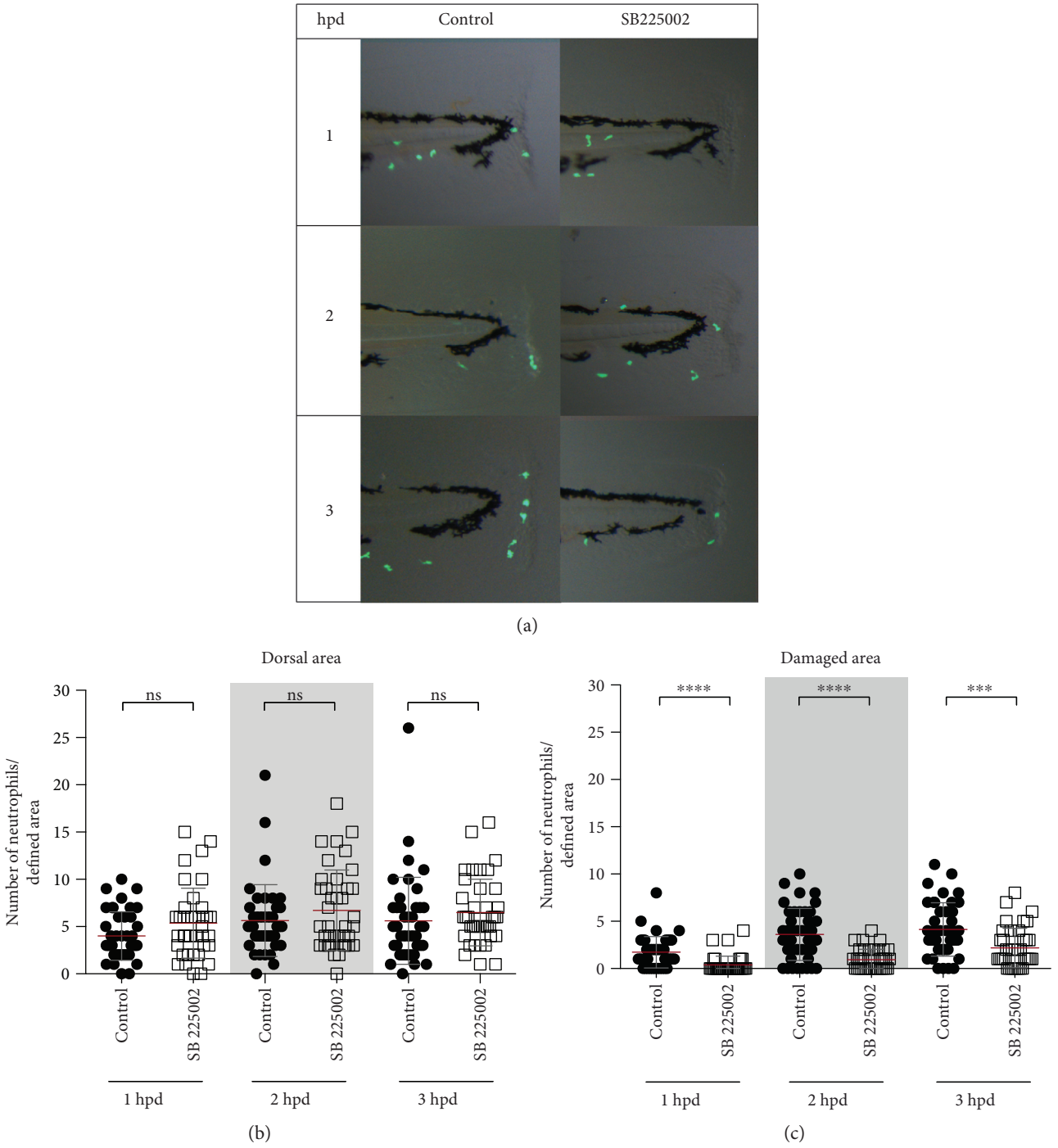

Figure 5: Cxcr2 inhibition decreases neutrophil infiltration in mild damage. (a) Lateral view of the caudal section of the embryo tail at 1, 2, and 3 hpd in the control and treated embryos. Demarked with a white rectangle are the two quantified areas. (b, c) Quantified neutrophils at the dorsal and damaged areas at 1, 2, and 3 hpd. ***p value < 0.005; ****p value < 0.0001.

by different chemokines. However, which chemokines and receptors involved in the regulation of these leukocytes' migration into blood circulation is unknown in both humans and, prior to this study, zebrafish. By using a series of methodological approaches and two different models of damages (severe and mild), a role for Cxcl8b and Cxcr2 in neutrophil entry to bloodstream was identified. Similarly, it was determined that Cxcl8a, but not Cxcl8b, attracts neutrophils to the wound area. Taken together, these data provide the first functional characterization of neutrophil migration by bloodstream after mechanical damage in zebrafish.

The transcriptional analysis showed that in a severe damage model, all the genes analyzed are increased, suggesting the participation of all of them in the inflammation process. On the other hand, during the mild damage, only *cxcr2* and *cxcl8a* were upregulated, implying that some events

occurring during the severe damage are not activated in this situation. One of these events is neutrophil migration by blood circulation, thus suggesting that Cxcl8a is only involved in the chemoattraction of neutrophils through the extracellular matrix. The lack of function assays developed confirmed these results. In the absence of Cxcl8a, the number on neutrophils present in the bloodstream is indistinguishable from control-damage embryos. In a preliminary analysis, it seems that these results do not agree with those obtained by the group of De Oliveira [26]. In their work, they indicate that the absence of either Cxcl8a or Cxcl8b decreases the number of neutrophils that reach the wound and conclude that both chemokines regulate neutrophil migration to the injury site. We observe the same in our severe damage model, the lack of each Cxcl8 paralogue affects the number of neutrophils that arrive at the wound area, but only the absence of Cxcl8b decreases the number of circulation neutrophils. Thus, we agree that the lack of each Cxcl8 paralogue affects the final number of neutrophils that reach the damage, but we think that the process altered in each case is different suggesting that Cxcl8a and Cxcl8b regulate different steps of the neutrophils' journey to the inflamed site. Also, they should be expressed in different tissues, Cxcl8a at the wound and Cxcl8b at the endothelium near the CHT.

On the other hand and although the function of CXCL8 in neutrophil entry to circulation is not clear in humans, the function of CXCL8 in a similar process, such as neutrophil extravasation, is well documented [33–36]. During neutrophil transendothelial migration, glycosaminoglycan-immobilized CXCL8 at the luminal surface of endothelial cells allows neutrophil adhesion and posterior emigration to surrounding tissue. This mechanism could shed light onto how neutrophils enter the bloodstream. Considering this and the present results regarding Cxcl8b, the CXCL8-endothelial cells-neutrophils interaction could also function in the opposite direction. In other words, zebrafish Cxlc8b could be immobilized and exposed to the abluminal endothelial surface, thereby allowing neutrophil contact with and entrance to the vasculature. Indeed, the entry of neutrophils to blood circulation occurs not only at the begging of the inflammatory process but also during resolution by reverse migration, a process that has been observed in vitro and in vivo in zebrafish and mice [37–41].

The function exerted by CXCL8 on neutrophils in humans can be divided into roles related to the vasculature and to the interstitial tissue. In zebrafish, CXCL8 orthologues contribute to both functions, but each role is performed by a separate paralogous gene, Cxcl8a or Cxcl8b. The existence of two orthologous CXCL8 genes in zebrafish is attributable to the genome duplication event that occurred near the base of the ray-finned fish evolutionary tree [42]. Indeed, the repertoire of chemokines present in zebrafish is twice that of humans (89 and 44, resp.) [43, 44].

On the other hand, in the current study, Cxcr2 was found to participate not only in the final neutrophil migration to the wound but also in neutrophil migration through the bloodstream. It is interesting that in the Cxcr2 lack of function assay, a low amount of neutrophils still circulate, suggesting that another chemokine receptor could also participate in the process but to a lesser extent. This is supported by the fact that in the Cxcl8b lack of function assay, no neutrophil was detected in the bloodstream. A receptor that is a good candidate to be involved in this process is Cxcr1, mainly because it interacts with CXCL8 in humans [8]. Finally, and not expected, we found that there is a neutrophil subpopulation that after injury migrates through the interstitial tissue in a Cxcr2-independent form. Moreover, these neutrophils did not migrate in wound direction but to the dorsal area and could or not be found later at the injury site.

The participation of CXCR2 in bone marrow neutrophil release is documented in mice, where neutrophils lacking CXCR2 are preferentially retained in the bone marrow, causing chronic neutropenia [7, 9, 45–47]. Several studies support the hypothesis that neutrophil release is antagonistically regulated by the CXCR2 and CXCR4 chemokine receptor system [7, 48]. Under homeostatic conditions, neutrophil retention signals are favored in the bone marrow since the CXCL12/CXCR4 pathway is dominant to the promigratory pathway mediated by the CXCR2/CXCL1-2 axis. When neutrophil release from the hematopoietic tissue is required, the levels of the promigration cytokines CXCL1 and CXCL2, as well as G-CSF, increase, thereby displacing the balance towards migration [7]. In a previous study, we demonstrated that zebrafish Gcsf-Chr19 regulates neutrophil migration by the bloodstream after mechanical damage [16]. In turn, the present study provided new details for how neutrophils are mobilized from the caudal hematopoietic tissue to the circulation after a sterile stimulus by addressing the role of Cxcr2 in this process and by confirming the evolutionary conservation of Cxcr2 function in lower vertebrates, such as fish. Furthermore, the present results suggest that Cxcr2 is the receptor for both Cxcl8 paralogues Cxcl8a and Cxcl8b.

In conclusion, and by consolidating previous and our present data, Cxcl8b and Cxcr2 are key regulators of neutrophil entrance into blood circulation in zebrafish. In more detail, we propose the following model regarding neutrophil migration during an inflammatory process in zebrafish (Figure 6). During homeostasis, neutrophils are retained in the caudal hematopoietic tissue by Cxcr4/Cxcl12 [49], meaning only a few neutrophils would be in the bloodstream (Figure 6(a)). After severe damage (Figure 6(b)), Gcsf-Chr19, Cxcl8b, and Cxcr2 expression would increase, and Cxcl8b would bind to Cxcr2. Considering the overexpression of Gcsf-Chr19, it is plausible to hypothesize that this molecule would functionally interact with its receptor, Gcsfr. Therefore, both the Cxcl8b/Cxcr2 and Gcsf-Chr19/Gcsfr signaling pathways would allow neutrophils to leave the caudal hematopoietic tissue and enter the bloodstream [16]. This would induce neutrophils to enter and remain in circulation until sensing an unknown signal (probably Cxcl8b) in the endothelium near the site of injury, where neutrophils would then leave the blood vessels. Furthermore, in the interstitial tissue, Cxcl8a would bind to Cxcr2 present in neutrophils to enable neutrophils to reach the wound [26, 28, 50].

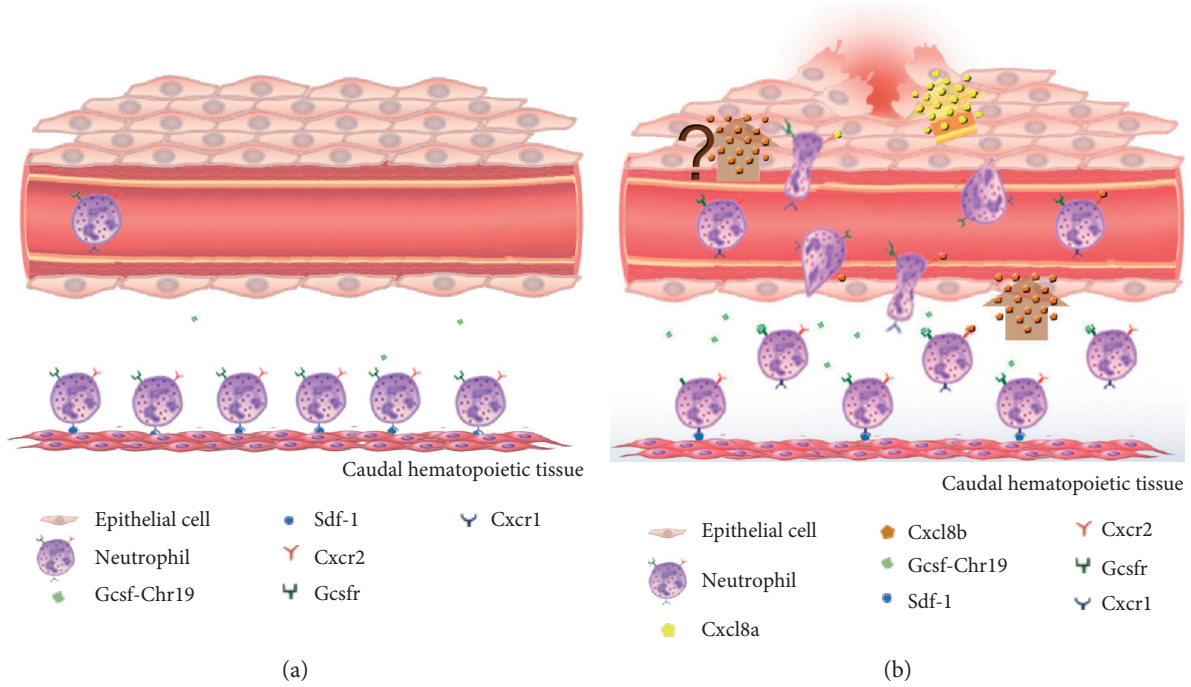

Figure 6: Model for the regulation of neutrophil migration to a wound. (a) During homeostasis, neutrophils are retained in the caudal hematopoietic tissue by Cxcr4/Cxcl12. (b) After damage, the expression of Gcsf-Chr19 would increase and interact with its receptor, Gcsfr. Likewise, mRNA levels of Cxcl8b and Cxcr2 would increase, and both proteins would interact. Therefore, both signaling pathways would allow neutrophils to leave the caudal hematopoietic tissue and enter the bloodstream. Neutrophils would stay in circulation until sensing an unknown signal (probably Cxcl8b) in the endothelium, then leaving the blood vessel. Finally, in the interstitial tissue, Cxcl8a/Cxcr2 would guide neutrophils to the wound.

Our results significantly contribute to fill the gap regarding the molecular signals that regulate inflammation and neutrophil recruitment from the hematopoietic tissue to the vasculature in zebrafish, a key step of the journey of this granulocyte during an inflammatory process. Considering the similarity in molecules between zebrafish and humans—which made this fish a suitable model for this study—our research provides new avenues for understanding neutrophil biology during homeostasis and pathologic conditions.

Conflicts of Interest

The authors declare no conflicts of interest in relation to the research and authorship.

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Review Article

Targets of Neutrophil Influx and Weaponry: Therapeutic Opportunities for Chronic Obstructive Airway Disease

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Neutrophils are important effector cells of antimicrobial immunity in an acute inflammatory response, with a primary role in the clearance of extracellular pathogens. However, in respiratory diseases such as asthma and chronic obstructive pulmonary disease (COPD), there is excessive infiltration and activation of neutrophils, subsequent production of reactive oxygen species, and release of serine proteases, matrix metalloproteinases, and myeloperoxidase—resulting in collateral damage as the cells infiltrate into the tissue. Increased neutrophil survival through dysregulated apoptosis facilitates continued release of neutrophil-derived mediators to perpetuate airway inflammation and tissue injury. Several target mechanisms have been investigated to address pathologic neutrophil biology and thereby provide a novel therapy for respiratory disease. These include neutrophil influx through inhibition of chemokine receptors CXCR2, CXCR1, and PI3Kγ signaling and neutrophil weaponry by protease inhibitors, targeting matrix metalloproteinases and neutrophil serine proteases. In addition, neutrophil function can be modulated using selective PI3Kδ inhibitors. This review highlights the latest advances in targeting neutrophils and their function, discusses the opportunities and risks of neutrophil inhibition, and explores how we might better develop future strategies to regulate neutrophil influx and function for respiratory diseases in dire need of novel effective therapies.

1. Introduction

Asthma and chronic obstructive pulmonary disease (COPD) are heterogeneous respiratory conditions characterized by airway inflammation, remodeling, and restricted pulmonary air flow—principally distinguished by reversible airway hyperreactivity in asthma. Together, asthma and COPD represent a major proportion of airway disease burden, where asthma affects 235 million people worldwide, COPD affects 384 million people worldwide, and 3 million deaths every year are caused by COPD globally (WHO http://www.who.int/respiratory/copd/en/, [1]). The global prevalence of COPD has been estimated to be 11.7% [2], and the global prevalence of adult asthma has been estimated to be 4.3% [3]. Current therapeutic strategies focus upon symptom relief and control using as-needed short-acting β_2-agonist (SABA), inhaled corticosteroids (ICS), and long-acting β_2-agonist (LABA) for asthma [4] with the addition of long-acting muscarinic antagonists (LAMA) and phosphodiesterase type 4 (PDE4) inhibitors for COPD [5]. Restricted air flow is treated by bronchodilators and the inflammatory response by ICS in well-controlled mild asthma. Despite the use of a broad selection of specific and nonspecific immune regulatory therapies (e.g., ICS, emerging anticytokine antibodies), no treatment other than glucocorticoids targets the underlying cause of inflammation; hence, both asthma and COPD still represent a significant unmet medical need. Indeed, only half of asthma patients respond adequately to current therapies [4].

The most common cause of COPD is cigarette smoking, but some patients develop COPD from inhaling smoke through combustion of biomass fuel or other irritants. Chronic inflammation of the lung, particularly in peripheral airways and parenchyma, is the hallmark of disease in COPD and may be the underlying cause for small airway destruction that progresses with disease. The underlying inflammation

then increases during acute exacerbations. COPD is also associated with systemic inflammation which may lead to comorbidities. There is a characteristic inflammation pattern with increased numbers of macrophages, T lymphocytes, and B lymphocytes, together with increased numbers of neutrophils in the airway lumen [6]. The inflammatory response in COPD involves both innate and adaptive immune responses, which are linked through the activation of dendritic cells. While endothelial cells and macrophages are the key cells responsible for triggering the immune response in COPD, classical adaptive immunity is the key driver in asthma. Airway inflammation in asthma is typically associated with Th2 cytokines, produced by activated CD4+ T cells polarized in the presence of interleukin (IL) 4. Cytokines produced by Th2 cells comprise of IL-4, IL-5, and IL-13 [6]. Asthmatic airways exposed to environmental stimuli such as allergens, viruses, pollutants, and bacteria lead to the epithelial damage which activate cells of the innate immune system such as dendritic cells, basophils, mast cells, eosinophils, and macrophages. Dendritic cells then direct the adaptive immune responses, promoting differentiation of Th2 cells and isotype switching of B cells to produce IgE.

However, both severe asthma and COPD, as well as bronchiectasis and cystic fibrosis, also have features of dysregulated neutrophil recruitment, activation, and survival that result in release of toxic proteases and reactive oxygen species perpetuating airway inflammation and tissue injury. Importantly, none of the currently available medical therapies selectively target neutrophils, even though neutrophils appear to have a role in disease pathogenesis and are causative for tissue damage in severe disease [7]. Thus, innovative therapeutic approaches are needed to treat poorly controlled asthma and COPD patients with sustained neutrophilic inflammation.

Neutrophils are the most abundant leukocytes in blood and are part of our native or innate immunity, and together with NK cells, platelets and macrophages, they mainly act as part of our defense to protect against microbes. Specifically, neutrophils are the final effector cells of antimicrobial immunity of an acute inflammatory response, with a primary role in the clearance of extracellular pathogens [8]. Microorganisms and particles reaching the airways and lung evoke a massive influx of neutrophils. However, in airway diseases such as severe asthma and COPD, there is excessive neutrophil recruitment, activation, and defective apoptosis. Neutrophil production of reactive oxygen species and release of serine proteases, matrix metalloproteinases, myeloperoxidase, and lysozymes contribute to lung tissue damage and airway remodeling. COPD and severe asthma are both characterized by sustained neutrophilic inflammation of the airways [7, 9–14], and the number of viable neutrophils in sputum is negatively correlated with lung function as measured by forced expiratory volume in 1 second (FEV1) [13, 15–18].

This review therefore sets out to describe the role of neutrophils in mediating inflammation and tissue damage in obstructive airways diseases and reviews potential therapeutic targets (Table 1) for measuring/modulating neutrophil presence and activity in the lung.

1.1. Targeting Neutrophil Influx

1.1.1. Chemokine Receptor Antagonism. There are several proteins involved in the chemoattraction, rolling, tight adhesion, and transmigration of neutrophils. Neutrophil trafficking out of the circulation into the lung is a multistep process, and each step can be targeted by a different mechanism. Neutrophils must first exit the circulation by rolling on the endothelium mediated by selectins, then tight adhesion using integrins, followed by migration via chemokine receptors. Migration into the inflamed tissues of the lung involves both transendothelial and transepithelial migration. During the first step in neutrophil emigration from the circulation, the adhesion to the vascular endothelial cells is mediated by selectins and these are similar between the intestine and lung, for example, L-, E-, and P-selectins, P-selectin glycoprotein ligand, and $\alpha 4\beta 1$ integrin. Transepithelial migration follows a similar pattern of adhesion, migration, and postmigration events, the difference being that neutrophil adhesion to the epithelium occurs on the basolateral as opposed to the apical surface. In the first stage of transepithelial migration, neutrophils adhere to the basolateral epithelial surface via $\beta 2$ integrins, and in most epithelial cell types, it is mediated via the CD11b/CD18 molecule. CD11b/CD18 is present both in intestinal and in bronchial epithelium while CD11a/CD18 is exclusive to bronchial and alveolar epithelium and CD11c/CD18 exclusive to bronchial epithelium. After firm adhesion to the basolateral surface of the epithelium, neutrophils begin to migrate across the epithelial monolayer through the paracellular space by mechanisms using the cell surface molecules CD47, SIRPα, and SIRPβ. Once the neutrophils have completed migration, they are retained on the luminal side as a defense barrier to clear pathogens [19]. The process is propagated by circulating leukocytes entering into inflamed tissue in response to inflammatory mediators. The process by which neutrophils enter into the tissue are directed through chemotactic processes regulated by several families of proteins including inflammatory cytokines, adhesion molecules, matrix metalloproteases, and chemokines. Four subfamilies of chemokines can act on chemokine receptors that are expressed on different inflammatory cells. For neutrophils, the chemokines GROα (CXCL1) and IL-8 (CXCL8) are potent chemoattractants and activate G protein-coupled receptors (GPCRs) CXCR1 and CXCR2 [20]. In patients with moderate to severe asthma, increased expression of CXCL8 has been shown to correlate with raised neutrophil numbers in sputum, which in turn is associated with an increase in the frequency of exacerbations of acute asthma [21, 22]. Activation of CXCR2 by, for example, CXCL8 mediates migration of neutrophils to sites of inflammation. Neutrophilic airway inflammation has been shown to be significantly reduced in animal studies when antagonizing this receptor. In addition, CXCR1 and CXCR2 are also expressed by other cell types associated with chronic inflammation, including macrophages, lymphocytes, mast cells, dendritic cells, and endothelial cells [23–27]. Ligand binding to CXCR1 is mainly responsible for the degranulation of neutrophils, whereas CXCR2 regulates recruitment of neutrophils from blood into tissues. CXCR2 is a receptor for a number of chemokines such

as the GRO family (CXCL1-3) and CXCL8, all of which are elevated in respiratory inflammatory diseases such as COPD, severe asthma, and acute respiratory distress syndrome. CXCR1 and CXCR2 have similar signaling mechanisms [28], and CXCL8 can potentiate several neutrophil functions triggered through both of its receptors, including phosphoinositide hydrolysis, intracellular Ca2+ mobilization, and chemotaxis. However, CXCR1 has been specifically implicated in phospholipase D activation, respiratory burst activity, and the bacterial-killing capacity of neutrophils [29], suggesting that CXCR1 and CXCR2 might have different physiological roles under inflammatory conditions. CXCL8 signals through both CXCR1 and CXCR2 [28]. Furthermore, CXCL1 may play a homeostatic role in regulating neutrophil egress from bone marrow to blood [30]. Therefore, targeting CXCR2 would be expected to effectively reduce neutrophilic inflammation, mucus production, and neutrophil proteinase-mediated tissue destruction in the lung [22].

Several small molecule C-X-C chemokine receptor antagonists have been developed as a potential therapeutic approach for the treatment of inflammatory disease, including repertaxin, navarixin, and danirixin [14] and AZD5069. CXCR2 selective small-molecule antagonists [31] have been shown not to adversely impact neutrophil effector host defense [32, 33]. These are in different stages of drug development and have been shown to reduce neutrophil recruitment to the lung in clinical studies [34–37]. Effects of inhibiting neutrophil recruitment have been shown by clinical biomarkers and endpoints indicative of disease efficacy in cystic fibrosis, severe asthma, and COPD [38–40]. However, O'Byrne et al. showed that 6 months treatment with AZD5069 did not reduce the frequency of severe exacerbations in patients with uncontrolled severe asthma, thereby questioning the role of CXCR2-mediated neutrophil recruitment in the pathobiology of exacerbations in severe refractory asthma [41]. Intriguingly, CXCR2 antagonists seem mainly to be of clinical benefit in patients who have ongoing exposure-induced stimulation of neutrophil recruitment to the lungs, such as oxidative stress due to tobacco smoking [40]. The only active CXCR2 antagonist trial (using danirixin, formerly called GSK-1325756, currently in clinical phase II trials for COPD (NCT02130193, TrialTroveID-208293, and TrialTroveID-267696)) may provide proof of concept efficacy.

1.1.2. PI3K Inhibition. Phosphoinositide 3-kinase (PI3K) family signaling can influence a multitude of cells and pathologic processes, including those in which neutrophils play a dominant role (reviewed Hawkins et al. [42]). Class I PI3K isoforms (α, β, γ, and δ) function by phosphorylating $PI(4,5)P_2$ to generate PI(3,4,5)P3 at the plasma membrane following receptor engagement [43] and are the most evolved as targets of drug discovery. Whereas PI3Kα and β isoforms are ubiquitously expressed, PI3Kδ is largely restricted to myeloid and lymphoid cells [44]. PI3Kγ is expressed highly in myeloid cells downstream of GPCRs and is an important regulator of neutrophil effector responses, thus making both γ and δ PI3K isoform inhibition the focus of modulating neutrophil movement.

Initial studies used knockout mice to study neutrophils, where Hirsch et al. showed chemoattractant-stimulated PI3K$\gamma^{-/-}$ neutrophils could not produce PI(3,4,5)P3 or downstream activation of pAkt, and displayed impaired respiratory burst and motility [45]. These findings were further confirmed through confocal imaging of knockout neutrophils which indicated PI3Kγ-mediated control of cell direction via colocalization of AKT and F-actin to the leading edge [46]. A role for PI3Kδ was discovered in neutrophil migration when trapping of cells in vessels following leukotriene B_4 (LTB_4) infusion was observed in PI3Kδ knockout mice, whereas wild-type controls showed neutrophil transmigration into tissue [47]. The first PI3Kδ-selective inhibitor studies, using IC87114, also demonstrated blockade of both N-formyl-methionyl-leucyl-phenylalanine- (fMLP-) and tumor necrosis factor-alpha- (TNF-α-) induced neutrophil superoxide generation and elastase exocytosis from neutrophils in a mouse model of inflammation [48]. The comparative roles of PI3Kγ versus δ were further investigated in knockout animals of each isoform sensitized with lipopolysaccharide (LPS), indicating a dominant role for PI3Kγ in neutrophil migration [49]. A key paper from Condliffe et al. made two important observations. Firstly that stimulation of TNF-α-primed human neutrophils with fMLP results in biphasic activation of PI3K; the initial phase is largely dependent on PI3Kγ, whereas the secondary phase is largely dependent on PI3Kδ (and the first phase itself) [50]. They also showed that murine cells can behave differently to human within their mechanistic systems [50]. Studies from Stephens and colleagues [43] further elucidated roles for PI3K in neutrophil movement, demonstrating PI3Kγ-mediated PIP_3 accumulation at the leading edge of the cell to be a vital step in chemokinesis, thus determining the proportion of cells able to move toward a chemokine gradient [51]. Also, studies using both short-term and long-term in vitro neutrophil migration assays showed that PI3K can enhance early responses to the bacterial chemoattractant fMLP, but that it is not required for migration towards this chemoattractant [51]. However, sensing the gradient itself was shown to be PI3Kγ independent, despite a role for the γ isoform in integrin-based adhesion and neutrophil polarization [52]. Yet, a recent bronchiectasis clinical trial where neutrophil chemotaxis was inhibited via CXCR2 antagonism failed to confer therapeutic benefit, thus suggesting that inhibition beyond GPCR/PI3Kγ-mediated cell movement is needed [37]. It was studies such as these which drove us to investigate our novel PI3Kγ and PI3Kδ inhibitors in a human neutrophil chemotaxis assay (Figure 1(a)). Here, we show dose response inhibition curves of low nM potent, >100-fold selective molecules to investigate chemotaxis to fMLP (and other GPCR ligands) and PI3Kγ versus δ isoform signaling. PI3Kγ-dominated inhibition showed a 3-log advantage in potency, thus confirming the dominance of PI3Kγ on GPCR-mediated neutrophil movement.

Translational evidence for class 1 PI3K signaling in severe neutrophilic asthma shows that neutrophil chemotaxis triggered by airway epithelial-conditioned media from severe

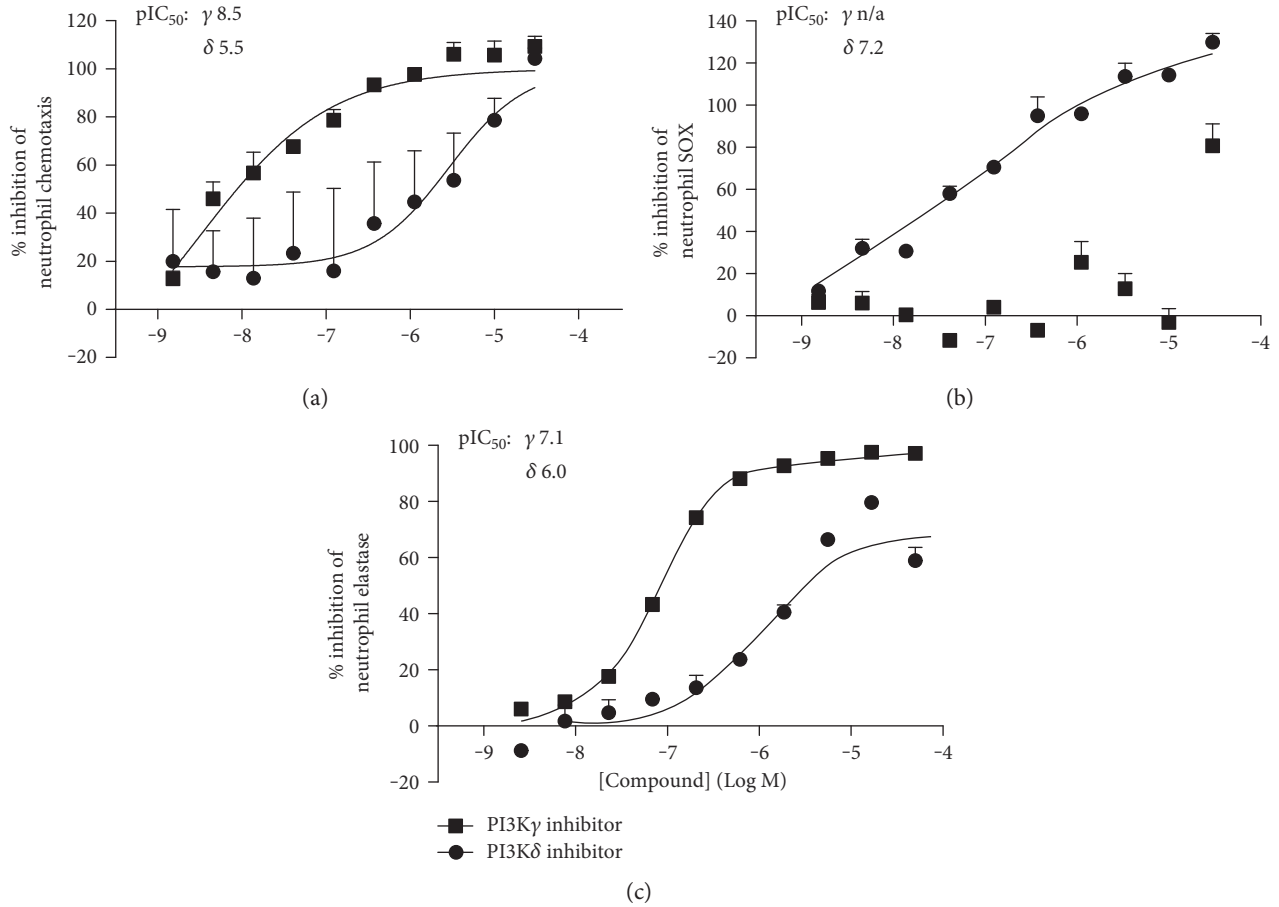

Figure 1: Comparison of PI3Kγ versus PI3Kδ inhibition on neutrophil functions. Novel inhibitors with >100-fold selectivity (versus other class 1 PI3K isoforms) for PI3Kγ (squares) or δ (circles) were compared across 3 neutrophil mechanisms. (a) Neutrophil chemotaxis to fMLP. (b) Neutrophil superoxide (SOX) generation following LPS priming and stimulation with fMLP. (c) Neutrophil degranulation (assessed via elastase release) following cytochalasin b priming and stimulation with fMLP. Mean ± standard error of $n > 3$ experiments are plotted as % inhibition. pIC_{50} ($-\log IC_{50}$) values for both γ and δ inhibitors are indicated.

asthmatics can be reduced by a PI3Kγ-selective inhibitor, whereas the same neutrophil migratory response is insensitive to PI3Kδ inhibition [53]. However, an inhaled PI3Kδ inhibitor is currently in early clinical trials for primary immune deficiency, activated PI3K-delta syndrome (APDS) caused by gain of function mutations in PIK3CD, and progressing into both asthma and COPD indications (NCT02294734, ClinicalTrials.gov). The therapeutic hypothesis is based upon rejuvenation of effective directionality in neutrophil movement and therefore a reduction in "collateral damage" observed in a neutrophil with upregulated PI3Kδ [54]. This hypothesis is intriguing, as it aims to retain effective neutrophil function in the lung and thus minimize any potential for liabilities attributed to immune suppression. The risk of increased infections has been recently identified through a 2016 safety review for idelalisib in three clinical trials, which showed increased numbers of fatal cases related to infections in the treatment arm [55]. Importantly, we are yet to understand the significance of systemic activity of PI3Kδ inhibitors, thereby affecting lymph node function, versus lung tissue biology and the relative pathologic roles for both PI3Kγ and δ isoforms.

There is clearly an association of chemokine-guided neutrophilic inflammation in disease pathogenesis, but the balance between beneficial control of the disease and maintaining host defense may be limiting the development of drugs targeting chemokine receptors. Alternatively, many complex inflammatory conditions may rely on multiple, interconnected chemotactic stimuli which resist the antagonism of a single pathway. To date, there are only two marketed products targeting chemokine receptors: plerixafor, a small molecule antagonist of CXCR4 used as an immunostimulant in cancer patients, and maraviroc, an antagonist of CCR5 used as treatment of HIV infection [56] despite strong associations of chemokine involvement in disease. Future strategies for inhibiting neutrophil migration may benefit from a more subtle modulatory mechanism aiming to retain host defense (e.g., PI3Kδ inhibition) or may require a more broad approach targeting multiple stimuli in the lung (e.g., PI3K$\gamma\delta$ dual inhibition).

1.2. Targeting Neutrophil Weaponry. The granules of neutrophils are rich in an array of different antimicrobial molecules that are released in a controlled manner to protect the host

from invading pathogens. During chronic neutrophilic inflammation, an increasing number of activated neutrophils secrete granule contents into the extracellular space, where the focal excess of normally protective proteases in the absence of pathogens can become destructive [18]. Intracellularly, neutrophil serine proteases (NSPs) help to destroy ingested bacteria within the phagolysosome. The family of NSPs include neutrophil elastase (NE), proteinase 3 (PR3), and cathepsin G (CG), all located in the primary azurophilic granules, and are together capable of degrading most of the extracellular matrix components such as elastin and collagen [57, 58]. The most studied of these proteases as a drug target is neutrophil elastase, the net activity of which is increased in patients with alpha-1-proteinase deficiency (A1ATD). The genetic loss of this gene results in early-onset emphysema [59]. The hypothesis that COPD is caused by a protease-antiprotease imbalance is further strengthened by studies with exogenous instillation of elastase (or other neutrophil serine proteases) into animal lungs that leads to emphysema [60, 61]. NSPs are amongst the most potent known stimulants of mucus secretion from epithelial cells [62, 63], hypersecretion of which is a common feature across the neutrophilic diseases including cystic fibrosis, bronchiectasis, and chronic bronchitic COPD. Neutrophil elastase may worsen mucus-driven airway obstruction via two processes: activation of the sodium channel ENaC on the apical surface of epithelial cells (via degradation of SPLUNC1, the endogenous inhibitor of ENaC [64]) and indirect degradation of the cystic fibrosis transmembrane conductance regulator (CFTR) [65]. This would lead to dehydration of the airway surface and further weaken the ability of the airways to effectively clear not only mucus but any pathogens present therein.

Of increasing interest is the role of proteinase (PR) 3 in disease, due to the subtle differences in its biological effects. Present in increasing amounts in stable and exacerbating respiratory disease [66], it is capable of influencing the inflammatory milieu by modifying key proinflammatory cytokines such as IL-8, leading to its enhanced stability and potency [67], and release of IL-1β and TNF-α from monocytic cells [68]. An ever-increasing number of proinflammatory cytokines are being shown to be modulated by not just PR3 [69] but also NE and CG [70]. The inactivation of the IL-6 trans-signaling pathway by NSPs reported by McGreal and colleagues is especially interesting as this mechanism is postulated to be necessary for recruitment of monocytes [71] and neutrophil apoptosis [72], leading to the resolution of inflammation.

Dysregulation of constitutive neutrophil apoptosis may delay the resolution of airway inflammation and is implicated in acute respiratory distress syndrome (ARDS) [73], cystic fibrosis [74], and severe asthma [13] whilst conflicting data exist in COPD [75, 76]. Efferocytosis of apoptotic neutrophils by macrophages is also required for resolution, before they become necrotic and release their cell contents into the inflamed tissue. A significant recognition ligand in this process is the apoptotic neutrophil cell surface-bound phosphatidylserine [77]. Cleavage of this receptor by NE has been reported in vitro using sputum from bronchiectasis and CF patients [78] which may explain why timely clearance of dying neutrophils is defective in the disease. In addition, it has been reported that in vitro NE is capable of creating an "opsonin-receptor mismatch" by cleaving complement receptor 1 (CR1) from the neutrophil surface and C3bi of opsonized *Pseudomonas aeruginosa* [79], impairing clearance of this bacteria commonly found in the CF airway and associated with mortality [80]. An important observation to note is that inhibitors of *Pseudomonas elastase* are reported to not inhibit this degradation in vitro [79]. Additional beneficial effects of blocking NSPs may arise through inhibition of neutrophil extracellular traps (NETs). Formation of NETs has been observed in the airways of patients with asthma [81] and in stable or exacerbated COPD [82, 83]. NET formation itself being an innate immune response can also further affect innate and adaptive immune responses [84, 85]. In addition, NET formation also displays direct cytotoxic effects on alveolar epithelial and endothelial cells [86]. NETs are fibres of chromatin released from neutrophils in an active process named NETosis. Flattening of the cells, chromatin decondensation with histone modifications, and citrullination of histone H3 by peptidylarginine deiminase 4 (PAD4) are a major modification during NETosis and result in DNA released from the cell [87]. Extracellular DNA alters the biophysical properties of mucus and has been correlated with airflow obstruction in CF patients [88].

Links between the neutrophil and the adaptive immune system are being steadily reported, such as inhibition of dendritic cell maturation [89] and the impairment of NK cell activity [90]. Impairment of T cell function via surface antigen cleavage by NSPs [91] could lead to a blunting of the immune response during chronic inflammation. Together, these observations point to the excess neutrophilia and their NSPs potentially having a pivotal role in the cycle of damage and inflammation in neutrophilic respiratory disorders than previously thought.

1.2.1. Neutrophil Elastase Inhibition. A wide variety of synthetic small molecule NE inhibitors have been studied for use in neutrophilic pulmonary disorders with varying degrees of clinical success [92]; however, no compound has progressed further for respiratory indications than phase 2 other than sivelestat which is approved only for acute respiratory indications such as acute respiratory distress syndrome (ARDS). In separate phase 2 trials in bronchiectasis [93], COPD [94, 95], and cystic fibrosis patients [96], the selective NE inhibitor AZD9668 [97] resulted in some beneficial effects, especially in the 4-week bronchiectasis study. Four weeks oral dosing of AZD9668 in these 20 bronchiectasis patients resulted in greatly improved lung function (FEV1 and SVC) and significant decreases in some sputum and plasma inflammatory markers such as IL-6 [93]. These effects were not confirmed in a larger study performed by Bayer (BAY 85-01, NCT01818544, ClinTrials.gov). The effects of another NE inhibitor, MR889, in a small COPD study resulted in no overall changes in the levels of lung destruction markers, but a subset of treated subjects (having shorter than average disease duration of 13.7 years) showed lower urinary desmosine, a marker of elastin degradation [98]. Due to

adverse liver effects, another NE inhibitor ONO-6818 was stopped in phase 2. The limited clinical success of NE inhibitors may be in part due not only to inadequate patient phenotype selection but also to the inability to attain stoichiometric equivalent ~mM concentrations of inhibitor at the sites of neutrophil degranulation within the tissue. This issue, coupled with the presence of exclusion zones created when neutrophils are in close contact with extracellular matrix [99], may be solved by inhibiting the protease activation before neutrophils are released into the circulation, rather than inhibit the protease activity. Neutrophil serine proteases are activated early in the promyelocyte stage of neutrophil development via cleavage of a dipeptide, by the cysteine protease dipeptidyl peptidase 1 (DPP1, also known as cathepsin C [100]). Redundancy is absent in this process as illustrated by individuals with inactivation mutations in the gene encoding DPP1, leading to the absence of NSPs [101]. Interestingly, neutrophils from these Papillon-Lefèvre syndrome (PLS) patients who show no generalised immonodeficiency seem incapable of forming NETs [102].

Only two potent and selective DPP1 inhibitors, AZD7986 (NCT02303574, ClinTrials.gov) and GSK2793660 (NCT02058407, ClinTrials.gov), have entered clinical development. Preclinical studies with AZD7986 showed decreased NSP activities in differentiating primary human neutrophils in vitro and in bone marrow neutrophils from treated rats in vivo [103]. In a recent study, DPP1 was found in bronchoalveolar lavage fluid (BALF) from CF patients and patients with neutrophilic asthma as well as in LPS treated macaques but was absent in healthy individuals and untreated macaques [98], the functional significance of which is as yet unknown.

1.2.2. Matrix Metalloprotease (MMP) Inhibition. MMPs, including the highly neutrophil-expressed MMP-8 (neutrophil collagenase) and MMP-9 (gelatinase B), have also been proposed to be involved in the pathophysiology of COPD [104–107]. In the healthy lung, MMPs regulate extracellular matrix turnover and can degrade matrix components such as elastin [108], but again, an excess of these proteases or the cells producing them leads to tissue destruction. It may be that MMPs from other sources may play a more significant role in the development of respiratory diseases such as MMP-12 from macrophages [109] or MMP-7 from hyperplastic epithelial cells in idiopathic pulmonary fibrosis [110, 111]. Whilst many MMPs are expressed by other immune and structural cells, often in greater amounts, the excessive active neutrophilia present in certain chronic lung disorders would add to an increasingly destructive and inflammatory proteolytic milieu. The protease-antiprotease balance might also be adversely altered by the degradation of endogenous MMP inhibitors, such as tissue inhibitor of MMPs (TIMPs), by NE [112]. There are also further possible interconnections between NSPs and MMPs, such as the inactivation of alpha-1-proteinase by MMP-9 [113] and the activation of MMP-9 by NE [114]. Less is known of the role of MMPs in other respiratory disease such as asthma, with MMP-9 and MMP-12 being reported to increase in the airway smooth muscle of fatal asthmatics [115] and mouse knockout studies indicating that several MMPs may be involved in fibrosis [116, 117]. Efforts to develop MMP inhibitors as therapeutic agents have been largely focused outside of respiratory disease and have proved fruitless, largely due to lack of efficacy or the musculoskeletal toxicity that has limited the clinical utility of unselective MMP inhibitors. In a short exploratory study, the dual MMP-9 and MMP-12 inhibitor AZD1236 provided no clinical benefit in moderate/severe COPD patients [118]. However, due to the mechanism of action, significant changes in lung function would not be expected over this time scale in such a small number of stable COPD patients.

1.2.3. PI3K Inhibition. The roles of PI3Kγ and δ isoforms have also been investigated neutrophil degranulation. In Figures 1(b) and 1(c), we show dose-response inhibition curves of low nM potent, >100-fold PI3K-selective molecules to investigate superoxide generation and elastase release, respectively. Interestingly, we saw superoxide generation following LPS priming and stimulation with fMLP was heavily dependent upon PI3Kδ activity. However, neutrophil degranulation assessed via elastase release following cytochalasin b priming and stimulation with fMLP proved to be a PI3Kγ-dominated process. And thus, it seems that the differential use of PI3Kγ and δ isoforms is dependent on the priming and the stimuli used. These data build upon a wealth of literature which point toward the value of dual PI3K$\gamma\delta$ inhibition for the treatment of neutrophil-mediated pathology.

Disease applications for PI3Kγ &/or δ inhibitors span those for which neutrophils are important and beyond—a reflection of the pleiotropic effects anticipated for such molecules. So far, oral systemic inhibitors of PI3Kδ, exemplified by idelalisib developed for oncology, show target-related toxicity primarily in the gut which hinders therapeutic utility [119]. One could further postulate therapeutic benefit in other pulmonary diseases from neutrophil-mediated bronchiectasis, where sputum neutrophil elastase activity is a biomarker of disease severity [120]. Furthermore, autoimmune activation of neutrophils in Churg-Strauss syndrome has been shown to be PI3Kγ dependent [121]. However, given our evolving mechanistic understanding of PI3K isoforms in neutrophil function, such diseases would gain far greater therapeutic benefit from inhibition of both PI3Kγ and δ together, where PI3Kδ controls release of neutrophil stimuli and PI3Kγ reduces responsiveness to them. Indeed, initial attempts to generate PI3K$\gamma\delta$ dual inhibitors for inhalation have shown some preclinical success. Doukas et al. induced lung neutrophilia via chronic smoke administration in mice—steroid resistant pathology which could be attenuated by aerosolized TG100-115 [122]. The forthcoming generation of PI3K inhibitors look to improve both potency and selectivity in order to offer a novel therapeutic option for neutrophil-driven diseases. An inhaled PI3Kδ inhibitor is currently in early clinical trials for activated PI3K delta syndrome (APDS) caused by gain of function mutations in PIK3CD, with the intent of expanding into both asthma and COPD indications.

Table 1: Overview of key neutrophil related targets with association to chronic respiratory disease as potential therapeutic targets.

Target	Drug name	Selectivity	Company	Indication	Last reported status	Reference	Subjects	Duration (weeks)
CXCR2	AZD5069	CXCR2	Astrazeneca	Asthma	Phase 2	NCT01704495	640	26
				Bronchiectasis	Phase 2	NCT01255592	52	4
	Danirixin	CXCR2	Glaxosmithkline	COPD	Phase 2	NCT02130193	102	2
	Elubrixin	CXCR2	Glaxosmithkline	CF	Phase 2	NCT00903201	146	4
	Navarixin	CXCR1/2	Merck	Asthma	Phase 2	NCT00632502	37	4
				Asthma	Phase 2	NCT00688467	19	1.3
				COPD	Phase 2	NCT01006616	616	102
	QBM076	CXCR2	Novartis	COPD	Phase 2	NCT01972776	48	8
	SX-682	CXCR1/2	Syntrix	Asthma	Preclinical			
DPP1	AZD7986		Astrazeneca	COPD	Phase 1	NCT02303574	237	4
	GSK2793660		Glaxosmithkline	Bronchiectasis	Phase 1	NCT02058407	33	2
MMP	AZD1236	9/12	Astrazeneca	COPD	Phase 2	NCT00758706	55	6
	AZD2551	12	Astrazeneca	COPD	Phase 1	NCT00860353	81	2
	AZD3342	8/9/12	Astrazeneca	COPD	Phase 1		49	2
	RBx 10017609	12	Glaxosmithkline & Ranbaxy	COPD	Phase 1			
NE	AZD9668		Astrazeneca	Bronchiectasis	Phase 2	NCT00769119	38	4
				CF	Phase 2	NCT00757848	56	4
				COPD	Phase 2	NCT00949975	838	12
				COPD	Phase 2	NCT01023516	615	12
	BAY 85-8501		Bayer	Bronchiectasis	Phase 2	NCT01818544	94	4
	ONO-6818		Ono	COPD	Phase 2			
PI3K	GSK2269557	δ	Glaxosmithkline	Asthma	Phase 2	NCT02567708	50	4
				COPD	Phase 2	NCT02294734	126	4
				COPD	Phase 2	NCT02522299	35	12
	GSK2292767	δ	Glaxosmithkline	Asthma	Phase1	NCT03045887	44	2
	IPI-145	δ (/γ)	Infinity	Asthma	Phase 2	NCT01653756	46	2
	RV1729	δ (/γ)	RespiVert	Asthma	Phase 1	NCT01813084	63	2
				Asthma	Phase 1	NCT02140320	49	4
				COPD	Phase 1	NCT02140346	48	4
	RV6153	δ (/γ)	RespiVert	Asthma	Phase 1	NCT02517359	55	4

2. Conclusions and Future Outlook

The current therapeutic pharmacological target paradigm for asthma and COPD is not adequately controlling disease in many patients. There is a need for innovative therapeutic approaches to treat severe disease and ultimately modify the underlying pathological changes in asthma and COPD. Although neutrophils appear to play a pathogenic role in severe disease, no neutrophil targeting approaches have been approved to date. Modulating the activity and numbers of neutrophils locally in the affected organs and systemically has been suggested for several chronic inflammatory conditions (e.g., asthma, ulcerative colitis, and rheumatoid arthritis).

Emerging evidence points to the existence of distinct neutrophil subsets in humans that could be phenotypically discriminated based on the surface expression of the markers, FcγRIII (CD16) and L-selectin (CD62L). Mature neutrophils ($CD16^{bright}/CD62L^{bright}$) display a normal-shaped nucleus, immature neutrophils ($CD16^{dim}/CD62L^{bright}$) have a banded-shaped nucleus, whereas neutrophils with a hypersegmented shape have a diminished expression of CD62L ($CD16^{bright}/CD62L^{dim}$) [123]. Whilst the mature phenotype was found to display a proinflammatory potential, the hypersegmented neutrophils were shown to suppress T cell proliferation in a Mac-1 and H_2O_2-mediated fashion and, therefore, may possess a potential immunomodulatory role [123]. It has been speculated that selective blockade of a specific neutrophil subset, notably the disease-promoting mature phenotype, without impacting on the immunoprotective hypersegmented phenotypes, could preserve neutrophil-mediated host-protective immunity [124].

Clinical challenges in using a neutrophil-targeted therapeutic approach have been related to concerns of compromising the patients host defense with an associated increased risk of serious sequelae on opportunistic infections.

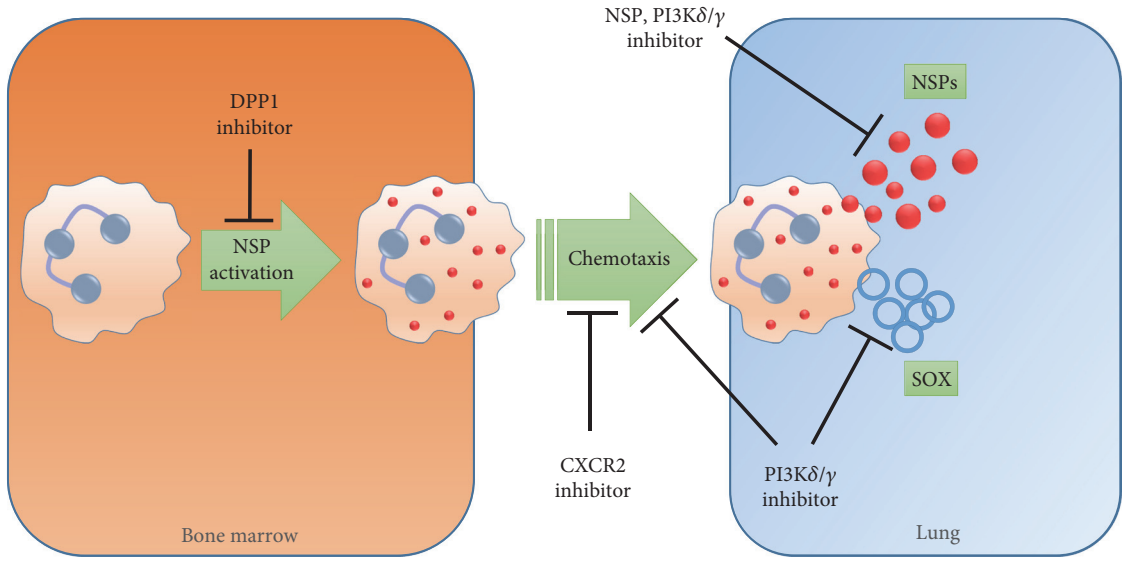

Figure 2: Summary illustration of neutrophil targets in chronic lung disease. Activation of NSPs during neutrophil maturation in the bone marrow is via DPP1. Chemotaxis to the lung can be modulated by targeting CXCR2 or PI3Kδ/γ, the latter of which can also inhibit SOX and NSP release.

Furthermore, the unresolved question of whether neutrophils are principal pathogenic drivers or bystanders in more complex inflammatory conditions has also resulted in less effort to target neutrophils selectively. Clearly, reduced neutrophil migration has been shown to reduce hazard exacerbation risk in COPD patients [40]. Significant effect was shown on time to first exacerbation and lung function (FEV1) after 6 months treatment using a 50 mg dose of navarixin, but only in a subpopulation of current smokers, and no effect was shown in the broad COPD population. A possible explanation for response only in active smokers is not clear, and it is conceivable that neutrophils are actually doing their intended job in such circumstances. Furthermore, clear dose-response relationships have been difficult to show and significant dropout of patients at higher doses due to reduction of neutrophil count in blood impacts data interpretation. Local inhibition of neutrophil function (PI3Kγ/δ antagonism) or strategies which spare host defense mechanisms (PI3K δ antagonism) may offer effective neutrophil-targeted therapies in the future.

Another explanation may be that antineutrophil therapies (illustrated in Figure 2) need an environment of active damage/challenge to show efficacy. Chronic bronchitic COPD patients have been linked to active smoking and neutrophilic airway inflammation. Chronic cough and sputum production are present in the majority of COPD patients (74.1% of COPD patients) [125] and are associated with frequent exacerbations and hospitalizations. Therefore, selecting patients such as these may improve success in therapeutic development.

In conclusion, targeting the neutrophil weaponry by blocking the activation of proteases via DPP1 inhibition, or neutrophil-mediated NETosis, or multiple neutrophil functions via dual blockade of PI3K$\gamma\delta$ may show promise as future therapies to address such pressing unmet medical needs.

Disclosure

The authors are employees of AstraZeneca.

Conflicts of Interest

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

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Review Article

Advanced Role of Neutrophils in Common Respiratory Diseases

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Respiratory diseases, always being a threat towards the health of people all over the world, are most tightly associated with immune system. Neutrophils serve as an important component of immune defense barrier linking innate and adaptive immunity. They participate in the clearance of exogenous pathogens and endogenous cell debris and play an essential role in the pathogenesis of many respiratory diseases. However, the pathological mechanism of neutrophils remains complex and obscure. The traditional roles of neutrophils in severe asthma, chronic obstructive pulmonary diseases (COPD), pneumonia, lung cancer, pulmonary fibrosis, bronchitis, and bronchiolitis had already been reviewed. With the development of scientific research, the involvement of neutrophils in respiratory diseases is being brought to light with emerging data on neutrophil subsets, trafficking, and cell death mechanism (e.g., NETosis, apoptosis) in diseases. We reviewed all these recent studies here to provide you with the latest advances about the role of neutrophils in respiratory diseases.

1. Introduction

With the changing of global environment, especially the increased air pollution worldwide, respiratory diseases are becoming a main killer to human health. According to recent researches, asthma is ranked as the 14th most important chronic disease, affecting 334 million individuals of all ages worldwide [1]. Lung is the leading cancer site in males, comprising 17% of the total new cancer cases and 23% of the total cancer deaths [2]. As for COPD, affecting 64 million people all over the world, it would be the third most common cause of death by 2030 [3, 4]. Community-acquired pneumonia is a common cause of sepsis, leading to 10 million deaths annually [5]. While epidemiology data of idiopathic pulmonary fibrosis (IPF) worldwide cannot be obtained, IPF incidence is still increasing and carries a high risk of respiratory failure and death [6]. Respiratory diseases not only increase the economic burden of global health care but also cause a terrible effect on the quality of daily life. Although the precise treatment of respiratory diseases has made a great progress [7–9], the pathogenesis of them still needs further elucidation.

Innate together with adaptive immunity, as natural systematic defensive barrier, is composed of immune organs, cells, and cytokines [10, 11]. The innate immunity is a natural defense that shapes in the process of long-time biological evolution [12, 13]. As the first barrier to defend infection, innate immunity participates in the resistance to pathogenic invasion and the clearance of aging, injured and even mutant cells nonspecifically. Innate immunity was firstly reported in the development of immunology and was becoming the focus of immunological research in recent twenty years, especially after the discovery of various kinds of pattern recognition receptors (PRRs) and innate lymphoid cells (ILCs) [14–17]. When the exogenous threats cannot be removed by innate immunity successfully, adaptive immunity will take part in the important defensive battle. Adaptive immunity system including humoral and cellular immunity often plays a leading role in the final clearance of invasive pathogens. The executors are T lymphoid cells and B lymphoid cells,

who can both recognize antigens specifically. Different immune cells can exert protective and defensive effect synergistically with the help of multiple cytokines and protein molecules. The mechanism of adaptive immunity has been being gradually clarified since the birth of immunology. Various monoclonal antibody medicines related with adaptive immunity such as rituximab and infliximab have brought a wonderful curative effect in many refractory diseases including respiratory diseases [18, 19].

Traditionally, neutrophils, originating in bone marrow stem cells, had only been considered as a kind of innate immune cell [20]. As an essential component of innate immunity, neutrophils play an important role in killing pathogens and removing cellular debris [21]. The migration and activation of neutrophils could cause inflammation and sensitization directly or indirectly. Inflammation caused by self-immune system is really important for the solution of infection and clearance of pathogens. But the persistent inflammation in respiratory system frequently leads to some adverse diseases such as asthma, COPD, and pulmonary fibrosis. In addition, neutrophils can synergize with lymphocytes and other granulocytes, such as Th2/Th17 and eosinophils, to participate in not only innate but also adaptive immune process and promote airway inflammation [22, 23]. The interaction between neutrophils and other immune cells, endogenous composition, and foreign matter is very complex and being clarified thoroughly.

There have been more and more studies on the role of neutrophils in respiratory diseases. Recently, exosomes, neutrophil extracellular traps (NETs), deriving from neutrophil and the higher autophagy of neutrophils have been reported in multiple respiratory diseases [24, 25]. Despite that the pathogeneses of respiratory diseases are being studied extensively, there is still a long way to go to clarify the complexity and heterogeneity, especially the participation of various immune components in the development of respiratory diseases. In this review, the latest progress of neutrophils in respiratory disease such as asthma, COPD, pneumonia, lung cancer, pulmonary fibrosis, bronchitis, and bronchiolitis will be summarized.

2. Asthmatic Neutrophils

2.1. What Is Neutrophilic Asthma? As Global Initial for Asthma (GINA, updated in 2017) elucidated [26], asthma is a heterogeneous disease, always characterized by expiratory airflow limitation and chronic inflammation. Asthma is usually categorized as different phenotypes and endotypes according to its different clinical characteristics and distinct pathological mechanism. Traditionally, asthma was classified as four different phenotypes [27], eosinophilic, neutrophilic, mixed granulocytic asthma, and paucicellular asthma according to the cellular counts of sputum, bronchoalveolar lavage fluid (BALF), or peripheral blood [28]. For example, Jodie et al. distinguished asthmatics with neutrophil proportion in sputum over 61% as neutrophilic asthma [29]. However, more and more researches have demonstrated the instability of asthma phenotypes [30–32]. Neutrophil as an essential granulocyte has been reported by many investigators to play a critical role in many immunity-associated diseases including asthma, especially steroid-refractory severe asthma [33, 34]. High blood neutrophils counts are associated with an increased risk of moderate, but not severe asthma exacerbation [35]. At the same time, the neutrophil-predominant asthmatics also tend to show a lower bronchial lability [36].

2.2. How Neutrophils Participate in Asthma?

2.2.1. Chemotactic Activity of Neutrophils. Lower respiratory tracts used to be considered as sterile. But more and more evidence had already showed us the conflicted results [37]. *Moraxella catarrhalis* or a member of the Haemophilus or Streptococcus genera was discovered colonizing in the lower airways of asthmatics [38]. These species' colonization was associated with more differential sputum neutrophil counts and worse clinical disease status. The altered colonization would participate in the development of asthma phenotype. Infection of *H. influenza* could synergize with allergic airway diseases to induce Th17 immune responses that drive the development of neutrophilic asthma. The process above is mediated by IL-17 responses [39]. In addition, subclinical infection likely contributes to neutrophilic inflammation in airways [40].

Microbial components, which contain LPS and β-glucan, could synergistically cause neutrophilic asthma mediated by TLR-4 and dectin-1 [41], whose deficiency could significantly attenuate the recruitment of neutrophils induced by house dust mite (HDM) into airways [42]. Blood neutrophils from allergic asthma also show the chemotactic and phagocytic activities towards LPS and asthmatic serum [43]. Asthmatics challenged with inhaled *Dermatophagoides pteronyssinus* (DP) would promote the production of neutrophil chemotaxis [44]. Siew et al. demonstrated that neutrophil chemotaxis induced by smoke and other environmental stimulations could also be helpful for developing inflammation in airways [45].

As described above, smoking and other infectious factors can cause the accumulation of neutrophils in BALF. This is associated with the activation of phosphatidylinositol 3-kinase (PI3K) signal [46, 47]. Phosphatidylinositol 3-kinases (PI3Ks), as the key elements in the signaling cascades, play an important role in the chemotaxis of neutrophils [48]. In particular, PI3Kδ and PI3Kγ isoforms contribute to inflammatory cell recruitment and subsequent activation [49]. The traditional role of different PI3K isoforms in the chemotaxis of neutrophils had already been reviewed previously [50, 51]. PI3Kγ deficiency could significantly reduce the influx of neutrophils into BALF [52]. PI3Kδ inhibition may also prevent recruitment of neutrophils [53]. The PI3K-related inflammation and steroid insensitivity should partly be attributed to microRNA-21/PI3K/histone deacetylase 2 (HDAC2) axis as Kim et al. reported [54]. In addition, the activation of PI3K is accompanied with the release of all kinds of chemokines and cytokines, such as IL-6 and IL-8, which are related with the increased chemotactic activity of neutrophils towards the inflamed sites [55, 56]. Not only

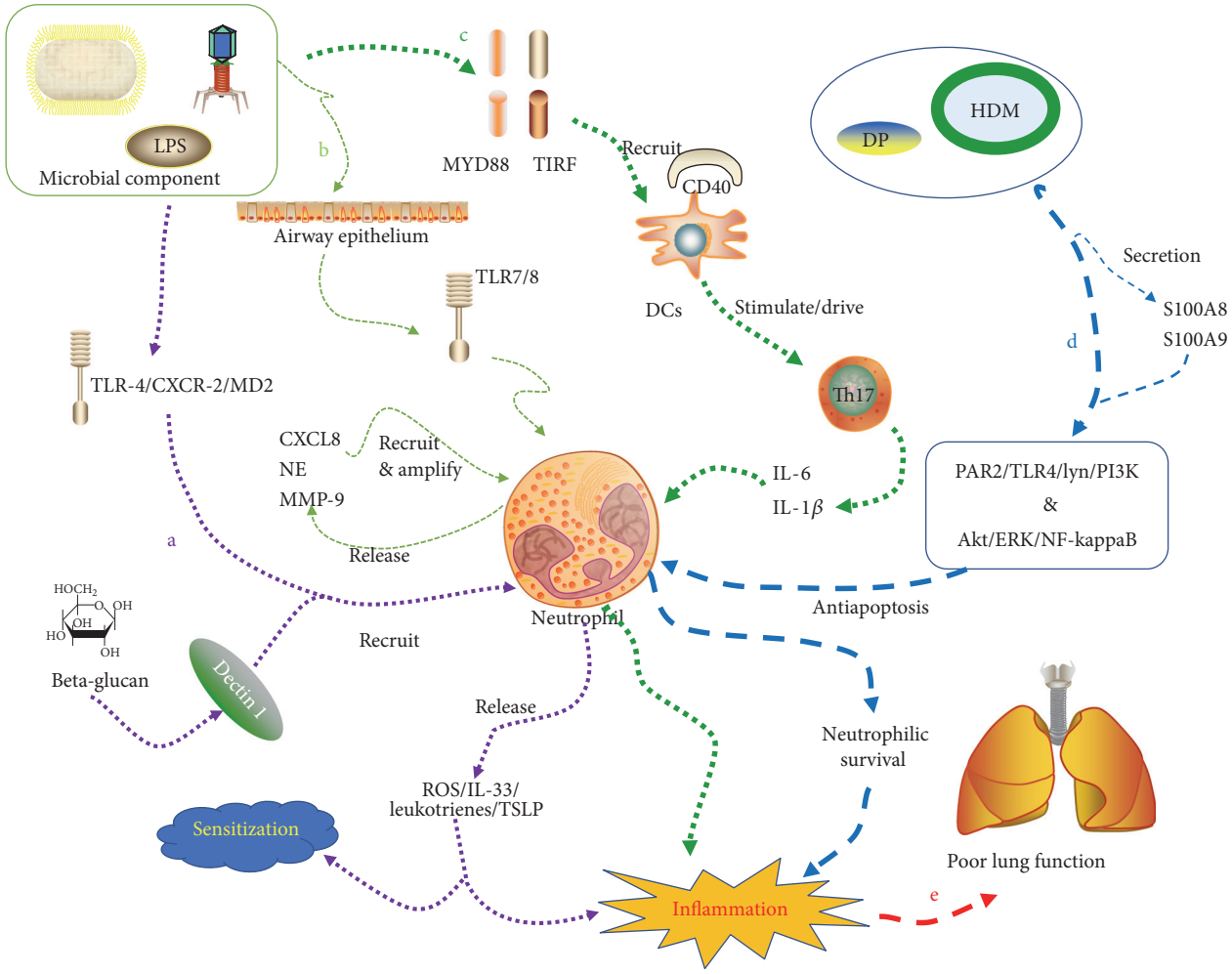

Figure 1: a: microbial component and environmental allergens such as LPS and β-glucan could recruit neutrophils via TLR-4/CXCR-2/MD2 and dectin-1. The recruited neutrophils could produce ROS/leukotrienes/IL-33/TSLP and cause inflammation or sensitization. b: the foreign allergens can act on airway epithelium and induce neutrophils to release CXCL8/NE/MMP-9, which is mediated by TLR-7/8. The released substance could amplify the recruitment of neutrophils in a positive feedback manner. c: microbial products and inhaled allergens can induce Th17 activated through TIRF and MYD88 by activating IRF-3, inducing I interferons, upregulating CD40 on DC, and finally releasing IL-6 and IL-1β to act towards airway neutrophils. TIRF also contribute to Th17 responses to inhaled allergens by increasing recruitment of DCs and to drive Th17 cell differentiation. d: HDM-DP could induce the secretion of S100A8/S100A9. The combination of S100A8/S100A9 might activate TLR4/Lyn/PI3K/Akt/ERK/NF-kappaB pathway to produce an antiapoptotic effect on neutrophils. e: the stimulated or survival neutrophils can cause the persistence of inflammation. As a result, the lung function is getting poorer and poorer.

the chemotactic activity of neutrophils but also the concrete details of neutrophil activation mechanism have been making progress in recent years.

2.2.2. Immune Interaction Related with Neutrophil Activation. As previously reviewed [57], TIR-domain-containing adapter-inducing interferon-beta (TRIF) played a key role in the induction of inflammatory mediators which could contribute to antiviral innate immune. Hsia et al. discovered that TRIF, as an essential component together with MYD88, could mediate microbial products (inhaled allergen) to induce Th17 activated. The process may include an activation of IRF-3, induction of I interferons, upregulation of CD40 on DCs or CD40L on T cells, and finally, the release of IL-6 and IL-1β, which are required for airway neutrophils [58]. The environmental stimulations could promote the production of IL-17. The expression level of IL-17 is also correlated with the expression of IL-8 and neutrophil numbers [45]. It was revealed that TRIF-CD40-Th17 axis participated in the IL-17-associated neutrophilic asthma. In addition, TRIF might also contribute to Th17 responses to inhaled allergens by increasing the recruitment of DCs to lung with potential to drive Th17 cell differentiation [58] (Figure 1, c). Similar to that, Siew et al. discovered that CSE (cigarette smoke extract), IL-17, and aeroallergens could act on human tracheal epithelial cells and further increase IL-6 and IL-8 production [45]. CD11b + DCs could sense some molecules in HDM extract and play a key role in the

induction of HDM-induced allergic airway inflammation by inducing the expression of chemokine or chemokine receptors in DCs by expressing dectin-1 [42].

Rhinovirus infection could induce bronchial mucosal neutrophilia in subjects with asthma. Airway neutrophils during infection are positively related to virus load [59]. Recently, it has been demonstrated that LPS and viral infection could also promote the release of CXCL8/NE/MMP-9 from neutrophils, which is mediated by TLR7/8. CXCL8, as a potent chemoattractant for neutrophils released by a variety of cells including neutrophils, could amplify the recruitment in a positive feedback manner [60] (Figure 1, b). Several studies have also reported that CXCL8 levels in the airways inversely correlate with FEV1 in asthmatics [38, 61]. However, N-formyl-methionyl-leucyl-phenylalanine (fMLF), a bacterial-derived protein and ligand for fMLF receptor [62] could stimulate neutrophils from asthmatics to produce CXCL8, whose production is positively correlated with FEV_1 and FEV_1/FVC. It showed that circulating neutrophils might be related to airflow limitation. These exteriorly conflicting reports tell us that neutrophils may be not the main source of CXCL8 in BALF [63]. In addition, Page et al. reported that challenging with German cockroach feces towards airway could migrate neutrophils into the airways and activate neutrophil cytokine production via TLR2 [64].

2.2.3. Inflammation Caused by Neutrophils. The inferior management of asthma in clinic practice is attributed to the persistent uncontrolled inflammation. Hosoki et al. report that allergens, such as pollen and cat dander, recruit neutrophils in a TLR-4/CXCR-2/MD2-dependent manner to the airways. The recruited neutrophils could produce ROS/leukotrienes/IL-33/TSLP (thymic stromal lymphopoietin) (Figure 1, a). All of these could cause the airway inflammation and allergic sensitization [65]. TLR4 expressed on hematopoietic cells is critical for neutrophilic airway inflammation following LPS exposure and for Th17-driven neutrophilic responses to the HDM lysates and ovalbumin (OVA). But at airway epithelial cells, TLR4 could also participate in the eosinophilic airway inflammation [66]. As described above, challenging with inhaled DP in asthmatics would not only increase the chemotaxis of neutrophils but also promote the production of ROS and the phagocytic activity, reflecting an enhanced systemic inflammation [44]. Moreover, apoptotic neutrophils in airway tissues could undergo secondary necrosis as the cause of inflammation [67].

TLR4 and cytokines seem to participate in the inflammatory process. The function of neutrophils from asthma patients was impaired with the lower levels of IL-8, IL-1β, and TNF-α and decreased Tlr4 gene expression [68]. All of these changes would lead to increased susceptibility and severity of infections. But this was conflicted with the discovery reported by Baines et al. which claimed that noneosinophilic asthma had an elevated level of IL-8 [69]. Simpson et al. reported that clarithromycin, which had additionally been used to reduce neutrophilic inflammation in asthma, can significantly reduce airway concentrations of IL-8 and neutrophil accumulation and activation in the airways of patients with refractory asthma [70]. Corresponding to the reports above, the withdrawal of inhaled corticosteroid (ICS) can lead to an increase of neutrophils and IL-8 in sputum [71]. The distinct role of IL-8 reported by researchers can be attributed to the different clinical research group design. In detail, noneosinophilic asthma is not equal to the neutrophilic asthma completely as previously described.

Recent investigation reported that a series of secretory protein (CCSP) could significantly reduce oxidative burst activity and increase phagocytosis of neutrophils [72]. CCSP could also enter neutrophils and alter their function. Secretoglobin protein, as a sort of CCSPs, has anti-inflammatory properties. Cote et al. reported that secretoglobin 1A1A could increase neutrophil oxidative burst and phagocytosis. Neutrophilic extracellular traps (NETs) are significantly reduced by secretoglobins 1A1 and 1A1A. Their functional difference may contribute to the pathogenesis of recurrent asthma obstruction [73]. These proteins have the potential to be novel therapeutic targets in the future.

2.2.4. Prosurvival of Neutrophils. Granulocytes, including eosinophils and neutrophils, have the significant capacity to evoke tissue inflammation and remodeling. The removal of these granulocytes would contribute to the resolution of airway inflammation in asthma. Tian et al. discovered that promoted apoptosis of inflammatory cells, such as eosinophils and neutrophils would be essential for the clearance of allergen-induced airway inflammation, especially for corticosteroid-insensitive neutrophilic airway inflammation [74]. However, noneosinophilic asthma shows an enhancement of blood neutrophil chemotaxis and survival [69]. Uddin et al. enumerated the apoptotic neutrophils in sputum from asthmatic patients with different disease severities and found amongst a subset of neutrophilic asthmatics (>65% PMNs) their sputum neutrophils inversely correlated with lung function (FEV1, % predicted) due to unidentified factors present in sputum [75]. HDM-DP, as an allergen from our environment, could induce the secretion of myeloid-related protein 8 (MRP8, S100A8) and MRP14 (S100A9). The combination of them might activate TLR4/Lyn/PI3K/Akt/ERK/NF-kappaB pathway so as to produce an antiapoptotic effect on neutrophils [76] (Figure 1, d). The similar discovery was also reported by Lee et al. [77]. The survival of neutrophils in BALF would bring up a poor lung function, consistent with the decline of FEV1, as Sikkeland et al. discovered [78] (Figure 1, e).

2.3. Where Is the Potential Targeted Therapy? It is believed that the failure of targeting neutrophils could be attributed to an incomplete understanding of underlying mechanism of neutrophilic asthma [79]. New insights into emerging neutrophil biology and underlying mechanisms of neutrophil phenotype might come to be the evidences of precision-based medicine. ICS is still the first-line medicine for ameliorating syndromes. Coinhalation of roflumilast and fluticasone, which reduces the counts of both neutrophils and eosinophils in BALF, could significantly improve the inflammatory condition in OVA-induced mice compared with the combination of formoterol and fluticasone [80].

Manually synthetic chemical drugs have an important curative effect on various diseases all along. Simvastatin, as an effective serum cholesterol-lowering agent could reduce the percentage of neutrophil in BALF and improve airway inflammation and remodeling in obese asthma mice [81]. Tamoxifen had a direct action on equine peripheral blood neutrophils and dampened the respiratory burst production [82]. Rosiglitazone (RSG), a peroxisome proliferator-activated receptor-γ agonist, has been reported to attenuate airway inflammation by inhibiting the proliferation of effector T cells in a murine model of neutrophilic asthma in vivo [48]. It can also downregulate the ratio of Treg and Th17 cells, inhibit the secretion of Th2 cytokines, and further inhibit the airway inflammatory response in asthma mice effectively [83]. AZD5069 as an antagonist of CXCR2, a receptor promoting neutrophils back to the inflamed airways, could reversibly reduce circulating neutrophils' count [84]. SCH527132, a selective CXCR2 receptor antagonist, can reduce sputum neutrophils and tend to improve the Asthma Control Questionaire scores of asthma [67].

Medicine from various plants has composed a large part in health care filed. Ligustrazine [85], water extract of *Helminthostachys zeylanica* (L.) Hook [86], astragalin as an anti-inflammatory flavonoid present in persimmon leaves and green tea seeds [87], hydroethanolic extract (70%) of *M. longiflora* (HEMI) [88], bufalin [89], and cordycepin [90] could target to the neutrophils, intervene the different inflammatory signaling pathways, and improve the prognosis of asthma. Biopharmaceutical has become a new treatment for asthma in recent years. Recombinant human activated protein C (rhAPC) could attenuate HDM + LPS-induced neutrophil migration in allergic asthma [91]. Another recombination protein, recombinant human IL-4, could inhibit airway inflammation in bronchial asthma by reducing the cytokines and inflammatory cells including neutrophils [92]. Medicine from plants and targeted biopharmaceuticals has a huge potential to play a major role in future medical field.

3. Neutrophils in COPD

3.1. Neutrophils Participate in COPD. The role of neutrophils in COPD is different from that in asthma [93]. In asthma, neutrophils are important only in some relatively rare and severe subtypes. However, neutrophils seem to always play a major role in COPD. The activation of neutrophils in the lung is directly correlated with the severity of symptoms [93]. The elevated neutrophil-lymphocyte ratio (NLR) can be used as a marker in the determination of increased inflammation and early detection of potential acute exacerbations in COPD patients. [94]. Although neutrophils in COPD patients' airway mucosa play a key role in antimicrobial defense theoretically as described in present review, a high number of neutrophils in patients' lungs are believed to correlate with poor prognosis [95]. It is well known to us all that neutrophil is an important part in maintaining our host defense and responding to injury and microbial infection. The removal of neutrophils can promote a return to homeostasis after its short-lived journey from circulation to injured or infected site. As Bratton et al. described, if not being removed, the dying neutrophils may even contribute to the ongoing inflammation, tissue damage, or autoimmune diseases by disintegrating and releasing various phlogistic cargoes [96].

3.2. The Role of Pathogen Infection and Proteases from Neutrophil in COPD. The pathogens' colonization, such as microbiota in lower respiratory airway, could interact with immune system. Airway bacterial loading at baseline correlates with sputum percent neutrophil count [97]. In COPD, exposure to bacterial pathogens could cause characteristic innate immune responses in peripheral blood monocytes and polymerphonuclear neutrophils (PMN), accompanied with the elevated protein expression of IL-8/IL-6/TNF-α/IFN-γ [98] (Figure 2, b). At the same time, Guiot et al. also observed that COPD patients had higher IL-6, IL-8, TNF-α, and MMP-9 in their induced sputum [99]. Treatment with levofloxacin in stable COPD could reduce the bacterial loading in a short time. This reduction is associated with the decrease of neutrophilic airway inflammation in patients with high airway bacterial loads [97].

Neutrophils contain a great deal of proteases, inflammatory mediators, and oxidants [100]. IL-22/IL-22R signaling pathway plays a pivotal role in antimicrobial defense. Influenza virus can promote the expression of IL-22R in human bronchial epithelia cells (Figure 2, a). Neutrophil-derived proteases may contribute to COPD by impairing the antimicrobial IL-22/IL-22R signaling pathway and decreasing the expression of antimicrobial effectors such as β-defensin-2. This process probably enhances pathogen replication and ultimately causes COPD exacerbations [95].

Neutrophil elastase (NE), one of the main proteases produced by neutrophils, plays an important role in inflammatory process. NE is able to increase the release of chemokines from epithelial cells with the activation of p38-α-MAP-kinase. The production of these chemokines can be blocked by roflumilast N-oxide combined with prostaglandin E_2 [101]. Neutrophil elastase-generated fragments of elastin (EL-NE) is different between stable and exacerbation of COPD patients. The serum level of EL-NE is associated with lung function [102]. Elastin breakdown mediated by neutrophil elastase is associated with COPD-induced inflammation [103]. α-1-Antitrypsin (A1AT), as an endogenous inhibitor of NE, can limit lung damage. Its effect had been already reviewed by Meijer et al. [93]. Deficiency of it is known as a risk factor for lung function [93]. Geraghty et al. reported that bioactive A1AT could modulate phosphatase 2A, which played a key role in COPD and expressed on the neutrophils to prevent inflammatory and proteolytic responses triggered by TNF-α simulation in the lung [104]. Whether A1AT or its unknown analogues can be used as a potential novel medicine needs further scientific research and clinical validation.

3.3. Adverse Effect of Cigarette Smoke via Neutrophils. Cigarette smoking (CS), a main environmental trigger of COPD [93], can decrease sputum neutrophils significantly. Experiments in vitro showed that CS could induce necrotic

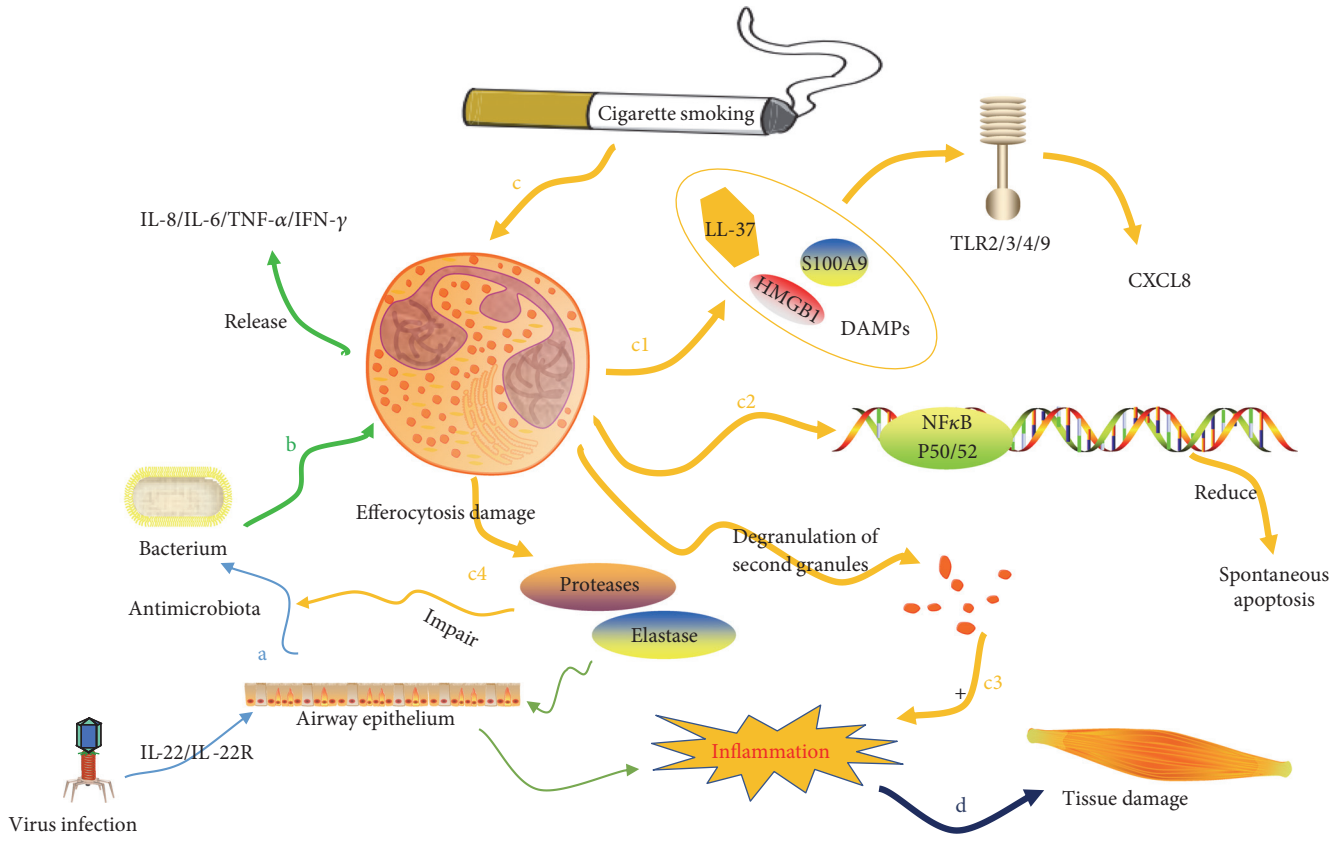

Figure 2: a: infection of virus can stimulate airway epithelium and produce antimicrobiota defense through IL-22/IL-22R signaling pathway. b: the colonization of bacterial pathogens could cause the release of IL-8/IL-6/TNF-α/IFN-γ. c1: cigarette smoking (CS) could induce the release of damage-associated molecular patterns (DAMPs). DAMPs can activate the innate immune system by binding to pattern recognition receptors (PRRs), such as TLR2, TLR4, and TLR9, and further induce CXCL8 release via TLR9 activation. c2: CS increase the expression of both p50 and p65 subunits of NF-kappaB in neutrophils and reduce the spontaneous apoptosis of neutrophils. c3: CS could cause the degranulation of secondary granules. This contributes to the accumulation of neutrophils and inflammation within the airways of smokers. c4: the efferocytosis damage caused by CS can increase the release of proteases and elastases. It not only impairs the antimicrobial defense but also (d) promotes the pulmonary inflammation and tissue degradation.

neutrophil cell death through mitochondrial dysfunction, apoptosis inhibition, and damage-associated molecular pattern (DAMP) release [105, 106]. DAMPs can activate the innate immune system by binding to pattern recognition receptors (PRRs), such as TLR2, TLR4, and TLR9. DAMP signaling plays an important role in the activation of neutrophils during COPD exacerbations. Serum level of DAMP gene expression is increased during COPD exacerbations [106]. TLR2, TLR4, and NLRP3 expressions in neutrophils are increased during acute exacerbations of COPD compared with stable disease. The activation of TLR2/TLR4 can induce the activation and the migration of neutrophils and may thus contribute to the elevated airway inflammation during COPD exacerbations [107]. The detailed positive feedback loop between DAMPs and TLR4 and the function of neutrophils' TLR4/TLR3 in respiratory diseases had already been reviewed by other researchers [106–108]. The novel therapeutic strategies aimed at DAMPs and its receptors need more attention.

Normal human bronchial epithelial cells could release more CXCL-8 significantly when stimulated with the supernatants from CS-treated neutrophils [105]. While in COPD patients, it was showed that there was an increase of inflammatory response and a decrease of PMNs apoptosis, which is independent from antiapoptotic cytokines such as CXCL-8 [109]. Mortaz et al. reported that cigarette smoking extract (CSE) could induce CXCL8 release via TLR9 activation [110] (Figure 2, c1). In addition, CSE could cause the degranulation of secondary granules. This may contribute to the accumulation of neutrophils and inflammation within the airways of smokers (Figure 2, c3). Furthermore, it promotes the pulmonary inflammation and tissue degradation [111].

Many kinds of membrane moleculars expressed on the surface of neutrophils are able to take part in mediating the biological activity resulted from cigarette smoke. Hoonhorst et al. reported that young individuals susceptible to COPD showed a significantly higher increase in the expression of Fc-γ-RII (CD32) in its active forms (A17 and A27) on neutrophils after smoking. This may indicate that systemic inflammation could participate in the early induction phase of COPD [112]. CD9, a transmembrane protein of the tetraspanin family, facilitates some pathogens and other foreign matters. HDAC and Rac have already been demonstrated

that they are required for efferocytosis [100]. Noda et al. reported that smoking could impair efferocytosis of neutrophils via inhibition of HDAC/Rac/CD9 pathways (Figure 2, c4). The following release of toxic intracellular contents from apoptotic neutrophils could cause tissue damage [100] (Figure 2, d).

The ability of ingesting respiratory pathogen is compromised in CSE-exposed neutrophils. As a result, it contributes to persistent existence of bacterium in the smokers' lungs and promotes further recruitment of neutrophils. Guzik et al. discovered a lack of apoptotic neutrophil populations in smokers' lungs, and these smokers exhibited an increased susceptibility to bacterial infections [111].

Human neutrophils share typical cell death features, including apoptosis, autophagy, and necrosis after exposure to CSE. These neutrophils could be effectively recognized and phagocytized by monoderived macrophages [111]. Neutrophils could also undergo a spontaneous and phagocytosis-induced apoptosis in a caspase-3-dependent manner. The suppression of caspase-3 activity induced by CSE does not alter spontaneous apoptosis but impair the phagocytic activity. The complex functions of caspase-3 may contribute to the persistent existence of neutrophils in smokers' lungs. At the same time, caspase-3 could also cause a higher incidence of community-acquired pneumonia [113]. In addition, percentage of sputum neutrophils undergoing spontaneous apoptosis in the subjects with COPD reduced significantly. The increased expression of both p50 and p65 subunits of NF-kappaB in neutrophils from COPD individuals may explain the phenomenon above [114] (Figure 2, c2). Interestingly, Makris et al. observed an increase of sputum apoptotic neutrophils' percentage in ex-smoking patients with COPD by the way of in situ detection with TUNEL assay technique [115]. Whether these conflict results are related with the distinct exclusion criteria of subjects needs further investigation.

3.4. Study on the Extracellular Traps of Neutrophils. Polymorphonuclear neutrophils have attracted new attention due to its ability of releasing web-like extracellular structures, which has been named as neutrophil extracellular traps (NETs) recently [116]. NETs' formation has been identified to be an essential part of innate immunity [117]. These NETs deriving from nuclear chromatin may have an ambiguous two-side effect on antimicrobial defense and host tissue damage. Microbicidal and cytotoxic proteins decorate the NETs, which are constituted with DNA strands of varying thickness. Their principal chemical structures have been characterized at molecular and ultrastructural levels in recent years. Nonetheless, many features relevant with cytotoxicity are still not clear completely [116]. Pedersen et al. observed a significant upregulation of NET formation, which was associated with significantly higher concentration of extracellular DNA in sputum supernatant of COPD [112]. Astrid et al. studied the genesis and structure of NETs from sputum of COPD patients. It was concluded that the genesis of NET was an integral part of COPD pathology. Moreover, the release of "beads-on-a-string" DNA studded with noncitrullinated histones, as Astrid et al. described, is a common feature of genesis of NETs in vivo. All of these are relevant to the antimicrobial and cytotoxic effects of NETs [116].

3.5. Pharmacological Mechanism of Neutrophil Targeted Therapy in COPD. Bronchodilators have always been a clinical first-line medication to ameliorate the symptoms in COPD [93]. Anderson et al. tested the effect of three β2-agonists (formoterol, indacaterol, and salbutamol) on the inhibition to the proinflammatory activity. The results showed that formoterol and indacaterol could effectively decrease the potential harm produced by stimulated neutrophils in vitro, while the effect of salbutamol was weaker. The anti-inflammatory actions of formoterol and indacaterol could contribute to the therapeutics in COPD [118]. Tanabe et al. reported that thioredoxin-1 could improve neutrophilic inflammation by suppressing the release of GM-CSF and enhancing the expression of MAP kinase phosphatase 1. All these indicate that thioredoxin-1 is a novel potential therapeutic agent for treating the exacerbation of COPD [119]. Keratin sulfate disaccharide repeating unit designated L4 could significantly attenuate inflammation in the lung by reducing neutrophil influx as well as the levels of inflammatory cytokines, tissue-degrading enzymes (matrix metalloproteinases), and myeloperoxidase in BALF [120]. More clinical trials are needed to further reveal the potential of these related medicines.

Similar to asthma, ICSs are also the common therapy strategy in clinical practices to control COPD. However, the discontinuation of ICS can increase the inflammation in COPD showing as an increased sputum cell counts including neutrophils [121]. At the same time, the effect of ICS in COPD patients is also dependent on the genotype of glucocorticoid-induced transcript 1 rs37973 in COPD patients. The effect of dexamethasone-mediated neutrophil apoptosis is impaired in homozygous GG genotype. In addition, CSE-induced neutrophil apoptosis could only be slightly attenuated by a relatively higher concentration GG genotype. As for AA and AG genotypes, dexamethasone can reduce the proapoptotic effect of CSE in a concentration-dependent manner [122]. Besides, aminophylline and theophylline, two typical medicines used in therapy for COPD, could restore the impairment caused by trichostatin A (TSA), a Rac inhibitor. This protective effect may have the potential to develop a novel therapeutic strategy to restore efferocytosis in COPD patients [100].

4. Neutrophils in Infectious Pneumonia

Pneumonia is a common infectious lung disease accompanied with inflammation in airway and alveolar and interstitial lung. Pneumonia is usually categorized as community-acquired pneumonia (CAP) and hospital-acquired pneumonia (HAP). Bacterial infection is the most common etiology of pneumonia as observed in our clinical practice. Neutrophils can function with other immune cells synergistically to control the pathogenic infection in pneumonia [123]. Evidence on the participation of neutrophil in the pathogenesis of pneumonia has been accumulated in recent years. For example, the neutrophil percentage in BALF was higher in the

relapse group of organizing pneumonias and may also be considered as a predictive factor of organizing pneumonia relapse as Onishi et al. reported [124]. But, on the contrary, CAP had significant low neutrophil counts in peripheral blood [125]. In addition, NLR together with red blood distribution can even be used as adjuncts to distinguish HAP and CAP [126].

4.1. Pseudomonas aeruginosa Pneumonia. Pseudomonas aeruginosa (PA) as an important opportunistic human pathogen can take part in the pathogenesis of pneumonia. PA lives in biofilm-like cell and aggregates at sites of chronic infection. During growth in a biofilm, *P. aeruginosa* dramatically increases the production of filamentous Pf bacteriophage (Pf phage). The Pf phage can trap within the lung PA by preventing the dissemination of *P. aeruginosa* from the lung in pneumonia and inhibit bacterial invasion of airway epithelial cultures. Importantly, the production of Pf phage was also associated with reduced neutrophil recruitment [127].

Mechanical ventilation is routinely used to treat patients with respiratory distress. However, a number of patients on ventilators exhibit enhanced susceptibility to infections and develop ventilator-associated pneumonia (VAP). PA is one of the most common species of bacteria found in these patients [128]. Mechanical ventilation after supernatant from PA-stimulated macrophages can induce more neutrophil sequestration in the lungs in wild-type mice than JNK1-deficient mice. Moreover, the pathogenesis mechanism of PA-VAP may involve the production of TNF-α through activation of IKK/NF-kappaB pathways in alveolar macrophages and JNK signaling pathway in the lungs [129]. A recent research indicates that IRF-3 can exacerbate PA-induced mortality in mice by inhibiting neutrophil adhesion and recruitment to the lungs [130]. In addition, the inhibition of full-length receptor for advanced glycation end product (FL-RAGE) shedding can be a novel mechanism for controlling inflammation to acute PA pneumonia [131]. Deepening the exploration of its pathogenesis will contribute to the next future clinical application.

The persistent presence of PA and huge recruitment of neutrophils in the lung are associated with the elevated level of high mobility group box 1 (HMGB1) in airways in respiratory diseases [132]. Exposure to hyperoxia leads to a significant elevation in HMGB1 and increased mortality in C57BL/6 mice infected with PA. Treatment of these mice with a neutralizing anti-HMGB1 monoclonal antibody can result in a reduction in bacterial counts, injury, and numbers of neutrophils in the lungs and an increase in leukocyte phagocytic activity [133]. This finding reveals a potential medical target. More related clinical trials are needed to validate it.

4.2. Streptococcus pneumoniae Pneumonia. Streptococcus pneumoniae (SP) is a common cause of pneumonia and infective exacerbations of chronic lung disease [134]. During lung infection, mice colonized with SP had an increased early neutrophil recruitment and reduced bacterial colony-forming units in the lungs and BALF. Colonization-induced protection was lost when experiments were repeated in B-cell-deficient or neutrophil-deficient mice [134]. Yet how neutrophils specifically prevent SP lung infection has been complex and still unclear till now.

Nucleotide-binding oligomerization domain-containing (NOD) 2 as a pattern recognition receptor can detect peptidoglycan fragments of SP. Nod2-deficient blood neutrophils displayed a reduced capacity to internalize pneumococci in vitro. But NOD2 does not contribute to host defense during pneumococcal pneumonia. Pneumococcal capsule can impair recognition of SP by NOD2 as Hommes et al. discovered [135]. Triggering receptor expressed on myeloid cells-1 (TREM-1) is a receptor on phagocytes known to amplify TLR- and NOD-like receptor inflammatory signaling [136, 137]. TREM-1/3 deficiency leads to an increased lethality, accompanied by enhanced growth and dissemination of SP. Trem-1/3-deficient mice demonstrated a strongly impaired innate immune response in the airways reflecting as a delayed influx of neutrophils [138]. However, the influx of endothelial protein C receptor positive neutrophils into lung tissue can cause pneumonia after the infection of SP, because endothelial protein C receptor can impair antibacterial defense in pneumococcal pneumonia [139].

Moreover, IL-1 can contribute to the host defense against SP independent on the recruitment and the bacteria-killing ability of neutrophils [140]. Alveolar neutrophils with single immunoglobulin IL-1 receptor-related molecule (SIGIRR) deficiency exhibit an increased capacity to phagocytose viable pneumococci but no impact on neutrophil recruitment. Besides, SIGIRR as a negative regulator of TLR signaling can impair the antibacterial host defense during pneumonia caused by SP [141].

4.3. Staphylococcus aureus Pneumonia. Staphylococcus aureus (SA) is a Gram-positive bacterium that persistently colonizes about 20% of the human population [142]. This high prevalence of SA is responsible for various illnesses in humans and animals worldwide, including the respiratory diseases [143, 144]. Similar to SP, infection with SA can exhibit an early increase in neutrophils that did not persist despite continued presence of the bacteria in neonatal mice. However, adult mice exhibited an increase in neutrophil recruitment that coincided with reduced bacterial titers [145]. SA, as an important pathogen, can efficiently cleave the pulmonary surfactant protein-A (SP-A), a major component of immune functions during SA infections. This degradation appears to result in a decrease or complete abolishment of SP-A biological activity, including the promotion of SA phagocytosis by neutrophils [146]. Recently, Dietert et al. reported that in SA pneumonia murine model, the deficiency of calcium-activated chloride channel regulators (mCLCA3) can lead to a decrease of neutrophil infiltration during infection. Moreover, mCLCA3 appears mainly to modulate leukocyte response via IL-17 and murine CXCL-8 homologs in acute SA pneumonia [147].

4.4. Klebsiella pneumoniae Pneumonia. Nosocomial infection with *Klebsiella pneumoniae* (KP) is a frequent cause of gram-negative bacterial sepsis [148]. C-type lectin receptor is an

innate immunity-related receptor, which can interact with pathogenic-associated molecular patterns [149]. Mincle and macrophage galactose-type lectin-1 (MGL1) are C-type lectin receptors. The deficiency of them can lead to a massive accumulation of neutrophils and a severe hyperinflammation in the lungs of KP-infected pneumonia. Importantly, Mincle-deficient neutrophils had an impaired ability to phagocytize bacteria and to form extracellular traps (NETs), which could clear the invading KP [148]. Similarly, MGL1-deficient neutrophils exhibited an increased influx in pneumonic lungs of KP-infected mice. Neutrophilic inflammation resolution relies on MGL1 during KP infection [150]. As previously described, NLRC4 belongs to the NOD-like receptor family and is involved in the assembly of the inflammasome complex [151]. NLRC4 can participate in the neutrophil chemoattractant in the lungs infected by KP. NLRC4 signaling contributes to KP-induced lung inflammation and neutrophil accumulation, which can be partially rescued by exogenous IL-1β in the lungs of NLRC4-deficient mice [151]. Myeloid-related protein 8 (MRP8, S100A8) and MRP14 (S100A9) are the most abundant cytoplasmic proteins in neutrophils. MRP8/14 heterodimers can inhibit bacterial dissemination and prevent the growth of KP in vitro. Mrp14 can take part in the genesis of neutrophils NETs to inhibit KP growth. Taken together, MRP8/14 is a key player in protective innate immunity during KP pneumonia [152].

4.5. Mycoplasma pneumoniae Pneumonia and Recent Therapy Advances in Pneumonia. Mycoplasma pneumoniae is also a significant cause of respiratory diseases including CAP for all ages [153]. *Mycoplasma pneumoniae* pneumonia may be altered by the level of host cell-mediated immunity [154]. *Mycoplasma pneumoniae* pneumonia has an increase of neutrophils and IL-6, especially in severe group. Compared to acute stage, a decreased percentage of neutrophils and IL-6 level was observed at the recovery stage in children with severe *Mycoplasma pneumoniae* pneumonia [155]. However, treatment with prednisolone or cyclosporin-A leads to marked neutrophils and exudates in the alveolar lumen.

Some traditional antibiotic therapeutic strategies such as macrolide containing regimen showed no statistical difference between cytokine levels or neutrophil activity for CAP patient [156]. Targeted and precise therapy has made an advanced progress in recent years. Vaccine as a typical biological therapy strategy had been attracting a huge attention since it was created. For example, recombinant Bacillus Calmette-Guerin (BCG) vaccine can decrease the infiltration of neutrophils within airways and reduce the viral loads in BALF in mice infected with respiratory syncytial virus (RSV) showing a potential to prevent pneumonia [157]. Trivalent pneumococcal protein recombinant vaccine vaccination results in a reduction in SP-induced lethality, enhanced early clearance of SP in lungs due to more rapid and thorough phagocytosis of SP by neutrophils, and correspondingly, a reduction in lung inflammation and tissue damage [158]. In addition, cathelin-related antimicrobial peptide appears to be protective in models of pneumonia [159]. α-Tocopherol form of vitamin E can reverse age-associated susceptibility to SP lung infection by decreasing pulmonary neutrophil recruitment [160].

5. Neutrophils Participate in Other Respiratory Diseases

5.1. Neutrophils in Lung Cancer. Lung cancer is still a common killer in all kinds of cancers and is also one of the most common cancers diagnosed globally [161]. The traditional role of neutrophils in pathogenesis of many kinds of tumors had already been reviewed by Zhang et al. briefly [162]. Here, we focus on the recent advances about the participation of neutrophils in lung cancer. It has been demonstrated that neutrophils could participate in the carcinogenesis in murine lung cancer model [163]. Lung squamous cell carcinoma mouse models contain more tumor-associated neutrophils compared to mouse adenocarcinomas [164]. Elastase from neutrophils could involve in lung cancer by inducing mitogenesis after entering the cells [165]. BALF from lung cancer patients contained higher level of neutrophils and lower percentage of total macrophages [166]. BALF of lung cancer patients had markedly higher levels of VEGF and IL-8, which was positively correlated to the numbers of neutrophils and lymphocytes. Tumor-associated neutrophils represent an important source of MMP-9, whose expression in tumor region is increased in non-small-cell lung cancer [167]. According to their results, the detection of infiltrating inflammatory cells and proangiogenic factors have the potential to be diagnosis indexes for cancerous inflammation in lungs [166].

Neutrophil-lymphocyte ratio (NLR), reflecting host immunity and systemic inflammation that facilitates tumor growth, could be an independent prognostic index for lung adenocarcinoma patients who undergo the complete resection [168]. NLR can also be an independent prognostic factor for overall survival. The evaluation of NLR can help identify patients with poor prognosis and appears to be a useful prognostic marker in clinical practices [169]. The model established by Jiang et al. utilizing multiple immunological markers, such as monocyte ratio, NLR, PD-L1 immunostaining score and PD-1-positive stained tumor-infiltrating lymphocyte counts, can offer a novel tool for survival prediction. This model has important clinical implications for patients with squamous non-small-cell lung cancer [170]. At the same time, NLR, together with other parameters such as age, gender, and smoking history, can be used to predict the prognosis of small cell lung cancer [171]. Consistent with the findings from Jeong et al. [172], Derman et al. found that the progressive increases of NLR are associated with the progressive disease, inferior overall survival, and weight loss in non-small-cell lung cancer patients [173]. The precise identification and prediction to the prognosis of all lung cancers with different histotypes is beneficial for future individualized therapy [170].

More recently, the role of myelomonocytic siglecs has attracted a greater attention [174, 175]. It is a receptor engaging lineage with sialic acids with dual functions towards cancer progression depending on the different stages of tumor growth and the microenvironment. Neutrophils

involved in this process play an important role. Neutrophils can express siglec-9 and interact with tumors. Tumors could interact and suppress the activation of neutrophils utilizing the ligand expressed on the tumor cells and acting on the siglec-9. In keeping with this, human polymorphism of the related gene that reduced siglec-9 binding to carcinomas could improve the survival of patients with early non-small-cell lung cancer [176], which accounts for 85 percent of all lung cancer according to their histotypes [177]. As for lung cancer histotypes, it has been demonstrated that lung cancer could exhibit a pronounced heterogeneity and differential immunological characteristics. For example, adenosquamous carcinoma showed a histotype-specific recruitment of $CD11b^{+}Gr\text{-}1^{+}$ tumor-associated neutrophils [178].

Metastases are the major cause of death from cancer. Lung is the most common metastatic site for many other cancers [179]. Impaired type I IFN signaling could develop more lung metastases. The higher metastasis is accompanied with massive neutrophil accumulation in the lungs. This is most likely due to elevated G-CSF levels in serum and enhanced CXCR2 expression on neutrophils. Reduced neutrophilic cytotoxicity against tumor cells can enhance metastasis [179]. Lung-infiltrating neutrophils facilitate an improved premetastatic niche formation [180–182]. Developing premetastatic niche can enhance metastasis [179]. In addition, intranasal delivery of CCL2 increases CD4+ T cell recruitment to the premetastatic niche of the lung, and this correlates with enhanced seeding and growth of tumor cells [183], while CCL2 shows a potential antitumor activity in tumor-entrained neutrophil-mediated tumor killing in vitro. In addition, $\gamma\delta$-T-cell could indirectly act on systemic expansion and polarization, suppress CD8+ T cell, and then cause a sequent metastasis formation. All of these indicate that the interaction between $\gamma\delta$-T-cell and neutrophil may contribute to the metastasis of carcinoma [184].

There are some interesting researches about how neutrophils participate into the pathogenesis of metastatic lung cancer. Bald et al. reported that ultraviolet (UV) exposure of primary cutaneous melanomas could promote metastasis dependent on the recruitment and activation of neutrophils which is initialed by HMGB1 [185]. It is also reported that an inhibitory host protein member of B7 family called as B7x may promote cancer cells to metastasize through interacting with innate and adaptive immune systems. The presence of B7x is correlated with an increased infiltration of tumor-associated neutrophils into tumor-bearing lungs [186]. Colorectal cancer can process a lung metastasis, which is dependent on CCL15-CCR1 axis. Their immunofluorescent staining results showed that most CCR1+ cells around lung metastases were tumor-associated neutrophil [167]. Although the role of neutrophils in metastatic lung cancer is still unidentified clearly, more and more emerging researches will tell a systematic integral story in the future.

5.2. Neutrophils in Pulmonary Fibrosis and Cystic Fibrosis. Pulmonary fibrosis is a common interstitial lung disease [187]. Traditionally, pulmonary fibrosis is tightly associated with immune component in the lung. A greater number of neutrophils in the BALF were associated with the increased early mortality of pulmonary fibrosis [188]. The end-stage cystic fibrosis (CF) explant lung tissue showed an increase of neutrophils. At the same time, there was a disproportionate increase of neutrophils around the airway in CF [189].

Pulmonary fibrosis murine model is always established with bleomycin injection. Neutrophils can take part in the acute inflammatory process. The occurrence of the acute inflammation is accompanied with the production of collagen in parallel [190]. Interestingly, bleomycin-induced lung fibrosis can be relieved by the reduction of soluble glycosaminoglycan (GAG), which could reduce the neutrophil transmigration and decrease the CXCL-8/neutrophil-mediated inflammation [191]. Carbohydrate antigen sialyl Lewis is secreted from the bronchial gland apically. Obayashi et al. reported that carbohydrate antigen sialyl Lewis in BALF could participate in the process of lung injury and repair in pulmonary fibrosis by modifying the function of neutrophils [192].

Serum amyloid P (SAP) is a pattern recognition molecule and could interact with pathogens and cell debris to promote their removal by macrophages and neutrophils [193]. Cox et al. reported that SAP could strongly affect several aspects of innate immune system and reduce fibrosis by binding to SAP-binding receptor (DC-SIGN), which is present on mouse lung epithelial cells. Binding of DC-SIGN receptor with SAP could reduce neutrophil accumulation in the acute lung inflammatory model and alleviate pulmonary fibrosis by increasing levels of immunosuppressant IL-10 [194]. In addition, they also reported that SAP could inhibit fibrocyte differentiation and reduce neutrophil adhesion by binding to Fc-γ-RI on monocytes and binding to Fc-γ-RIIa on neutrophils, respectively [195].

Cystic fibrosis (CF) lung disease as a genetic disease is displayed as a chronic and nonresolving activation of innate immune system, accompanied with the release of neutrophil-derived oxidants and proteases and chemokines and an infiltration of neutrophils into the airways [196]. Subjects with stable CF had not only significant elevated levels of proinflammatory genes and its products but also an elevated MMP8/9 and neutrophil elastase [197]. In addition, patients with CF and small airway disease had pronounced sputum neutrophil counts and elevated level of IL-6 [198]. The traditional role of immunity in CF had already been reviewed by Rieber et al. [196]. PMNs, which is recruited massively into the cystic fibrosis lumen, could modulate arginase 1 and suppress the early PMN-driven T cell in CF. All of these might hamper the resolution of infection and inflammation in CF airway lumen [199].

HMGB1 is an alarmin released from macrophages after infection or inflammation and is a biomarker of lung disease progression in patients with cystic fibrosis [200]. Entezari et al. demonstrated that the elevated levels of HMGB1 in CF airways were essential for neutrophil recruitment and persistence of PA in the lung, which could significantly contribute to mortality in cystic fibrosis [132]. The infection of PA can secrete epoxide hydrolase, a kind of CF transmembrane conductance regulator (CFTR) inhibitor, which could cause neutrophil activation and tissue inflammation. The hydrolase could also increase IL-8 concentration, which

drives neutrophils' transepithelial migration in vitro as illustrated above. Finally, the lung function of CF patients is impaired [201].

CF is a fatal recessive genetic disease. Ng et al. demonstrated that CF could be attributed to the mutations in the CFTR gene. In detail, the mutation of the gene could compromise the phagocytic capacity of neutrophils and contribute to the infection of the CF lung [202]. Recently, Duchesneau et al. found that bone marrow cell delivery therapy can contribute to the restoration of CFTR expression in airway epithelium by recruiting neutrophils and macrophages [203].

Moreover, other related gene polymorphisms or the difference of expressive level can also have effect on the pathogenesis of CF or other lung fibrotic diseases. Hector et al. demonstrated that interferon-related development regulator-1(IFRD1) expression of neutrophils was systemically upregulated in CF. This regulation was related to the production of ROS and was modulated by chemokines in airway fluids, such as CXCL-8 and CXCL-2. The decrease of lung function was associated with the genotype of IFRD1 [204]. Forkhead transcription factor 3 (Foxp3) is a critical regulator of Treg [205]. The overexpression of Foxp3 in radiation-induced lung inflammation also showed a significant inhibition of neutrophilic infiltration in BALF. At the same time, overexpression of Foxp3 can decrease the expression of inflammatory and fibrosis-related genes [206]. Extracellular superoxide dismutase 3 (SOD3) is the only extracellular enzymatic defense against the free radical, superoxide. Impaired SOD3 activity is implicated in inflammatory and fibrotic lung and vascular diseases as Mouradian et al. reviewed. However, the redistribution of superoxide dismutase 3 as a result of R213G single-nucleotide polymorphism could protect mice from bleomycin-induced fibrosis by resolving the neutrophil infiltration in BALF [207].

The potential therapy of pulmonary fibrosis is rarely reported in recent years. Recently, Yang et al. found that glaucocalyxin A (GLA) could exert antipulmonary fibrosis activity in mice. GLA could significantly improve survival in bleomycin-treated mice and reduce the weight loss caused by fibrosis. At the same time, GLA could alleviate the infiltration of neutrophils in the lungs and attenuate the increase of proinflammatory cytokines in lung tissue and BLAF. In addition, GLA could inhibit the activation of NF-kappaB in fibrotic lungs [208]. Acebilustat, as a potential leukotriene A4 hydrolase inhibitor, could reduce sputum neutrophil counts by 65% in CF patients treated with 100 mg dosage [209]. Targeting chemotaxis of neutrophils has been a promising therapeutic direction [210]. Intracellular secretory leukoprotease inhibitor can exert an anti-inflammatory effect on neutrophils of individuals with CF and COPD by inhibiting the excessive influx of neutrophil [211]. PA401 as a recombinant therapeutic protein can disrupt the CXCL8:GAG complexes. And then, the chemokine CXCL8 is degraded. As a result, the chemotaxis of neutrophil and the inflammation decreased [212]. All in all, with the development of the detailed pathogenesis of pulmonary fibrosis, the targeted modulation of the related pathways may be of therapeutic benefit to patients.

5.3. Neutrophils in Bronchitis and Bronchiolitis. Acute and chronic bronchitis and bronchiolitis are common respiratory diseases [213]. Despite that most patients have a good prognosis, a few people still may be persistently unhealed. Immunity plays a critical but unidentified role in this process. The related inflammatory mechanism of neutrophils in airway inflammatory diseases and the role of proteases, mediators, and TLR2 in the incidence of the illness had already been reviewed [214]. But science has never stopped unraveling its nature. Recently, it has been demonstrated that neutrophilic infiltration in nasopharyngeal aspirate (NPA) samples was positively correlated with the degree of airway tissue injury in infants hospitalized with acute bronchiolitis [215].

Increased level of IL-8, a potent neutrophilic chemokine, is often strongly correlated with an increase in cellular infiltration [215]. For example, Dixon et al. reported that breastfed infants had lower level of IL-8 in their nasal compared to the formula-fed controls hospitalized with severe bronchiolitis. Meanwhile, there is a decrease in cellular infiltration, whose predominance is mature, secondary granule-laden neutrophils [216]. Chronic bronchitis is a risk factor for COPD. Patients with chronic bronchitis and COPD exhibit reduced immune regulation and increased innate immunity response in the lung [217]. Sahlander et al. have observed an increase of blood neutrophils in farmers exposed to the organic material. These farmers often suffered from the chronic bronchitis [218]. But in a more recent experiment, they only found that both the expression of CD62L and CD162 on blood neutrophils and the expression of CD14 on sputum neutrophils decreased. All of these indicated that chronic exposure of organic material may participate in the pathogenesis of chronic airway diseases, such as chronic bronchitis. This may involve the participation of neutrophils. In addition, they also discovered the exposure could increase the presence of bacteria in airways [219].

The presence of potentially pathogenic bacteria is positively correlated the severity of bronchiolitis. The percentage of neutrophils is higher in patients with potentially pathogenic bacteria [220]. Protracted bacterial bronchitis also had a marked neutrophil infiltration and more respiratory bacterial pathogens load, especially *Haemophilus influenza*. This may be related with activated innate immunity [221]. PA is very common in respiratory airways. Club cell secretory protein (CCSP) is a regulator, which could exert an immunosuppressive, anti-inflammatory, antiproteinase, and antiphospholipase A2 activities. Chronic PA inflammation can lead to chronic bronchitis in the CCSP-deficient mice. Neutrophils are increased in the BALF from the CCSP-deficient mice in comparison to wild-type mice [222]. In addition, colonization with Gram-negative bacteria was associated with higher levels of proinflammatory cytokines. The colonization would increase the severity of disease [223]. Borthwick et al. reported that conditioned media from PA-infected epithelial cells induced a potent proinflammatory phenotype in fibroblasts via an IL-1α-/IL-1R-dependent signaling pathway. The evaluated level of IL-1α is significantly correlated with IL-8 and neutrophil percentage in BALF [224].

Viral etiology could potentially contribute to the pathogenesis of bronchiolitis [215]. Human respiratory syncytial virus (HRSV) infections have a close relationship with many respiratory diseases, such as bronchiolitis, asthma, and pneumonia [225]. Neutrophils together with its proteases, NETs, and cytotoxic and direct interaction with infected epithelial cell play an important role in the pathological process [225]. RSV could induce NET formation in vitro. Conversely, NETs can capture RSV virions, indicating an antiviral role [226]. Bronchiolitis, usually requiring hospitalization in infants, is caused predominantly by RSV. It is one of the main causes of infant mortality and morbidity in developed world [216]. Suarez et al. reported that infants with RSV bronchiolitis had more systemic inflammatory cells, such as neutrophils and leukocytes, and more pathogenic bacterial colonization in nasopharyngeal. Besides, neutrophil infiltration is independent on the viral etiology nor the degree of viral coinfection, providing support for the neutrophil as a target of therapeutic intervention for the treatment of bronchiolitis in all virus-positive infants as Cavallaro et al. reported [215]. It is pleasuring that BPZE1, as a kind of attenuated Bordetella pertussis vaccine, could markedly attenuate RSV by inducing the efflux of neutrophils and increasing the production of IL-17 by CD4+ T cells [227].

In a special case, patients undergoing lung transplantation often suffer from obliterative bronchiolitis (OB). Clinical research showed us that the percentage of lymphocytes and neutrophils increased and the percentage of macrophages reduced in BALF of patients with OB [228]. This is similar with the reports, which suggest an involvement of neutrophils in OB from both Vandermeulen et al. and Eckrich et al. [229, 230]. Tiriveedhi et al. reported that neutrophil can participate in the obliterative airway disease and cause the early injury after passive transfer of CD8+ T cells [231]. They also reported that antibodies to MHC class I of the transplanted lung could induce both innate and adaptive cellular immune responses, which is characterized by a predominance of Th17. Their data indicated that Treg cells could suppress "anti-MHC induced IL-8-mediated neutrophil infiltration," which is critical for the development of obliterative airway disease [232]. VEGF-C/VEGFR-3 signaling can be involved in the pathogenesis of OB [233]. Upregulation of VEGF-C/VEGFR-3 signaling could induce epithelial activation, neutrophil chemotaxis, and significant neutrophilia. Both neutrophils and neutrophil chemoattractant human IL-8 contribute to the development of OB for its inflammatory infiltration [233].

Hyaluronan is an extracellular matrix component, which has been demonstrated to activate innate immunity, regulate inflammation, and could accumulate in BALF and blood of lung transplant recipients with OB. The low-molecular-weight form of hyaluronan can abolish the tolerance and promote the rejection of lung transplant through a mechanism dependent on innate immunity and neutrophils [234]. More details about its mechanism need further illustration. Adenosine is produced to protect tissues from injury when ischemia or inflammation occurs [235]. Zhao et al. described the role of $A_{2B}R$, a receptor of adenosine in the pathogenesis of OB. Neutrophil infiltration was decreased in $A_{2B}R$ knockout OB model on day 3 and day 21 but increased in wild-type models in the same time point. The results showed us that neutrophils could also take part in the pathogenesis of OB depending on $A_{2B}R$ to some extent [235]. In contrast, $A_{2A}R$ could also participate in the inflammatory process in OB. Neutrophil infiltration was increased in $A_{2A}R$ knock-out OB model [236]. Whether the exogenous pharmaceuticals of adenosine can be helpful to relieving OB needs future clinical validation.

6. Conclusions

The critical role of neutrophils in immunity-associated diseases including respiratory diseases cannot be overlooked. The colonization of microbiota in airway acts as a trigger of neutrophilic inflammation. Different stimulations from our environment could produce a chemotactic activity towards the inflammatory sites for neutrophils. Innate and adaptive immune component could participate in the activation of neutrophils in many different respiratory diseases. Neutrophils often cooperate with lymphocytes synergistically and constitute a huge immune regulatory network. Different PRRs, such as TLR and NLR families, are indispensable to interact with DAMPs from the dying cells. After the immunological mission of neutrophils is accomplished, the apoptosis of neutrophils is very important for the withdrawal of inflammation or tissue damage. But the prosurvival of neutrophils usually aggravates the injury. Proteases, as key functional component from neutrophils, could exert a double-sided effect on the pathogenesis of respiratory diseases. They could clear the adverse factors to maintain the homoeostasis. On the other hand, their excessive secretion also contributes the injury of normal tissue. In addition, neutrophils have developed a unique NET system to play their role of double-edged sword. It can not only trap the pathogens but also amplify the inflammatory cascade. However, there are still many unanswered questions on the role of neutrophils in lung cancer, pulmonary fibrosis, cystic fibrosis, bronchitis, and bronchiolitis. Despite that the detailed pathogeneses of these diseases are fragments for now, it is believed that more and more scientific researches will connect them. Different pharmaceutical intervention had been investigated to act upon the neutrophils and manifested a promising effect to some extent. The clarification on the mechanism of traditional drugs and the development of new precise medicine, such as monoantibody and vaccine will be the following research direction. Neutrophils have the potential to be a new therapeutic target as expected in the future. Finally, understanding how neutrophils cooperate with other immune component to integrate the disease pathogenic mechanisms, and exploring how to develop novel avenues for therapeutic strategies aimed at the key pathway involved of neutrophils, will offer further insights and inform better treatment of respiratory diseases.

Conflicts of Interest

The authors all declare that there is no any conflict of interests regarding the publication of this review.

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Review Article

Neutrophilic Inflammation in the Immune Responses of Chronic Obstructive Pulmonary Disease: Lessons from Animal Models

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Chronic obstructive pulmonary disease (COPD) is a major cause of mortality worldwide, which is characterized by chronic bronchitis, destruction of small airways, and enlargement/disorganization of alveoli. It is generally accepted that the neutrophilic airway inflammation observed in the lungs of COPD patients is intrinsically linked to the tissue destruction and alveolar airspace enlargement, leading to disease progression. Animal models play an important role in studying the underlying mechanisms of COPD as they address questions involving integrated whole body responses. This review aims to summarize the current animal models of COPD, focusing on their advantages and disadvantages on immune responses and neutrophilic inflammation. Also, we propose a potential new animal model of COPD, which may mimic the most characteristics of human COPD pathogenesis, including persistent moderate-to-high levels of neutrophilic inflammation.

1. Introduction

Chronic obstructive pulmonary disease (COPD) is a major public health problem that is currently the fourth cause of death globally and affects about 10% of the adult population worldwide [1–3]. It is generally accepted that the neutrophilic inflammation observed in the lungs of COPD patients is intrinsically linked to the tissue destruction and alveolar airspace enlargement, leading to disease progression. Cigarette smoke injures epithelial cells and then releases "danger signals" which act as ligands for toll-like receptors (TLRs) in the epithelium. These actions contribute to the production of chemokines and cytokines, which results in an innate immunity. Products from the inflammatory cells may injure the extracellular matrix, leading to the release of TLR ligands and TLR activation, which in turn promote further inflammation and damage of lung parenchyma. Moreover, chronic cigarette smoke can induce an adaptive immune response, including $CD4^+$ T cells, cytolytic $CD8^+$ T cells, and B cells, leading to cellular necrosis and apoptosis, immune and complement deposition, tissue injury with airway remodeling, and emphysema [4]. Animal models act as a bridge between in vitro studies in the laboratory and studies in humans. As such, they have exerted a great impact on the investigation of many medical conditions. This review first gives an overview of the main experimental models of COPD to discuss their advantages and disadvantages in studying the neutrophilic inflammation in COPD and then try to propose a new, effective, and useful model.

2. Protease-Induced Emphysema Models

In 1965, Gross et al. [5] firstly proposed a model of pulmonary emphysema by instilling papain into trachea of rats. With the diagnosis of emphysema and the genetic deficiency of the protease inhibitor alpha-1-anti-trypsin [6], this animal

Table 1: Acute tobacco smoke exposure.

Treatment	Time	Response	Reference
Twice a day, 1 hour per section, 3 days	3 days	The numbers of neutrophils and the levels of proinflammatory mediators, keratinocyte chemoattractant (KC), macrophage inflammatory protein 2 (MIP-2), and interleukin 1 beta (IL-1β) are all increased in bronchoalveolar lavage fluid (BALF).	[26]
Twelve cigarettes a day, three times a day	5 days	Acute exposure to cigarette smoke causes oxidative stress and increases the counts of leukocytes and macrophages and the levels of several proinflammatory cytokines, such as tumor necrosis factor alpha (TNF-α), IL-1β, interleukin 6 (IL-6), and KC.	[27]
Twenty cigarettes a day, four times a day	7 days	Acute exposure to cigarette smoke increases the number of total cells, neutrophils, macrophages, and lymphocytes in BALF and increases the levels of KC and monocyte chemotactic protein 1 (MCP-1).	[28]
Five cigarettes a day	3 days	The numbers of mobilizing neutrophils and differentiating macrophages are significantly increased in BALF, and the levels of IL-1β, IL-6, interferon gamma (IFN-γ), TNF-α, and MCP-1 in BALF and lung are also increased.	[29]

model provided the basis for the proteinase-anti-proteinase hypothesis of human emphysema. According to this theory, various proteases, such as porcine pancreatic elastase, papain, or neutrophil elastase [7–9], have been subsequently instilled into trachea to test for the function to induce emphysema in animals. After intratracheal protease instillation, airway inflammation in the lung parenchyma, airspace enlargement, and pulmonary dysfunction (such as air trapping, alveolar destruction, and increased lung volume) have been observed [10, 11]. These features are similar to human emphysema although the speed of occurrence and development is drastically increased.

There are a number of protocols for the induction of emphysema by proteases. Authors have shown that the severity of the protease-induced emphysema is related to the dosage and frequency of the protease [12]. In a murine model, the damage caused by protease persists after 4 weeks of administration, and the application of repeated doses can result in severe cases of the disease [13–15].

Protease-induced emphysema models have a number of advantages, such as simple operation, inexpensive, and high efficiency. The characteristics similar to COPD appear quickly compared to other methods [13], such as the use of cigarette smoke. Moreover, the severity of disease depends on the dosage and frequency of the enzyme, and protease-induced emphysema is related to the epithelial and endothelial cell apoptosis, extracellular matrix degradation, and presence of oxidative stress, which make it suitable to study every phase in emphysema and to investigate neutrophilic inflammation in COPD. Other interesting aspects of this model are that it can reproduce various features of the human disease [16–18], especially the morphology of the lung parenchyma destruction, and that the induced morphological and functional changes are detectable for a long time.

Generally, it takes quite a long time for the development of COPD in human. Despite that the model reproduces many characteristics of human emphysema, this protease-induced emphysema model does not mimic a continuous low-level inflammatory process, especially the adaptive helper T cell immune responses induced by tobacco smoke, which is the most risk factor of COPD. This model neither provides the exact mechanism of alveolar destruction and the sequence of pathological events [17].

3. Tobacco Smoke-Induced Emphysema Models

Tobacco smoke is the most risk factor for COPD [19]. Epidemiology studies demonstrate that the incidence of COPD in smokers is higher than that in nonsmokers. Exposure to tobacco smoke can continuously induce inflammation (inflammatory cell influx and increases of cytokines and chemokines in the airway and parenchyma), mucus hypersecretion (goblet cell metaplasia), emphysema (alveolar enlargement and increased lung volume), airway remodeling (smooth muscle deposition, matrix deposition, and fibrosis), and lung dysfunction [20]. Thus, the use of tobacco smoke-induced animal models can reproduce the real process in the development of COPD, especially for investigating the pathophysiological mechanisms.

Since Huber et al. [21] have firstly described a detailed study regarding smoke-induced emphysema in animals, there are a wide variety of exposure protocols for this model. Using tobacco smoke-induced model with single, multiple, or chronic exposure regimes can have different insights into the disease pathology. Acute tobacco smoke exposure (Table 1) has been demonstrated the presence of inflammatory infiltrate in the pulmonary parenchyma (the increased number of inflammatory cells and cytokines) [22], whereas chronic exposure (Table 2) probably provides the most similar animal model of human COPD because it induces disease (emphysema, epithelial cell metaplasia, airway remodeling, and decline in lung function) with the same stimulus, rather than just inflammation [23]. At present, there are two major exposure modes, whole-body smoke exposure [24] and nose-only smoke exposure [25].

Smoke-induced animal models of emphysema can not only reproduce the pathological process in the development of COPD but also provide the opportunity to test the effect of viral or bacterial infection in the presence of emphysema development which contributes to the acute exacerbation of COPD. Meshi et al. [33] have found that in guinea pigs, chronic cigarette smoke exposure caused lesions similar to human centrilobular emphysema and that latent adenoviral

Table 2: Chronic tobacco smoke exposure.

Treatment	Time	Response	Reference
Four cigarettes a day, 5 days a week	6 months	Both Th1 and Th17 cells are significantly increased, and the levels of IL-6 and IL-17 are also increased.	[30]
Twelve cigarettes a day, 5 days a week	6 months	An increase in the total number of inflammatory cells and macrophages in BALF of mice exposed to cigarette smoke. The release of IL-1β and TNF-α is increased as well.	[31]
Four times a day, 5 minutes per section, 5 days a week	4 months	Functional IL-17A protein secreted in the lung likely establishes an autocrine loop that further induces TH17 differentiation, thereby exacerbating the effect of smoke-induced TH1 and TH17 inflammation in the lungs.	[32]

infection combined with cigarette smoke exposure caused an excess increase in lung volume, air-space volume, and lung weight and a further decrease in surface-to-volume ratio compared with smoke exposure alone. In addition, tobacco smoke exposure faithfully recapitulates the predominant lung TH1 and TH17 responses that have previously been demonstrated as the characteristic of human emphysema. Thus, this model is suitable for studying COPD pathogenesis, especially for the T cell immunity.

Despite the smoke-induced animal models of emphysema are widely accepted worldwide, they still have a lot of disadvantages [23]. Firstly, the model is time- and energy-consuming (about 6 months). Secondly, there is no uniform standard for exposure method, since the dose, time, and animal strains may lead to different conclusions with same stimulator. Thirdly, the airway inflammation in this model is weak and the mucus expression is not obvious, which is not suitable to study about the mechanisms, especially for the neutrophilic inflammation in COPD [23]. The major limitation of this methodology, however, is the fact that even the COPD patients have left the smoking habit, the progression of disease still occurs. In experimental models, this phenomenon is not observed, since the end of exposure results in stable and nonprogressive emphysema [34]. Furthermore, species and strain differences must be taken into consideration when selecting an appropriate model. For instance, it appears that guinea pigs will acquire vascular alterations with smoke that are not found in standard rat models [35, 36].

4. Chemical Drug-Induced Airway Inflammation Models

Authors have found that various kinds of chemical drugs could induce inflammation and emphysema in pulmonary parenchyma, including lipopolysaccharides (LPS) [37], cadmium chloride, sulfur dioxide, and so on.

4.1. LPS-Induced Models. LPS is a major proinflammatory glycolipid component in the gram-negative bacterial cell wall. It can present as a contaminant on airborne particles and exist in cigarette smoke and air pollution. A single large dose of LPS instillation causes an inflammatory response that is combination with mucus hypersecretion and bronchoconstriction [38, 39], which mimic acute exacerbations, either given alone or given to animals also receiving cigarette smoke.

Long-term usage of bacterial LPS alone or together with short periods of exposure to cigarette smoke can induce emphysema in animals. For instance, a hamster emphysema model induced by installation of LPS twice per week for 4 weeks produced enlarged air spaces and remodeled airways with thickened walls and increased goblet cells. These changes resemble human emphysema and small airway remodeling [40].

4.2. Cadmium-Induced Models. Several reports have suggested that cadmium chloride (CdCl2) can reproduce experimental emphysema in animals [41, 42]. A few days after CdCl2 instillation, it causes an increase in vascular permeability with enhanced migration of polymorphonuclear leucocytes (PMN) and macrophages. Polymorphonuclear leucocyte recruitment plays an important role in enhancing inflammatory process and impairing the oxidant–antioxidant balance. Moreover, proinflammatory cytokines, such as matrix metalloprotease (MMP), are also related to cadmium-induced emphysema [43–45]. Kirschvink et al. [46] have shown that cadmium-induced emphysema in rats is dependent of pulmonary inflammation as well as of MMP production, as the increased MMP-2 and MMP-9 production contributes to the development of emphysema.

4.3. Sulfur Dioxide-Induced Models. As an irritant gas, sulfur dioxide can melt in water and become H_2SO_3 after intratracheal inhalation. H_2SO_3 can damage airway epithelium, and chronic exposure of rats to high concentrations of SO_2 gas causes lesions similar to those seen in human chronic bronchitis. Shore et al. [47] have found that rats exposed to 250 ppm SO_2 gas, 5 hours a day, 5 days a week, for a period of 4 weeks caused a small but significant increase in pulmonary resistance (RL) and a decrease in dynamic compliance (Cdyn). Kodavanti et al. [48] have shown that SO_2-induced model could produce emphysema and bronchitis similar to human COPD through pulmonary function test.

COPD is a chronic pathological process, and the accumulation of neutrophils and macrophages in trachea can contribute to airway remodeling, which causes ventilatory disorder gradually [49]. Chemical drug-induced airway inflammation model can only mimic lesions of airway and pulmonary parenchyma in COPD rather than reproduce the chronic pathological process, and these models are not recommended for investigation of COPD pathogenesis. However, the airway inflammation in this model is strong enough and the observed inflammatory and pathologic

Table 3: Natural mutant emphysema models.

Mouse and gene	Phenotypes	Reference
Beige (Bg)	Impaired alveolar septation because of its deficiency in endosome biogenesis	[51, 52]
Blotchy (Blo)	Disruption of elastic fibers	[53]
Pallid (Pa)	Progressive emphysema because of increased collagen degradation	[54]
Tight skin (Tsk +/−)	Airspace enlargement because of impaired alveolar septation. Mice also have lower serum elastase inhibitory capacity	[55]

Table 4: Knockout mutant emphysema models.

Mouse and gene	Phenotypes	Reference
Tissue inhibitor of metalloproteinases-3 (TIMP-3)	Progressive emphysema from two weeks old with evidence of collagen degradation and increased MMP activity	[56, 57]
Surfactant protein D (SP-D)	Progressive airspace enlargement with 3 weeks of life. Increased macrophages with activated MMPs. The gene influences the response of alveolar epithelial type II cells to the injurious events	[58, 59]
Lysosomal acid lipase (LAL)	LAL is a key enzyme in the metabolic pathway of neutral lipids. Areas of alveolar destruction because of neutrophil influx, foamy macrophages, and Clara cell hypertrophy	[60, 61]
Klotho	Klotho is an "antiageing" hormone and transmembrane protein	[62]
Integrin beta-6 (Itgb6)	Inhibition of TGF-β signaling causes increased expression of MMP-12 by macrophages.	[63]
Gamma retinoic acid receptor (RARγ)	Airspace enlargement because of impaired alveolar septation	[64, 65]
Platelet-derived growth factor A (PDGF-A)	Impaired alveolar septa lake of tropoelastin expression and lack lung alveolar smooth muscle cells	[66]
Growth factor receptor 3 and 4(GFR 3-4)	Absence of secondary alveoli	[67]
Fibulin-5/DANCE	It is a secreted extracellular matrix protein that functions as a scaffold for elastin fiber assembly. The model is due to the interruption of elastin synthesis	[68, 69]
Elastin	Deficient formation of air sacs	[70]
Tumor-necrosis alpha-converting enzyme (TACE)	Disabled saccular structures	[71]
Adenosine deaminase	Increased adenosine levels impair alveolar septation and induce inflammation	[72, 73]
POD-1	Hypoplastic lungs	[74]

changes mimic those observed in human subjects with COPD, suggesting that this murine model could be applicable to dissect the role of inflammation in the pathogenesis of these disease conditions.

5. Genetic Models

Epidemiology studies have found that not all smokers are equally susceptible to toxicants (toxic particles and gases, mostly tobacco smoke) and only a percentage of them develop the disease. Another interesting aspect of observation is that COPD shows familial aggregation, suggesting that the genetic background of the smoker is a key element for COPD development [4, 50]. According to the importance of gene, various studies produced emphysema models using either naturally occurring mouse strains or laboratory-produced animals that either overexpress or knock out particular genes (Tables 3–5). We summarized briefly herein their types, advantages, and disadvantages.

Genetically altered animals can not only allow research of the effects of a specific gene/protein on almost all different anatomic lesions of COPD but also potentially be useful in designing therapeutic agents. In order to link genetic predisposition and environmental factors, genetic models have also been used in combination with cigarette smoke exposure to mimic the natural condition of the onset of COPD [82, 83].

However, different mouse strains have a variety of genetic differences and they correspond to the phenotype of extreme monogenic individuals that probably does not adequately reproduce the most common forms of COPD, thus presenting limitations in terms of the translation of the results to the human disease [84, 85]. Moreover, genetic operation is a difficult and lengthy process, since the inactivation of a gene sometimes causes lethal effects and in some cases do not produce a phenotype due to overlapping functional gene [86, 87]. In view of this point, pulmonary cell specifically genetic alternations are highly encouraged to demonstrate the function of a specific gene in COPD pathogenesis. In addition, the studies of genetically altered animals usually focus on

Table 5: Overexpression mutant emphysema models.

Mouse and gene	Phenotype	Reference
Metalloproteinase-1 (MMP-1)	Progressive airspace enlargement because of degradation of collagen type III	[75]
Placenta growth factor (PLGF)	PLGF is an erythroblast-secreted factor. Airspace enlargement because of increased alveolar epithelial cell apoptosis	[76, 77]
Interleukin-13 (IL-13)	Increased MMP and cathepsin expression leading to emphysema	[78]
Interferon gamma (IFN-γ)	Progressive emphysema and increased lung compliance. Increased expression of MMPs, cathepsins, and caspases	[79, 80]
Tumor necrosis factor alpha (TNF-α)	Nonprogressive emphysema after 1–3 months of life	[81]

the different anatomic lesions in mouse lung rather than the chronic pathological process, so that these animal models may be inappropriate to study the neutrophilic inflammation in COPD.

6. Emphysema Models Based on Autoimmunity

Some COPD patients never have cigarette smoking, and the abnormal inflammatory response in patients does not resolve after quitting smoking. Furthermore, recent advances in our understanding of disease pathogenesis indicate that COPD patients exhibit many of the same features as patients suffering from classical autoimmune diseases. For instance, COPD is typified by familial predilections, the frequent presence of systemic abnormalities, and the persistence of intrapulmonary inflammation (and clinical progression) despite removal of the stimulant (e.g., tobacco smoke) [88]. In general, these observations suggest that in some patients, the pathogenesis of COPD may involve an autoimmune component that contributes to the enhanced and persistent inflammatory response [88]. It has been shown that the presence of lymphoid aggregates rich in T and B cells correlated with the severity of airflow obstruction in COPD. Also, in these patients, infiltrating $CD8^+$ cell counts were related to the severity of emphysema, airway flow limitation, and the increased apoptotic epithelial and endothelial cells [89]. It has also been shown that the $CD4^+$ INF-γ producing cells were related to the degree of airway obstruction [90], and that the $CD4^+$ T cells from smokers with emphysema showed a Th17 profile, as they were able to secrete IL-17A [91]. According to the significant role of autoimmunity in progressive emphysema, Taraseviciene-Stewart et al. [92] have found that nude rats injected intraperitoneally with human umbilical vein endothelial cells (HUVECs) could produce an antibody against ECs (anti-EC humoral response), which subsequently leads to the influx of CD4 lymphocytes into the lung, apoptosis of alveolar septal cells, activation of MMPs, and eventual emphysema. In 2007, these authors also used CSE (cigarette smoke extract) intraperitoneal injection instead of xenogeneic endothelial cells to induce emphysema, and they hypothesized that CSE could act as an antigen triggering an immune response as well as oxidative stress that induced emphysema [93]. These models can reproduce emphysema, however, they use xenogeneic cells and external antigen, which cannot mimic the real pathogenesis regarding homologous autoimmunity in COPD patients. Moreover, Lee et al. have shown that the lung extracellular matrix proteins elastin, collagen, and decorin can be auto-antigen that induces the occurrence and development of COPD [94, 95]. This exciting new breakthrough may open new avenues for developing effective animal models of COPD.

7. Conclusion and a Perspective

As summarized above, a variety numbers of animal models have been created to attempt to reproduce human COPD, but there are still some controversial and divergent aspects regarding some methodological variables in these models. There is no model that can totally mimic the whole features in human COPD at present. Also, the lack of golden standard whether the model has been built successfully makes it difficult to analyze conclusions from different models. Thus, it is important to establish a useful and effective model which can highly mimic the human COPD characteristics.

An ideal animal model of COPD should be induced by cigarette smoke or other pathogens related to human disease, with persistent moderate-to-high levels of neutrophilic airway inflammation, typical T cell immune responses, clearly evidenced mucus hyperproduction, progressed destruction of the lung parenchyma eventually leading to epithelial apoptosis and airspace enlargement, and declined lung functions, and if possible, less time and energy-consuming.

Given the fact that cigarette smoke-induced COPD could be a Th1/Th17 predominant autoimmune disease, and there should be certain self-antigens mediate such an autoimmune adaptive response, we propose here a novel emphysema model by referencing to a standard allergen-induced asthma model [96, 97]. For such a model, we should first find the effective self-antigen which is likely produced in the lungs by cigarette smoke exposure and mediates the COPD autoimmune response. With this self-antigen, we may sensitize the mice and challenge the mice to build up an adaptive immune response model, likely exhibiting a high level of neutrophilic airway inflammation and a Th1/Th17 predominant immune response. It will be more appreciated if this model could clearly display a mucus hyperproduction as in the allergen-induced asthma model. If this could be realized, then a long-time self-antigen challenge should lead to the airway remodeling and emphysema-like airspace enlargement and eventually result in declined lung function, as does by allergens in the models of asthmatic airway remodeling [98, 99].

Nevertheless, a perfect animal model should provide a wide range of information on the pathophysiology of COPD

and, as a consequence, support the development of new therapeutic approaches, resulting in a better quality of life for patients. Also, it should help us to understand more and better about the underlying mechanisms in COPD pathogenesis.

Conflicts of Interest

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

Authors' Contributions

Gang Huang and Xu-Chen Xu wrote the manuscript. Jie-Sen Zhou, Zhou-Yang Li, Hai-Pin Chen, Yong Wang, and Wen Li participated in the preparation of the manuscript and discussion. Hua-Hao Shen and Zhi-Hua Chen supervised the work.

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Research Article

Regulation of Discrete Functional Responses by Syk and Src Family Tyrosine Kinases in Human Neutrophils

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Neutrophils play a critical role in innate immunity and also influence adaptive immune responses. This occurs in good part through their production of inflammatory and immunomodulatory cytokines, in conjunction with their prolonged survival at inflamed foci. While a picture of the signaling machinery underlying these neutrophil responses is now emerging, much remains to be uncovered. In this study, we report that neutrophils constitutively express various Src family isoforms (STKs), as well as Syk, and that inhibition of these protein tyrosine kinases selectively hinders inflammatory cytokine generation by acting posttranscriptionally. Accordingly, STK or Syk inhibition decreases the phosphorylation of signaling intermediates (e.g., eIF-4E, S6K, and MNK1) involved in translational control. By contrast, delayed apoptosis appears to be independent of either STKs or Syk. Our data therefore significantly extend our understanding of which neutrophil responses are governed by STKs and Syk and pinpoint some signaling intermediates that are likely involved. In view of the foremost role of neutrophils in several chronic inflammatory conditions, our findings identify potential molecular targets that could be exploited for future therapeutic intervention.

1. Introduction

Neutrophils have long been known to play a critical role in host defense against infectious agents. However, the contribution of neutrophils to host immunity extends well beyond their traditional depiction as professional phagocytes specialized in pathogen clearance. It is now widely recognized that neutrophils and their products condition the onset and evolution of both innate immunity and the ensuing immune response [1, 2]. This occurs in good part through the generation of numerous proinflammatory mediators (chemokines, cytokines, and lipid mediators) by activated neutrophils [3], in conjunction with the increased persistence of neutrophils in inflamed tissues (relative to their circulating counterparts). We and the others have shown that the production of inflammatory cytokines by neutrophils, as well as their delayed apoptosis, are controlled by discrete signaling cascades, including the TAK1, IKK, p38 MAPK, MEK/ERK, and PI3K pathways [4–11]. We further showed that these signaling pathways control cytokine production through downstream effectors such as NF-κB, CREB, and C/EBP transcription factors [8, 12–14]. We also showed that the above signaling cascades affect cytokine generation and delayed apoptosis translationally [8, 10, 15]. Thus, a picture is gradually emerging, of how various kinases and their downstream targets govern key neutrophil responses.

Whether other kinases also participate in controlling the aforementioned neutrophil responses remains to be explored. In this regard, one of the most immediate events occurring following neutrophil activation is the phosphorylation of cellular proteins by nonreceptor tyrosine kinases such as members of the Src family (including Hck, Fgr, and Lyn), as well as Syk [16–22]. Accordingly, the Src family of protein tyrosine kinases (STKs) and Syk have been reported to couple Fc receptors, adhesion receptors (integrins and selectins), and chemoattractant receptors to several classical effector functions of neutrophils, including phagocytosis, degranulation, ROS production, and leukotriene synthesis, though these tyrosine kinases seem to affect neutrophil migration to a lesser extent [23–25]. In contrast, a role for Src family

and Syk tyrosine kinases in controlling inflammatory cytokine production or delayed apoptosis has yet to be described in human neutrophils.

We now report that inhibition of STKs or Syk selectively hinders inflammatory cytokine generation by acting posttranscriptionally. Accordingly, STK or Syk inhibition decreases the phosphorylation of signaling intermediates (e.g., eIF4B, S6K, and MNK1) involved in translational control. By contrast, delayed apoptosis appeared to be independent of either STKs or Syk. Our data therefore significantly extend our understanding of which neutrophil responses are governed by STKs and Syk and pinpoint some of the likely mechanisms involved.

2. Materials and Methods

2.1. Antibodies and Reagents. Antibodies raised against STK isoforms and β-actin were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); antibodies against Syk, as well as all phospho antibodies, were from Cell Signaling (Beverly, MA, USA). Ficoll-Paque Plus was from GE Biosciences (Baie-d'Urfé, QC, Canada); endotoxin-free (<2 pg/ml) RPMI 1640 was from Wisent (St-Bruno, QC, Canada). Recombinant human cytokines were from R&D Systems (Minneapolis, MN, USA), and UltraPure LPS (from *E. coli* 0111:B4) was from InvivoGen (San Diego, CA, USA). Dimethyl sulfoxide (DMSO), N-formyl-methionyl-phenylalanine (fMLP), and phenylmethanesulphonyl fluoride (PMSF) were from Sigma-Aldrich (St. Louis, MO, USA). Diisopropyl fluorophosphate (DFP) was from Bioshop Inc. (Burlington, ON, Canada). The protease inhibitors, aprotinin, 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), leupeptin, and pepstatin A, were all from Roche (Laval, QC, Canada). Kinase inhibitors were all purchased through Cedarlane Labs (Mississauga, Canada). All other reagents were of the highest available grade, and all buffers and solutions were prepared using pyrogen-free clinical grade water.

2.2. Cell Isolation and Culture. Neutrophils were isolated from the peripheral blood of healthy donors, following a protocol that was duly approved by an institutional ethics committee. The entire procedure was carried out at room temperature and under endotoxin-free conditions, as described previously [26]. Purified neutrophils were resuspended in RPMI 1640 supplemented with 5% autologous serum, at a final concentration of 5×10^6 cells/ml (unless otherwise stated). As determined by Wright staining and FACS analysis, the final neutrophil suspensions contained less than 0.1% monocytes or lymphocytes; neutrophil viability exceeded 98% after up to 4 h in culture, as determined by trypan blue exclusion and by annexin V/propidium iodide FACS analysis.

2.3. Immunoblots. Cells were incubated at 37°C in the presence or absence of stimuli. Incubations were stopped by adding equivalent volumes of ice-cold PBS supplemented with DFP (2 mM, final concentration) and phosphatase inhibitors (10 mM NaF, 1 mM Na_3VO_4, and 10 mM $Na_4P_2O_7$). For whole-cell samples, boiling 2X sample buffer was added directly to cell pellets, which were briefly vortexed and placed in boiling water for a further 5 min. Samples thus prepared were sonicated to disrupt chromatin and stored at −20°C prior to analysis. For subcellular fractions, cells were resuspended in relaxation buffer prior to disruption by nitrogen cavitation, as described previously [27, 28]. Denatured samples were electrophoresed, transferred onto nitrocellulose, and processed for immunoblot analysis as previously described [27].

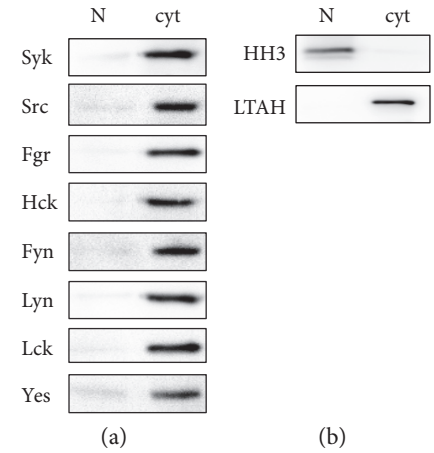

Figure 1: Expression and cellular distribution of Src family and Syk tyrosine kinases in human neutrophils. (a) Neutrophils (pmn) were disrupted by nitrogen cavitation; cytoplasmic and nuclear fractions were then processed for immunoblot analysis of Src family and Syk kinases (0.5×10^6 cell equivalents were loaded per lane). (b) Neutrophil subcellular fractions prepared as above were processed for immunoblot analysis of the cytosolic marker leukotriene A4 hydrolase (LTAH) or the nuclear marker histone H3 (HH3). The experiment shown in this figure is a representative of three.

2.4. Real-Time PCR Analyses. Procedures and primers used are exactly as described before [13].

2.5. ELISA Analyses. Neutrophils were cultured in 24-well plates at 37°C under a 5% CO_2 atmosphere, in the presence or absence of stimuli and/or inhibitors, for the indicated times. Culture supernatants were carefully collected, snap-frozen in liquid nitrogen, and stored at −80°C. Samples were analyzed in ELISA using commercially available capture and detection antibody pairs (R&D Systems, BD Biosciences).

2.6. Determination of Neutrophil Apoptosis. After the desired culture period, neutrophils (5×10^5 cells) were washed twice in ice-cold PBS containing 5 mM EDTA, then once more in cold PBS, and incubated on ice for 15 min with FITC-conjugated annexin V. Cells were then counterstained with propidium iodide and analyzed (minimum of 10,000 cells) on a Cytoflex instrument (Beckman Coulter) using the CytExpert software.

2.7. Data Analysis. All data are represented as the mean ± SEM of at least three independent experiments. Statistical differences were analyzed by Student's *t*-test for paired data,

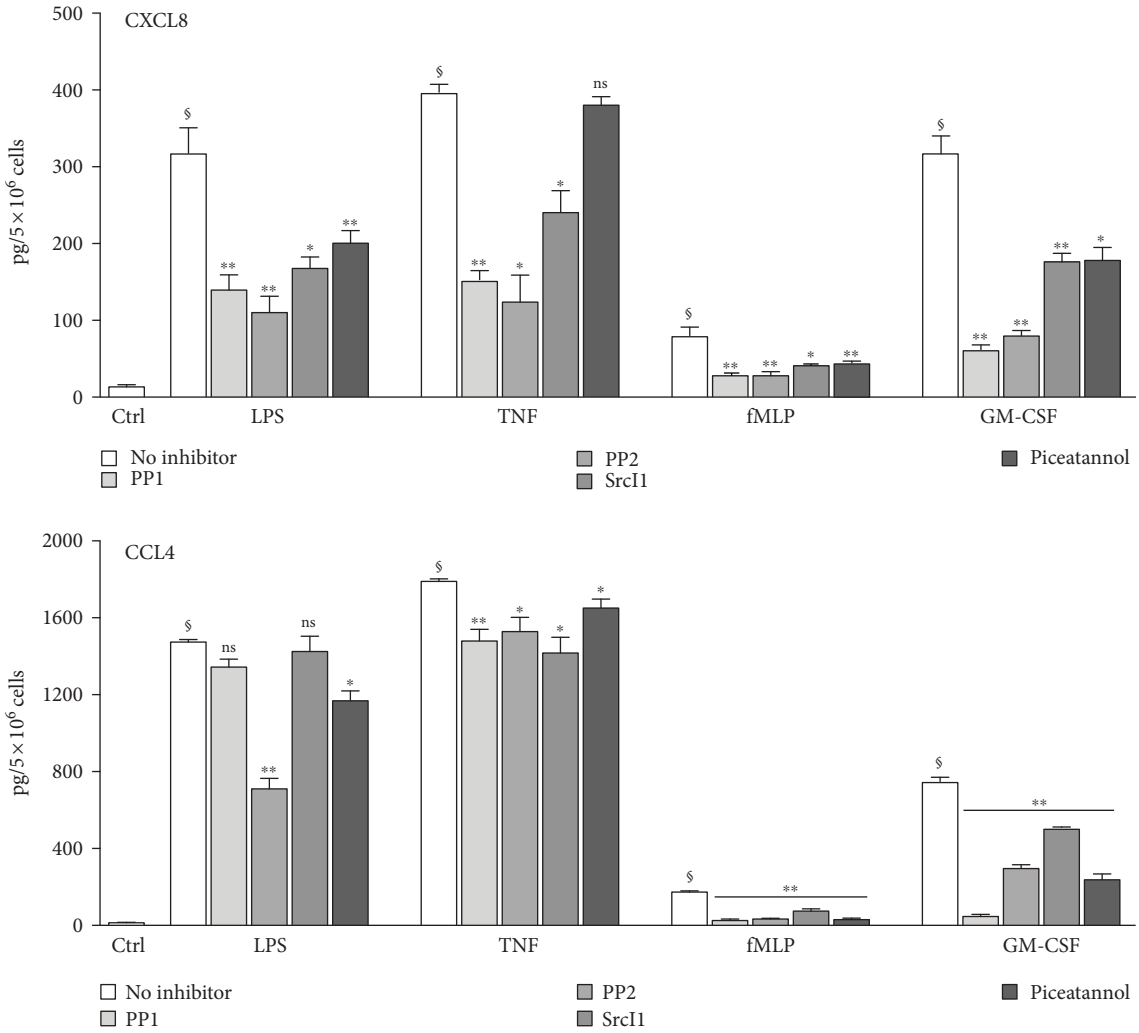

Figure 2: Effect of STK and Syk inhibition on inflammatory cytokine generation in human neutrophils. Cells were pretreated for 30 min in the absence or presence of STK inhibitors (10 μM of PP1, PP2, or Srcl1) or of a Syk inhibitor (10 μM piceatannol), prior to stimulation for 6 h with 1 μg/ml LPS, 100 U/ml TNFα, 30 nM fMLP, or 1 nM GM-CSF. Culture supernatants were analyzed in ELISA. Results are expressed as mean ± SEM from at least 3 independent experiments, each performed in duplicate. §, $p < 0.001$ versus unstimulated cells ("ctrl"); $^{*}p < 0.05$ versus matched condition without inhibitor; $^{**}p < 0.01$ versus matched condition without inhibitor.

using Prism 7 software (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Expression and Distribution of Src Family and Syk Tyrosine Kinases in Human Neutrophils. Previous studies have established that at least three STK isoforms are present in neutrophils (e.g., Fgr, Hck, and Lyn), as well as Syk [18–22]. To gain a more complete understanding of which isoforms are present (and where they localize), resting neutrophils were disrupted by nitrogen cavitation, and cytoplasmic and nuclear fractions were processed for immunoblot analysis. Figure 1(a) shows that all STKs investigated, as well as Syk, are strictly cytoplasmic in human neutrophils. The purity of our subcellular fractions was ascertained for the presence of cytosolic and nuclear markers. As shown in Figure 1(b), strictly cytosolic proteins, such as leukotriene A4 hydrolase [29], were only detected in cytoplasmic fractions; conversely, histone H3 was exclusively nuclear, as expected.

3.2. Effect of STK and Syk Inhibition on Cytokine Expression and Release in Human Neutrophils. Whereas Src family and Syk tyrosine kinases play an important role in various classical functions of neutrophils, such as phagocytosis, ROS production, and leukotriene synthesis [23], the potential involvement of these kinases in cytokine generation has not been studied to date. To address this issue, neutrophils were pretreated with Src family inhibitors (i.e., PP1, PP2, and SrcI1) or a widely used Syk inhibitor (piceatannol), prior to stimulation with physiological stimuli; cytokine production was then assessed by ELISA. As shown in Figure 2, all inhibitors strongly repressed the inducible secretion of CXCL8 in response to LPS, TNFα, fMLP, or GM-CSF, with the notable exception of TNF-elicited

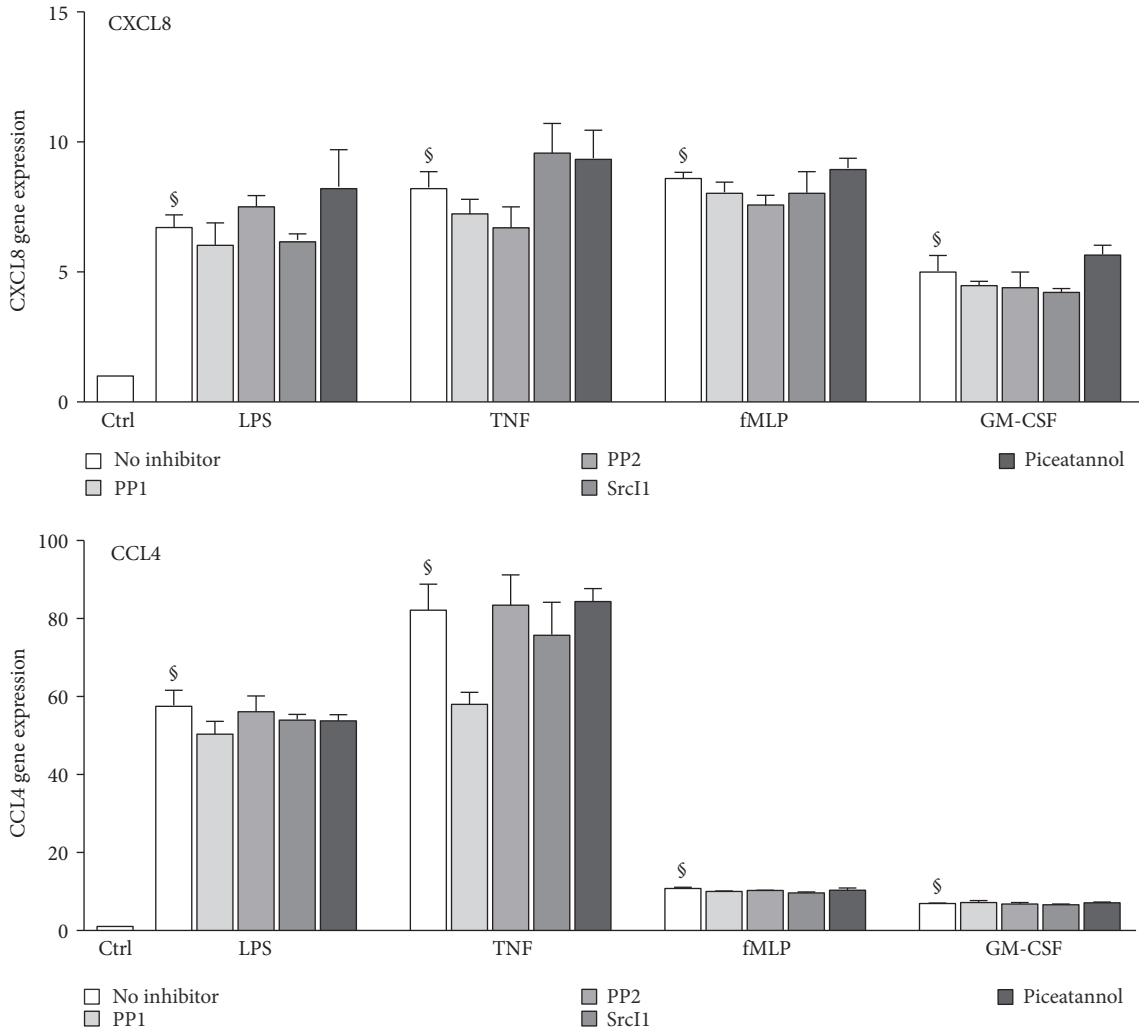

Figure 3: Effect of STK and Syk inhibition on inflammatory cytokine gene expression in human neutrophils. Cells were pretreated with STK or Syk inhibitors, prior to a 30 min stimulation with LPS, TNFα, fMLP, or GM-CSF, as described for Figure 2. Total RNA was isolated, reverse-transcribed, and analyzed for cytokine gene expression by real-time qPCR. Values were normalized over RPL32 and are represented as fold increase relative to unstimulated cells. Mean ± SEM from at least 3 independent experiments, each performed in duplicate. §, $p < 0.007$ versus unstimulated cells ("ctrl").

CXCL8 release, which was consistently unaffected by Syk inhibition. The release of another chemokine, CCL4, was likewise hindered by all inhibitors tested in fMLP- and GM-CSF-stimulated cells; in LPS- or TNF-stimulated neutrophils, however, CCL4 release was only modestly affected by Syk or STK inhibitors (even though the inhibition was often statistically significant).

Because inflammatory chemokine generation is typically preceded by an accumulation of the corresponding transcripts in neutrophils, we also investigated whether inhibition of Src family and Syk kinases would yield similar outcomes. In contrast to cytokine release, STK and Syk inhibition failed to significantly alter CXCL8 or CCL4 gene expression induced by LPS, TNFα, fMLP, or GM-CSF (Figure 3). Taken together, the above data indicate that in response to physiological agonists, STKs and Syk affect cytokine generation posttranscriptionally.

3.3. Translational Targets of STKs and Syk in Human Neutrophils. We previously identified several signaling intermediates involved in the translational control of inflammatory cytokine production in neutrophils [8, 10, 15]. Since our present data indicates that STKs and Syk act posttranscriptionally towards this response, we investigated whether these tyrosine kinases might affect some of the translational events, which we identified in previous studies. As shown in Figure 4 and Figure S1 available online at https://doi.org/10.1155/2017/4347121, neutrophil pretreatment with STK or Syk inhibitors clearly diminished the LPS- or TNF-elicited phosphorylation of MNK1; of ribosomal S6 kinase and its substrate, the S6 ribosomal protein; and to a lesser extent, of 4E-BP1. A similar inhibition profile was observed in neutrophils activated with fMLP or GM-CSF, though the extent of inhibition was less pronounced than in LPS- or TNF-treated cells. Thus, Src and Syk kinases

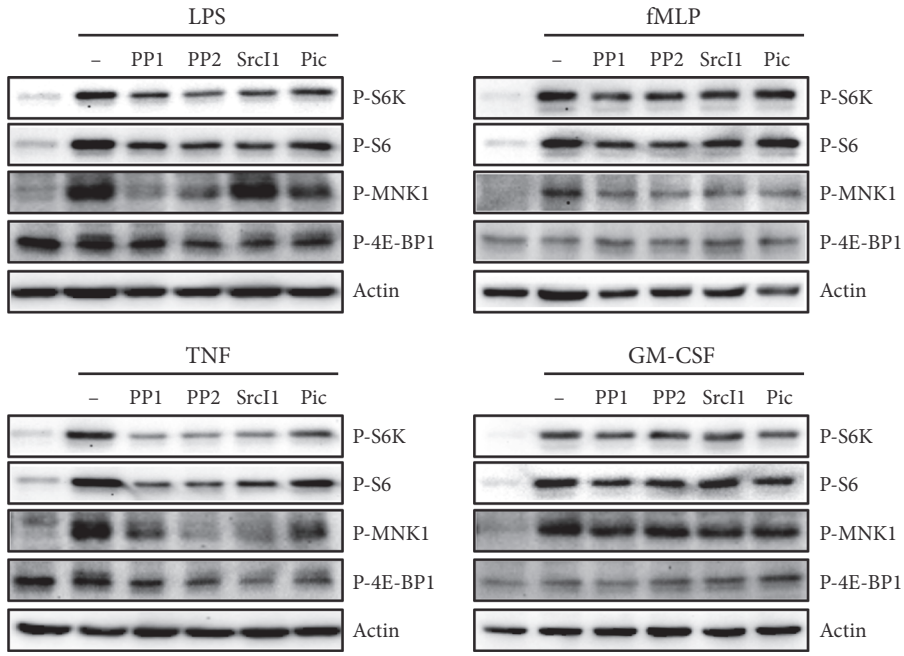

Figure 4: Potential translational targets of Syk and Src family tyrosine kinases in human neutrophils. Cells were pretreated with STK or Syk inhibitors, prior to a 10 min stimulation with LPS, TNFα, fMLP, or GM-CSF, as described for Figure 2. Samples were then processed for immunoblot analysis; membranes were also blotted for β-actin (as a loading control). Data are representative of at least 3 independent experiments.

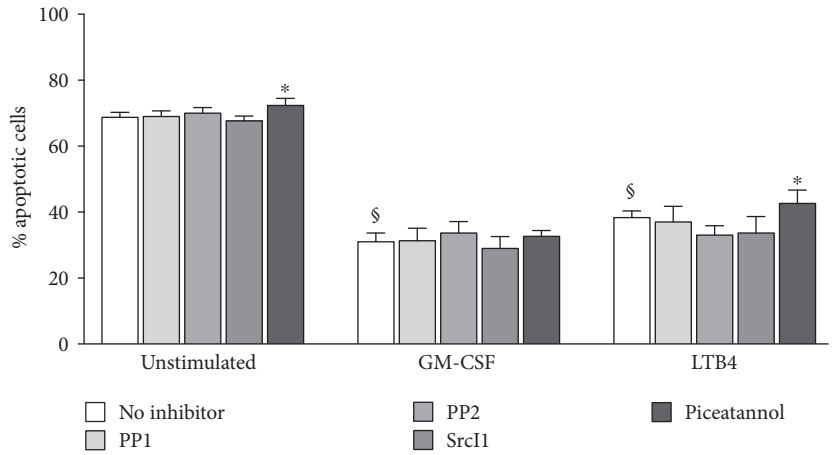

Figure 5: Effect of STK and Syk inhibition on delayed apoptosis in human neutrophils. Cells were pretreated with STK or Syk inhibitors, prior to a 20 h culture with LPS, TNFα, fMLP, or GM-CSF, as described for Figure 2. Cells were then processed for FACS analysis of annexin V binding; a minimum of 10,000 cells were processed for each sample. Results are expressed as mean ± SEM of at least 3 independent experiments. §, $p < 0.001$ versus unstimulated cells ("ctrl"); $^*p < 0.05$ versus matched condition without inhibitor.

have the potential to modulate the translation of inflammatory cytokines by controlling discrete signaling intermediates.

3.4. Impact of STKs and Syk Kinases on Delayed Apoptosis in Human Neutrophils. We finally determined whether Src family and Syk kinases might contribute to the apoptosis-delaying effect of GM-CSF or LTB4 in neutrophils. The reason for using LTB4 as a chemoattractant is that fMLP does not retard constitutive apoptosis in neutrophils. Neutrophils were pretreated with inhibitors of STKs or Syk and cultured overnight in the presence of GM-CSF or LTB4 before apoptosis assessment. As shown in Figure 5, piceatannol by itself exerted a small effect on spontaneous apoptosis ($p = 0.04$), whereas both GM-CSF and LTB4 potently countered the spontaneous apoptotic rate, as expected (Figure 5). In stimulated neutrophils, the STK inhibitors failed to affect the prosurvival effect of either GM-CSF or LTB4, whereas piceatannol slightly affected that of LTB4 ($p = 0.04$) but not

that of GM-CSF (Figure 5). Thus, STKs or Syk exerts little or no effect on the delayed apoptotic response of neutrophils to growth factors or chemoattractants.

4. Discussion

A large number of inflammatory and immune processes have been reported to be influenced by neutrophils and their products in vivo, whence the sustained interest in the underlying signaling events. Following neutrophil exposure to various stimuli, protein tyrosine kinases such as STKs and Syk rapidly become activated and influence several classical functions of these cells, including phagocytosis, degranulation, ROS production, and leukotriene synthesis [23]. In this report, we show that STKs and Syk also participate in the control of another major functional response of neutrophils, that is, inflammatory cytokine generation, whereas they have little or no effect on either spontaneous or delayed apoptosis in these cells.

We first found that STK or Syk inhibition strongly represses the secretion of CXCL8 elicited by physiological neutrophil agonists (i.e., LPS, TNFα, fMLP, and GM-CSF), with the exception of TNF-induced CXCL8 release, which appeared to occur independently of Syk. Similarly, CCL4 secretion was hindered by STK and Syk inhibitors in fMLP- and GM-CSF-activated neutrophils but was only moderately unaffected by the inhibitors in LPS- or TNF-stimulated cells. Thus, depending on the cytokine and the stimulus, inflammatory cytokine generation can be controlled by STKs or Syk in neutrophils. These tyrosine kinases appeared to act posttranscriptionally, since the induction of inflammatory cytokine gene expression was unaffected by STK or Syk inhibition. Accordingly, several signaling intermediates known to affect cytokine translation were found to be under the control of STKs and Syk. Among these downstream targets, MNK1 is particularly relevant, as we recently showed it to participate in the translational regulation of cytokine generation in neutrophils [15]. As observed for MNK1, the inducible phosphorylation of the S6 kinase (and of its substrate, the S6 ribosomal protein) and to a lesser extent the inducible hyperphosphorylation of 4E-BP1 were impaired following STK or Syk inhibition. This again points towards translational control, given the known involvement of S6K, S6, and 4E-BP1 in cap-dependent protein translation in various cell types [30, 31]. Thus, it appears that STKs and Syk control inflammatory cytokine generation translationally in primary neutrophils.

In contrast to cytokine production, STKs and Syk affect other neutrophil responses only moderately, as in the case of chemotaxis [23–25], or not at all, as we found here in the case of constitutive and delayed apoptosis. In the latter instance, this was somewhat surprising, given that cytokine generation and delayed apoptosis have been shown to share many upstream signaling events in neutrophils. Our results therefore identify some of the differences in how these key neutrophil responses are governed.

Collectively, our data significantly extends our understanding of which neutrophil responses are governed by STKs and Syk (and which are not). More importantly, we uncovered some of the likely mechanisms involved in the translational control of cytokine generation by STKs and Syk. In view of the foremost role of neutrophils in several chronic inflammatory conditions, our findings identify potential molecular targets that could be exploited for future therapeutic intervention.

Conflicts of Interest

The authors declare no competing financial interests.

Authors' Contributions

Olga Tatsiy carried out most of the experiments and compiled most of the data. Thornin Ear initiated the project, completed some experiments, and wrote the first draft. Frédérick L. Allard carried out the apoptosis experiments. Patrick P. McDonald designed the research, mentored the other authors, and wrote the final version of the paper. Thornin Ear and Olga Tatsiy contributed equally to this study and are listed alphabetically.

Acknowledgments

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Review Article

Neutrophil Extracellular Traps and Microcrystals

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Neutrophil extracellular traps represent a fascinating mechanism by which PMNs entrap extracellular microbes. The primary purpose of this innate immune mechanism is thought to localize the infection at an early stage. Interestingly, the ability of different microcrystals to induce NET formation has been recently described. Microcrystals are insoluble crystals with a size of 1–100 micrometers that have different composition and shape. Microcrystals have it in common that they irritate phagocytes including PMNs and typically trigger an inflammatory response. This review is the first to summarize observations with regard to PMN activation and NET release induced by microcrystals. Gout-causing monosodium urate crystals, pseudogout-causing calcium pyrophosphate dehydrate crystals, cholesterol crystals associated with atherosclerosis, silicosis-causing silica crystals, and adjuvant alum crystals are discussed.

1. Neutrophil Extracellular Traps

NET formation is a breathtaking mechanism by which neutrophil granulocytes (PMNs) trap extracellular pathogens (Figure 1) [1]. This innate immune mechanism involves remarkable cellular and molecular changes in PMNs. The membranes of granules and the nucleus dissolve, and the cytosolic and nuclear contents fuse [2]. The tightly packed, multilobulated nucleus of stimulated PMNs decondenses and will be released in the extracellular space (Figure 1) [1, 2]. The released DNA is associated with a variety of proteins, mainly histones and primary granule components. In fact, protein-DNA complexes have been used to define NET-derived extracellular DNA (ecDNA) and to distinguish it from DNA released from PMNs by other mechanisms [3, 4]. In addition to PMNs, eosinophil granulocytes, mast cells, and macrophages have also been shown to release extracellular traps, and ET formation has been documented in several species including humans [5–7]. Although the signaling steps in PMNs leading to NET formation remain largely unknown, a few steps are accepted. The NADPH oxidase was identified first as an enzyme essential for the extrusion of NETs [2]. Later on, the critical contributions of myeloperoxidase and neutrophil elastase were also revealed [8, 9]. A milestone in the process of understanding the mechanism of NET formation was the discovery that citrullination of histones by peptidylarginine deiminase 4 (PAD4) is also crucial [10–12]. Although these molecules are important in mediating NET formation, more recent results indicate that their contribution to the process is likely stimulus-, species-, and context-dependent [13–16]. These observations are also in line with the notion that the complicated process of NET formation is unlikely mediated by a single signaling pathway but rather by a complex network of molecular and cellular events. A wide range of stimuli has been described that stimulate NET release in PMNs including whole microbes (bacteria, viruses, fungi, and parasites), soluble molecules (microbial and host), and microcrystals of different origin [17, 18]. Trapping microorganisms is definitely a major function of NETs but might not be the only one. Considering the variety of agents triggering NETs under sterile inflammatory conditions including microcrystals discussed here, it is likely that NETs play a main role in the general inflammatory cascade, no matter what the stimulus. A novel role for NETs in limiting inflammation has already been proposed in gout, for instance [19]. Future research needs to clarify their exact physiological role, mechanism, and regulation. Microcrystals represent a unique set of NET-inducing stimuli (Figure 1) since they are particulate, can be phagocytosed, and form under different pathological conditions. In this review

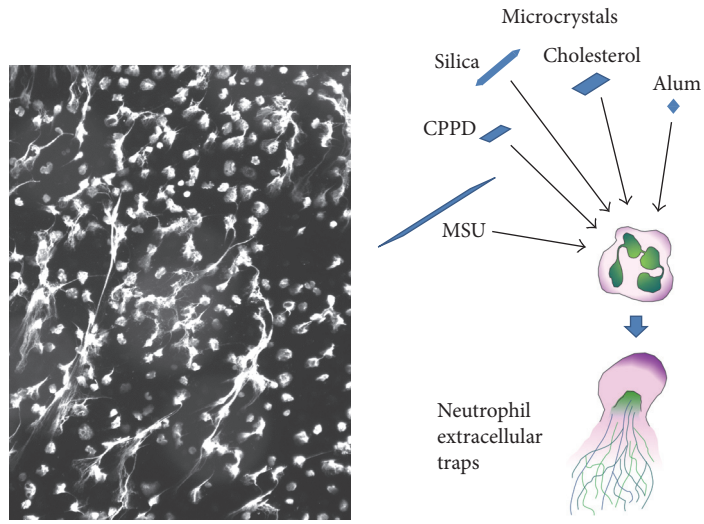

Figure 1: Neutrophil extracellular traps. (a) This fluorescent image depicts NETs released from human PMNs following CPPD crystal stimulation (50 μg/mL, 3 hrs, unpublished data). PMN DNA was stained by DAPI and the color was artificially turned into white for better visibility. (b) Scheme demonstrating different types of microcrystals that were documented to release DNA from PMNs.

current knowledge on microcrystal-induced formation of NETs is summarized.

2. Monosodium Urate Crystals (MSU)

MSU crystals are the causative agents of the autoinflammatory condition, gout [21]. MSU crystals are negatively birefringent, needle-shaped, and generally 5–25 μm (sometimes 100 μm) in length [22, 23]. Uric acid is a degradation product of nucleic acid metabolism and crystallizes in the joints of gout patients in the form of needle-shaped crystals [21]. MSU crystals irritate the innate immune system including macrophages and PMNs leading to acute, painful attacks and chronic joint destruction [21, 24]. MSU crystal-induced PMN activation is a critical step in this inflammatory cascade and understanding its mechanism is crucial to developing novel anti-inflammatory therapies for gout.

PMNs attempt to phagocytose MSU crystals and produce reactive oxygen species (ROS) by the NADPH oxidase in response to them [33–35]. The first observation that MSU crystals induce NET release in PMNs was made by Mitroulis et al. showing that autophagy, PI3K signaling, and endosomal acidification are required for NET formation by MSU crystals [25]. The authors also described that gout synovial cells and peripheral PMNs of gout patients spontaneously release NETs, and gout synovial fluid and gout serum promote NET formation of PMNs obtained from healthy volunteers [25]. This observation was further expanded by Schorn et al. reporting that histones colocalize with DNA in MSU crystal-elicited NETs, and not only PMNs, but also basophil and eosinophil granulocytes also release NETs in response to MSU crystals [7]. They proposed that NETs immobilize the crystals, similarly how NETs would entrap bacteria [26]. The biological relevance of this finding was characterized in the landmark paper written by Schauer et al. suggesting that MSU crystal-induced formation of aggregated NETs (aggNETs) limits inflammation [19]. The high concentration of PMN proteases found in aggNETs was proposed to degrade several proinflammatory cytokines and put an end to recruitment of new leukocytes [19]. The authors showed that aggNETs formed in vitro and in vivo strongly reduced the amount of detectable proinflammatory cytokines [19]. They also found that mice deficient in the NADPH oxidase and incapable of making NETs developed an exacerbated, prolonged, chronic inflammation in contrast to control mice with normal NET-forming ability that had a restricted inflammatory response [19]. This phenomenon could be reversed by adoptively transferring aggNETs into NETosis-deficient mice [19].

Based on this study, the following role of NETs in gout pathogenesis has been proposed (Figure 2) [36]. First, PMNs recruited in large numbers to the joints of gout patients following inflammasome activation encounter MSU crystals (Figure 2) [36]. Activation of PMNs is accompanied with inflammation-associated pain in acute gout [36]. Whether NETs contribute to this phase of gout attack remains to be elucidated but is likely since by forming NETs PMNs also release their dangerous granule content. Second, at high PMN densities present at later stages of acute attacks, NETs form aggNETs that degrade proinflammatory cytokines and densely pack crystals to stop inflammation (Figure 2) [36]. AggNETs were proposed to form the basis for gouty tophi [19], a long-described white material that typically appears at the end of acute attacks and is characteristic for the chronic phase of gout (Figure 2) [19, 37]. Overall, aggNET formation was proposed to stop the acute inflammatory response at the expense of forming tophi that have been associated with symptoms of chronic gout [19, 36]. Recently, some of these data have been challenged [38]. Future studies are required to

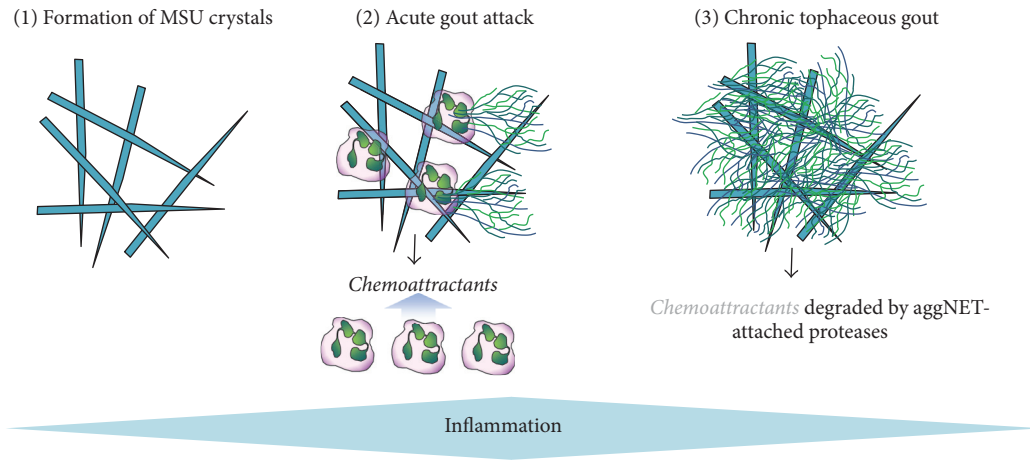

Figure 2: The proposed role of PMNs in the immunopathogenesis of gout. Phase (1) shows the deposition of needle-shaped MSU crystals. Phase (2) depicts PMNs phagocytosing crystals and releasing chemoattractants and NETs. Phase (3) shows the formation of aggregated NETs (aggNET) that provide the structural basis of gouty tophi and contain high concentration of PMN proteases degrading PMN chemoattractants.

work out all the details of this mechanism [39]. Whether the general PMN-mediated inflammatory cascade has a built-in breaking mechanism identical or similar to the one described in gout remains an exciting, open question.

Despite its proposed novel role in gout pathogenesis, less is known about the cellular and molecular mechanism and regulation of MSU crystal-elicited NET formation. The requirement of a functional NADPH oxidase for MSU crystal-evoked NET release has been shown [19]. PMNs of patients suffering from chronic granulomatous disease (CGD) are unable to release NETs in response to PMA, bacteria [2], and MSU crystals [19]. NADPH oxidase deficient murine PMNs stimulated with MSU crystals do not release NETs and aggNETs, neither in vitro, nor in vivo [19]. Interestingly, soluble uric acid, not its crystallized form, stimulates NET release in an NADPH oxidase-independent manner [40]. These results indicate that NET release in gout must be complex, and multiple mechanisms could be responsible for mediating it. Authophagy has also been proposed to mediate NET formation induced by MSU crystals and other stimuli [25, 41, 42]. In a study by Desai et al. the involvement of RIPK1-RIPK3-MLKL signaling has been proposed in MSU crystal- and PMA-induced NET formation suggesting that NETosis is actually a PMN-specific necroptotic pathway [27]. This has been challenged by Amini et al. showing that NET release can occur independently of RIP3K and MLKL signaling, in response to PMA at least [43]. Thus, the relationship between NET formation and PMN necroptosis remains to be studied in more detail. In a recent study performed by Sil et al., we found that PMNs need to attempt to phagocytose MSU crystals in order to perform subsequent NET release and to form aggNETs [23]. PMNs do not really phagocytose MSU crystals since most of the crystals are far longer than PMNs themselves [23]. Our data indicated that only a small fraction of PMNs engaged in attempting MSU crystal phagocytosis but NET-releasing PMNs were all associated with MSU crystals [23]. This let us conclude that MSU crystal phagocytosis is a prerequisite for NET formation [23]. We proposed the involvement of the purinergic P2Y6 receptor in this mechanism based on a strong reduction of MSU crystal-induced NET release by general purinergic receptor inhibitors and the P2Y6-specific inhibitor MRS2578 [23]. Interestingly, exonucleotides alone failed to induce NET release in human PMNs [23]. On the other hand, MRS2578 reduced MSU crystal-stimulated ROS production, cytokine release, and PMN migration suggesting the involvement of these steps in MSU crystal-promoted NET extrusion [23]. In a separate study we revealed that interleukin-1β (IL-1β) derived from macrophages enhances NET release triggered by MSU crystals [28]. IL-1β promotes NET formation but NETs degrade cytokines including IL-1β; what could be the relevance of these two, opposite mechanisms in vivo in acute gout? They are most likely separated in time during the inflammatory process. While, at the early stage of gout flares, IL-1β drives inflammation, PMN recruitment and activation (proinflammatory segment), NETs become important later when sufficient levels accumulated capable of aggNET formation and cytokine degradation (anti-inflammatory phase). The details of this complex in vivo mechanism are, however, not well-understood. We and others also showed that anakinra, a potent IL-1 receptor antagonist, and antibodies neutralizing IL-1β inhibit the NETosis-enhancing effect of macrophages and gout synovial fluid [25, 28]. These results add a novel mechanism by which anakinra works and describe IL-1β as a potentiator of NET formation linking two significant arms of the inflammatory cascade in gout, inflammasome activation in macrophages, and NET formation in PMNs. A recent work by Pieterse et al. emphasized the critical role of phagocytes engulfing small urate microaggregates (SMA) in hyperuricemic blood [44]. These SMAs form first before they grow into long, needle-shaped MSU crystals that are known to trigger NET release [44]. Phagocytes take up SMAs and prevent the formation of MSU crystals and NETs in the circulation [44].

Table 1: Microcrystals that trigger NET formation.

Crystal name	Clinical relevance	Requirement of the following				References
		NADPH oxidase	PAD4	MPO	NE	
Monosodium urate (MSU)	Gout	Yes	?	?	No	[7, 19, 23, 25–28]
Calcium pyrophosphate dehydrate crystals (CPPD)	Pseudogout	No	?	?	?	[29]
Cholesterol crystals	Atherosclerosis	Yes	No	?	Yes	[30]
Silica crystals	Silicosis	?	?	?	?	[31]
PMA (in comparison)	—	Yes	?	Yes	Yes	[2, 9, 32]

3. Calcium Pyrophosphate Dehydrate Crystals (CPPD)

Pseudogout is a condition similar to gout also characterized by periodic acute joint attacks that potentially turn into a chronic disease. Pseudogout is, however, caused by a different inflammatory microcrystal, calcium pyrophosphate dihydrate (CPPD) crystals [45]. CPPD crystals are typically shorter than MSU crystals and have a more rhomboid shape in contrast to the needle-like form of MSU crystals [29]. The pathomechanism of pseudogout is less studied than that of gout but PMN accumulation and its coincidence with painful attacks are also characteristic [46]. In a paper by Pang et al. we described robust in vitro NET formation of human PMNs in response to CPPD crystals [29]. CPPD crystals represent a much stronger NET-inducing signal for PMNs than MSU crystals [23, 28, 29]. We found that PMNs phagocytose CPPD crystals that is also a requirement for CPPD crystal-triggered NET release [29]. PMN nuclei underwent the same, characteristic morphological changes following CPPD crystal stimulation [29] as after PMA challenge [47]. The nucleus of PMNs undergoing NET formation first loses its segmented nature and lobi [1, 2, 29, 47]. Next, the nuclear material decondenses leading to the appearance of diffuse NETs followed by the formation of full-blown spread NETs [29, 47]. NADPH oxidase activity was not needed for CPPD crystal-elicited extrusion of NETs (Table 1) while it has been reported to be essential for MSU crystal-stimulated NET formation [19]. The NET-inducing ability of CPPD crystals required the activity of the heat shock protein 90, PI3K, and CXCR2 [29]. These results indicate that while both crystals induce NET release in human PMNs, different signaling pathways might be responsible for mediating the process.

4. Alum

Alum is the most successful vaccine adjuvant used in the history of human medicine [48]; its exact mechanism of action remains, however, largely unknown to this day. Alum is composed of microcrystals and is thought primarily to enhance the efficacy of vaccines by increasing antigen phagocytosis by antigen presenting cells and by serving as an antigen depot [49]. Although PMNs are not the first cell type that comes to our mind when thinking of the mechanism of action of adjuvants, recent publications suggest that PMNs could play an important role in mediating or fine-tuning the immune response in the presence of adjuvants [50–52]. PMNs are rapidly recruited to the site of vaccination in large numbers; therefore, studying their interaction with adjuvants is clinically relevant since they could significantly alter the immune response at this early stage. PMNs have already been shown to release fibrin-like extracellular traps in the presence of aluminium adjuvants in vivo in mice [53]. No study has been performed though on how human PMNs interact with alum crystals in vitro. We therefore isolated human PMNs from the peripheral blood of healthy volunteers according to previously described protocols [20, 29] and stimulated them with aluminium adjuvant (Alhydrogel, InvivoGen) to detect extracellular DNA release using the DNA-binding, membrane-impermeable dye, Sytox Orange [4]. As our previously unpublished data show in Figure 3, PMNs responded to increasing concentrations of Alhydrogel with extracellular DNA release. This alum-induced DNA release was independent of reactive oxygen species production since the NADPH oxidase inhibitor diphenyleneiodonium (DPI) was without any effect (Figure 3). These data suggest that PMNs release their DNA upon alum crystal exposure. Future experiments are required to reveal the exact nature of this cell death mechanism.

5. Cholesterol Crystals

The important role of IL-1β in the pathogenesis of atherosclerosis has been well known but the mechanism by which macrophages release this cytokine remained poorly understood. Warnatsch et al. demonstrated recently that PMNs and NETs are crucial for both priming and stimulating macrophages to secrete IL-1β that will recruit additional PMNs to the atherosclerotic lesions [30]. PMNs have been previously implicated in the pathogenesis of atherosclerosis but their exact role has been unclear [54, 55]. These researchers showed that cholesterol crystals induce NET release in vitro in human PMNs in a concentration range that also activates the inflammasome [30]. Cholesterol crystals stimulated ROS production in PMNs and NET formation was blocked by the NADPH oxidase inhibitor DPI (Table 1) [30]. Neutrophil elastase translocated to the nucleus during cholesterol crystal-triggered NET formation but the PAD4 inhibitor Cl-amidine was without any effect [30]. NETs were also detected in vivo in lesions but were entirely absent in ApoE/PR3/NE-deficient mice lacking apolipoprotein E, neutrophil elastase, and proteinase 3 [30]. NET-deficient animals on high fat diet exhibited a reduced lesion size after 8 weeks proposing that NETs promote lesion formation in atherosclerosis [30]. NETs were required for enhanced cytokine production by macrophages in presence of cholesterol crystals

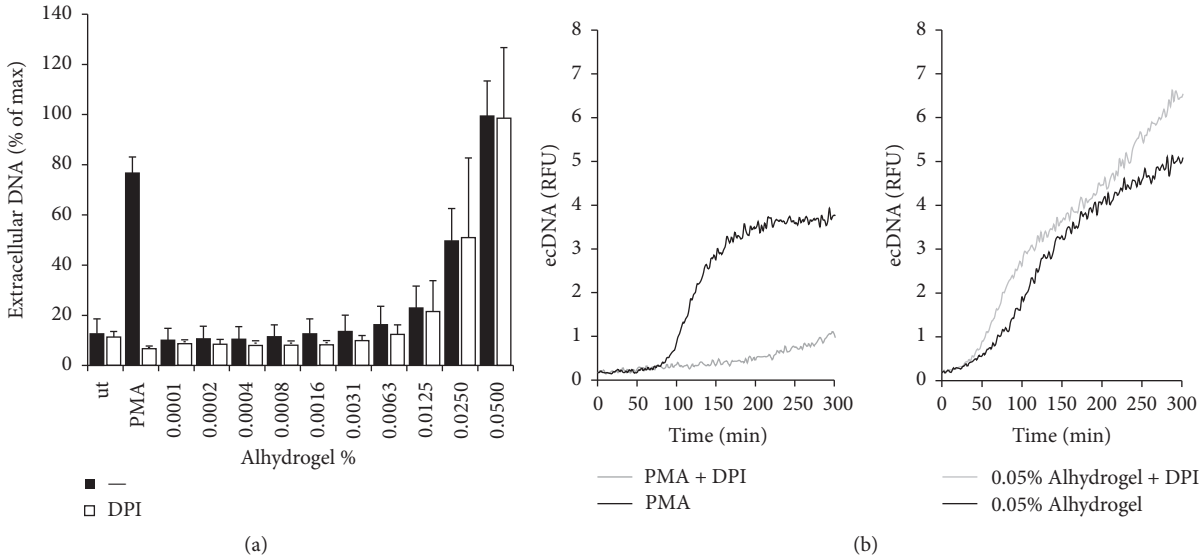

Figure 3: PMNs release extracellular DNA in response to Alhydrogel in vitro. Human PMNs seeded on a 96-well black microplate were incubated for 30 minutes in the presence or absence of 10 μM DPI prior stimulation with increasing doses of commercially available Alhydrogel (InvivoGen, cat#: vac-alu-50) or 100 nM PMA. Increase in fluorescence due to extracellular DNA (ecDNA) release was measured in presence of 10 μM Sytox Orange DNA-binding dye for 5 hours with a microplate fluorimeter. DNA release is presented as either relative fluorescence units (RFU) or percentage of maximal DNA released achieved by saponin treatment [4, 20]. (a) Summary of three independent experiments using PMNs obtained from independent human donors. Mean +/− SEM. (b) Representative kinetics of fluorescence results (n = 3). Ut, untreated; PMA, phorbol myristate acetate.

that activated Th17 cells and amplified leukocyte recruitment [30]. The authors concluded that danger signals fuel sterile inflammation in atherosclerosis via PMNs [30].

6. Silica Crystals

Chronic exposure to silica crystals leads to pulmonary silicosis or chronic obstructive pulmonary disease and also relates to vasculitis or chronic renal failure [56, 57]. Silica crystals activate the inflammasome and can be phagocytosed by immune cells including PMNs [58]. NETs have also been associated with glomerulonephritis and small vessel vasculitis as the source of antineutrophilic cytoplasmic antibodies [59, 60]. Although silica crystal stimulation of murine PMNs leads to ROS release, the in vivo relevance of this finding has not been established yet [61]. Brinkmann et al. described extracellular DNA release in human PMNs challenged with different doses of silica crystals suggesting that silica crystal-promoted NETs could play an important role in the establishment of lung disease [31]. PMNs are known to be recruited in large numbers to the lungs in silicosis animal models and human patients [62–64]. While silica crystal-stimulated DNA release from PMNs was comparable to that induced by MSU crystals [31], eosinophils did not release ETs in the presence of silica crystals [7].

7. Conclusion

Despite their different origin and structure, microcrystals activate PMNs leading to an inflammatory response. PMNs attempt to engulf microcrystals that is required for launching their effector responses including ROS production and NET release. Although a young and specific field, PMN-microcrystal interactions are clinically relevant to study due to their involvement in diverse biological processes ranging from disease pathologies of sterile autoinflammatory and infectious diseases to vaccination.

Competing Interests

The author has no conflict of interests to report.

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Review Article

Thrombosis, Neuroinflammation, and Poststroke Infection: The Multifaceted Role of Neutrophils in Stroke

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Immune cells can significantly predict and affect the clinical outcome of stroke. In particular, the neutrophil-to-lymphocyte ratio was shown to predict hemorrhagic transformation and the clinical outcome of stroke; however, the immunological mechanisms underlying these effects are poorly understood. Neutrophils are the first cells to invade injured tissue following focal brain ischemia. In these conditions, their proinflammatory properties enhance tissue damage and may promote ischemic incidences by inducing thrombus formation. Therefore, they constitute a potential target for therapeutic approaches and prevention of stroke. Indeed, in animal models of focal brain ischemia, neutrophils have been targeted with successful results. However, even in brain lesions, neutrophils also exert beneficial effects, because they are involved in triggering immunological removal of cell debris. Furthermore, intact neutrophil function is essential for maintaining immunological defense against bacterial infections. Several studies have demonstrated that stroke-derived neutrophils displayed impaired bacterial defense capacity. Because infections are known to impair the clinical course of stroke, therapeutic interventions that target neutrophils should preserve or even restore their function outside the central nervous system (CNS). This complex situation requires well-tailored therapeutic approaches that can effectively tackle immune cell invasion in the brain but avoid increasing poststroke infections.

1. Introduction

Stroke is one of the leading causes of death in the world. Most stroke-related deaths result from thrombotic occlusion of brain vessels. Infections are a known risk factor for acute stroke [1]. This enhanced risk is at least in part due to activated immune cells that interact with platelets and release coagulation factors, which amplify thrombus formation. In this review, Section 2 describes the deleterious role of neutrophils in initiating thrombosis. Stroke treatment has been limited to a strategy of rapid revascularisation, initiated within 4.5 h of onset, by inducing thrombolysis with recombinant tissue plasminogen activator (rtPA). In the setting of intracranial large artery occlusion (iLAO) this treatment is associated with low rates of recanalization and high rates of neurological morbidity and severe disability. Here endovascular therapy, particularly mechanical thrombectomy, is a promising therapeutic adjunct to rtPA [2]. The early multicentre trails IMS III [3], MR RESCUE [4], and SYNTHESIS Expansion [5] failed to show a benefit from endovascular intervention. However, quite recently, a series of studies with improved protocols demonstrated that mechanical recanalization in combination with rtPA administration was a superior treatment strategy in patients with iLAO compared to rtPA treatment alone [6–11].

The control of inflammation at the site of the ischemic lesion is a potential therapeutic target that has resulted in promising results in experimental stroke studies and may allow for a longer therapeutic window. Cellular invasion and the resulting proinflammatory response develop within days rather than hours and contribute to secondary lesion growth, which enhances ischemic brain tissue destruction. The role of neutrophils in these central events and corresponding therapeutic approaches are discussed in Section 3.

Another aspect of stroke is the systemic immune suppression that predisposes patients to systemic bacterial infections. Infection by itself is an independent risk factor of an

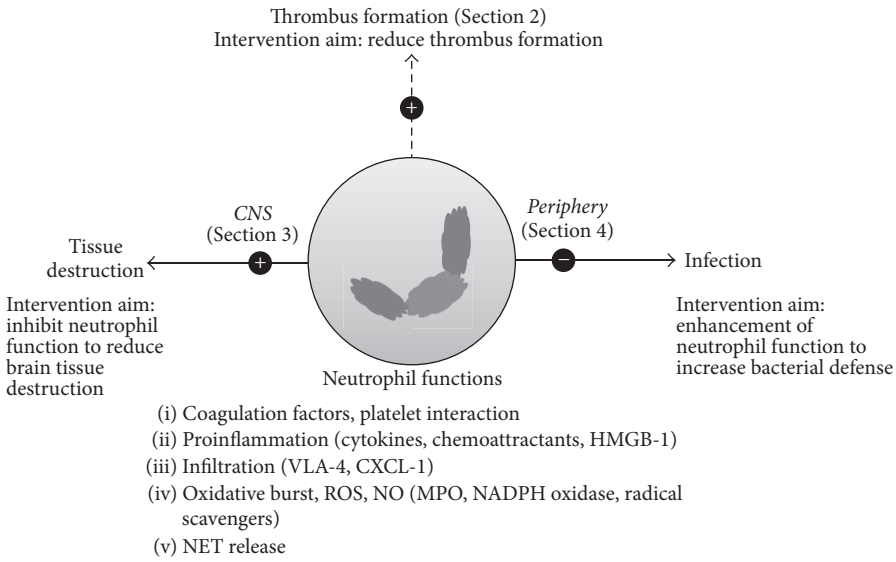

Figure 1: Neutrophil functions that can be targeted to reduce brain tissue destruction after stroke. Targets include factors involved in proinflammation, infiltration of immune cells, production of reactive oxygen species (ROS) and nitric oxide (NO), enzymatic functions of myeloperoxidase (MPO) and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, and the release of neutrophil extracellular trap (NET) components. Inhibiting these pathways may also reduce thrombus formation and prevent recurrent stroke. In addition, after a stroke, patients undergo poststroke immune suppression, which includes impaired oxidative burst and NET formation, induced by catecholamines. Enhancing bacterial defense by targeting these mechanisms could decrease the risk of secondary infections. Therefore, poststroke immune modulation must take into account the fact that immune suppression has opposing effects in the central nervous system and in the periphery. HMGB-1: high mobility group protein box 1; VLA-4: very-late-antigen 4; CXCL-1: chemokine (C-X-C motif) ligand 1.

unfavorable outcome in ischemic stroke [12, 13]. Infections contribute to worse clinical outcome measures, increased risk of recurrent stroke, and death. We discuss the function of neutrophils in bacterial defense, after stroke, and the associated therapeutic targets in Section 4. Figure 1 summarizes the aspects of stroke and the therapeutic targets evaluated in this review.

2. Neutrophils Promote Thrombosis

Neutrophils promote thrombus formation through different mechanisms, including the release of molecules involved in neutrophil extracellular trap (NET) formation, the release of proteases, and direct interactions with platelets. This prothrombotic process might (i) increase the risk of ischemic stroke and (ii) promote further thrombosis during acute stroke. The percentage of neutrophil-platelet interactions was enhanced in patients with symptomatic carotid stenosis [14]. However, the formation of leukocyte-platelet aggregates was reduced by inhibiting GPIIb/IIIa and selectin adhesion molecules. Leukocyte-platelet aggregate formation after ischemic stroke and reperfusion may be a useful biomarker and potential therapeutic target, since these aggregates also promote intravascular thrombus formation [15].

Neutrophils adhere to injured vessels immediately, preceding platelets, by binding to the activated endothelium through an interaction between leukocyte function-associated antigen and ICAM-1. This is an important step for the activation and accumulation of thrombocytes, and blocking this step might be an efficient strategy for reducing cerebral thrombosis. Both candesartan and dipyridamole were found to inhibit the adhesion of neutrophils to vascular endothelium in patients with ischemic stroke, but not in patients with chronic stroke or healthy individuals [16].

The protease, cathepsin G, is released by neutrophils and acts on coagulation factors to promote clot formation. Inhibition of cathepsin G decreased thrombus formation and reduced brain injury. The result was an improvement in the neurobehavioral outcome in a mouse model of ischemic stroke [17]. On the other hand, neutrophils also release the protease ADAMTS13, which cleaves hyperactive ultralarge von-Willebrand-factor and, thus, reduces acute cerebral inflammation after ischemic stroke [18].

An additional neutrophil contribution to thrombus formation results from the prothrombotic activity of NETs [19]. NETs have a web-like structure composed of DNA, histones, and specific granule proteins, such as neutrophil elastase and MPO, which can be released in response to various stimuli. Their primary function is to trap bacteria and exert bactericidal effects [20]. Platelets can bind to released NETs and get activated by histones [19, 21]. Interaction with neutrophils takes place through P-selectin and neutrophil P-selectin glycoprotein ligand-1 [22]. Upon activation platelets also express HMGB-1 and expose it on their surface promoting additional NET release by neutrophils [23], a self-energizing process that even activates the extrinsic coagulation pathway and may be responsible for further thromboinflammation observed after stroke [24]. Administration of DNase I resulted in the resolution of NETs. This strategy had a protective in vivo effect, in murine models of ischemic stroke [25].

3. Inflammatory Role of Neutrophils within the Brain

Necrotic cell death within the infarcted area leads to the release of proinflammatory cytokines and a rapid infiltration of immune cells. Neutrophils are the first cells recruited into the brain within minutes after stroke. The mechanism of neutrophil entry into the brain after stroke was investigated in permanent and transient experimental stroke models with in vivo imaging. Blood-borne neutrophils immediately migrate, even against blood flow, and then transmigrate out of blood vessels to reach the injured brain area [26]. The zenith of neutrophil invasion is reached between 48 and 72 h after stroke [27]. In physiological conditions, the blood brain barrier (BBB) controls the entry of immune cells into the brain. However, neutrophil entry is facilitated by a local BBB breakdown induced by ischemia [28].

The impact of immune cell invasion is a controversial issue. Although these cells might play a role in the initiation of tissue repair, their detrimental effects dominate. This was demonstrated in experimental stroke settings, where invading neutrophils enhanced ischemic neurotoxicity through different actions [29]. When neutrophils are activated, they produce reactive oxygen species (ROS), like superoxide radicals and hydrogen peroxide. In addition, they release enzymes from different granules, like cathepsin G, collagenase, gelatinase, and heparinase, which contribute to ROS-mediated extracellular matrix breakdown and vascular damage. As described in Section 2 neutrophils can activate complement and release cell content, like DNA, during suicidal extracellular trap formation. This antibacterial defense mechanism also includes the release of neutrophil elastase, which was shown to increase vascular permeability [30–32]. In addition, neutrophil release of proinflammatory mediators initiates a self-energizing cascade of proinflammation and destruction. Resident microglia can fight this detrimental destruction to a minor extent, by engulfing neutrophils [29].

These detrimental effects of neutrophils make them a prime target in novel therapies for stroke. Indeed, in experimental focal brain ischemia models, a variety of therapeutic interventions successfully reduced lesion size. One approach was to block proinflammatory cytokines and mediators that act as chemoattractants. For example, antagonization of C-X-C motif chemokine receptor 2 (CXCR-2) prevented recruitment of neutrophils to the infarct area [33]. Another neutrophil chemoattractant, chemokine (C-X-C motif) ligand 1 (CXCL-1), is induced by interleukin 17 (IL-17), which is released by $\gamma\delta$ T-cells. Blocking this pathway with an anti-IL-17-antibody reduced the experimental stroke lesion size [34]. In addition, neutrophil extravasation was shown to be mediated by very-late-antigen 4 (VLA-4) in an experimental stroke model. Accordingly, blocking VLA-4 reduced lesion size [26].

A different approach, which does not interfere with neutrophil invasion, is to block the neutrophil proinflammatory function. Oxidative stress, caused by an overload of ROS, contributes to various acute, chronic, and inflammatory diseases. Thus, this mechanism has been suggested as a target of stroke therapy. In the preclinical setting, beneficial effects were achieved by inhibiting type 4 nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX4). In experimental stroke models, brain damage was also ameliorated by inhibiting myeloperoxidase oxidant (MPO) production, with N-acetyl lysyltyrosylcysteine amide or with the flavonoid, eriodictyol [35, 36]. Moreover, neutrophil infiltration, assessed by measuring MPO activity, and infarct volume were significantly reduced following the administration of AM-36 (1-(2-(4-chlorophenyl)-2-hydroxy)ethyl-4-(3,5-bis-(1,1dimethyl-ethyl)-4-hydroxyphenyl) methylpiperazine). This arylalkyl-piperazine is a neuroprotectant with combined antioxidant and Na(+) channel-blocking actions [37].

Nitric oxide (NO) produced by inducible NO synthase (iNOS) contributes to ischemic brain injury. iNOS expression is predominantly found in invading neutrophils after stroke. When iNOS(+/+), but not iNOS(−/−), neutrophils were transferred into iNOS(−/−) mice, infarct volume increased. That result identified iNOS as an important mediator of secondary tissue destruction [38]. The inhibition of oxidative radical production was reported to be a favorable strategy in lacunar infarctions [39]. In contrast, the administration of Edaravone, a free radical scavenger, in patients with cardiogenic embolism increased hemorrhagic transformation [40]. In patients receiving rtPA treatment hemorrhagic complications and particular symptomatic intracerebral hemorrhage are more frequent in blacks and Asians. Since Mehta et al. administered Edaravone to patients with an Asian background it is possible that the higher bleeding rate was caused by ethnic-related reasons [41].

Uric acid, another free radical scavenger, was thought to protect the brain from oxidative injury. Until now studies investigating the neuroprotective effect of UA after stroke remain controversial [42]. While descriptive studies find that higher concentrations of UA in serum are beneficial in patients with stroke treated by thrombolysis [43, 44] the results of the URICO-ICTUS (study of intravenous uric acid administered during alteplase treatment for ischemic stroke) showed only a beneficial outcome for selected patient groups, for example, women [45].

Nevertheless it is known that different additional factors like advanced age, increased time to treatment, the extent of ischemic injury prior to administration of therapy, higher baseline National Institutes of Health Stroke Scale (NIHSS) score, high systolic blood pressure, or diabetes mellitus increase the risk of hemorrhagic incidence after stroke [46]. Therefore, treatment options might depend on the combination of individual factors.

Another molecule discussed in the modulation of post-stroke immune response is the HMGB-1. This DNA-binding protein is passively released during stroke from cells undergoing necrosis. This damage-associated molecular pattern molecule can also be actively secreted by immune cells and is released and exposed by platelets promoting thrombus formation as described in Section 2. In clinical studies, elevated plasma HMGB-1 levels were detected in patients with acute ischemic stroke. A correlation between HMGB-1 levels and circulating leukocytes was verified [47]. It was also shown that HMGB-1 contributed to tissue destruction by recruiting neutrophils and inducing extracellular trap

formation [48, 49]. Reductions in plasma HMGB-1 levels with cannabinoids were associated with reductions in infarct size and in the number of activated neutrophils [50]. The rapid early changes observed in experimental stroke models could be prevented by blocking β-adrenoceptors with propranolol or by neutralizing HMGB-1 activity with antibodies or an antagonist of its receptor, the receptor for advanced glycation end-products (RAGE) [51, 52]. These treatments were applied before and directly after stroke induction. To the best of our knowledge, no studies have reported delayed treatment regimens. Therefore, it remains unknown how the timing of catecholamine and HMGB-1 actions affects the development of stroke-induced immune alterations.

In addition to enhancing ischemic injury and the subsequent signaling cascades, neutrophils are also involved in reperfusion injury. Risk of hemorrhagic transformation is increased by as much as tenfold after intravenous rtPA administration, largely due to reperfusion injury and the toxic effects of rtPA [27]. High neutrophil counts and a high neutrophil-to-lymphocyte ratio were independently associated with worse outcomes at 3 months, in patients with stroke that were treated with rtPA [53, 54]. Similar results were found for patients with intracerebral hemorrhage [55]. Interestingly, treatment with rtPA induced neutrophil degranulation in vitro. In a cohort of 60 patients that underwent thrombolysis, during the first hours after drug administration, a peak of neutrophil degranulation products was observed, including matrix metalloproteinase- (MMP-) 9, MMP-8, neutrophil elastase, and MPO. Even though tissue destruction by neutrophils seems more pivotal, also protective molecules like tissue inhibitor of metalloproteinase- (TIMP-) 1 and TIMP-2 are elevated in serum [56].

Granulocyte colony stimulating factor (G-CSF) had a neuroprotective effect in several models of experimental stroke. Administration of G-CSF decreased infarct size and improved motor function recovery [57]. A recent meta-analysis of several small clinical trials concludes, though, that G-CSF did not improve stroke outcome in patients suffering from stroke [58]. In experimental stroke models applying rtPA, no beneficial effects of additional G-CSF administration were observed; instead an increased risk of hemorrhage occurred within the infarct area at 72 h after stroke [59]. In these models neutrophil blood counts were increased and neutrophilic activation occurred within 15 min after reperfusion, and it remained evident after 24 h [60]. Neutrophils might be mediators of hemorrhagic complications after thrombolysis; thus, they could represent new targets for neuroprotective strategies in patients treated with rtPA.

4. Anti-Infective Role of Neutrophils in the Periphery

Even before the appearance of stroke, neutrophils might play a role as a predictive marker of stroke risk. The neutrophil-to-lymphocyte ratio (NLR) was directly associated with the risk of stroke in patients with atrial fibrillation. As a predictor of stroke, the NLR appeared to be important for refining the risk of stroke and for improving the management of patients with atrial fibrillation and a low CHA2DS2-VASc score, a score for atrial fibrillation stroke risk [61]. The NLR was also increased in symptomatic intermediate carotid artery stenosis. It was shown that an elevated NLR was an independent variable associated with carotid artery plaques becoming symptomatic [62, 63].

In peripheral blood of patients with stroke, lymphocyte number and HLA-DR expression on monocytes decline, but neutrophil numbers increase. Animal studies have described an immediate reduction in spleen volume following cerebral ischemia. The change due to cerebral ischemia in human spleens was described as a biphasic process; splenic volumes initially decreased over time, reached a nadir at 48 h after stroke onset, and then increased thereafter. This process was positively correlated to the percentage of peripheral blood neutrophils [64]. Additionally, experimental stroke led to an activation of the hematopoietic system, via increased stimulation of the autonomic nervous system. This stimulation resulted in increased hematopoiesis and greater output of neutrophils from the bone marrow [65].

As previously discussed, despite an increase in granulocyte numbers, infections can occur in patients within days after a stroke [66]. A diagnosis of infection after stroke can be very difficult, because the hallmark signs, like fever and inflammation, can be present in patients as a consequence of neurological damage that disrupts homeostatic regulation of body temperature [67]. A method for identifying patients prone to subsequent infection could promote early interventions that reduce poststroke bacterial burden and improve clinical outcome. However, powerful prognostic biomarkers remain to be identified.

The role of neutrophils in infections after stroke is controversial. In the Enlimomab study protocol, patients with stroke were treated with anti-ICAM-1 antibodies to diminish neutrophils; however, those patients experienced an increased rate of infection, particularly pneumonia on day 5 after Enlimomab administration (2.2% versus 1.6% in placebo patients) as serious adverse event. Moreover, the infections were associated with a worse outcome [68]. In that study, neutrophils seemed to be important preventers of poststroke infections; inhibiting neutrophils with neutrophil inhibitory factors in humans did not increase the rate of infections, but this intervention neither reduced infarct volume nor improved stroke outcome [69].

Neutrophils obtained from patients that underwent neurosurgical interventions for hemorrhagic stroke showed significantly lower levels of oxygen species than neutrophils from healthy controls [70]. An earlier report suggested that alterations in neutrophil function occurred in patients with stroke, and these alterations were indicated by measurements of the granulocyte antisedimentation rate [71]. In ischemic stroke, ROS was impaired in monocytes and granulocytes, but no alterations were found in phagocytosis, migration, or the amounts of human neutrophil peptides 1 to 3 (HNP 1–3). However, patients with infections after stroke showed lower amounts of ROS than patients without a poststoke infection. Therefore, phagocyte dysfunction seemed to be associated with stroke-associated infections [72].

In patients with stroke, NETs were impaired in the early phase of stroke (day 1 of admission) and recovered function on day 5 after admission. Because these phagocyte dysfunctions were present upon admission to the stroke unit, they might contribute to the susceptibility to stroke-associated infections [72].

According to our current understanding of stroke-induced immune alterations, immediately after a stroke, a "storm" of stress hormones, particularly catecholamines, are released by the adrenal gland and via direct sympathetic innervation of the lymphoid organs (reviewed in [13, 73, 74]). This concept was derived from the original observation that a β-blockade at the time of stroke could reverse most effects of stroke-induced immune alterations observed in an experimental stroke model [51]. It is currently known that neutrophils express different receptors that are regulated by glucocorticoids and catecholamines. Interestingly, in vitro experiments have attributed stroke-induced neutrophil impairments to the influence of catecholamines [72].

5. Conclusions

Neutrophils are a promising target in stroke therapy. However, the development of novel, neutrophil-based therapies must take into account the opposing effects that immune suppression has in the CNS and in the periphery. Although bacterial defense must be maintained or enhanced in the periphery, the immune response in the brain is largely detrimental and should be inhibited. This quandary has been reflected in clinical trial results that could not reproduce preclinical experimental successes.

Competing Interests

The authors declare no competing interests.

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Review Article

Neutrophils and Immunity: From Bactericidal Action to Being Conquered

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The neutrophil is the major phagocyte and the final effector cell of the innate immunity, with a primary role in the clearance of extracellular pathogens. Using the broad array of cytokines, extracellular traps, and effector molecules as the humoral arm, neutrophils play a crucial role in the host defense against pathogen infections. On the other hand, the pathogen has the capacity to overcome neutrophil-mediated host defense to establish infection causing human disease. Pathogens, such as *S. aureus*, have the potential to thwart neutrophil chemotaxis and phagocytosis and thereby succeed in evading killing by neutrophils. Furthermore, *S. aureus* surviving within neutrophils promotes neutrophil cytolysis, resulting in the release of host-derived molecules that promote local inflammation. Here, we provide a detailed overview of the mechanisms by which neutrophils kill the extracellular pathogens and how pathogens evade neutrophils degradation. This review will provide insights that might be useful for the development of novel therapies against infections caused by antibiotic resistant pathogens.

1. Introduction

The immune system protects the body from microbes that invade and harm the host. In humans roughly 100 billion neutrophils enter and leave circulating blood every day [1] and constitute the dominant leukocyte population in the circulation, mediate the earliest innate immune responses to infection, and play a pivotal role in the resolution of microbial infections. Neutropenia, an acquired or inherited neutropenia, and neutrophil malfunction result in recurrent, life-threatening infections with bacteria [2].

Neutrophils originate and mature in the bone marrow and are subsequently released into the peripheral vasculature. After a pathogen has breached the epithelial barriers, neutrophils are the first innate immune cells that are rapidly recruited from the bloodstream to sites of infection. Pathogens entry and replication in host tissues lead to the release of exogenous products, such as formyl peptides, lipoproteins, or peptidoglycan. Moreover, the invasive pathogen can also damage body tissues that produce inflammatory signals, for example, chemoattractants and cytokines [3]. These pathogenic products and inflammatory signals are detected by neutrophils via Toll-like receptors (TLRs), G protein-coupled receptors (GPCR), and cognate immune receptors. By sensing the receptor signal, neutrophils will respond to these stimuli, extravasate from blood vessels, and migrate towards the site of infection to phagocytose pathogens. This multistep process encompasses rolling adhesion of neutrophils on endothelial cells, firm adhesion of neutrophils, extravasation through the endothelium, chemotactic migration, and subsequent killing of invading bacterial pathogens. Following migration to the site of infection and phagocytosis, neutrophils have a repertoire of antimicrobial arsenal at their disposal to fulfil this function [4]. Neutrophils utilize a combination of NADPH oxidase-derived reactive oxygen species (ROS), cytotoxic granule components, antimicrobial peptides, and neutrophil extracellular traps (NETs) to generate a highly lethal environment that is essential for efficient microbe killing and degradation [5, 6].

On the other hand, many pathogens have evolved efficient strategies to outfox the weaponry of neutrophils. The main strategies can be divided into five categories: evading extravasation and chemotaxis, preventing opsonization and phagocytosis, surviving inside the neutrophil, inducing cell

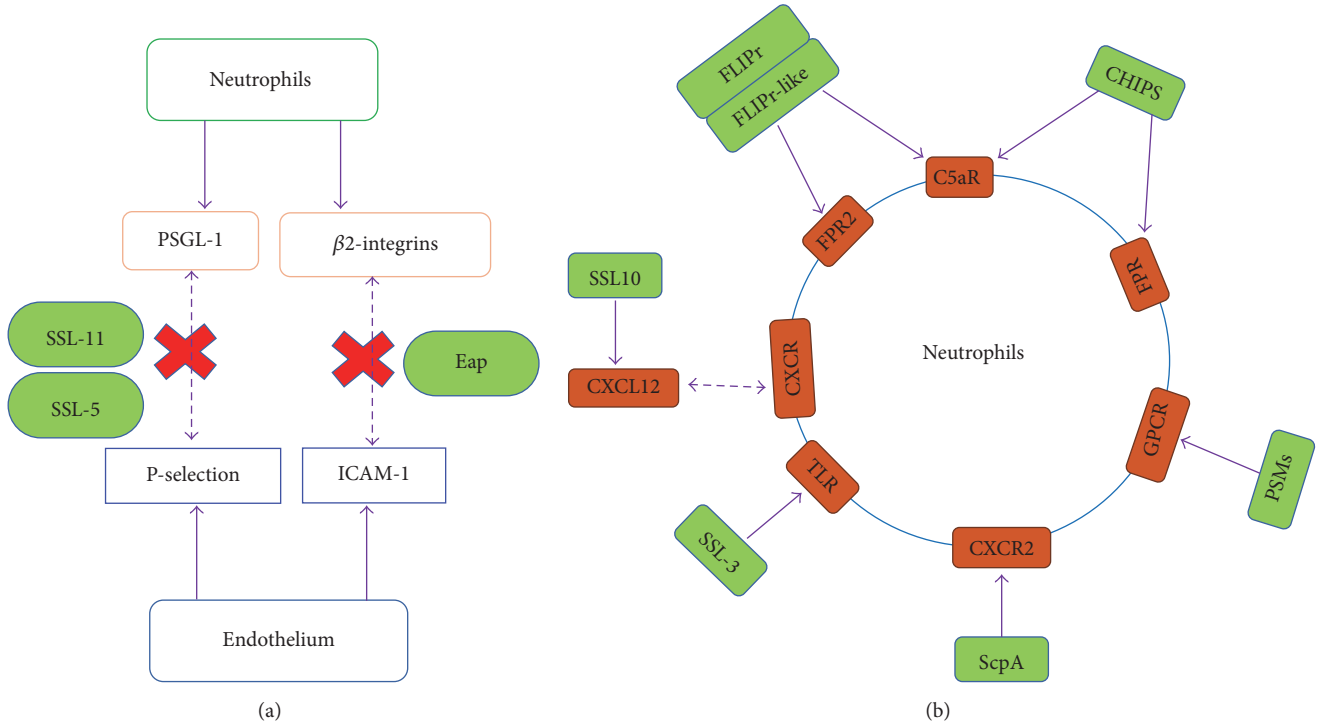

Figure 1: Evasion of neutrophil adhesion and transmigration. (a) Mechanisms by which *Staphylococcus aureus* subverts neutrophil extravasation. (b) Neutrophil attack and evasion of activation.

death, and avoiding killing in NETs [7, 8]. In this review, we will highlight the suite of mechanisms employed by neutrophils to clear bacterial infections and the corresponding counterattack mounted by bacterial pathogens.

2. Neutrophil-Mediated Phagocytosis of Pathogenic Microorganism

Initial elimination of invading pathogenic microorganism from human tissue is mediated by professional phagocytes. For efficient phagocytosis, neutrophils first need to leave the bloodstream and reach the site of infection, termed neutrophil recruitment. Furthermore, initiation of phagocytosis requires decoration of bacteria with opsonins that are recognized by specific surface receptors, of which process is termed opsonization of microbes. Lastly, neutrophils express numerous receptors that recognize microbe via binding its specific molecules and host proteins (such as IgG and complement), termed pathogen recognition.

2.1. Neutrophils Migrate from the Bloodstream to the Site of Infection. Upon the breach of epithelium by pathogens, as the first responder to microbial invasion, neutrophils leave the bloodstream and move to the site of infection. This recruitment process consists of three major steps: initiation of adherence to activated endothelial cells and rolling, neutrophil arrest caused by firm attachment to the endothelium, and finally migrating across the endothelial barrier to the infection site.

The initial step occurs through the interaction between the glycoprotein P-selectin glycoprotein ligand-1 (PSGL-1) of neutrophils and P-selectin/E-selectin of endothelial cells [9] (Figure 1(a)). Owing to this loose adhesion, neutrophils can roll along the endothelial cells. The second step is dependent on the interaction between $\beta 2$ integrins (such as LFA-1 and Mac-1) present on the surface of neutrophils and intercellular adhesion molecule 1 (ICAM-1) present on endothelial cells (Figure 1(a)). The final step is triggered by chemokines released by host cells and bacterial products. Host-derived chemokine, such as IL8, GRO-α, granulocyte chemotactic protein 2, and complement component C5a/C3a, are potent proinflammatory mediators that are used to recruit additional neutrophils to areas of infection. Furthermore, neutrophils migration also can be elicited by bacteria-derived chemokine, such as lipoteichoic acid or N-formyl peptides (fMLP).

2.2. Neutrophil Phagocytosis Is Dependent on Opsonization of Microbes. Initiation of neutrophil phagocytosis is dependent on opsonization of the target microbes that are recognized by specific surface receptors of neutrophils. Complement components and immunoglobulins (Igs) are the predominant factor in serum that enables efficient opsonization. The human complement system is composed of more than 30 proteins and is activated by any one of three routes: the classical pathway, the lectin pathway, and the alternative pathway (Figure 2). Complement system uses three independent pathways to distinguish bacteria from host cells and then can rapidly

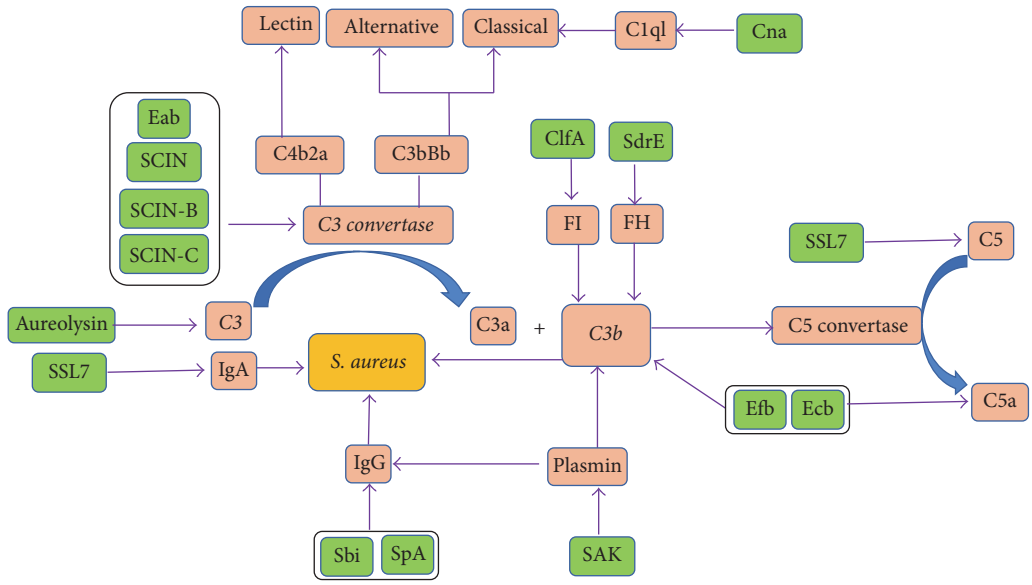

Figure 2: *Staphylococcus aureus* was interfered with chemotaxis and activation of complement.

recognize and opsonize bacteria or kill gram-negative bacteria directly by formation of the membrane attack complex [10]. All three pathways converge in the assembly of a C3 convertase, which are enzyme complexes that consist of C4b2a and C3bBb (C4b2a for the classical and lectin pathways and C3bBb for the alternative pathway). The C3 convertase catalyzes the key reaction in complement activation: cleavage of complement protein C3 results in release of anaphylactic agents C3a and C3b. Most of C3b is further processed into iC3b by complement factor H and complement factor I (Figure 2). At high local concentrations of C3b, the C3 convertase is changed into a C5 convertase, which cleaves C5, resulting in release of the potent chemoattractants C5a, and C5b, which initiates the lytic pathway when deposited on gram-negative bacteria, thereby amplifying the opsonization process.

Igs, which are the second most abundant protein in serum/plasma, play an important role in opsonization of bacteria and subsequent recognition by specific Fc receptor present on the surface of neutrophils. Several Ig subtypes (IgG, IgM, and IgA) have roles in microbial infection control (Figure 2). Different subclasses of Igs display distinct differences in complement activation and Fcγ receptor (FcγR). Normal human neutrophils express two major FcγRs, FcγRII and FcγRIIIB, and do not express the FcγR1 [11]. IgG can activate the classical complement pathway and neutralize toxins or other bacterial virulence factors. IgM, owing to its polymeric nature, is particularly effective at complement activation and opsonization. In contrast to IgG and IgM, IgA does not activate the complement system.

2.3. Receptor-Mediated Pathogen Recognition and Phagocytosis. Once neutrophils migrate to the site of infection, the opsonized pathogen can be recognized and phagocytized via receptor-mediated uptake into a vacuole within the cell. Similar to other phagocytes, such as macrophages, neutrophils express a large number of receptors including pattern-recognition receptors (PRRs), G protein-coupled receptors (GPCRs), and opsonic receptors. These receptors can recognize microbe-associated molecular patterns (MAMPs) and host proteins (such as IgG and complement) which were used to opsonize the microbe (Figure 1(b)). The PRRs can recognize pathogen-associated molecular patterns (PAMPs), such as bacterial DNA, lipopolysaccharide, peptidoglycan, and lipoteichoic acids. The major types of PRRs on neutrophils include Dectin-1 (recognizing fungal β-glucan), triggering receptor expressed on myeloid cells-1 (TREM-1, recognizing bacteria and fungi) [12], and toll-like receptors (TLRs, recognizing lipids, carbohydrates, and peptide). GPCRs, which are expressed in the surface of neutrophils, can recognize bacterial products as well as endogenous molecules released during inflammation. The formyl peptide receptors 1 (FPR1) and its homologue FPR2 belong to the GPCRs family, recognize N-formylated proteins and peptides (fMLP), and consequently induce and potentiate chemotaxis, phagocytosis, and the generation of oxidative burst in neutrophils (Figure 1(b)). Invasive bacterial pathogens are opsonized with complement (e.g., C3b) and antibody (e.g., IgG), recognized by opsonic receptors, including FcγRs and the complement receptors (CRs), respectively. Activation of opsonic receptors rapidly enhances the efficiency of phagocytosis and is critical for neutrophil-mediated pathogen killing [13].

3. Pathogen Killing by Neutrophils

Neutrophils are the first line of innate immune cells arriving at the site of bacterial inoculation, where they exert diverse antimicrobial mechanisms to prevent pathogen dissemination to normally sterile sites. The process by which neutrophils kill invading pathogens depends on three primary mechanisms [14]: production of highly toxic reactive oxygen

species (ROS) in the pathogen-containing vacuole; fusion of neutrophil granules, containing various antimicrobial mediators to the vacuole; NETs formation. These steps may also contribute to inflammatory diseases in which ligands are deposited on tissue components.

3.1. Phagocytic Uptake of Bacteria Triggers Production of ROS. Coincident with phagocytosis of bacteria, neutrophils produce an oxidative burst resulting in the rapid release of high levels of bactericidal reactive chemical species under the catalyzation of NADPH oxidase, myeloperoxidase (MPO), or nitric oxide (NO) synthetase [15]. NADPH oxidase is responsible for the generation of ROS, such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($HO^\bullet$). The NADPH oxidase functions by shuttling electrons across the phagosomal membrane from cytosolic NADPH to molecular oxygen to produce O_2^-. By superoxide dismutase (SOD), the superoxide anion is readily converted to hydrogen peroxide. H_2O_2 and O_2^- can combine to generate the highly reactive $HO^\bullet$ via the Haber-Weiss reaction, which requires a metal such as iron. As a microbicidal agent, $HO^\bullet$ was probably not found in intact cells because that lactoferrin inhibits the generation of $HO^\bullet$ and other free radical reactions by binding free copper and iron. Against certain pathogens, such as *Aspergillus*, NADPH oxidase is critical for host defense independently of proteinases, and its importance is revealed in that patients who lack any one of the oxidase subunits suffer from chronic granulomatous disease (CGD) [16].

MPO converts hydrogen peroxide to primarily hypochlorous acid (HOCl). HOCl is the most bactericidal oxidant in neutrophils. Notably, hydrogen peroxide and other secondary oxygen derivatives such hydroxyl radical, chloramines, and HOCl can inactivate iron-sulphur proteins, membrane proteins, and the origin of replication site for DNA synthesis, which play a critical role in the killing of pathogenic bacteria [17]. Indeed, some patients, whose neutrophils lacked MPO, were thought to be immunodeficient [18]. And MPO knockout mice have also shown an undue susceptibility to bacterial and fungal infections [19].

Oxidative deamination of L-arginine by nitric oxide (NO) synthetase generates NO that together with superoxide anion forms reactive nitrogen intermediates with antimicrobial activity [20]. NO, a short-lived (half-life of a few seconds), highly reactive molecule, is produced by inducible nitric oxide synthase (iNOS), which is present in primary granules and is induced upon neutrophil priming (via TNF, IL-1, or IFN-γ) and during bacterial infection. NO production complements ROS production by neutrophils to exert antibacterial functions.

3.2. Phagocytic Uptake of Bacteria Triggers Production of Degranulation. Pathogens sequestered by neutrophils are trafficked to and fused with the phagosome in a process called degranulation, leading to the killing of invading pathogens in a process involving the release and action of proteinases and peptidases (Table 1). Functionally, the granules can be subdivided into three different classes based on the contents of their matrices and their integral membrane proteins: azurophilic granule, specific granule, and gelatinase granule. Neutrophils are "prepacked" with multiple types of granules that fuse with phagocytic vacuoles to facilitate pathogens destruction. Moreover, granules also help to initiate an inflammatory response and contain alkaline phosphatase, lactoferrin, lysozyme, and NADPH oxidase.

Azurophil (or primary) granules are the first to be produced and contain MPO and a spectrum of neutrophil serine proteases (NSPs): cathepsin G (CG), neutrophil elastase (NE), proteinase 3 (PR3), and the recently discovered neutrophil serine protease-4 (NSP4) [30, 90]. NSPs are critical for the effective functioning of neutrophils and greatly contribute to immune protection against bacterial infections [27]. NSPs are currently believed to have three functions. (1) NSPs can directly kill bacterial cell. NE has been shown to directly kill the gram-negative bacteria *E. coil* by cleavage of its outer membrane protein A, resulting in loss of membrane integrity and cell death. In vivo, the concerted action of NE, CG, and PR3 can kill *S. pneumonia* within phagocytic vacuole. (2) NSPs can cleave host proteins to generate antimicrobial peptides. The best-known example is that PR3 that has been shown to cleave hCAP-18 to generate the antimicrobial peptide LL-37. (3) NSPs can attenuate bacterial virulence by inactivating factors required for pathogenesis. *Shigella flexneri* mobility proteins IcsA and IpaA-C can be cleaved by NE, consequentially preventing its dissemination into the cytoplasm of neutrophils. Similar to NE, CG can cleave the *S. aureus* adhesin clumping factor A and remove its active domain. Together, these NSPs are critical for the effective functioning of neutrophils and immune protection against bacterial infections [27].

In addition, neutrophils also contain a full-length cationic antimicrobial protein, bactericidal/permeability-increasing protein (BPI) in azurophil granules [91]. BPI possesses three types of anti-infective activities: direct antimicrobial activity, neutralizing endotoxin activity through direct binding of LPS, and opsonic activity. BPI binding to LPS results in increased bacterial permeability, hydrolysis of bacterial phospholipids, and death of the bacterium. In addition to its well-documented anti-infective properties, BPI has also been shown to possess additional bioactivities, such as accelerating apoptosis, binding the vascular endothelial growth factor (VEGF), and inhibiting migration of human umbilical vein endothelial cells.

The specific granules are smaller with 0.1 μm diameter and formed after azurophilic granules. These granules do not contain MPO and are characterized by the presence of the glycoprotein lactoferrin. They primarily contain a wide range of antimicrobial compounds including calprotectin, lactoferrin, neutrophil gelatinase-associated lipocalin (NGAL), hCAP-18, and lysozymes. Calprotectin, also called S100A, is a critical factor in the innate immune response to infection and has been shown to inhibit microbial growth through chelation of nutrient Mn^{2+} and Zn^{2+}, resulting in reprogramming of the bacterial transcriptome [92]. Lactoferrin, also called lactotransferrin, is an iron-binding glycoprotein present in most biological fluids of mammals and is released from neutrophil granules during inflammatory responses [93, 94]. Lactoferrin possesses a number of types of antibacterial activities: (1) blocking the entry of bacterial pathogens competitively

Table 1: Mechanism of action of neutrophil antimicrobial proteins/peptide.

Antimicrobial protein/peptide	Direct antimicrobial mechanism	Alternative antimicrobial mechanism	Subcellular localization	Ref.
α-Defensins	Membrane-active; inhibition of DNA, RNA, protein, bacterial cell wall synthesis	Opsonisation of bacteria/ROS formation	Primary granules, NETs	[21]
LL-37	Transmembrane pore-forming	ROS formation	Secondary granules, NETs	[22]
BPI	Hydrolysis of bacterial phospholipids by binding to LPS	Inhibiting cytokine liberation by binding to CD14	Primary granules	[23, 24]
Histones	Membrane-active	NETs formation	Nucleus, NETs	[25]
Lysozyme	Degrades bacterial cell wall	NETs formation	Lysosomes	[5, 26]
PR3	Proteolytic activity; degrading virulence factors	NETs formation	Primary granules/NETs	[27–29]
NE	Proteolytic activity; degrading virulence factors	NETs formation	Primary granules/NETs	[27–29]
CatG	Proteolytic activity	NETs formation; ROS formation	Primary granules/NETs	[28, 29]
NSP4	Trypsin-like activity	Unknown	Primary granules	[30–32]
Azurocidin	Membrane-active	Opsonisation of bacteria	Primary granules	[33]
Lactoferrin	Altering bacterial growth by binding to iron; increase in membrane permeability by binding to the lipid A	Decreasing the release IL-1, IL-2, and TNFα; Suppressing NETs release	Secondary granules/NETs	[34–37]
Calprotectin	Altering bacterial growth by sequestering Mn^{2+} and Zn^{2+}	Inhibition of Mn^{2+}-dependent bacterial superoxide defenses; NETs formation	Secondary granules	[38, 39]
PTX3	As a soluble pattern recognition receptor in innate immunity	NETs formation	Secondary granules/NETs	[40]
NADPH oxidase	Generation of superoxide anion	NETs formation	Lysosomes	[41]
MPO	Generation of hypochlorous acid	NETs formation	Lysosomes	[18, 42, 43]
Platelets	Activating neutrophils to release NETs	NETs formation	NETs	[44]
NGAL	Inhibit bacteria growth by capturing and depleting siderophores	Acting as a growth and differentiation factor in multiple cell type	Secondary granules	[45, 46]

binding onto cell receptors, such as glycosaminoglycans; (2) degrading protein virulence produced by bacteria, such as *H. influenza* and *E. coil*, through proteolysis; (3) preventing bacterial adhesion through competing bacterial adhesion sites on bacteria and host cells [95].

The tertiary granules, also named gelatinase granules, are smaller than specific granules and are both MPO- and lactoferrin-negative. These granules contain few antimicrobials but serve as a storage location for a number of metalloproteases, such as gelatinase and leukolysin. These granules may represent one end of the population of granules formed during neutrophil maturation.

3.3. Neutrophil Extracellular Traps Killing Bacteria. In addition to pathogens phagocytosis and subsequent reactive species- and enzyme-dependent pathogen destruction, neutrophils also exert antibacterial activity through neutrophil extracellular traps (NETs), which was first described by Brinkmann et al. in 2004 [96]. Sensing the entry of bacteria, neutrophils extrude a mesh-like structure consisting of DNA/histones and are peppered with granule-derived antimicrobial peptides and enzymes, a process termed NETosis. NETs are composed of DNA strands associated with histones and decorated with about 20 different proteins, including NE, CG, PR3, MPO, lactoferrin, pentraxin 3 [40], high mobility group protein B1, LL37, and buforin II [97]. Mitochondria can also serve as a source of DNA for NET formation. The NETs are capable of ensnaring microbes by localizing and trapping pathogens within a sticky meshwork of chromatin. Furthermore, NETs facilitate pathogen

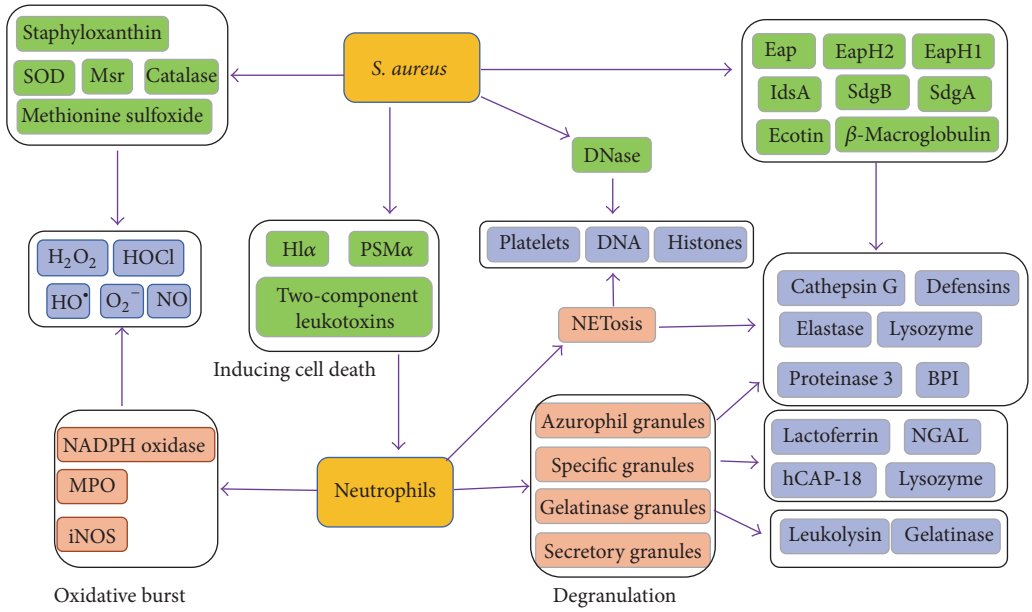

Figure 3: Direct antimicrobial mechanisms from neutrophils and the *S. aureus* counterattack. Neutrophils are equipped with multiple anti-infective strategies including the bacterial uptake (phagocytosis), the phagolysosomal degradation of bacteria via reactive oxygen species (oxidative burst), the release of antimicrobial molecules (degranulation), and the formation of a web-like structure composed of chromatin, histones, and antimicrobials (neutrophil extracellular traps, NETs). *S. aureus* is equipped with a magnitude of neutrophil resistance factors (green boxes) allowing the pathogen to uniquely counteract each antibacterial strategy of neutrophils.

exposing to highly concentrate antimicrobial peptides and enzymes, such as MPO, neutrophil elastase, LL-37, S100A, and lactoferrin-chelating proteins [98]. Along with the chromatin network, these antimicrobial agents are concentrated and the potential for synergistic action is enhanced. When neutrophils extrude a meshwork of chromatin to form NETs, it is not an end point for neutrophils and anuclear neutrophils can also migrate and retain the necessary components to kill bacteria through phagocytosis and formation of mature phagosomes [99].

The molecular mechanisms details of NETs formation are tightly linked to the production of ROS. The magnitude and duration of ROS production play an important role in promoting NETs formation and may be a major role in determining the fate of the neutrophil. In addition, individuals lacking MPO and NADPH oxidase, two key enzymes in the ROS cascade, are unable to make NETs and suffer from debilitating infections [100]. However, ROS are not the only vital roles in NETs formation and decondensation of chromatin is also critical for proper NETs formation. Neutrophil elastase was shown to partially degrade histones and further leads to decondensation of chromatin, which is also a pivotal event in the process of NETs formation [101].

NETosis also has the dark side: apart from this antimicrobial function, the cytotoxicity of NETs can be harmful to the host if their release is inappropriately controlled. Excessive NETs formation is linked to various neutrophil-mediated pathologies, including vasculitis, sepsis, and systemic lupus erythematosus nephritis. NETs also induce platelet procoagulant activation, which can lead to significant thrombosis and vascular injury. Excessive NETs formation and endothelial cell activation are also associated with preeclampsia of pregnancy [102].

4. "Catch Me If You Can": How Pathogens Evade Antibacterial Arsenal of Destruction by Neutrophils

To promote its own survival within the host, bacterial pathogens have evolved an array of specific mechanisms to overcome destructions by neutrophils (Figure 3, Table 2). *S. aureus*, the culprit of many types of infections, exhibits many characteristics of antineutrophils pathogens [103].

4.1. Inhibition of Neutrophil Recruitment. Counter measures adopted by pathogen may affect these steps to inhibit neutrophil recruitment. For instance, staphylococcal superantigen-like 5 (SSL5) can block neutrophil adhesion to endothelial cells by binding to PSGL-1 and consequently blocking its interaction with the natural ligand P-selectin [104]. SSL5 and other family members also inhibit leukocyte responses to chemokines, such as CXC, CC, CX3C, and CXCL12, and to the complement fragments C3a and C5a. Moreover, extracellular adherence protein (Eap) generated by *S. aureus* can bind and inhibit ICAM-1, a crucial molecule used to facilitate the neutrophils firm adhesion of endothelial cells. Furthermore, bacteria can secrete a variety of proteases, leading to degradation of chemokines. Chemotaxis inhibiting protein (CHIPS), a protein freely secreted by *S. aureus*, binds directly to the C5a receptor and formyl peptide receptors (FPRs) and thereby inhibits neutrophils recruitment [105, 106]. As a homologue of CHIPS in *S. aureus*, FPR-like 1 inhibitory proteins (FLIPr

Table 2: Neutrophil antibacterial functions subverted by *S. aureus*. *S. aureus* produces a large suite of virulence factors to counteract specific neutrophil clearance mechanisms during the pathogenesis of invasive infection.

Virulence factor	Targets	Function	Ref.
SSL-5	PSGL1/GPCRs	Recruitment/chemotaxis inhibition	[47, 48]
SSl-6	PSGL1	Recruitment inhibition	[49]
SSl-11	PSGL1	Recruitment inhibition	[50]
SSl-3	TLR2	Chemotaxis inhibition	[51, 52]
SEIX	PSSG1	Recruitment inhibition	[49]
ScpA	CXCR2	Chemotaxis inhibition	[53]
CHIPS	FPR1, C5aR	Chemotaxis inhibition	[54, 55]
FLIPr	FPR2	Chemotaxis inhibition	[56, 57]
FLIPrL	FPR1, FPR2	Chemotaxis inhibition	[56, 57]
PSMs	FPR2	Chemotaxis inhibition/neutrophils lysis	[56, 58]
Eap	ICAM1/C4b/NE/CG/PR3	Recruitment/phagocytic inhibition	[59]
Aureolysin	C3	Complement inhibition	[60]
SCIN	C3bBb	Complement inhibition	[61, 62]
SCIN-B/C	C3bBb	Complement inhibition	[61, 62]
Efb	C3b	Complement inhibition	[63]
Ecb	C3b	Complement inhibition	[64]
SSL7	IgA/C5	Phagocytosis/complement inhibition	[65]
SSL10	IgG	Phagocytosis inhibition	[66]
SAK	C3/IgG	Phagocytosis inhibition	[67]
Sbi	IgG/C3/factor H	Phagocytosis inhibition	[68, 69]
SpA	IgG	Phagocytosis inhibition	[70]
ClfA	Factor I	Phagocytosis inhibition	[56]
SOK	Unknown	Phagocytosis inhibition	[71]
CP	Unknown	Phagocytosis inhibition	[72]
SdrE	Factor H	Complement inhibition	[73]
IsdH	C3b	Complement inhibition	[74]
Cna	C1q	Complement inhibition	[75]
LukAB	αM integrin	Neutrophils lysis	[76]
LukED	CCR5/CXCR1/CXCR2	Neutrophils lysis	[77]
LukMF	Not known	Neutrophils lysis	[78]
PVL	C5aR	Neutrophils lysis	[79]
Hla	C5aR	Neutrophils lysis	[80]
Staphyloxanthin	Unknown	Resistance to ROS	[81]
KatA	Hydrogen peroxide	Resistance to ROS	[82]
AhpC	Hydrogen peroxide	Resistance to ROS	[82]
Msr	Hydrogen peroxide	Resistance to ROS	[83]
AdsA	Adenosine	Resistance to ROS	[84]
IsdA	Fibrinogen	Resistance to lactoferrin	[85]
OatA	Peptidoglycan	Resistance to lysozyme	[86, 87]
EapH1	NSPs	Resistance to NSPs	[88]
EapH2	NSPs	Resistance to NSPs	[88]
Nuclease	DNA	Resistance to NETs	[89]

and FLIPr-like) bind and inhibit FPR1 as well as C5aR and then impair neutrophil chemotaxis. Another cysteine protease secreted by *S. aureus* is staphopain A, which inactivates CXCR2 chemokines by cleaving its N-terminal domain and then inhibits neutrophil activation and recruitment [53]. In addition, SSL3 specifically binds and inhibits TLR2 activation, which is critical for host defense against *S. aureus* [107].

4.2. Preventing Phagocytosis. *S. aureus* has successfully developed ways to evade the complement system by secretion of specific complement inhibitors (Figure 2, Table 2). The

secreted factors described below allow bacteria to either diminish or delay the detrimental effects of an innate immune attack, thereby generating a window of opportunity to replicate and establish a microenvironment conducive to bacterial survival and disease pathogenesis [108, 109].

4.2.1. Cleavage of IgG. SSL7 binds host IgA and complement component C5, inhibiting generation of C5a, phagocytosis, and production of phagocyte reactive oxygen species. *S. aureus* expresses two surface-anchored proteins, staphylococcal protein A (SpA) and staphylococcal immunoglobulin-binding protein (Sbi), which impair IgG function. SpA possesses five immunoglobulin-binding repeat domains. Each domain can bind the Fc-part of IgG, thereby blocking the interaction with Fc receptors on neutrophils. Sbi consists of four small domains, of which two (Sbi-I and Sbi-II) can bind IgG [110–112].

4.2.2. Direct Inactivation of C3 Convertases. It has been shown that SCIN and its homologues (SCIN–B and SCIN–C), as strongly antiphagocytic molecules, modulate all the three complement pathways through the unique interaction with C3 convertases [61]. Extracellular fibrinogen-binding protein (Efb) and its homologue extracellular complement-binding protein (Ecb) can modulate the alternative pathway convertase by binding to the C3b molecule directly [113]. *S. aureus* secretes the 16 Kda Efb that binds two different plasma proteins using separate domains: the Efb N-terminus binds to fibrinogen, while the C-terminus binds complement C3b.

4.2.3. Binding or Cleavage of Human Convertase Regulators. *S. aureus* recruits the complement regulatory protein factor H (fH) and factor I (fI) to its surface to inhibit the alternative pathway of complement activation. The surface-associated protein SdrE, as an fH-binding protein, enhances recruitment of fH which resulted in increased iC3b generation [73]. The clumping factor A (ClfA) of *S. aureus* binds to complement regulator factor I and increases factor I cleavage of C3b [114–116]. Similar to ClfA, iron-regulated surface determinant protein H (IsdH) could act as a factor I-mimicking protease and directly trap factor I to the *S. aureus* surface, promoting cleavage of C3b [74].

4.2.4. Eliminating Opsonic Molecules from the Bacterial Surface. Staphylokinase (SAK) is a secreted protein that binds and activates surface-bound plasminogen into plasmin, which removes IgG as well as C3b from the bacterial surface, making this protein a unique antiopsonic molecule [67]. Aureolysin [60], a secreted metalloprotease, inhibits the deposition of C3b on *S. aureus* surfaces and the release of the chemoattractant C5a. It has been shown that aureolysin cleaves the central complement protein C3 specifically in the α-chain, close to the C3 convertase cleavage site, yielding active C3a and C3b. The antiphagocytic activity of the capsule is well established and the quantity of capsule is decisive for *S. aureus* and virulence. Overexpression of capsular polysaccharides type 8 renders *S. aureus* more resistant to phagocytosis by neutrophils in vitro [72, 117].

4.3. Surviving inside the Neutrophil. The combined action of ROS and antimicrobial proteins generated by granules creates a lethal environment for microbes. However, *S. aureus* harbored by neutrophils can survive in the presence of extreme environment, although not replication [118]. This is because *S. aureus* has evolved many means to resist oxidant damage and antimicrobial proteins degradation, as well as surviving within phagosomes.

First of all, *S. aureus* strains can express five types of enzymes or pigment promoting resistance to oxidative killing by stimulated neutrophils, including superoxide dismutase, catalase, staphyloxanthin, methionine sulfoxide reductases (Msr), and adenosine synthase A (AdsA). Superoxide dismutase produced by *S. aureus* can convert superoxide anion to H_2O_2, which is then consumed to yield O_2 and H_2O by catalase, thereby eliminating oxidants generated by stimulated neutrophils. Furthermore, *S. aureus* strains also produce the pigment staphyloxanthin, which consumes oxidants and renders bacteria resistant to oxidant-dependent killing, protecting bacteria from singlet oxygen via an undefined mechanism. Msr is a highly conserved enzyme that repairs oxidative damage incurred within neutrophils, contributing to survival of bacteria within neutrophils. AdsA, a cell wall-anchored enzyme, can convert adenosine monophosphate to adenosine [119]. As a critical virulence factor, AdsA promotes staphylococcal synthesis of adenosine in blood, escaping from phagocytic clearance [84]. Adenosine is also known to inhibit neutrophil degranulation, adhesion to vascular surfaces, and superoxide burst [120]. These findings indicate that phagocytosed *S. aureus* devote significant energy and effort to self-preservation rather than to growth and replication.

Bacteria pathogens have evolved two strategies to counteract human NSPs. The first one is modifications of bacterial NSPs substrates. For gram-positives, such as *S. epidermidis* and *S. aureus*, glycosyltransferases (SdgA and SdgB) are expressed to modify the serine-aspartate dipeptide repeats (SDR) of GLcNAc, which will protect these bacteria from proteolytic degradation by CG. The LPS of Gram-negatives, such as *Neisseria meningitidis*, are anchored to the outer membrane by lipid A. The lipid can be modified by phosphoethanolamine transferase to prevent proteolysis-independent killing by CG.

The second strategy is production of NSPs inhibitors. A recent study reports that *S. aureus* has evolved three highly specific NSPs inhibitors: extracellular adherence protein (Eap) and its smaller homologues EapH1 and EapH2 [88]. These proteins are very potent and specific inhibitors of NSPs and imply a crucial role for NSPs in the defense against *S. aureus*. Iron-regulated surface determinant protein H (IsdH) is present in the surface of *S. aureus*, which binds to lactoferrin, the most abundant antistaphylococcal polypeptide [121]. IsdA confers resistance to killing by lactoferrin. In addition, recombinant IsdA was a competitive inhibitor of lactoferrin protease activity. Thus, IsdA can protect *S. aureus* against lactoferrin and acts as a protease inhibitor [122].

4.4. Inducing Cell Death by Cytolytic Toxins. Following phagocytosis of bacteria pathogens, neutrophils would kill most bacteria and have initial features typical of apoptosis.

However, *S. aureus* can survive within these neutrophils and ultimately cause cytolysis. Recent studies have provided evidence that cytolytic toxins produced by *S. aureus* contribute to neutrophil lysis after phagocytosis [123]. Cytolytic toxins produced by *S. aureus*, including the phenol soluble modulins (PSMs), alpha-hemolysin (Hlα), and two-component leukotoxins, facilitate neutrophil killing after phagocytosis [124]. PSMs were first identified in 1999 by hot phenol extraction from *S. epidermidis* culture filtrate, in which three peptides termed PSMα, PSMβ, and PSMγ were identified. PSMs do not have uniform charge characteristics. PSMαs of *S. aureus* are positively charged, while PSMβ peptides are all negatively charged, and the PSMγ is neutral [125]. In *S. aureus*, PSMα peptides have a pronounced ability to lyse human neutrophils, in which PSMα3 has by far the strongest activity. However, PSMγ (also named δ-toxin) has moderate cytolytic activity and the PSMβ peptides are non-cytolytic. At the micromolar concentrations, PSMα has the pronounced capacity to kill human neutrophils after phagocytosis by disrupting the cytoplasmic membrane [126]. While at nanomolar concentrations, PSMα may stimulate neutrophils and initiate proinflammatory responses including neutrophil chemoattraction, activation, and the release of IL-8 [127]. Neutrophils sense PSMs via formyl peptide receptor 2 (FPR2), which may sense the amphipathic, α-helical structure of PSMs rather than a specific amino acid sequence motif.

Panton-Valentine Leukocidin (PVL) is a prophage-encoded pore-forming exotoxin, which mainly acts on neutrophils as a crucial virulence factor in necrotizing diseases. PVL is a staphylococcal bicomponent pore-forming toxin comprising the protein subunits LukS-PV and LukF-PV [128]. Initial binding of LukS-PV to the surface of target cells triggers secondary binding of LukF-PV and subsequently induces the assembly of lytic pore-forming [129]. PVL-induced pore formation is mediated by the human C5aR, which determines species specificity of PVL [79]. The C5aR can bind LukS-PV, which is a potent inhibitor of C5a-induced immune cell activation.

S. aureus α-hemolysin (α-toxin, Hla) belongs to the class of small β-barrel pore-forming cytotoxins [130]. As a water soluble monomer, α-hemolysin is capable of binding and oligomerization into a heptameric structure on neutrophils. Then, α-hemolysin exhibits the main action on pore formation and neutrophils lysis after phagocytosis [131]. In other studies, α-hemolysin has been suggested to directly disrupt the *S. aureus* phagosome and promote *S. aureus* escape to and replication in the cytoplasm [131]. *S. aureus* α-hemolysin facilitates the secretion of newly synthesized CXC chemokines into the airway and stimulates neutrophil homing in staphylococcus aureus pneumonia [132].

4.5. Avoiding Killing in NETs. In addition to phagocytosis and intracellular killing, neutrophils release NETs that capture and kill microbes in the extracellular space. Several bacterial pathogens have evolved sophisticated mechanisms to suppress, escape, and/or resist NETs. Expression of nucleases is one highly conserved anti-NET factor among bacteria, which can degrade NETs indicating that the chromatin functions as a scaffold and is a major component of the fibres. Interestingly, extracellular nucleases are found in several pathogenic bacteria including *S. aureus*, *Clostridium perfringens*, and *S. pyogenes* (group A *Streptococcus*, GAS) [133].

In addition to produce nucleases, GAS can also suppress NETs formation by degrading the neutrophil stimulatory chemokine IL-8 with peptidase SpyCEP or HA capsule engagement of the inhibitory neutrophil receptor Siglec-9. Other GAS resistance factors, including M1 protein, Scl-1 protein, and the GLcNAc side chain, contribute to GAS resistance to antimicrobial components.

5. Conclusion

The interaction between neutrophils and pathogens remains a fascinating subject. The host requires the action of neutrophils to fight invaders and the pathogens in turn must cope with neutrophil attacks in order to colonize the host [134]. Several pharmacological agents can be used to enhance neutrophil energy generation, antimicrobial activities, and treatment outcomes. For instance, hypoxia-inducible factor 1 (HIF-1), innate defense regulator peptides (IDRs), and vitamin B3 all enhance antimicrobial activities to provide prophylactic and therapeutic activity against bacterial pathogens in vivo. And tamoxifen [135] or anacardic acid [136] could boost NETs formation and bacterial killing of neutrophils. To overcome antibiotic resistant pathogens which harness the multifaceted antimicrobial properties of neutrophils, these host-directed strategies provide a critical new element to boost neutrophil function and minimize the risk for development of antibiotic resistance during infection.

Competing Interests

The authors declare that they have no competing interests.

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