ROLE OF NEUTROPHILS IN INFLAMMATORY DISEASES

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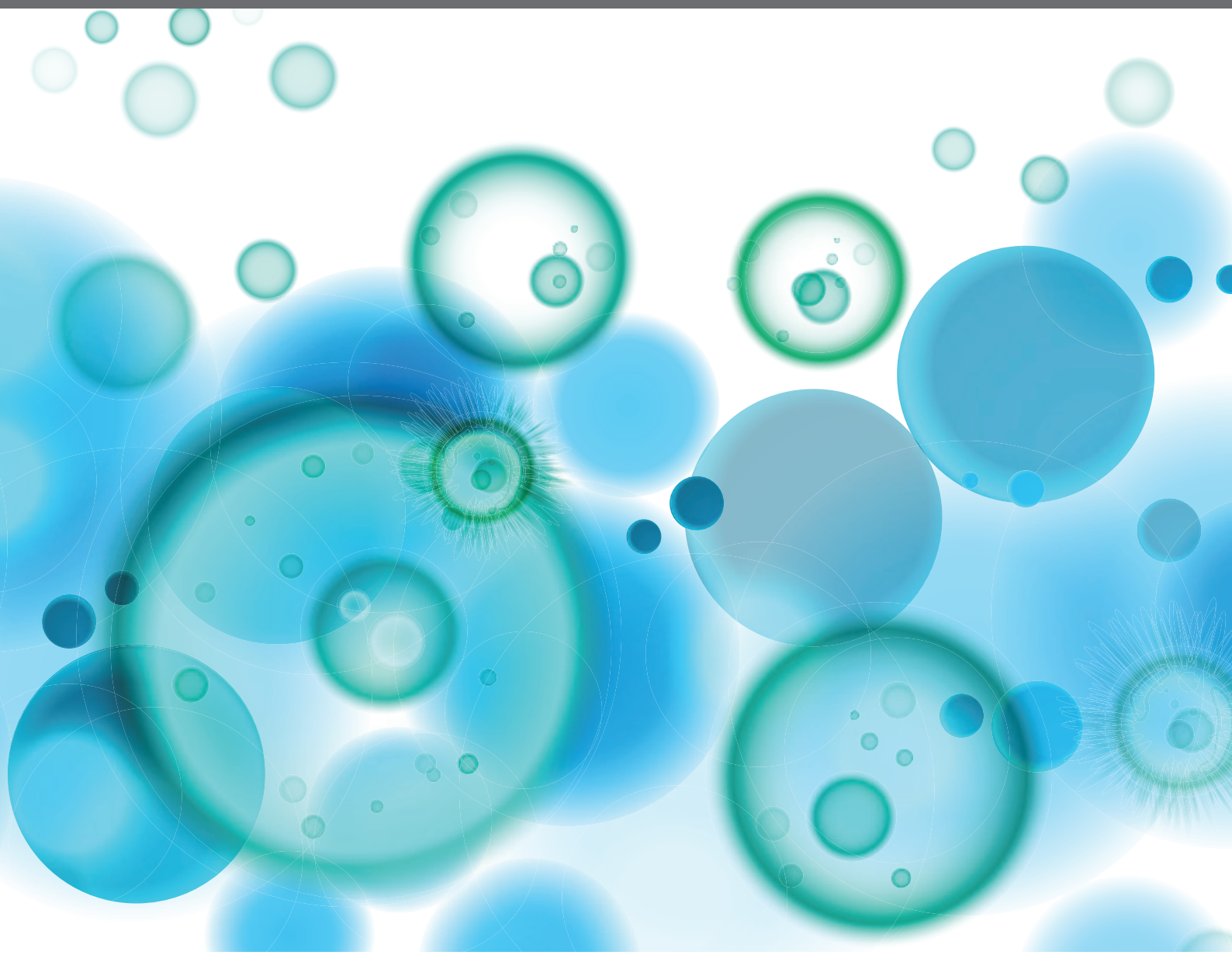

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ROLE OF NEUTROPHILS IN INFLAMMATORY DISEASES

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Table of Contents

04 ***Editorial: Role of Neutrophils in Inflammatory Diseases***
Thomas Marichal, Onur Boyman and Laurent L. Reber

07 ***Role of Neutrophil Extracellular Traps and Vesicles in Regulating Vascular Endothelial Permeability***
Yonggang Ma, Xiaoyuan Yang, Victor Chatterjee, Jamie E. Meegan, Richard S. Beard Jr. and Sarah Y. Yuan

17 ***Cooperative Action of Oxidized Low-Density Lipoproteins and Neutrophils on Endothelial Inflammatory Responses Through Neutrophil Extracellular Trap Formation***
Takashi Obama, Hitomi Ohinata, Takashi Takaki, Sanju Iwamoto, Naoko Sawada, Toshihiro Aiuchi, Rina Kato and Hiroyuki Itabe

31 ***Neutrophils in Psoriasis***
Chih-Chao Chiang, Wei-Jen Cheng, Michal Korinek, Cheng-Yu Lin and Tsong-Long Hwang

43 ***Short-Term Fever-Range Hyperthermia Accelerates NETosis and Reduces Pro-inflammatory Cytokine Secretion by Human Neutrophils***
Irene A. Keitelman, Florencia Sabbione, Carolina M. Shiromizu, Constanza Giai, Federico Fuentes, David Rosso, Camila Ledo, Maximiliano Miglio Rodriguez, Mauricio Guzman, Jorge R. Geffner, Jeremías Galletti, Carolina Jancic, Marisa I. Gómez and Analía S. Trevani

56 ***The Regulatory Effects of Interleukin-4 Receptor Signaling on Neutrophils in Type 2 Immune Responses***
Cecilie Egholm, Lukas E. M. Heeb, Daniela Impellizzieri and Onur Boyman

70 ***The Prolyl Isomerase Pin1 Controls Lipopolysaccharide-Induced Priming of NADPH Oxidase in Human Neutrophils***
Min Liu, Samia Bedouhene, Margarita Hurtado-Nedelec, Coralie Pintard, Pham My-chan Dang, Shiyuan Yu and Jamel El-Benna

82 ***Neutrophil Extracellular Traps in Autoimmunity and Allergy: Immune Complexes at Work***
Vanessa Granger, Marine Peyneau, Sylvie Chollet-Martin and Luc de Chaisemartin

92 ***IL8 and PMA Trigger the Regulation of Different Biological Processes in Granulocyte Activation***
Roxane L. Degroote, Maria Weigand, Stefanie M. Hauck and Cornelia A. Deeg

105 ***Mouse Models and Tools for the in vivo Study of Neutrophils***
Julien Stackowicz, Friederike Jönsson and Laurent L. Reber

124 ***Detection and Quantification of Histone H4 Citrullination in Early NETosis With Image Flow Cytometry Version 4***
Emilia A. Barbu, Venina M. Dominical, Laurel Mendelsohn and Swee Lay Thein

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EDITORIAL
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Editorial: Role of Neutrophils in Inflammatory Diseases

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Keywords: neutrophils, inflammation, neutrophil extracellular trap, immunity, models

Editorial on the Research Topic

Role of Neutrophils in Inflammatory Diseases

Neutrophils are best known for their role in host defense against microbes (1). However, many recent discoveries highlight the fact that neutrophils also act as key regulators of both acute and chronic sterile inflammatory conditions (2). Neutrophils can mediate their functions through different mechanisms, including generation of reactive oxygen species (ROS) and neutrophil extracellular traps (NETs), and the release of a broad range of mediators, including cytokines, alarmins, and proteases (1). A better knowledge of the mechanisms controlling these processes might thus help design new therapeutic strategies for the treatment of inflammatory diseases.

This Research Topic brings together original and review articles that explore the role of neutrophils and neutrophil-derived products in various inflammatory diseases, and assess some molecular mechanisms through which these cells are activated in inflammatory conditions.

It is known that lipopolysaccharide (LPS) from gram negative bacteria can "prime" neutrophil for ROS production in combination with other agonists. Liu et al. used pharmacological approaches to show that the prolyl *cys/trans* isomerase Pin1 plays an important role in LPS-induced priming of human neutrophils. These data suggest that Pin1 could be a therapeutic target in sepsis and other diseases involving ROS generation by neutrophils.

Chiang et al. present an overview of the potential roles of neutrophils in psoriasis, a chronic inflammatory skin disease in which high numbers of neutrophils are observed in skin lesions. Their review focuses mostly on clinical observations, including evidence of neutrophil activation in skin lesions, with generation of ROS and formation of NETs. The authors also discuss the potential contribution of NETs in driving Th17 inflammation in psoriasis.

Lastly, the potential role of NETs in infectious and inflammatory diseases has emerged as a major field of research (3, 4). Not surprisingly, several manuscripts of this special issue focus on the mechanisms of NET formation, and on the role of NETs in various inflammatory conditions. Barbu et al. describe a new method for the detection and quantification of histone H4 citrullination (H4cit3) during NET formation using imaging flow cytometry (IFC). Using this method, the authors quantify H4cit3 in human blood neutrophils stimulated with NET-triggering molecules. They suggest that their method can be particularly suitable for studies looking at NETs as potential biomarkers for diseases. The method is fast and requires only minimal handling of neutrophils,

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thereby reducing potential unspecific cell activation. However, the authors also emphasize that detection limitations might be associated with some stimuli.

Many infectious and inflammatory diseases are characterized by an increase in vascular permeability. In a review article, Ma et al. present a detailed overview of current knowledge on the potential role of neutrophils in regulating vascular endothelial permeability. The authors particularly focus on the role of NETs and the molecular pathways leading to NET formation, and on the potential role of neutrophil-derived extracellular vesicules, which are heterogenous membrane-enveloped structures released by many cell types, including neutrophils.

Fever is a hallmark of both infectious and inflammatory diseases, and hyperthermia is known to increase neutrophil recruitment to the affected sites. Keitelman et al. studied the impact of hyperthermia on key neutrophil functions including NET release, production of ROS, and release of pro-inflammatory cytokines. The authors propose that fever that occurs during bacterial infections might trigger an emergency response in neutrophils in order to enhance some of their antimicrobial functions, but could at the same time reduce their capacity to release proinflammatory cytokines in order to limit potentially damaging inflammatory processes.

Obama et al. investigate the mechanisms of neutrophil activation in the context of cardiovascular diseases. The authors focused on the effects of oxidatively modified low-density lipoprotein (oxLDL), which are known as a risk factor for the progression of cardiovascular diseases. They show that while oxLDL alone does not induce NET formation, it enhances phorbol myristate acetate (PMA)-induced release of NETs and myeloperoxidase (MPO). The authors suggest that oxLDL may contribute to vascular endothelial inflammation, at least in part by NET-dependent mechanisms.

Granger et al. review the available literature on the role of IgG immune complexes (ICs) in triggering NET release through activation of IgG Fc gamma receptors (FcγRs) on neutrophils. They focus on two autoimmune diseases, systemic lupus erythematosus in which NETs can be induced by DNA/anti-DNA IgG ICs, and rheumatoid arthritis for which citrullinated protein/anti-citrullinated protein IgG ICs might mediate NET formation. In addition to autoimmunity, they also review available data on the potential role of ICs-induced NETs in anaphylaxis, a systemic and potentially lethal hypersensitivity reaction. Finally, the authors discuss the potential applications of NETs as biomarkers and therapeutic targets in these diseases.

Most of the aforementioned studies focus on the pro-inflammatory and tissue-damaging potential of neutrophils. However, it was reported that neutrophils also have important regulatory functions, and might participate in the resolution of inflammation (5). In this context, Egholm et al. review existing literature on the regulatory effect of the receptor IL-4R (shared by the cytokines IL-4 and IL-13) on neutrophils during type 2 immune responses. They highlight the fact that engagement of IL-4R on neutrophils can inhibit several neutrophil effector functions, including the formation of NETs. The authors thus propose that, during type 2 immune responses as in other immune responses, neutrophils are the first non-resident cells to arrive at the site of inflammation, but that subsequent IL-4/IL-13 signaling can rapidly shut off neutrophil effector functions to prevent damage to healthy tissues.

Many tools and models are now available to study the role of neutrophils during biological responses *in vivo*. However, each model has its own advantages and limitations. In the review by Stackowicz et al., the authors provide a side-by-side comparison of the main existing genetic and pharmacological approaches to assess the functions of neutrophils and neutrophil-derived products in mice. The authors recommend that, given the known or potential limitation(s) of each model, at least two different approaches should be employed to legitimately conclude on neutrophil functions.

Finally, illustrating the variety of models which can be used to study neutrophils, the work by Degroote et al. focuses on primary neutrophils from equine whole blood. The authors analyzed these neutrophils by mass spectrometry and identified a total of 2,032 proteins forming the whole proteome of these cells. Furthermore, they identified a total of 58 or 207 proteins differentially regulated after stimulation with interleukin-8 (IL-8, also known as CXCL8) or PMA, respectively.

In conclusion, the papers included in this Research Topic highlight the diverse role of neutrophils in the regulation of inflammatory responses, and the potential of future therapies targeting neutrophils or neutrophil-derived products for the management of inflammatory diseases.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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REFERENCES

1. Mayadas TN, Cullere X, Lowell CA. The multifaceted functions of neutrophils. *Annu Rev Pathol* (2014) 9:181–218. doi: 10.1146/annurev-pathol-020712-164023
2. Soehnlein O, Steffens S, Hidalgo A, Weber C. Neutrophils as protagonists and targets in chronic inflammation. *Nat Rev Immunol* (2017) 17(4):248–61. doi: 10.1038/nri.2017.10
3. Jorch SK, Kubes P. An emerging role for neutrophil extracellular traps in noninfectious disease. *Nat Med* (2017) 23(3):279–87. doi: 10.1038/nm.4294
4. Papayannopoulos V. Neutrophil extracellular traps in immunity and disease. *Nat Rev Immunol* (2018) 18(2):134–47. doi: 10.1038/nri.2017.105
5. Mantovani A, Cassatella MA, Costantini C, Jaillon S. Neutrophils in the activation and regulation of innate and adaptive immunity. *Nat Rev Immunol* (2011) 11(8):519–31. doi: 10.1038/nri3024

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REVIEW
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Role of Neutrophil Extracellular Traps and Vesicles in Regulating Vascular Endothelial Permeability

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The microvascular endothelium serves as the major barrier that controls the transport of blood constituents across the vessel wall. Barrier leakage occurs during infection or sterile inflammation, allowing plasma fluid and cells to extravasate and accumulate in surrounding tissues, an important pathology underlying a variety of infectious diseases and immune disorders. The leak process is triggered and regulated by bidirectional communications between circulating cells and vascular cells at the blood-vessel interface. While the molecular mechanisms underlying this complex process remain incompletely understood, emerging evidence supports the roles of neutrophil-endothelium interaction and neutrophil-derived products, including neutrophil extracellular traps and vesicles, in the pathogenesis of vascular barrier injury. In this review, we summarize the current knowledge on neutrophil-induced changes in endothelial barrier structures, with a detailed presentation of recently characterized molecular pathways involved in the production and effects of neutrophil extracellular traps and extracellular vesicles. Additionally, we discuss the therapeutic implications of altering neutrophil interactions with the endothelial barrier in treating inflammatory diseases.

Keywords: cell-cell junction, endothelial barrier, extracellular vesicles, glycocalyx, inflammation, neutrophil extracellular traps, permeability

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INTRODUCTION

Serving as the blood-tissue interface, the vascular endothelial barrier plays a critical role in regulating host defense against infection or injury. Endothelial hyperpermeability is considered an important cause, as well as consequence, of inflammatory/immune responses associated with sepsis, trauma, ischemia-reperfusion injury, diabetes, and metastatic tumor development (1, 2). This pathological process involves complex cell-cell communications and molecular signaling. Among the multiple subtypes of leukocytes in the circulation, polymorphonuclear granulocytes (PMNs), or neutrophils, are the most impactful cells to vascular permeability, as they can alter endothelial barrier properties via direct contacts (adhesion and transmigration) and/or by secreting bioactive products capable of disrupting the barrier structure. Below, we discuss the effects of neutrophil-endothelium contact and neutrophil-derived factors on endothelial permeability.

PMN-ENDOTHELIUM INTERACTIONS

Neutrophils comprise the innate immune system providing the first line of defense against invading bacteria and acute injury (1). Traditionally, the life span of mature neutrophils is thought to be short, as they normally stay in the circulation for 5–10 h and subsequently infiltrate into tissues and die within the next 8–16 h (1, 2). Challenging this dogma, recent studies using *in vivo* labeling with ^2H_2O reveal that the life span of human circulating neutrophils lasts as long as 5.4 days (3), at least 10 times longer than previously reported. Another interesting finding is that after diapedesis, neutrophils can live in tissues for up to 7 days in the proinflammatory microenvironment (4). Whether and how neutrophil interaction with the microvascular endothelium affects their life span in the circulation, or in tissues, remain as a puzzle; however, evidence is accumulating that endothelial cells have the ability to educate neutrophils and modify their behavior during diapedesis (5, 6).

Adhesion and Transendothelial Migration (TEM)

Neutrophil diapedesis is a tightly regulated process initiated with cell rolling along the microvascular (mainly venular) wall, followed by adhesion to endothelial surface and migration across the endothelium. The process is mediated by adhesion molecules whose expression is rapidly upregulated by inflammatory cytokines, including tumor necrosis factor (TNF)-α and interleukin (IL)-1β. In particular, ligation of neutrophil P-selectin and endothelial E-selectin slows down neutrophils and enables their rolling under relatively high shear stress (7, 8). Subsequently, firm adhesion is secured via the binding of neutrophil CD11/CD18 integrins to endothelial adhesion molecules (7, 9). Transmigration occurs through the paracellular route via endothelial cell-cell junctions (6), or through the transcellular route across endothelial cell body (10); the former is considered the predominant pathway (∼70–90%) (11). In 2004, Carman and colleagues identified microvilli-like projections on endothelial cell surface that form "transmigratory cup" to provide directional guidance for leukocyte trafficking.

Reverse TEM (RTEM)

To prevent excessive inflammation and secondary tissue injury, activated neutrophils at sites of inflamed tissue have to be timely cleared, which can happen in several ways (12). Apoptosis and subsequent clearance by macrophage phagocytosis are thought to be a common fate to innate immune cells, such as neutrophils, eosinophils, and basophils (12–14). However, a growing body of evidence suggests that neutrophils can re-enter the circulation through RTEM (15–17). Some mechanisms have already been revealed. For instance, leukotriene (LT)B4 can disrupt the junctional adhesion molecule-C and facilitate neutrophil reverse migration (18). Macrophages are shown to promote reverse migration through neutrophil redox-Src family kinase signaling, whereas Src deficiency impairs neutrophil RTEM (19). This might represent another mechanism of macrophage clearance of neutrophils, in addition to macrophage phagocytosis of apoptotic neutrophils. Interestingly, reverse transmigrated neutrophils display high expression of intercellular adhesion molecule (ICAM)-1, which is minimally expressed in circulatory neutrophils (20); the functional implication of this phenotype change is unclear. It is suggested that RTEM assists in the dissemination of systemic inflammation (18). Therefore, neutrophil RTEM contributes to not only resolution, but also propagation, of inflammation. More work is warranted to establish the pathophysiological significance of neutrophil TEM/RTEM. Of particular interest is how these processes affect endothelial barrier property.

ENDOTHELIAL BARRIER

The endothelial barrier of exchange microvessels (capillaries and post-capillary venules) has three major components (**Figure 1A**): cell-cell junctions, luminal surface glycocalyx, and basolateral focal adhesions (9). These components act in concert to determine the barrier permeability.

Cell-Cell Junctions

In the microvasculature, at least two types of junctions are identified: tight junctions (TJs) and adherens junctions (AJs) (**Figures 1A,C**). TJs have been extensively studied with respect to blood-brain barrier (BBB) and blood-retinal barrier due to their predominant expression in cerebral and retinal microvasculature, respectively. TJs are composed of occludins and claudins, tetraspanning molecules linked to the actin cytoskeleton through cytoplasmic adaptor proteins, zonula occludens (ZO) (21). AJs are considered the primary junctions in the peripheral microvasculature. They mainly consist of the transmembrane homophilic dimers, vascular endothelial (VE)-cadherin, which are anchored to the actin cytoskeleton through catenins (α-, β-, γ-, and p120-catenin) (22, 23).

Disassembly or opening of AJs can lead to increased paracellular permeability (24). Given the rapid nature of leak responses to inflammatory agonists, such as histamine and vascular endothelial growth factor (VEGF), which occur in minutes following stimulation (25), dynamic changes of barrier conformation via post-translational modification (PTM) of junction molecules are considered to be an important underlying mechanism. Protein phosphorylation is a commonly studied PTM. It is generally accepted that tyrosine phosphorylation of VE-cadherin triggers its dissociation from catenins, thereby weakening the junction anchorage to cytoskeleton (26, 27). VE-cadherin can be phosphorylated by tyrosine kinase Src or protein kinase C (PKC) (28). Phosphatases also regulate VE-cadherin de-phosphorylation thereby altering barrier function (27). There is evidence that VE-cadherin phosphorylation and dephosphorylation at different sites differentially regulate vascular permeability (29). Other junction proteins, such as β-catenin, can be phosphorylated by proline-rich tyrosine kinase (Pyk)-2 (26), promoting its dissociation from the VE-cadherin junction (30). In addition to junction molecules, cytoskeleton molecules undergo conformational changes upon phosphorylation. For example, myosin light chain kinase

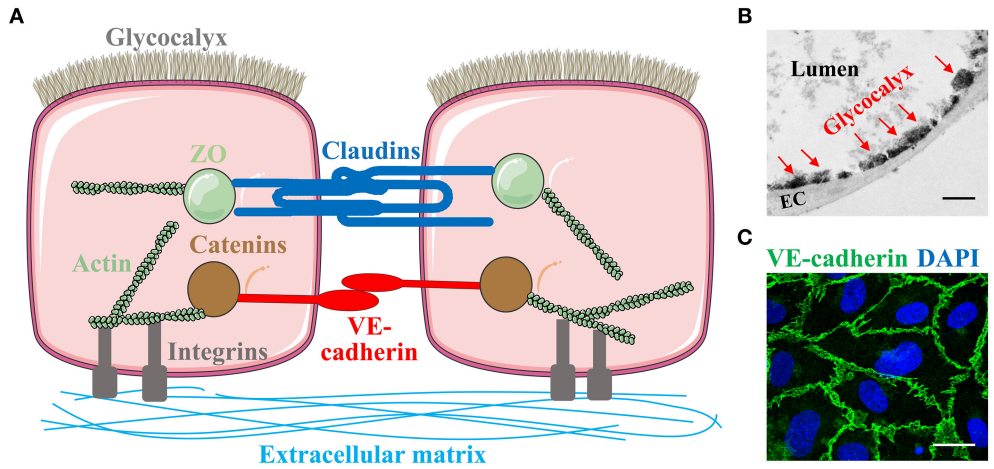

FIGURE 1 | Endothelial barrier structure**. (A)** The endothelial barrier of exchange microvessels is composed of endothelial cells connected to each other via junctions, with its luminal surface protected by glycocalyx and basolateral side anchored to the extracellular matrix in the basement membrane through focal adhesions. Endothelial cell-cell adhesion is mediated by two types of junction: the claudin-based tight junction which is linked to the actin cytoskeleton through zonula occludens (ZO), and the VE-cadherin-based adherens junction which binds actin through catenins. Some images of cells or organelles were obtained from Servier Medical Art (www.servier.com). **(B)** Glycocalyx in mouse lung capillary under transmission electron microscopy. EC, endothelial cells. Red arrows indicate glycocalyx. Scale bar = 1 μm. **(C)** Immunofluorescent staining of VE-cadherin on human umbilical vein endothelial cells. Green, VE-cadherin. Blue, DAPI. Scale bar = 20 μm.

(MLCK) phosphorylates myosin light chain and triggers actin-myosin contraction, pulling away the neighboring cells and leading to intercellular gaps (31).

Additional PTM mechanisms implicated in junction permeability include nitrosylation (32) and lipidation. We have recently identified a new lipidation pathway, endothelial protein palmitoylation mediated by palmitoyl acyltransferase DHHC21, in promoting neutrophil-endothelium adhesion and microvascular permeability (33).

Glycocalyx

Covering the luminal surface of endothelial barrier is a matrix meshwork, glycocalyx, constituted with glycosaminoglycans (GAG), proteoglycans, and glycoproteins (**Figures 1A,B**) (34). The GAG chains contain heparan sulfate, chondroitin sulfate, and hyaluronic acid; the latter binds to a transmembrane glycoprotein, CD44 (35). Proteoglycans, the core transmembrane proteins, include syndecans and glypicans. Glycoproteins include selectins and integrins, which participate in neutrophil adhesion and other intravascular processes such as coagulation and fibrinolysis (36). A dynamic equilibrium exists between the biosynthesis and shedding of endothelial glycocalyx constituents, which determines glycocalyx thickness, morphology, and function (37).

An important function of endothelial surface glycocalyx is providing a protective layer to prevent the endothelium from being exposed to circulating cells or agents (38). It participates in a number of biological events, including neutrophil-endothelium cross-talk (36, 37). Glycocalyx disruption contributes to compromised endothelial barrier integrity and increased microvascular permeability (39). Shedding of glycocalyx constituents occurs via enzymatic digestion by metalloproteases and hyaluronidase, or non-enzymatic stimulation such as oxidative stress (40, 41); both are activated during neutrophil-mediated innate immune response. Our recent study revealed an important role of a disintegrin and metalloproteinase 15 (ADAM15) in glycocalyx destruction (35). In particular, ADAM15 is upregulated during infection, and it cleaves glycocalyx constituents, including CD44. The cleaved products target endothelial cells in a paracrine manner inducing barrier dysfunction and microvascular leakage (35). We also show that syndecan-3/4 can be cleaved by thrombin to produce ectodomain fragments, and these fragments trigger AJ disorganization and stress fiber formation, causing elevated para-cellular permeability (42). Consistent with our findings, other studies show that in septic lungs, glycocalyx degradation leads to increased availability of endothelial surface receptors for neutrophil adhesion molecules and thereby facilitating neutrophil infiltration (43).

Focal Adhesions

At the basolateral side, endothelial cells are attached to extracellular matrix (ECM) through focal adhesions, complex transmembrane structures consisting of integrins, focal adhesion kinase (FAK), and adaptor proteins (44). While they are essential to the maintenance of endothelial barrier properties under basal conditions (28, 31), their activation or redistribution contributes to paracellular leakage (45). Studies have shown that both FAK and β1/3 integrins are required for microvascular leak responses to blood clot fibrinolysis products (46).

FAK is a non-receptor tyrosine kinase that controls focal adhesion assembly and distribution. We have previously reported that FAK mediates endothelial barrier dysfunction caused by C5a-activated neutrophils, an effect dependent on FAK signaling activity (9). Certain inflammatory mediators secreted by neutrophils can activate FAK by inducing its

phosphorylation (47, 48). FAK phosphorylation at tyrosine-925 exposes the SH2-binding site for Grb2, which triggers downstream signals involving Ras-ERK1/2 and MLCK-dependent actomyosin contraction (9). FAK inhibition alleviates venular hyperpermeability caused by neutrophils or VEGF (48, 49).

PMN REGULATION OF ENDOTEHLIAL PERMEABILITY

Neutrophils regulate endothelial permeability by altering the structure and function of the aforementioned barrier components: cell-cell junctions, glycocalyx, and focal adhesions. During inflammation, activated neutrophils exert detrimental effects to these structures via direct contacts established during adhesion and transmigration, or via secretion of barrier-disrupting molecules (**Figure 2**). Neutrophil respiratory burst produces reactive oxygen species (ROS), and neutrophil degranulation produces myeloperoxidase, elastase, cathepsin G, and metalloproteases; all are capable of cleaving glycocalyx. Glycocalyx injury results in the loss of protective layer and exposure of endothelial surface receptors for neutrophil adhesion, further activating neutrophil-endothelium interactions. In endothelial cell-cell junctions, VE-cadherin is particularly susceptible to enzymatic degradation, and its cleavage by metalloproteases, elastase, and cathepsin G leads to impaired junction integrity (27, 50). At the basal lateral site, FAK activation and integrin engagement in response to neutrophil TEM, or their secreted products, promote focal adhesion assembly and redistribution in alignment with contractile cytoskeleton, providing support for endothelial cells to undergo conformational changes. Below we discuss further details on how neutrophils regulate endothelial barrier function, focusing on adhesion-dependent and secretion-dependent pathways.

PMN Adhesion and Endothelial Permeability

Traditionally, neutrophil adhesion followed by TEM is thought to physically damage the endothelial barrier. Challenging this dogma, transmission electron microscopic studies show that no tracer leakage is coupled with neutrophil TEM, and that TEM can occur without impairing the junctional structure (9, 51). While the physical attachment of neutrophils to the endothelium can exacerbate barrier injury, it is not required for hyperpermeability responses.

Several studies suggest the importance of adhesion molecule engagement in barrier regulation. In particular, ICAM-1 engagement in the absence of leukocytes is sufficient to increase endothelial permeability (52). Ligation of endothelial ICAM-1 can directly increase permeability (26, 52), and antibodies blocking ICAM-1 alleviate endothelial injury during

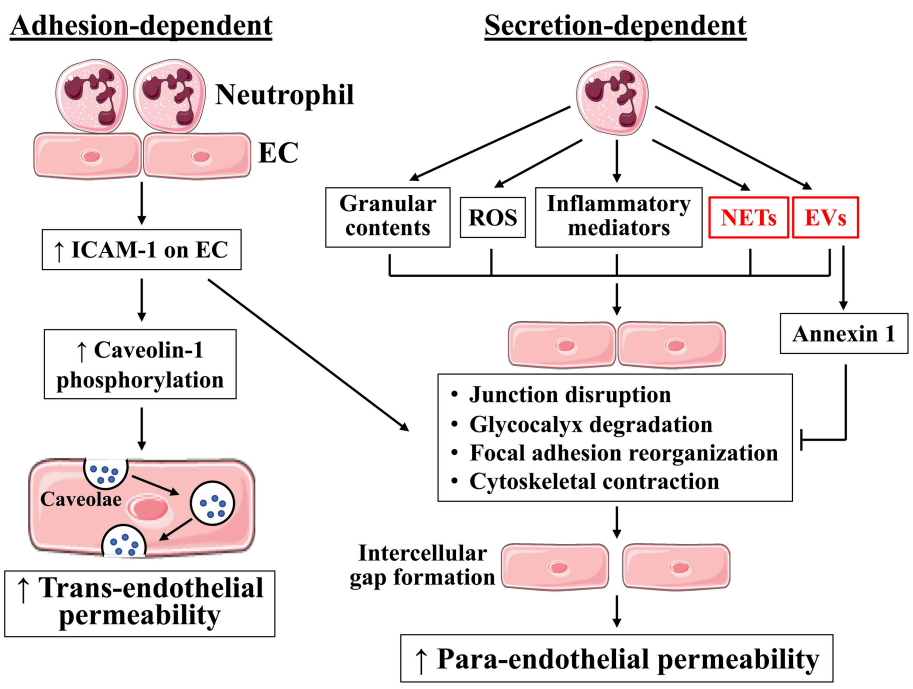

FIGURE 2 | Neutrophils regulate endothelial barrier function through adhesion-dependent and secretion-dependent mechanisms. Neutrophil adhesion to endothelial cells activates ICAM-1 signaling, which increases permeability through both para-endothelial and trans-endothelial routes. In addition, neutrophils can generate ROS, inflammatory mediators, granular contents, neutrophil extracellular traps (NETs), and extracellular vesicles (EVs), which in turn cause junction disruption, glycocalyx degradation, focal adhesion reorganization, and cytoskeletal contraction, leading to intercellular gap formation and increased para-endothelial permeability. Neutrophils also release barrier-protecting factors, including annexin 1. EC, endothelial cells. Blue dots, blood constituents. Images of cells were obtained from Servier Medical Art (www.servier.com).

inflammation (53). The signal transduction downstream from ICAM-1 engagement involves Rac, which subsequently activates NADPH oxidases and causes ROS production. ROS activate Src or Pyk2, which phosphorylate VE-cadherin molecules and promote their dissociation. Src also activates FAK and initiates focal adhesion redistribution. Moreover, ICAM-1 ligation induces dissociation of vascular endothelial protein tyrosine phosphatase from VE-cadherin, promoting VE-cadherin phosphorylation (5). In the cytoskeleton, MLCK is also activated after ICAM-1 ligation (54). Together, these signaling reactions lead to a focal adhesion-supported cytoskeleton contraction and junction opening (9, 55).

In addition to its role in paracellular permeability, neutrophil adhesion also activates the trans-endothelial route of protein transport through ICAM-1 signaling (**Figure 2**) (53). ICAM-1 ligation facilitates Src phosphorylation of caveolin-1, a major component of caveolae. Caveolae serve as the primary mechanism of moving albumin across the endothelial cell body, from the luminal side of cell membrane toward basement membrane. The relative contributions of ICAM-1 to paracellular vs. transcellular permeability remains to be established.

PMN Secretion and Endothelial Permeability

It is well-known that neutrophils cause barrier dysfunction by producing ROS, secreting inflammatory mediators, and releasing granular contents (**Figure 2**) (1, 56). The mechanisms by which ROS induce vascular hyperpermeability include junction disruption and endothelial cell contraction mediated by MLCK, MAPK, PKC, tyrosine kinases, and Rho GTPases (56, 57). Other permeability-increasing agents released by neutrophils include TNF-α, IL-1β, and chemokines CXCL1, 2, 3, and 8 (5, 58). Through degranulation, neutrophils release metalloproteases, elastase, cathepsin G, and proteinase 3; these proteolytically active enzymes can breakdown junctional complexes, glycocalyx constituents, and focal adhesion components (59, 60). Additionally, neutrophil-derived LTA4 induces the synthesis of biologically active LTB4 (61), LTB4 then further activates neutrophils to release heparin-binding protein (a granule component), causing endothelial cell contraction (62). TNF-α can stimulate neutrophil release of elastase and cathepsin G, which cleave VE-cadherin and disrupt the junction integrity (63). These findings suggest a synergistic action of neutrophil-derived agents in regulating barrier property. While these mechanisms represent the traditional pathways of neutrophil secretion-induced permeability, below we discuss two newly characterized barrier-altering factors produced by neutrophils.

Neutrophil Extracellular Traps (NETs)

Neutrophils can release nuclear components (DNA and histones) and cytoplasmic granular proteins (elastase, myeloperoxidase, cathepsin G, and metalloproteases) into the extracellular environment, which form NETs to trap invading microorganisms. This pathogen-killing mechanism was first described by Brinkmann et al in 2004 (64). Peptidylarginine deiminase (PAD)4 plays a key role in NET formation by converting arginyl residues on chromatin histones to citrulline (which lacks positive charge), releasing the ionic bonds that constrain nuclear DNA to nucleosomes and thus freeing the strands of DNA to unfurl (65). In parallel, neutrophil elastase translocates to the nucleus and degrade histones, facilitating chromatin decondensation (66). Subsequently, decondensed chromatin fused with granule components is released to extracellular space.

NETs are originally thought to be generated by neutrophils undergoing cell death, a process known as suicidal NETosis. Non-suicidal vital NETosis was subsequently described, which occurs via blebbing of the nuclear envelope and vesicular exportation, thus displaying intact plasma membrane and viable neutrophils (67, 68). Additionally, Yousefi et al. identified mitochondrial NET, which is formed in living neutrophils and contains mitochondrial, but not nuclear, DNA (69). It is unclear whether and how these different types of NETs coexist, and what distinct functions they exert.

NET production can be induced by biological and chemical agents, including live bacteria, ROS, inflammatory cytokines (e.g., IL-1β, IL-8, and TNF-α), phorbol 12-myristate 13-acetate (PMA), and calcium ionomycin (70). NET formation is enhanced in infection and inflammation-associated diseases. The primary function of NETs is to trap pathogens and prevent dissemination of infection, being protective (64). Subsequently, NETs have been shown to be detrimental in multiple diseases including lung disease, thrombosis, cancer, and autoimmune disease (71). For instance, NETs exhibit the pro-inflammatory feature in chronic airway disease (72). The finding that PAD4 inhibition decreases arterial thrombosis in apolipoprotein-$E^{-/-}$ mice indicates the pro-coagulant nature of NETs (73). NETs are recently shown to awaken dormant cancer cells and facilitate tumor metastasis through the activation of integrin and FAK/ERK/MLCK/YAP signaling by laminin fragments generated by neutrophil elastase and MMP-9 cleavage (74). NETs associated components are potential inducers of autoantibody production, a hallmark for auto-immune diseases. Not surprisingly, blocking NET formation decreases disease severity in a mouse model of systemic lupus erythematosus (75). A recent study demonstrates that partial PAD4 deficiency ($PAD4^{+/-}$ or DNase I treatment) reduces lung injury and improves survival in a murine model of bacterial pneumonia, while $PAD4^{-/-}$ mice show increased bacterial load and inflammation (76). This finding highlights the pleiotropic roles of NETs in pro-inflammation and anti-inflammation, which needs to be taken into consideration when targeting NETs as therapeutics.

With respect to NET regulation of microvascular permeability, we are at the very early stage of understanding NET's effects on endothelial barriers and their underlying mechanisms. *In vitro*, NETs increase the flux of albumin or 10-kDa dextran across endothelial cell monolayers (77, 78). Neutralizing NET components by DNase 1, or inhibition of NET formation by PAD2/4 inhibitor or PAD4 gene deletion, reduces lung vascular permeability in murine models of transfusion-related acute lung injury and LPS-induced endotoxemia, respectively (76–78). Our recent study reveals that citrullinated histone 3, a major protein component of NETs, causes microvascular leakage and barrier dysfunction

by disrupting AJs and rearranging contractile cytoskeleton in endothelial cells (79). Consistent with our finding, others show that NETs increase albumin permeability through disrupted AJs (80). Serine proteinases (e.g., neutrophil elastase) and MMPs, enriched in NETs, can cleave VE-cadherin and compromise junction integrity (63, 81). MMPs further activate barrier-disrupting cytokines and chemokines, such as IL-1β, TNF-α, and CXCL8 (82, 83), which may amplify the hyperpermeability signaling. **Figure 3** depicts effects of specific NET components on endothelial permeability.

The NET pathway is also under negative regulation. Lactoferrin, an iron-binding protein present in secondary granules of neutrophils, is released during neutrophil degranulation. A recent study by Okubo and colleagues demonstrates that lactoferrin suppresses NET formation, acting as an intrinsic inhibitor of NETs (84). Mice deficient in the lipoxin receptor 2 generate more NETs, leading to elevated lung injury and mortality after pneumonia (76). Thus, lipoxin receptor 2 may negatively regulate NET formation. Additionally, activated protein C (APC), a natural anticoagulant, is known to protect barrier function and decrease vascular permeability. Recent evidence shows that APC inhibits NET formation (85). It would be interesting to investigate whether NET inhibition by APC contributes to its barrier-protective effects.

Neutrophil-Derived Extracellular Vesicles

Extracellular vesicles (EVs) are heterogeneous membrane enveloped structures released by a variety of cells into body fluids (86). Based on their size and formation pathways, EVs are divided into 3 types: apoptotic bodies, microparticles (also known as microvesicles), and exosomes. Apoptotic bodies are the largest EVs with a diameter of 800 to 5,000 nm. They are released during the last stage of apoptosis, characterized by a permeable plasma membrane with externalized phosphatidylserine. This process is mediated by caspase and Rho-associated kinase I. Microparticles, ranging 100–1000 nm in diameter, are formed by the outward blebbing of the cell membrane, a process called "ectocytosis." During its formation, the cytoskeleton is reorganized, and phosphatidylserine is redistributed to the outside of the plasma membrane, which involves multiple complex pathways, including calcium signaling and Rho-associated kinase I and II, nuclear factor-κB, p38MAPK, or TNF-related apoptosis-inducing ligand (87). Exosomes are the smallest EVs of 30–120 nm in diameter and exhibit a cup-like shape (88). In contrast to apoptotic bodies and microparticles that derive from plasma membrane, exosomes stem from the endosomal system. Exosomes are intraluminal vesicles contained in multivesicular bodies, which then fuse with the plasma membrane and are released into extracellular environment (89). The formation and secretion of exosomes are regulated by endosomal sorting complexes required for transport-dependent (90) and -independent manners (tetraspanins, lipid rafts, and Rab GTPases) (86, 91).

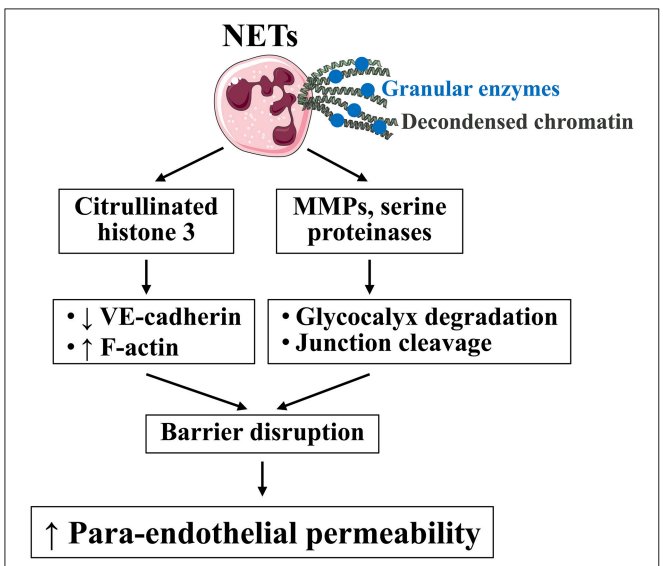

FIGURE 3 | Effects of specific NET constituents on endothelial barrier function. NETs are composed of decondensed chromatin (e.g., citrullinated histone 3) and granular enzymes (MMPs and serine proteinases). Citrullinated histone 3 induces actin stress fiber formation and VE-cadherin junction discontinuity; MMPs and serine proteinases cleave glycocalyx and other barrier molecules; both lead to increased para-endothelial permeability. Images of cells were obtained from Servier Medical Art (www.servier.com).

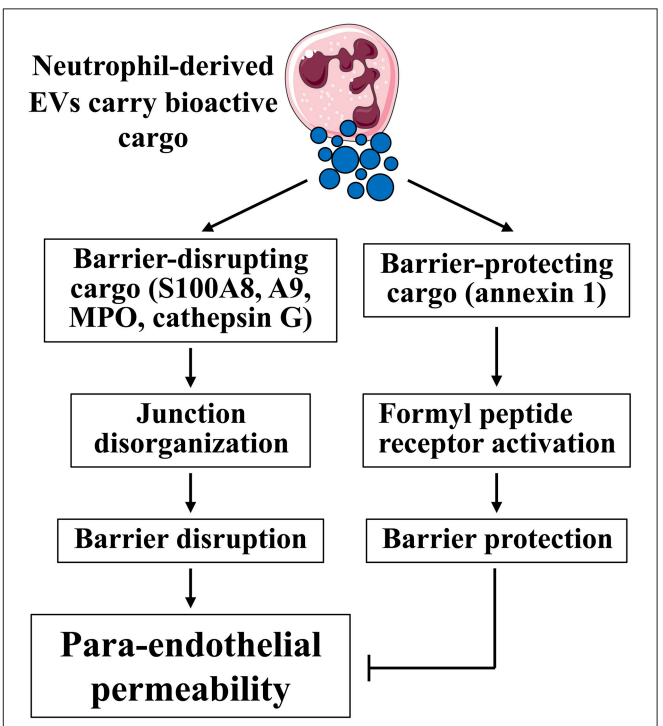

FIGURE 4 | Effects of neutrophil-derived EVs on endothelial barrier function. Neutrophil-derived EVs display either positive or negative impact on endothelial permeability depending on their cargo contents. Barrier-disrupting cargo, such as S100A8, A9, MPO, and cathepsin G, are able to disrupt junction integrity and increase permeability. In contrast, barrier-protecting cargo, such as annexin 1, maintain junction integrity, and decrease permeability. Images of cells were obtained from Servier Medical Art (www.servier.com).

Originally thought as inactive cell debris, EVs have been studied as biomarkers for cell injury. Recently, growing evidence has emerged showing that EVs are active players in cell-cell communication (86, 89). EVs carry nucleic acids (e.g., microRNA), peptides, proteins, carbohydrates, and lipids that act as bioactive molecules to regulate multiple biological processes, including angiogenesis, immune response, cell migration and differentiation. In addition, EV cargos can be exchanged between cells as a mean of cell-cell communication or recycling.

EVs in the blood mainly derive from blood cells (leukocytes, platelets) and vascular cells (endothelial cells) (86, 92); their molecular property and cargo contents vary depending on the origin and pathophysiological state of parent cells that produce them. Neutrophil-derived EVs are in small amounts under normal conditions, but significantly increased in the blood during sepsis and inflammation (93–95). Pathophysiological stimuli, such as bacteria, complements, inflammatory cytokines, calcium, and platelet activating factor, are able to induce neutrophil production of EVs (95). In general, these EVs are considered to be foe to the endothelial barrier because they contain pro-inflammatory cargo, although the distinct effects of specific cargo contents remain to be established.

We propose that neutrophil-derived EVs possess both barrier-disrupting and barrier-protecting capabilities depending on their cargo components (**Figure 4**). In particular, proteomic analysis reveals that microparticles generated from fMLP-activated neutrophils contain > 300 different proteins, including pro-inflammatory S100A8, S100A9, MPO, and cathepsin G (96). S100A8, S100A9, or S100A8/A9 complexes induce F-actin and ZO-1 disassembly; they also increase endothelial monolayer permeability through activating p38 and ERK1/2 signaling pathways via binding to receptors TLR4 and RAGE (97). MPO can bind to the glycocalyx heparan sulfate by ionic interaction (independent of its catalytic property), which induces the release of neutrophil granular proteinases to cause syndecan-1 shedding and glycocalyx impairment (98). Cathepsin G is known to increase endothelial permeability to albumin through the detachment of plasminogen activator inhibitor-1 from the subendothelial matrix, causing F-actin rearrangement (99). In addition, cathepsin G can degrade VE-cadherin and impair junction integrity (100). All these studies suggest that neutrophil-derived EVs may have permeability-enhancing effects.

Other studies suggest the beneficial role of EVs in vascular homeostasis and endothelial permeability (101–104). For example, neutrophils adhering to endothelial cells generate microparticles enriched of annexin 1, an anti-inflammatory and pro-resolving protein that inhibits neutrophil adhesion and recruitment (104). Annexin 1, also known as annexin A1 or lipocortin 1, is expressed by brain microvascular endothelial cells and mediates the anti-inflammatory effects of glucocorticoid hormones. Annexin $1^{-/-}$ mice display increased BBB permeability as a result of disrupted TJs and disorganized actin cytoskeleton, which could be rescued by exogenous annexin 1 administration (105). This indicates that annexin 1 maintains endothelial tight junctions and BBB homeostasis (**Figure 4**). Annexin 1 is also shown to prevent inflammation-induced impairment in cerebrovascular endothelial barrier function (106). Annexin 1 targets endothelial cells by binding to G protein-coupled receptor formyl peptide receptor (or lipoxin A4 receptor) and subsequently activating intracellular signaling (107, 108). Therefore, the net effect of neutrophil-derived EVs may depend on their cargo contents and balance between barrier-protecting and barrier-disrupting molecules.

THERAPEUTIC IMPLICATIONS

Vascular leakage is a common complication of various infectious or inflammatory diseases (109). The importance of protecting endothelial barriers and repairing leaky vessels has increasingly been appreciated, as evidenced by many recent trials aimed at targeting endothelial dysfunction. Molecules that demonstrate the capability to enhance barrier property include sphingosine 1-phosphate, APC, angiopoietins, PKC inhibitors, RhoA inhibitors, corticosteroids, histamine receptor blockades, anti-VEGF, and vasopressin type 1a agonists (110–113). While all of them display beneficial roles in animal models, many do not demonstrate clinical efficacy. For example, anti-neutrophil adhesion therapies using monoclonal antibodies against CD18 or ICAM-1 have failed to improve clinical outcomes in patients with burn injury, traumatic shock, and ischemia-reperfusion injury (114). The lack of endothelial barrier-specific therapies highlights the need for further studies to identify novel therapeutic targets. As accumulating evidence supports the important contribution of NETs and neutrophil-derived EVs to vascular barrier dysfunction, additional work is warranted to investigate whether altering their production, or interfering their mechanistic pathways, has clinical implications or therapeutic potential. In view of the complexity of human diseases, targeting specific molecular pathways key to barrier structure and function holds great promise to the treatment of diseases associated with aberrant immune/inflammatory response.

AUTHOR CONTRIBUTIONS

All authors have made a substantial and intellectual contribution to this work and approved it for publication.

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REFERENCES

1. Ma Y, Yabluchanskiy A, Lindsey ML. Neutrophil roles in left ventricular remodeling following myocardial infarction. *Fibrogen Tissue Repair.* (2013) 6:11. doi: 10.1186/1755-1536-6-11
2. Kubes P. The enigmatic neutrophil: what we do not know. *Cell Tissue Res.* (2018) 371:399–406. doi: 10.1007/s00441-018-2790-5
3. Pillay J, den Braber I, Vrisekoop N, Kwast LM, de Boer RJ, Borghans JA, et al. *In vivo* labeling with 2H2O reveals a human neutrophil lifespan of 5.4 days. *Blood.* (2010) 116:625–7. doi: 10.1182/blood-2010-01-259028
4. Kobayashi SD, Malachowa N, DeLeo FR. Influence of microbes on neutrophil life and death. *Front Cell Infect Microbiol.* (2017) 7:159. doi: 10.3389/fcimb.2017.00159
5. DiStasi MR, Ley K. Opening the flood-gates: how neutrophil-endothelial interactions regulate permeability. *Trends Immunol.* (2009) 30:547–56. doi: 10.1016/j.it.2009.07.012
6. Nourshargh S, Alon R. Leukocyte migration into inflamed tissues. *Immunity.* (2014) 41:694–707. doi: 10.1016/j.immuni.2014.10.008
7. Gerhardt T, Ley K. Monocyte trafficking across the vessel wall. *Cardiovasc Res.* (2015) 107:321–30. doi: 10.1093/cvr/cvv147
8. Yuan Y, Mier RA, Chilian WM, Zawieja DC, Granger HJ. Interaction of neutrophils and endothelium in isolated coronary venules and arterioles. *Am J Physiol.* (1995) 268:H490–8. doi: 10.1152/ajpheart.1995.268.1.H490
9. Yuan SY, Shen Q, Rigor RR, Wu MH. Neutrophil transmigration, focal adhesion kinase and endothelial barrier function. *Microvasc Res.* (2012) 83:82–8. doi: 10.1016/j.mvr.2011.06.015
10. Carman CV, Sage PT, Sciuto TE, de la Fuente MA, Geha RS, Ochs HD, et al. Transcellular diapedesis is initiated by invasive podosomes. *Immunity.* (2007) 26:784–97. doi: 10.1016/j.immuni.2007.04.015
11. Muller WA. Mechanisms of leukocyte transendothelial migration. *Annu Rev Pathol.* (2011) 6:323–44. doi: 10.1146/annurev-pathol-011110-130224
12. Burn T, Alvarez JI. Reverse transendothelial cell migration in inflammation: to help or to hinder? *Cell Mol Life Sci.* (2017) 74:1871–81. doi: 10.1007/s00018-016-2444-2
13. Leitch AE, Duffin R, Haslett C, Rossi AG. Relevance of granulocyte apoptosis to resolution of inflammation at the respiratory mucosa. *Mucosal Immunol.* (2008) 1:350–63. doi: 10.1038/mi.2008.31
14. Bratton DL, Henson PM. Neutrophil clearance: when the party is over, clean-up begins. *Trends Immunol.* (2011) 32:350–7. doi: 10.1016/j.it.2011.04.009
15. Mathias JR, Perrin BJ, Liu TX, Kanki J, Look AT, Huttenlocher A. Resolution of inflammation by retrograde chemotaxis of neutrophils in transgenic zebrafish. *J Leukoc Biol.* (2006) 80:1281–8. doi: 10.1189/jlb.0506346
16. Yoo SK, Huttenlocher A. Spatiotemporal photolabeling of neutrophil trafficking during inflammation in live zebrafish. *J Leukoc Biol.* (2011) 89:661–7. doi: 10.1189/jlb.1010567
17. Woodfin A, Voisin MB, Beyrau M, Colom B, Caille D, Diapouli FM, et al. The junctional adhesion molecule JAM-C regulates polarized transendothelial migration of neutrophils *in vivo*. *Nat Immunol.* (2011) 12:761–9. doi: 10.1038/ni.2062
18. Colom B, Bodkin JV, Beyrau M, Woodfin A, Ody C, Rourke C, et al. Leukotriene B4-neutrophil elastase axis drives neutrophil reverse transendothelial cell migration *in vivo*. *Immunity.* (2015) 42:1075–86. doi: 10.1016/j.immuni.2015.05.010
19. Tauzin S, Starnes TW, Becker FB, Lam PY, Huttenlocher A. Redox and Src family kinase signaling control leukocyte wound attraction and neutrophil reverse migration. *J Cell Biol.* (2014) 207:589–98. doi: 10.1083/jcb.201408090
20. Buckley CD, Ross EA, McGettrick HM, Osborne CE, Haworth O, Schmutz C, et al. Identification of a phenotypically and functionally distinct population of long-lived neutrophils in a model of reverse endothelial migration. *J Leukoc Biol.* (2006) 79:303–11. doi: 10.1189/jlb.0905496
21. Zihni C, Mills C, Matter K, Balda MS. Tight junctions: from simple barriers to multifunctional molecular gates. *Nat Rev Mol Cell Biol.* (2016) 17:564–80. doi: 10.1038/nrm.2016.80
22. Burridge K, Sastry SK, Sallee JL. Regulation of cell adhesion by protein-tyrosine phosphatases. I. Cell-matrix adhesion. *J Biol Chem.* (2006) 281:15593–6. doi: 10.1074/jbc.R500030200
23. Piedra J, Miravet S, Castano J, Palmer HG, Heisterkamp N, Garcia de Herreros A, et al. p120 Catenin-associated Fer and Fyn tyrosine kinases regulate beta-catenin Tyr-142 phosphorylation and beta-catenin-alpha-catenin Interaction. *Mol Cell Biol.* (2003) 23:2287–97. doi: 10.1128/MCB.23.7.2287-2297.2003
24. Vandenbroucke St. Amant E, Tauseef M, Vogel SM, Gao XP, Mehta D, Komarova YA, et al. PKCalpha activation of p120-catenin serine 879 phospho-switch disassembles VE-cadherin junctions and disrupts vascular integrity. *Circ Res.* (2012) 111:739–49. doi: 10.1161/CIRCRESAHA.112.269654
25. Yuan SY, Rigor RR. *Regulation of Endothelial Barrier Function. Integrated Systems Physiology: From Molecule to Function to Disease*. San Rafael, CA: Morgan and Claypool Life Sciences (2010).
26. Allingham MJ, van Buul JD, Burridge K. ICAM-1-mediated, Src- and Pyk2-dependent vascular endothelial cadherin tyrosine phosphorylation is required for leukocyte transendothelial migration. *J Immunol.* (2007) 179:4053–64. doi: 10.4049/jimmunol.179.6.4053
27. Dejana E, Orsenigo F, Lampugnani MG. The role of adherens junctions and VE-cadherin in the control of vascular permeability. *J Cell Sci.* (2008) 121:2115–22. doi: 10.1242/jcs.017897
28. Konstantoulaki M, Kouklis P, Malik AB. Protein kinase C modifications of VE-cadherin, p120, and beta-catenin contribute to endothelial barrier dysregulation induced by thrombin. *Am J Physiol Lung Cell Mol Physiol.* (2003) 285:L434–42. doi: 10.1152/ajplung.00075.2003
29. Wessel F, Winderlich M, Holm M, Frye M, Rivera-Galdos R, Vockel M, et al. Leukocyte extravasation and vascular permeability are each controlled *in vivo* by different tyrosine residues of VE-cadherin. *Nat Immunol.* (2014) 15:223–30. doi: 10.1038/ni.2824
30. Chen XL, Nam JO, Jean C, Lawson C, Walsh CT, Goka E, et al. VEGF-induced vascular permeability is mediated by FAK. *Dev Cell.* (2012) 22:146–57. doi: 10.1016/j.devcel.2011.11.002
31. Rigor RR, Shen Q, Pivetti CD, Wu MH, Yuan SY. Myosin light chain kinase signaling in endothelial barrier dysfunction. *Med Res Rev.* (2013) 33:911–33. doi: 10.1002/med.21270
32. Guequen A, Carrasco R, Zamorano P, Rebolledo L, Burboa P, Sarmiento J, et al. S-nitrosylation regulates VE-cadherin phosphorylation and internalization in microvascular permeability. *Am J Physiol Heart Circ Physiol.* (2016) 310:H1039–44. doi: 10.1152/ajpheart.00063.2016
33. Beard RS, Jr., Yang X, Meegan JE, Overstreet JW, Yang CG, Elliott JA, et al. Palmitoyl acyltransferase DHHC21 mediates endothelial dysfunction in systemic inflammatory response syndrome. *Nat Commun.* (2016) 7:12823. doi: 10.1038/ncomms12823
34. Salmon AH, Satchell SC. Endothelial glycocalyx dysfunction in disease: albuminuria and increased microvascular permeability. *J Pathol.* (2012) 226:562–74. doi: 10.1002/path.3964
35. Yang X, Meegan JE, Jannaway M, Coleman DC, Yuan SY. A disintegrin and metalloproteinase 15-mediated glycocalyx shedding contributes to vascular leakage during inflammation. *Cardiovasc Res.* (2018) 114:1752–63. doi: 10.1093/cvr/cvy167
36. Weinbaum S, Tarbell JM, Damiano ER. The structure and function of the endothelial glycocalyx layer. *Annu Rev Biomed Eng.* (2007) 9:121–67. doi: 10.1146/annurev.bioeng.9.060906.151959
37. Reitsma S, Slaaf DW, Vink H, van Zandvoort MA, oude Egbrink MG. The endothelial glycocalyx: composition, functions, and visualization. *Pflugers Arch.* (2007) 454:345–59. doi: 10.1007/s00424-007-0212-8
38. Rehm M, Zahler S, Lotsch M, Welsch U, Conzen P, Jacob M, et al. Endothelial glycocalyx as an additional barrier determining extravasation of 6% hydroxyethyl starch or 5% albumin solutions in the coronary vascular bed. *Anesthesiology.* (2004) 100:1211–23. doi: 10.1097/00000542-200405000-00025
39. Salmon AH, Ferguson JK, Burford JL, Gevorgyan H, Nakano D, Harper SJ, et al. Loss of the endothelial glycocalyx links albuminuria and vascular dysfunction. *J Am Soc Nephrol.* (2012) 23:1339–50. doi: 10.1681/ASN.2012010017
40. Ali MM, Mahmoud AM, Le Master E, Levitan I, Phillips SA. Role of matrix metalloproteinases and histone deacetylase in oxidative stress-induced degradation of the endothelial glycocalyx. *Am J Physiol Heart Circ Physiol.* (2019) 316:H647–63. doi: 10.1152/ajpheart.00090.2018

41. Hippensteel JA, Anderson BJ, Orfila JE, McMurtry SA, Dietz RM, Su G, et al. Circulating heparan sulfate fragments mediate septic cognitive dysfunction. *J Clin Invest.* (2019) 129:1779–84. doi: 10.1172/JCI124485
42. Jannaway M, Yang X, Meegan JE, Coleman DC, Yuan SY. Thrombin-cleaved syndecan-3/-4 ectodomain fragments mediate endothelial barrier dysfunction. *PLoS ONE.* (in press).
43. Schmidt EP, Yang Y, Janssen WJ, Gandjeva A, Perez MJ, Barthel L, et al. The pulmonary endothelial glycocalyx regulates neutrophil adhesion and lung injury during experimental sepsis. *Nat Med.* (2012) 18:1217–23. doi: 10.1038/nm.2843
44. Quadri SK. Cross talk between focal adhesion kinase and cadherins: role in regulating endothelial barrier function. *Microvasc Res.* (2012) 83:3–11. doi: 10.1016/j.mvr.2011.08.001
45. Wu MH. Endothelial focal adhesions and barrier function. *J Physiol.* (2005) 569:359–66. doi: 10.1113/jphysiol.2005.096537
46. Guo M, Daines D, Tang J, Shen Q, Perrin RM, Takada Y, et al. Fibrinogen-gamma C-terminal fragments induce endothelial barrier dysfunction and microvascular leak via integrin-mediated and RhoA-dependent mechanism. *Arterioscler Thromb Vasc Biol.* (2009) 29:394–400. doi: 10.1161/ATVBAHA.108.180950
47. Yuan Y, Meng FY, Huang Q, Hawker J, Wu HM. Tyrosine phosphorylation of paxillin/pp125FAK and microvascular endothelial barrier function. *Am J Physiol.* (1998) 275:H84–93. doi: 10.1152/ajpheart.1998.275.1.H84
48. Wu MH, Guo M, Yuan SY, Granger HJ. Focal adhesion kinase mediates porcine venular hyperpermeability elicited by vascular endothelial growth factor. *J Physiol.* (2003) 552:691–9. doi: 10.1113/jphysiol.2003.048405
49. Guo M, Wu MH, Granger HJ, Yuan SY. Focal adhesion kinase in neutrophil-induced microvascular hyperpermeability. *Microcirculation.* (2005) 12:223–32. doi: 10.1080/10739680590905251
50. Luplertlop N, Misse D, Bray D, Deleuze V, Gonzalez JP, Leardkamolkarn V, et al. Dengue-virus-infected dendritic cells trigger vascular leakage through metalloproteinase overproduction. *EMBO Rep.* (2006) 7:1176–81. doi: 10.1038/sj.embor.7400814
51. Lewis RE, Granger HJ. Diapedesis and the permeability of venous microvessels to protein macromolecules: the impact of leukotriene B4 (LTB4). *Microvasc Res.* (1988) 35:27–47.
52. Sumagin R, Kuebel JM, Sarelius IH. Leukocyte rolling and adhesion both contribute to regulation of microvascular permeability to albumin via ligation of ICAM-1. *Am J Physiol Cell Physiol.* (2011) 301:C804–13. doi: 10.1152/ajpcell.00135.2011
53. Hu G, Vogel SM, Schwartz DE, Malik AB, Minshall RD. Intercellular adhesion molecule-1-dependent neutrophil adhesion to endothelial cells induces caveolae-mediated pulmonary vascular hyperpermeability. *Circ Res.* (2008) 102:e120–31. doi: 10.1161/CIRCRESAHA.107.167486
54. Yuan SY, Wu MH, Ustinova EE, Guo M, Tinsley JH, De Lanerolle P, et al. Myosin light chain phosphorylation in neutrophil-stimulated coronary microvascular leakage. *Circ Res.* (2002) 90:1214–21. doi: 10.1161/01.RES.0000020402.73609.F1
55. Sarelius IH, Glading AJ. Control of vascular permeability by adhesion molecules. *Tissue Barriers.* (2015) 3:e985954. doi: 10.4161/21688370.2014.985954
56. Meegan JE, Yang X, Coleman DC, Jannaway M, Yuan SY. Neutrophil-mediated vascular barrier injury: Role of neutrophil extracellular traps. *Microcirculation.* (2017) 24:12352. doi: 10.1111/micc.12352
57. Krizbai IA, Bauer H, Bresgen N, Eckl PM, Farkas A, Szatmari E, et al. Effect of oxidative stress on the junctional proteins of cultured cerebral endothelial cells. *Cell Mol Neurobiol.* (2005) 25:129–39. doi: 10.1007/s10571-004-1378-7
58. Rigor RR, Beard RS Jr., Litovka OP, Yuan SY. Interleukin-1beta-induced barrier dysfunction is signaled through PKC-theta in human brain microvascular endothelium. *Am J Physiol Cell Physiol.* (2012) 302:C1513–22. doi: 10.1152/ajpcell.00371.2011
59. Yang Y, Estrada EY, Thompson JF, Liu W, Rosenberg GA. Matrix metalloproteinase-mediated disruption of tight junction proteins in cerebral vessels is reversed by synthetic matrix metalloproteinase inhibitor in focal ischemia in rat. *J Cereb Blood Flow Metab.* (2007) 27:697–709. doi: 10.1038/sj.jcbfm.9600375
60. Muradashvili N, Benton RL, Saatman KE, Tyagi SC, Lominadze D. Ablation of matrix metalloproteinase-9 gene decreases cerebrovascular permeability and fibrinogen deposition post traumatic brain injury in mice. *Metab Brain Dis.* (2015) 30:411–26. doi: 10.1007/s11011-014-9550-3
61. Folco G, Murphy RC. Eicosanoid transcellular biosynthesis: from cell-cell interactions to *in vivo* tissue responses. *Pharmacol Rev.* (2006) 58:375–88. doi: 10.1124/pr.58.3.8
62. Di Gennaro A, Kenne E, Wan M, Soehnlein O, Lindbom L, Haeggstrom JZ. Leukotriene B4-induced changes in vascular permeability are mediated by neutrophil release of heparin-binding protein (HBP/CAP37/azurocidin). *FASEB J.* (2009) 23:1750–7. doi: 10.1096/fj.08-121277
63. Hermant B, Bibert S, Concord E, Dublet B, Weidenhaupt M, Vernet T, et al. Identification of proteases involved in the proteolysis of vascular endothelium cadherin during neutrophil transmigration. *J Biol Chem.* (2003) 278:14002–12. doi: 10.1074/jbc.M300351200
64. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, et al. Neutrophil extracellular traps kill bacteria. *Science.* (2004) 303:1532–5. doi: 10.1126/science.1092385
65. Li P, Li M, Lindberg MR, Kennett MJ, Xiong N, Wang Y. PAD4 is essential for antibacterial innate immunity mediated by neutrophil extracellular traps. *J Exp Med.* (2010) 207:1853–62. doi: 10.1084/jem.20100239
66. Papayannopoulos V, Metzler KD, Hakkim A, Zychlinsky A. Neutrophil elastase and myeloperoxidase regulate the formation of neutrophil extracellular traps. *J Cell Biol.* (2010) 191:677–91. doi: 10.1083/jcb.201006052
67. Clark SR, Ma AC, Tavener SA, McDonald B, Goodarzi Z, Kelly MM, et al. Platelet TLR4 activates neutrophil extracellular traps to ensnare bacteria in septic blood. *Nat Med.* (2007) 13:463–9. doi: 10.1038/nm1565
68. Pilsczek FH, Salina D, Poon KK, Fahey C, Yipp BG, Sibley CD, et al. A novel mechanism of rapid nuclear neutrophil extracellular trap formation in response to *Staphylococcus aureus*. *J Immunol.* (2010) 185:7413–25. doi: 10.4049/jimmunol.1000675
69. Yousefi S, Mihalache C, Kozlowski E, Schmid I, Simon HU. Viable neutrophils release mitochondrial DNA to form neutrophil extracellular traps. *Cell Death Differ.* (2009) 16:1438–44. doi: 10.1038/cdd.2009.96
70. Hoppenbrouwers T, Autar ASA, Sultan AR, Abraham TE, van Cappellen WA, Houtsmuller AB, et al. In vitro induction of NETosis: comprehensive live imaging comparison and systematic review. *PLoS ONE.* (2017) 12:e0176472. doi: 10.1371/journal.pone.0176472
71. Jorch SK, Kubes P. An emerging role for neutrophil extracellular traps in noninfectious disease. *Nat Med.* (2017) 23:279–87. doi: 10.1038/nm.4294
72. Wright TK, Gibson PG, Simpson JL, McDonald VM, Wood LG, Baines KJ. Neutrophil extracellular traps are associated with inflammation in chronic airway disease. *Respirology.* (2016) 21:467–75. doi: 10.1111/resp.12730
73. Knight JS, Luo W, O'Dell AA, Yalavarthi S, Zhao W, Subramanian V, et al. Peptidylarginine deiminase inhibition reduces vascular damage and modulates innate immune responses in murine models of atherosclerosis. *Circ Res.* (2014) 114:947–56. doi: 10.1161/CIRCRESAHA.114.303312
74. Albrengues J, Shields MA, Ng D, Park CG, Ambrico A, Poindexter ME, et al. Neutrophil extracellular traps produced during inflammation awaken dormant cancer cells in mice. *Science.* (2018) 361:aao4227. doi: 10.1126/science.aao4227
75. Lood C, Blanco LP, Purmalek MM, Carmona-Rivera C, De Ravin SS, Smith CK, et al. Neutrophil extracellular traps enriched in oxidized mitochondrial DNA are interferogenic and contribute to lupus-like disease. *Nat Med.* (2016) 22:146–53. doi: 10.1038/nm.4027
76. Lefrancais E, Mallavia B, Zhuo H, Calfee CS, Looney MR. Maladaptive role of neutrophil extracellular traps in pathogen-induced lung injury. *JCI Insight.* (2018) 3:e98178. doi: 10.1172/jci.insight.98178
77. Caudrillier A, Kessenbrock K, Gilliss BM, Nguyen JX, Marques MB, Monestier M, et al. Platelets induce neutrophil extracellular traps in transfusion-related acute lung injury. *J Clin Invest.* (2012) 122:2661–71. doi: 10.1172/JCI61303
78. Liang Y, Pan B, Alam HB, Deng Q, Wang Y, Chen E, et al. Inhibition of peptidylarginine deiminase alleviates LPS-induced pulmonary dysfunction and improves survival in a mouse model of lethal endotoxemia. *Eur J Pharmacol.* (2018) 833:432–40. doi: 10.1016/j.ejphar.2018.07.005
79. Meegan JE, Yang X, Beard RS Jr., Jannaway M, Chatterjee V, Taylor-Clark TE, et al. Citrullinated histone 3 causes endothelial barrier dysfunction. *Biochem Biophys Res Commun.* (2018) 503:1498–502. doi: 10.1016/j.bbrc.2018.07.069

80. Pieterse E, Rother N, Garsen M, Hofstra JM, Satchell SC, Hoffmann M, et al. Neutrophil Extracellular Traps Drive Endothelial-to-Mesenchymal Transition. *Arterioscler Thromb Vasc Biol.* (2017) 37:1371–9. doi: 10.1161/ATVBAHA.117.309002
81. Carden D, Xiao F, Moak C, Willis BH, Robinson-Jackson S, Alexander S. Neutrophil elastase promotes lung microvascular injury and proteolysis of endothelial cadherins. *Am J Physiol.* (1998) 275:H385–92.
82. Yabluchanskiy A, Ma Y, Iyer RP, Hall ME, Lindsey ML. Matrix metalloproteinase-9: Many shades of function in cardiovascular disease. *Physiology.* (2013) 28:391–403. doi: 10.1152/physiol.00029.2013
83. Ma Y, de Castro Bras LE, Toba H, Iyer RP, Hall ME, Winniford MD, et al. Myofibroblasts and the extracellular matrix network in post-myocardial infarction cardiac remodeling. *Pflugers Arch.* (2014) 466:1113–27. doi: 10.1007/s00424-014-1463-9
84. Okubo K, Kamiya M, Urano Y, Nishi H, Herter JM, Mayadas T, et al. Lactoferrin suppresses neutrophil extracellular traps release in inflammation. *EBioMed.* (2016) 10:204–15. doi: 10.1016/j.ebiom.2016.07.012
85. Healy LD, Puy C, Fernandez JA, Mitrugno A, Keshari RS, Taku NA, et al. Activated protein C inhibits neutrophil extracellular trap formation *in vitro* and activation *in vivo*. *J Biol Chem.* (2017) 292:8616–29. doi: 10.1074/jbc.M116.768309
86. Yanez-Mo M, Siljander PR, Andreu Z, Zavec AB, Borras FE, Buzas EI, et al. Biological properties of extracellular vesicles and their physiological functions. *J Extracell Vesicles.* (2015) 4:27066. doi: 10.3402/jev.v4.27066
87. Dignat-George F, Boulanger CM. The many faces of endothelial microparticles. *Arterioscler Thromb Vasc Biol.* (2011) 31:27–33. doi: 10.1161/ATVBAHA.110.218123
88. Thery C, Amigorena S, Raposo G, Clayton A. Isolation and characterization of exosomes from cell culture supernatants and biological fluids. *Curr Protoc Cell Biol.* (2006) 3:22. doi: 10.1002/0471143030.cb0322s30
89. Todorova D, Simoncini S, Lacroix R, Sabatier F, Dignat-George F. Extracellular Vesicles in Angiogenesis. *Circ Res.* (2017) 120:1658–73. doi: 10.1161/CIRCRESAHA.117.309681
90. Colombo M, Moita C, van Niel G, Kowal J, Vigneron J, Benaroch P, et al. Analysis of ESCRT functions in exosome biogenesis, composition and secretion highlights the heterogeneity of extracellular vesicles. *J Cell Sci.* (2013) 126:5553–65. doi: 10.1242/jcs.128868
91. Andreu Z, Yanez-Mo M. Tetraspanins in extracellular vesicle formation and function. *Front Immunol.* (2014) 5:442. doi: 10.3389/fimmu.2014.00442
92. McVey M, Tabuchi A, Kuebler WM. Microparticles and acute lung injury. *Am J Physiol Lung Cell Mol Physiol.* (2012) 303:L364–81. doi: 10.1152/ajplung.00354.2011
93. Timar CI, Lorincz AM, Csepanyi-Komi R, Valyi-Nagy A, Nagy G, Buzas EI, et al. Antibacterial effect of microvesicles released from human neutrophilic granulocytes. *Blood.* (2013) 121:510–8. doi: 10.1182/blood-2012-05-431114
94. Johnson BL, III, Midura EF, Prakash PS, Rice TC, Kunz N, Kalies K, et al. Neutrophil derived microparticles increase mortality and the counter-inflammatory response in a murine model of sepsis. *Biochim Biophys Acta Mol Basis Dis.* (2017) 1863:2554–63. doi: 10.1016/j.bbadis.2017.01.012
95. Johnson BL, III, Kuethe JW, Caldwell CC. Neutrophil derived microvesicles: emerging role of a key mediator to the immune response. *Endocr Metab Immune Disord Drug Targets.* (2014) 14:210–7. doi: 10.2174/1871530314666140722083717
96. Dalli J, Montero-Melendez T, Norling LV, Yin X, Hinds C, Haskard D, et al. Heterogeneity in neutrophil microparticles reveals distinct proteome and functional properties. *Mol Cell Proteomics.* (2013) 12:2205–19. doi: 10.1074/mcp.M113.028589
97. Wang L, Luo H, Chen X, Jiang Y, Huang Q. Functional characterization of S100A8 and S100A9 in altering monolayer permeability of human umbilical endothelial cells. *PLoS ONE.* (2014) 9:e90472. doi: 10.1371/journal.pone.0090472
98. Manchanda K, Kolarova H, Kerkenpass C, Mollenhauer M, Vitecek J, Rudolph V, et al. MPO (Myeloperoxidase) Reduces endothelial glycocalyx thickness dependent on its cationic charge. *Arterioscler Thromb Vasc Biol.* (2018) 38:1859–67. doi: 10.1161/ATVBAHA.118.311143
99. Iacoviello L, Kolpakov V, Salvatore L, Amore C, Pintucci G, de Gaetano G, et al. Human endothelial cell damage by neutrophil-derived cathepsin G. Role of cytoskeleton rearrangement and matrix-bound plasminogen activator inhibitor-1. *Arterioscler Thromb Vasc Biol.* (1995) 15:2037–46.
100. Cohen-Mazor M, Mazor R, Kristal B, Sela S. Elastase and cathepsin G from primed leukocytes cleave vascular endothelial cadherin in hemodialysis patients. *Biomed Res Int.* (2014) 2014:459640. doi: 10.1155/2014/459640
101. Hutcheson JD, Aikawa E. Extracellular vesicles in cardiovascular homeostasis and disease. *Curr Opin Cardiol.* (2018) 33:290–7. doi: 10.1097/HCO.0000000000000510
102. Ridger VC, Boulanger CM, Angelillo-Scherrer A, Badimon L, Blanc-Brude O, Bochaton-Piallat ML, et al. Microvesicles in vascular homeostasis and diseases. *Thromb Haemost.* (2017) 117:1296–316. doi: 10.1160/TH16-12-0943
103. Mahmoud AM, Wilkinson FL, McCarthy EM, Moreno-Martinez D, Langford-Smith A, Romero M, et al. Endothelial microparticles prevent lipid-induced endothelial damage via Akt/eNOS signaling and reduced oxidative stress. *FASEB J.* (2017) 31:4636–48. doi: 10.1096/fj.201601244RR
104. Dalli J, Norling LV, Renshaw D, Cooper D, Leung KY, Perretti M. Annexin 1 mediates the rapid anti-inflammatory effects of neutrophil-derived microparticles. *Blood.* (2008) 112:2512–9. doi: 10.1182/blood-2008-02-140533
105. Cristante E, McArthur S, Mauro C, Maggioli E, Romero IA, Wylezinska-Arridge M, et al. Identification of an essential endogenous regulator of blood-brain barrier integrity, and its pathological and therapeutic implications. *Proc Natl Acad Sci USA.* (2013) 110:832–41. doi: 10.1073/pnas.1209362110
106. Maggioli E, McArthur S, Mauro C, Kieswich J, Kusters DH, Reutelingsperger CP, et al. Estrogen protects the blood-brain barrier from inflammation-induced disruption and increased lymphocyte trafficking. *Brain Behav Immun.* (2016) 51:212–22. doi: 10.1016/j.bbi.2015.08.020
107. Solito E, Romero IA, Marullo S, Russo-Marie F, Weksler BB. Annexin 1 binds to U937 monocytic cells and inhibits their adhesion to microvascular endothelium: involvement of the alpha 4 beta 1 integrin. *J Immunol.* (2000) 165:1573–81. doi: 10.4049/jimmunol.165.3.1573
108. McArthur S, Loiola RA, Maggioli E, Errede M, Virgintino D, Solito E. The restorative role of annexin A1 at the blood-brain barrier. *Fluids Barriers CNS.* (2016) 13:17. doi: 10.1186/s12987-016-0043-0
109. Wenceslau CF, McCarthy CG, Szasz T, Goulopoulou S, Webb RC. Mitochondrial N-formyl peptides induce cardiovascular collapse and sepsis-like syndrome. *Am J Physiol Heart Circ Physiol.* (2015) 308:H768-77. doi: 10.1152/ajpheart.00779.2014
110. Rodrigues SF, Granger DN. Blood cells and endothelial barrier function. *Tissue Barriers.* (2015) 3:e978720. doi: 10.4161/21688370.2014.978720
111. Kumar P, Shen Q, Pivetti CD, Lee ES, Wu MH, Yuan SY. Molecular mechanisms of endothelial hyperpermeability: implications in inflammation. *Exp Rev Mol Med.* (2009) 11:e19. doi: 10.1017/S1462399409001112
112. Alves NG, Yuan SY, Breslin JW. Sphingosine-1-phosphate protects against brain microvascular endothelial junctional protein disorganization and barrier dysfunction caused by alcohol. *Microcirculation.* (2019) 26:e12506. doi: 10.1111/micc.12506
113. Alves NG, Trujillo AN, Breslin JW, Yuan SY. Sphingosine-1-Phosphate reduces hemorrhagic shock and resuscitation-induced microvascular leakage by protecting endothelial mitochondrial integrity. *Shock.* (2018) doi: 10.1097/SHK.0000000000001280. [Epub ahead of print].
114. Yonekawa K, Harlan JM. Targeting leukocyte integrins in human diseases. *J Leukoc Biol.* (2005) 77:129–40. doi: 10.1189/jlb.0804460

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Cooperative Action of Oxidized Low-Density Lipoproteins and Neutrophils on Endothelial Inflammatory Responses Through Neutrophil Extracellular Trap Formation

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The function of oxidatively modified low-density lipoprotein (oxLDL) in the progression of cardiovascular diseases has been extensively investigated and well-characterized with regards to the activation of multiple cellular responses in macrophages and endothelial cells. Although accumulated evidence has revealed the presence of neutrophils in vascular lesions, the effect of oxLDL on neutrophil function has not been properly investigated. In the present decade, neutrophil extracellular traps (NETs) gained immense attention not only as a primary response against pathogenic bacteria but also due to their pathological roles in tissue damage in various diseases, such as atherosclerosis and thrombosis. In this study, we investigated if oxLDL affects NET formation and if it is a risk factor for inflammatory reactions in endothelial cells. HL-60-derived neutrophils were stimulated with phorbol 12-myristate 13-acetate (PMA) for 30 min to induce NET formation, followed by incubation with 20 μg/mL native or oxidized LDL for additional 2 h. Culture media of the stimulated cells containing released NETs components were collected to evaluate NET formation by fluorometric quantitation of released DNA and detection of myeloperoxidase (MPO) by western blot analysis. NET formation of HL-60-derived neutrophils induced by PMA was significantly enhanced by additional incubation with oxLDL but not with native LDL. Treatment of HL-60-derived neutrophils with oxLDL alone in the absence of PMA did not induce NET formation. Furthermore, the culture media of HL-60-derived neutrophils after NET formation were then transferred to human aortic endothelial cell (HAECs) culture. Treatment of HAECs with the culture media containing NETs formed by HL-60-derived neutrophils increased the expression of metalloproteinase-1 protein in HAECs when HL-60-derived neutrophils were incubated with native LDL, and the expression was accelerated in the case of oxLDL. In addition,

the culture media from NETs formed by HL-60-derived neutrophils caused the elongation of HAECs, which was immensely enhanced by coincubation with native LDL or oxLDL. These data suggest that oxLDL may act synergistically with neutrophils to form NETs and promote vascular endothelial inflammation.

Keywords: neutrophil extracellular traps, oxidized low-density lipoprotein, endothelial cells, inflammatory response, HL-60 cell

INTRODUCTION

The biological significance of oxidatively modified low-density lipoprotein (oxLDL) has been widely and extensively studied as a risk factor for the development and progression of cardiovascular diseases. Increase in oxLDL level is found not only in atherosclerotic lesions but also in the plasma and various organs (1, 2). The pathological process of vascular diseases induced by oxLDL has been explored in terms of the activation of multiple cellular responses in the macrophages and vascular endothelial cells. The underlying mechanisms of atherosclerotic plaque formation were strenuously analyzed via lipid accumulation in macrophage-derived foam cells and vascular endothelial cell dysfunction (3–5). It has also been demonstrated that neutrophils (polymorphonuclear leukocytes), the most abundant form of white cells, infiltrate into the atherosclerotic and thrombotic lesions (6, 7); however, the impact of neutrophils on the progression of atherosclerosis and its functional link with lipoproteins remains to be investigated.

Neutrophils have diverse functions, and they are crucial as the first line of defense responses against pathogenic bacteria due to their phagocytotic activity and ability to produce bactericidal reactive oxygen species (ROS). In 2004, Brinkmann et al. reported that neutrophil extracellular traps (NETs) cause mortality (8). NETs are evoked by pathological bacteria, lipopolysaccharide, cytokines, or other various stimuli that can induce decondensation of chromosome DNA via peptidyl arginine deiminase 4 (PAD4)-mediated histone citrullination, leading to the release of DNA, proteins, such as histones, myeloperoxidase (MPO), and elastase derived from nucleus and azurophil granules into the extracellular space. Signal transduction including ROS production by NADPH oxidase and mitochondria in neutrophils is involved in the process of NET formation (9). NETs have been studied with regards to the primary immune responses elicited against pathogenic bacteria via extracellular DNA trap. In the present decade, a growing evidence has revealed that NETs have pathophysiological functions in various diseases including rheumatoid arthritis (RA), systemic lupus erythematosus (10), cancer (11), Alzheimer's disease (12), metabolic diseases, and diabetes (13), indicating that NET formation may play a pivotal role in cellular dysfunction and histological damage leading to pathological conditions.

The presence of neutrophils in vasculature has been extensively investigated for cardiovascular diseases, such as abdominal aortic aneurysm (AAA) (14, 15), atherosclerosis, and thrombosis (6, 16, 17). Citrullinated histone known as a marker of NET formation is detected in the lesions of AAA (18, 19), thrombosis (20–22), atherosclerosis (23–25), and superficial erosion of atheroma linked with acute coronary syndrome (17). Several studies have demonstrated that citrullinated histone H4 and another NETs marker MPO-DNA complex in plasma are associated with the risk of cardiovascular diseases (26). The blockage of NET formation by Cl-amidine, a peptidyl arginine deiminase (PAD) inhibitor, reduced the atherosclerotic lesions in apoE-KO mice (27).

We have studied the pathological roles of oxLDL in vascular diseases (1, 2, 28, 29), and we investigated if oxLDL affects neutrophils in relation to vascular inflammation. It has already been established that both NETs and oxLDL independently act on the endothelial cells to evoke inflammatory responses and dysfunction; however, the role of native and modified lipoproteins in the progression of NET formation have remained unclear. In this study, we examined NET formation using HL-60-derived neutrophils in the presence of native LDL or oxLDL. Moreover, we assessed the possible synergistic effects of oxLDL treatment and NETs-forming cells on human aortic endothelial cells (HAECs).

MATERIALS AND METHODS

Materials

All-*trans* retinoic acid (AtRA) and phorbol 12-myristate 13-acetate (PMA) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Poly-L-lysine solution (P4707) and gelatin solution (G1393) were purchased from Sigma (Saint Louis, MO, USA). Anti-human MPO antibody (A0398) was purchased from Dako (Carpinteria, CA, USA). Anti-citrullinated histone H4 (citrulline 3) antibody (#07-596) and anti-histone H4 antibody (ab7311) were purchased from Merck KGaA (Darmstadt, Germany) and Abcam (Cambridge, UK), respectively. Horseradish peroxidase (HRP)-conjugated second antibody (anti-mouse IgG) (#3376) was purchased from Cell

Abbreviations: AAA, abdominal aortic aneurysm; apoB, apolipoprotein B; AtRA, all-trans retinoic acid; BSA, bovine serum albumin; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; FBS, fetal bovine serum; HAEC, human aortic endothelial cell; HRP, horseradish peroxidase; LDL, low-density lipoprotein; MMP, matrix metalloproteinase; MPO, myeloperoxidase; NETs, neutrophil extracellular traps; oxLDL, oxidatively modified low-density lipoprotein; PAD4, peptidyl arginine deiminase 4; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PVDF, polyvinylidene difluoride; RA, rheumatoid arthritis; ROS, reactive oxygen species; RT, room temperature; SEM, scanning electron microscope; TBS, Tris-buffered saline; TTBS, Tris-buffered saline (TBS) containing 0. 1% Tween-20.

Signaling Technology, Inc. (Beverly, MA, USA). Anti VE-cadherin antibody (sc-9989), control mouse IgG (sc-2025), and control rabbit IgG (sc-2027) were purchased from Santa Crus Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-matrix metalloproteinase-1 antibody (MMP-1; MAB901) was purchased from R&D Systems (Minneapolis, MN, USA). Anti-PKCβ antibody (GTX113252) was purchased from GeneTex Inc. (Irvine, CA, USA). SYTOX Green and second antibodies conjugated with Alexa Fluor 488 or 594 were purchased from Thermo Fisher Scientific (Rockford, IL, USA). Hoechst 33342 was purchased from Dojindo Laboratories (Kumamoto, Japan).

Cell Culture

The human promyelocytic leukemia cell line HL-60 was purchased from American Type Culture Collection (ATCC), and the cells were cultured in RPMI-1640 medium (Wako Pure Chemical Industries, Ltd.) containing 5% fetal bovine serum (FBS) supplemented with 50 U/mL penicillin and 50 μg/mL streptomycin. HL-60 cells were incubated with 2 μM AtRA for 4 days to facilitate differentiation into mature neutrophils, as previously reported (30). Neutrophils were isolated from freshly drawn blood of healthy donors as previously reported (31). HAECs purchased from Lonza (Walkersville, MD, USA) were cultured in EGM-2 medium (Lonza) or Endothelial Cell Growth Medium 2 (PromoCell GmbH, Heidelberg, Germany). HAECs from passage 4–7 were used for this study. Morphological change in HAECs was evaluated by VE-cadherin staining. Circularity ($4\pi \times [\text{Area}]/[\text{perimeter}]^2$) of the cells was calculated using ImageJ software (NIH, USA) from at least 60 cells in each experimental condition.

LDL Isolation and Oxidation

Human LDL was isolated from human plasma of healthy subjects, as described previously (32). Written informed consents in accordance with the Declaration of Helsinki were prepared and all human subjects voluntarily gave their signatures for participation in this study. The protocol was approved by the ethical committee of Showa University School of Pharmacy (No. 231). Briefly, the LDL fraction at a density of 1.019–1.063 was obtained via KBr density gradient ultracentrifugation, and was dialyzed against phosphate buffered saline (PBS) containing 250 μM EDTA in the dark to prevent divalent cation-mediated oxidation of LDL. Copper-mediated oxidation of LDL was performed by incubation of 0.2 mg/mL LDL with 5 μM $CuSO_4$ at 37°C for 3 h. The reaction was stopped by cooling on ice and by addition of 250 μM EDTA.

NETs Induction of HL-60-Derived Neutrophils or Human Neutrophils

HL-60-derived neutrophils or human neutrophils (1×10^5 cells) were seeded on a poly-L-lysine-coated 12-well plate and were stimulated with 50 nM PMA for 30 min. After removing the culture medium, cells were washed once with the medium, followed by incubation in a medium containing 20 μg/mL native LDL or oxLDL for additional 2 h. The culture medium was collected and centrifuged at 700 × g for 3 min at room temperature (RT), and the supernatant was used for the quantitation of NETs and the stimulation of HAECs.

Fluorometric Quantitation of NETs-DNA Release

Extracellular DNA released into the culture medium was quantified according to the previously reported protocol (33). Briefly, the culture medium collected as aforementioned was treated with 1 U/mL micrococcal nuclease (TAKARA Bio Inc. Kusatsu, Shiga, Japan) to partially digest NETs-derived DNA, and was centrifuged at 1,800 × g for 10 min. The DNA fragments recovered in the supernatant were quantified using 1 μM SYTOX Green and a fluorometer (VARIOSCAN, Thermo Fisher).

Visualization of NETs

HL-60-derived neutrophils were seeded on poly-L-lysine-coated chamber slide (Thermo Fisher) and were stimulated as aforementioned. Cells were treated with 1 μg/mL Hoechst 33342 and 5 μM SYTOX green for 15 min, fixed with 4% paraformaldehyde in PBS for 15 min, and then permeabilized with 0.5% Triton X-100 in PBS for 1 min. After blocking cells with 5% BSA in PBS, they were incubated with the primary antibody against citrullinated histone H4 at RT for 1 h. After further washing with PBS, the cells were incubated with Alexa Fluor 594-conjugated anti-rabbit IgG antibody at RT for 45 min. Following this, the cells were washed with PBS, mounted with SlowFade™ Diamond (Thermo Fisher Scientific), and observed using a fluorescence microscope (BZ-9000, KEYENCE, Osaka, Japan).

Moreover, the chamber slide was used in an electron microscope for the analysis of NET formation of HL-60-derived neutrophils. The cells fixed with 4% paraformaldehyde were treated with 0.5% OsO_4 for 30 min and were dehydrated by immersion in increasing concentrations of ethanol solutions (50, 60, 70, 80, 90, and 100%). The samples were dried using critical point dryer (HCP-2, HITACHI), and the surfaces of the samples were coated with 5 nm platinum layer using an auto fine coater (JEC-3000FC, HITACHI), followed by observation under a scanning electron microscope (SEM) (FlexSEM 1000, Hitachi High-Technologies Co., Tokyo, Japan).

Culture and Stimulation of HAECs

HAECs were cultured on 0.1% gelatin-coated 6-well plates until confluent. The cells were washed with fresh medium and cultured for 30 min. Culture medium of HL-60-derived neutrophils after NET formation (333 μL) was added to 1 mL of HAECs culture medium, and the cells were incubated for 24 h. Notably, the final concentration of lipoproteins was 5 μg/mL, and the HAECs were lysed in SDS-PAGE sample buffer.

Immunoblot Analysis

Cell lysates or culture medium was subjected to 15% gel SDS-PAGE or gradient gel native-PAGE, respectively, and then transferred to polyvinylidene difluoride (PVDF) membranes. After blocking the membrane with 2% skim milk (Wako Pure Chemical Industries, Ltd., Osaka, Japan) in Tris-buffered saline (TBS) containing 0.1% Tween-20 (TTBS) for 1 h, the membranes were treated with a primary antibody in the blocking solution at

4°C overnight. Furthermore, after washing the membrane with TTBS, the membrane was treated with a secondary antibody in TTBS containing 0.5% skim milk at RT for 2 h. The protein bands were detected using ECL-plus western blotting detection reagent (GE Healthcare UK Ltd., Buckinghamshire, UK) by ImageQuant LAS500 (GE Healthcare). Densitometric analysis was performed using ImageJ software.

Immunocytochemistry

HAECs were cultured on 0.1% gelatin-coated glass coverslip (Matsunami Glass Inc., Ltd., Osaka, Japan) placed on a 12-well plate. After stimulation, the cells were fixed with 4% paraformaldehyde in PBS for 15 min, permeabilized with 0.5% Triton X-100 in PBS for 1 min, and blocked with 5% BSA in PBS. The cells were then washed with PBS and incubated with the primary antibody against VE-cadherin (with shaking) at RT for 1 h. After washing with PBS, the cells were incubated with Alexa Fluor 488-conjugated anti-mouse IgG antibody (ThermoFisher Scientific) at RT for 45 min. After further washing with PBS, the cells were mounted with SlowFade™ Diamond using 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI; ThermoFisher Scientific), and observed under a fluorescence microscope.

Statistical Analysis

Data are presented as the mean ± standard deviation. The results were analyzed using Tukey–Kramer *post-hoc* test. Statistically significant difference was evaluated at $p < 0.05$.

RESULTS

OxLDL Enhances NET Formation of HL-60-Derived Neutrophils and Human Peripheral Blood Neutrophils Induced by PMA

Extracellular DNA release from HL-60-derived neutrophils under PMA treatment and treatments with native LDL or oxLDL (**Figure 1A**) was determined by fluorometric quantitation of DNA in the culture medium with SYTOX Green. DNA release was significantly induced by PMA treatment. The DNA release increased 3-fold by additional oxLDL treatment, but not by native LDL (**Figure 1B**). A similar response was observed when human peripheral blood neutrophils were assessed under the same experimental conditions (**Figure 1C**).

DNA release from the cells and histone citrullination in NETs structure were examined by immunofluorescent microscopy. Extracellular DNA and citrullinated histone H4 were microscopically visualized using SYTOX Green and anti-histone H4 (citrulline 3) antibody, respectively. When HL-60-derived neutrophils were incubated without PMA, slight staining of SYTOX Green and citrullinated histone H4 was observed (**Figure 2A**). When HL-60-derived neutrophils were stimulated with PMA, citrullinated histone H4 generated by the cells colocalized with extracellular DNA stained by SYTOX Green (**Figure 2B**). Neither native LDL nor oxLDL in the absence of PMA was sufficient for the induction of histone citrullination as well as DNA release, and this was demonstrated by the Hoechst 33342 staining of nuclei (**Figure 2A**). Strong overlapping signals of released DNA and citrullinated histones were detected when the neutrophils were treated with PMA followed by oxLDL. In similar conditions, loss of cell nuclei, released strings, and cellular debris were observed with SEM (**Figure 2B**).

Immunoblot analysis of HL-60-derived neutrophils was carried out to determine histone citrullination during NET formation (**Figure 3**). Citrullinated histone H4 was formed under PMA stimulation. The level of citrullinated histone in the cell lysate decreased with additional oxLDL treatment. Native LDL or oxLDL treatment alone did not cause histone citrullination in HL-60-derived neutrophils, indicating that the lipoproteins did not activate PAD4 in the cells to initiate histone citrullination.

These data suggest that extracellular release of DNA into the culture medium is induced by PMA and subsequent oxLDL treatment significantly enhanced the release of DNA and associating proteins.

OxLDL Treatment Following NET Formation Increased MPO Release From HL-60-Derived Neutrophils

Western blot analysis of the culture media was carried out to detect MPO. MPO is a heterotetrametric protein containing 60 kDa subunit, which can be recognized by the antibody used (34). MPO released from HL-60-derived neutrophils was not clearly visible in the absence of PMA stimulation. Notably, MPO release increased dramatically when the cells were stimulated with PMA and subsequently treated with oxLDL, whereas native LDL treatment of stimulated cells did not induce MPO release (**Figures 4A,C**). Intracellular MPO decreased by 40% after the stimulation of the cells with PMA and oxLDL (**Figures 4B,D**). MPO in the cell lysate was detected at 60 kDa as presented in **Figures 4B,D**; however, the band for extracellular MPO appeared larger than 250 kDa on a native PAGE (**Figures 4A,C**). The high molecular weight MPO band colocalized with apoB-100, a major protein component of LDL, on native PAGE, suggesting that released MPO interacts with oxLDL. Apparent size of the MPO band was not affected by treating the culture media with DNase I to cleave NETs DNA prior to native-PAGE analysis (**Supplemental Figure 1**). These data indicate that MPO released into the culture medium interacts with lipoproteins.

NETs Components Induced Inflammatory Responses in HAECs

To further investigate the possible effects of enhanced NET formation by oxLDL treatment, the culture media of HL-60-derived neutrophils were transferred to HAECs and incubated for 24 h. In this series of experiments, HL-60-derived neutrophils were treated with native LDL or oxLDL after washing the cells in order to remove PMA. Subsequently, HAECs were exposed to NETs components released from PMA-stimulated HL-60-derived neutrophils, with 5 μg/mL lipoproteins (**Figure 5A**). Induction of MMP-1

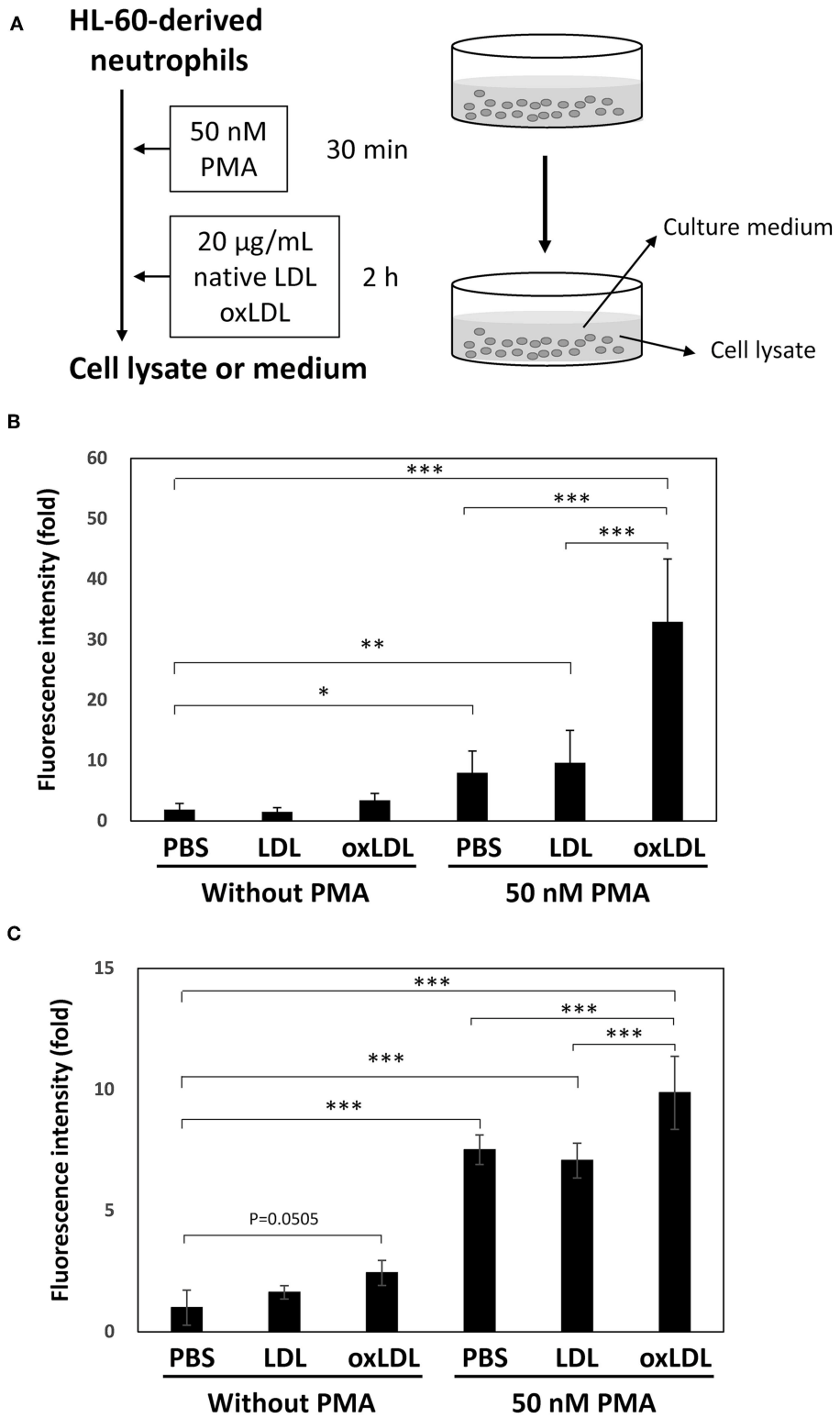

FIGURE 1 | NET formation was enhanced by oxLDL treatment after priming HL-60-derived neutrophils with PMA. **(A)** The experimental procedure is illustrated. HL-60 cells were stimulated with 2 µM all-trans retinoic acid for 4 days to differentiate into neutrophils. The cells were stimulated with 50 nM PMA for 30 min and then treated with 20 µg/mL native LDL or oxLDL for additional 2 h. **(B)** Fluorometric quantification of DNA release from HL-60-derived neutrophils and **(C)** Human peripheral blood neutrophils. Collected culture medium was treated with micrococcal nuclease and then stained with 1 µM SYTOX Green. The data are presented as the mean ± SD of four independent experiments. Each experiment contains triplicate wells. Asterisks indicate statistical significance. $^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.001$.

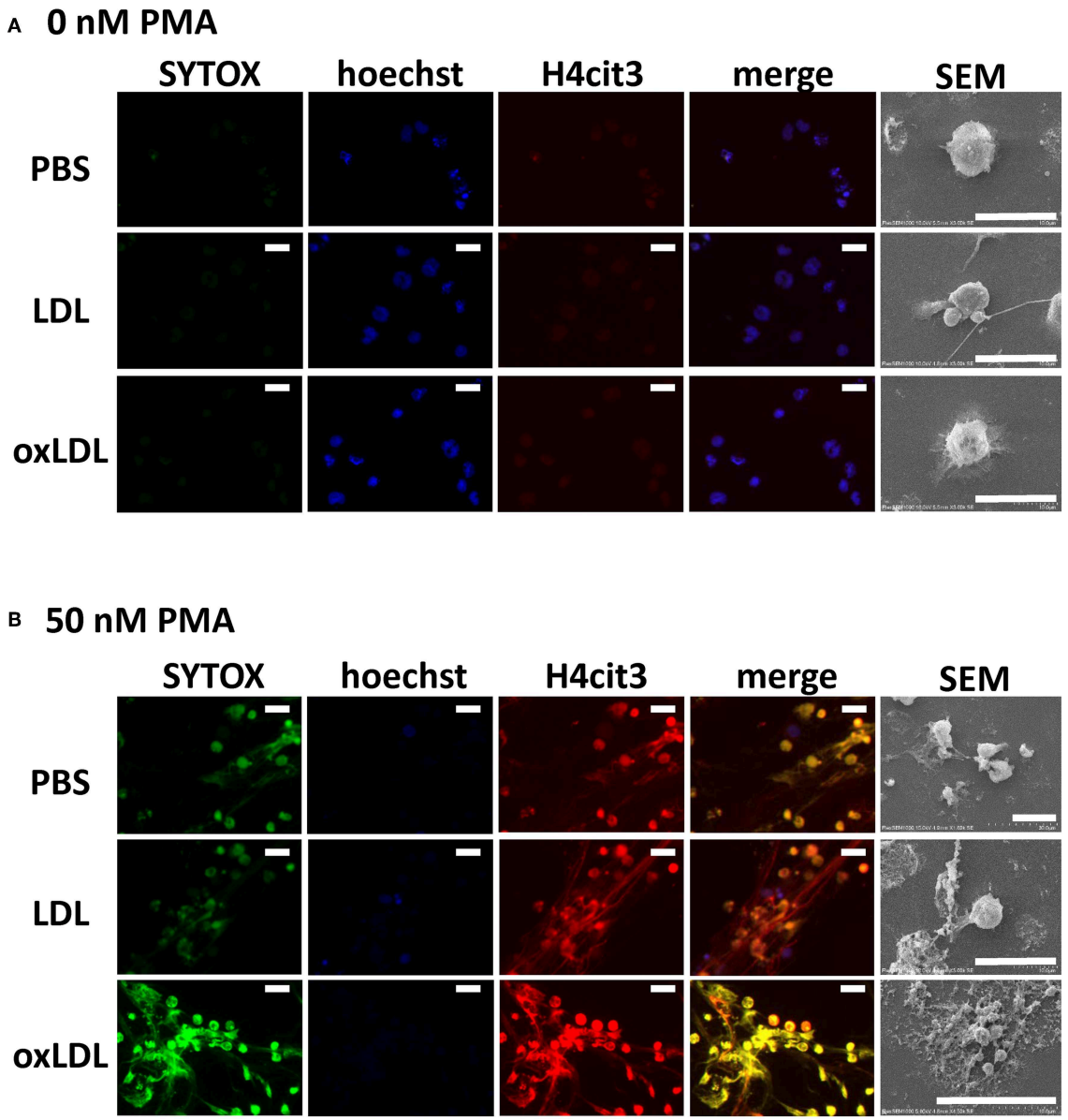

FIGURE 2 | Microscopic observation of NET formation of HL-60-derived neutrophils. Cells were stimulated **(A)** without or **(B)** with 50 nM PMA for 30 min, followed by 20 μg/mL native LDL or oxLDL for additional 2 h. Cells were fixed and stained with Hoechst 33342 (blue), SYTOX Green (green), and citrullinated histone H4 (red), and then visualized by fluorescence microscopy and scanning electron microscopy (SEM). The results are representative of more than three experiments. Scale bars indicate 20 μm.

protein expression in HAECs was observed after treatment with NETs components collectively with native LDL or oxLDL, whereas MMP-1 was not induced without NETs components even though HL-60-derived neutrophils were treated with native LDL or oxLDL (**Figure 5B**). The response of HAECs is presumably parallel to the release of NETs components from HL-60-derived neutrophils. Although PMA is also known as a powerful inducer of PKC expression in HAECs (35, 36), culture medium of NET-induced HL-60-derived neutrophils did not induce PKCβ protein expression in HAECs under our experimental conditions (**Supplemental Figure 2**), indicating that the transfer of PMA from culture medium of PMA-treated HL-60-derived neutrophils could be ignored.

Morphological Changes in HAECs Stimulated With Soluble NETs Components

Moreover, the morphological changes in HAECs were observed by carrying out immunostaining with VE-cadherin. Treatment with culture media from NETs-induced HL-60-derived neutrophils caused the elongation of HAECs which

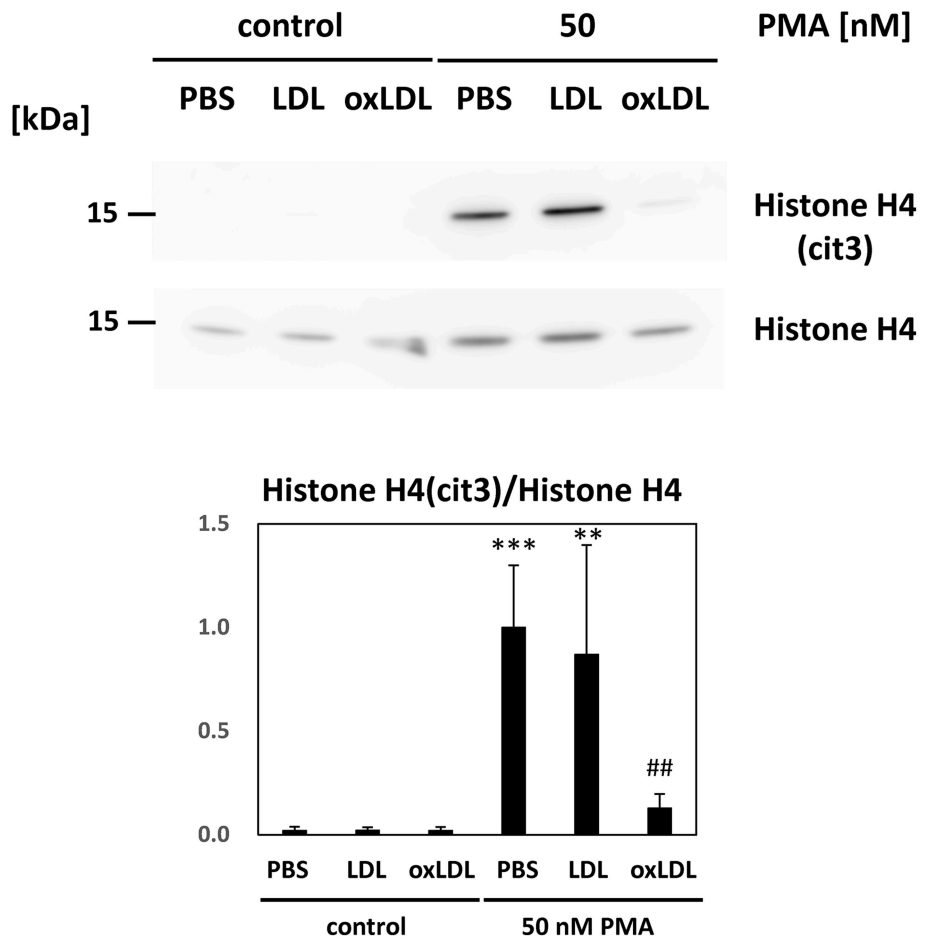

FIGURE 3 | Citrullination of histone H4 in the HL-60-derived neutrophils was evaluated. Cells were stimulated with 50 nM PMA for 30 min and followed by 20 μg/mL native LDL or oxLDL for additional 2 h. Citrullinated histone H4 in the cell lysate of HL-60-derived neutrophils was analyzed by western blot analysis. Each band was normalized by histone H4 of the corresponding sample. Quantitative data presented in the bar graph are the mean ± SD of three experiments. Asterisks indicate statistical significance against the condition "PBS without PMA"; $^{**}p < 0.005$, $^{***}p < 0.001$. Sharps indicate statistical significance against the condition "PBS with PMA"; $^{\#\#}p < 0.005$.

reduced cell circularity. The average circularity of HAECs was significantly decreased from 0.697 ± 0.122 to 0.600 ± 0.145 when the culture medium of HL-60-derived neutrophils stimulated with PMA was added to HAECs. Such effects on the cell shape of HAECs were immensely enhanced when HL-60-derived neutrophils were additionally treated with native LDL (from 0.701 ± 0.118 to 0.447 ± 0.153) or oxLDL (from 0.705 ± 0.119 to 0.457 ± 0.152; **Figure 6B**). In addition, decrease in HAEC circularity was associated with reduced fluorescence intensity of VE-cadherin-stained HAECs (**Figure 6A**). Notably, such changes were not observed in the cell shape of HAECs when they were incubated with native LDL or oxLDL alone at the same concentration (5 μg/mL as the final concentration). These data indicate that bioactive components generated during NET formation may act on HAECs, and the activity was greatly facilitated in the presence of native LDL and oxLDL. After incubating HAECs for 24 h, colocalization of MPO with apoB of native LDL increased (**Supplemental Figure 3**), indicating that the oxidative modification of native LDL may proceed to increase its electronegativity.

DISCUSSION

In this study we demonstrated that oxLDL significantly augmented NET formation by HL-60-derived neutrophils and human peripheral blood neutrophils, as well as NETs-dependent inflammatory activation in vascular endothelial cells. Neutrophil is one of the major cell types recruited in the lesions of vascular diseases. OxLDL accumulates in the atherosclerotic lesions and exhibits various biological activities against vascular cells to promote inflammatory responses and dysfunctions; however, the possible effect of oxLDL on neutrophils with regards to the promotion of vascular diseases has not been studied. To the best of our knowledge, this study was the first to

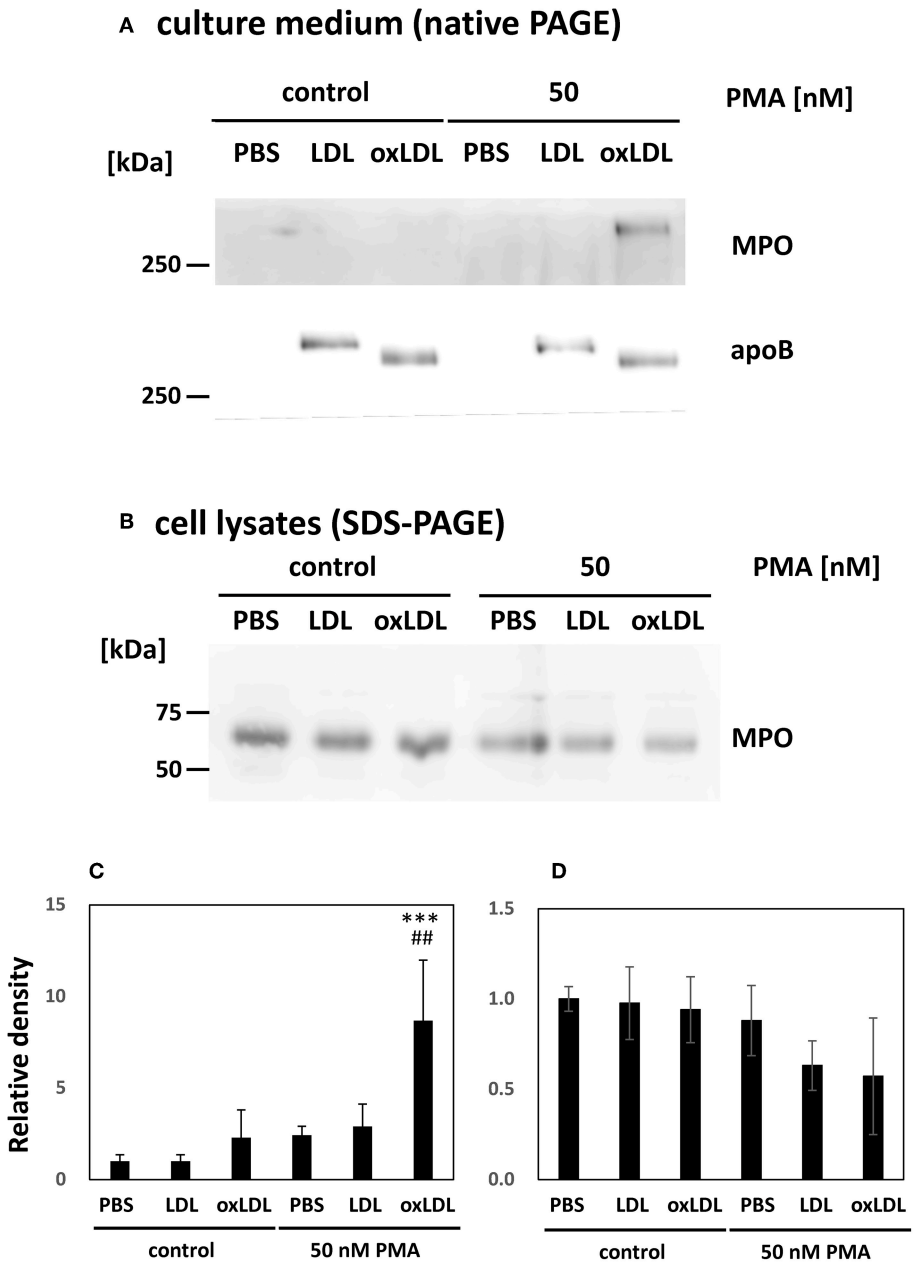

FIGURE 4 | Myeloperoxidase (MPO) released from PMA-stimulated neutrophils was enhanced by oxLDL treatment. HL-60-derived neutrophils were stimulated with 50 nM PMA for 30 min following 20 μg/mL native LDL or oxLDL for additional 2 h. **(A,C)** Western blot analysis of culture media of the cells to detect MPO and apolipoprotein B-100 (apoB) following native PAGE on a 3–20% gradient gel. **(B,D)** Western blot analysis of MPO in cell lysates of HL-60-derived neutrophils following 12% gel SDS-PAGE. Quantitative data presented in the bar graph are the mean ± SD of three experiments. Asterisks indicate statistical significance against the condition "PBS without PMA"; $^{***}p < 0.001$. Sharps indicate statistical significance against the condition "PBS with PMA"; $^{\#\#}p < 0.005$.

investigate the cooperative effect of neutrophils and oxLDL on vascular cells.

NETs were initially reported to act as one of the natural immune responses to bacterial infection by releasing their own chromosomal DNA collectively with the nuclear, cytoplasmic, and granular proteins; however, excess NET formation has been implicated in pathophysiological disruption in various organs. Neutrophil infiltration is detected in the intima of atherosclerotic lesions in human vessels, specifically in acute coronary syndrome (6), myocardial infarction, and thrombotic lesions (7). Certain studies demonstrated that NET formation is involved in the activation and dysfunction of endothelial cells (37), promotion of angiogenesis (38), and progression of atherosclerotic lesions and cardiovascular diseases (16).

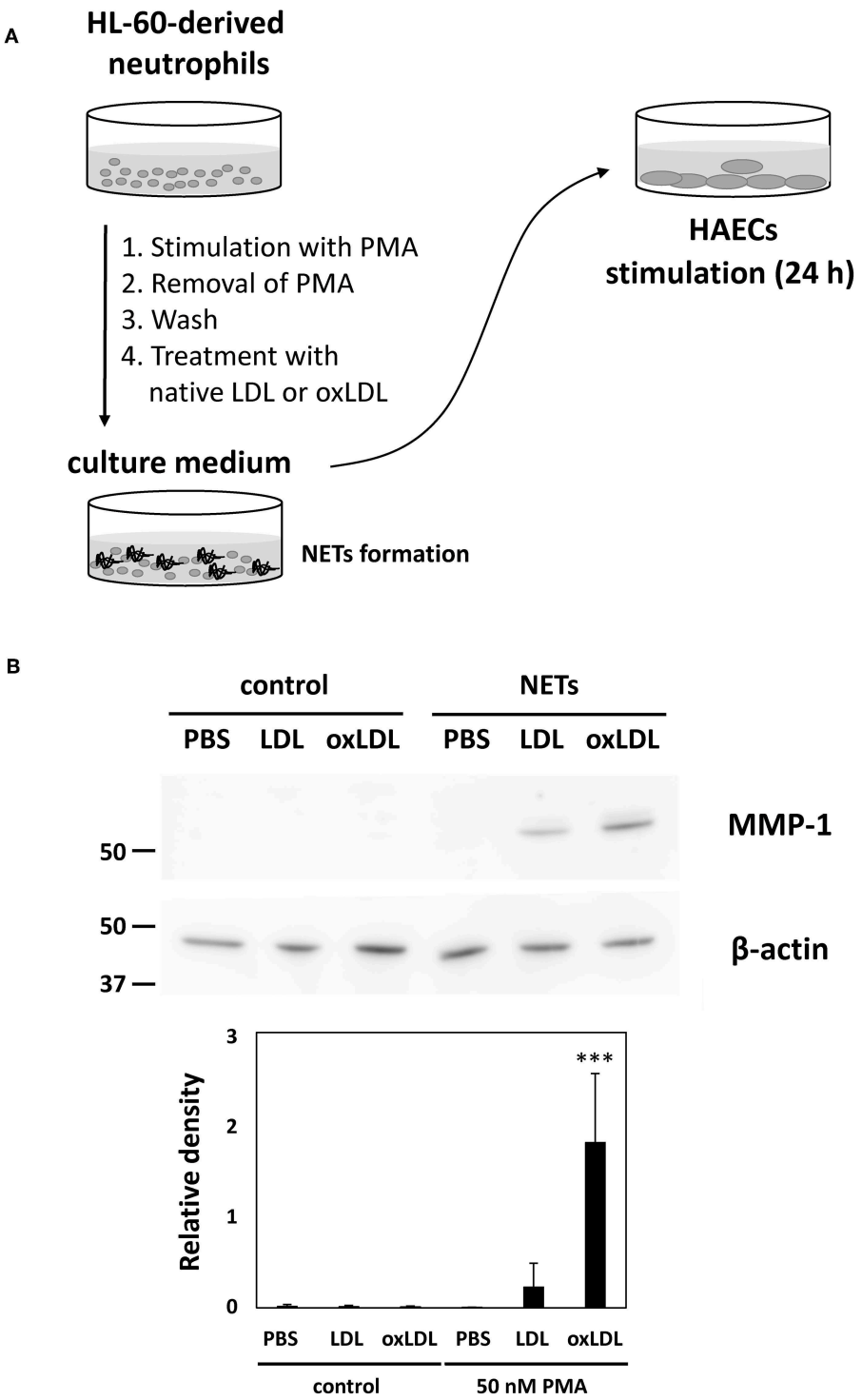

FIGURE 5 | Enhanced NETs release from HL-60-derived neutrophils by oxLDL treatment is associated with increased inflammatory responses in HAECs. **(A)** Experimental procedure for the activation of HAECs by NETs components. HL-60-derived neutrophils were stimulated with 50 nM PMA for 30 min. The culture supernatant was discarded, and the cells were washed with fresh medium to remove PMA, then the cells were subjected to subsequent treatment with 20 μg/mL LDL or oxLDL for 2 h. The culture medium of the HL-60-derived neutrophils (333 μL) was transferred to 1 mL of HAECs culture medium (final concentration of 5 μg/mL lipoproteins) and cultured for 24 h. **(B)** Expression of MMP-1 in HAECs was analyzed by using western blot. The intensity of each band was normalized by the corresponding band of β-actin. Quantitative data presented in the bar graph are the mean ± SD of three experiments. Asterisks indicate statistical significance against the condition PBS without PMA; ***$p < 0.001$.

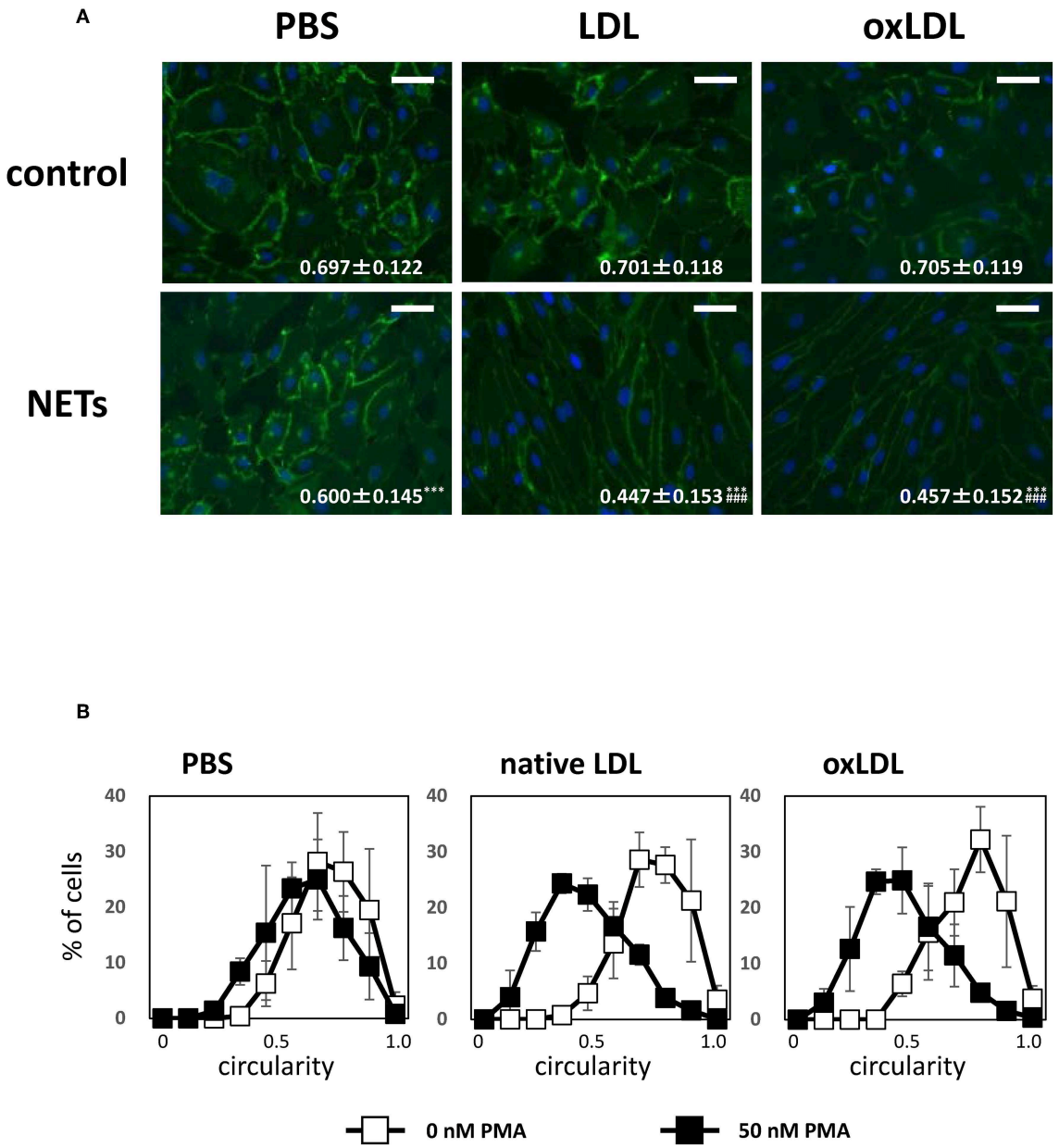

FIGURE 6 | NETs components induce morphological alteration of HAECs, which is enhanced by coincubation with LDL or oxLDL. HAECs were stimulated as indicated in the figure legends of **Figure 5**. **(A)** Changes in the cell shapes of HAECs were evaluated by VE-cadherin (green) staining. Scale bars indicate 100 μm. Circularity of each cells was calculated using Image J software. Average circularity in each experimental condition is presented at the bottom of the images as the mean ± SD of at least 60 cells. Asterisks indicate statistical significance against each condition without PMA; $^{***}p < 0.001$. Sharps indicate statistical significance against PBS with PMA; $^{\#\#\#}p < 0.001$. **(B)** Distribution of circularity of each HAECs is shown in white (control) and black (NETs) symbols. The data presented are the mean ± SD of three independent experiments.

Obesity-induced endothelial dysfunction was prevented by NETs inhibition (39). These studies would provide a promising new approach for the treatment of vascular diseases. Considering such information on the potential importance of NETs in vascular diseases and the presence of neutrophils in atherosclerotic lesions, we hypothesized that oxLDL is involved in NET formation.

Quantitative evaluation of NET formation is difficult; however, we introduced a fluorometric quantitation procedure to determine the extracellular DNA. Based on fluorometric quantitation of released NETs-DNA and western blot of citrullinated histone H4 in cell lysates, PMA-induced NET formation of HL-60-derived neutrophils was significantly accelerated by oxLDL at a concentration of 20 μg/mL, which

was insufficient for NETs induction without PMA stimulation. In addition, the immunofluorescent and electron microscopic analyses of released DNA confirmed the drastic change in the cells. In a previous study, Awasthi et al. reported that oxLDL was able to induce NET-like structure against neutrophils; however, they treated neutrophils with 100 μg/mL oxLDL, whereas histone citrullination was not assessed (40). In the previous study, the circulating oxLDL level in human plasma was estimated to be 7–35 μg/mL (41, 42). In our present study, we utilized 20 μg/mL oxLDL at physiologically relevant concentration and confirmed that histone citrullination was not induced by stimulation with oxLDL alone. Thus, the effect of oxLDL on HL-60-derived neutrophils represents the enhancement of NET formation. According to a previous report, the blocking of toll like receptor-2 and -6 partially reduced oxLDL-induced NET formation (40), and these receptors may recognize and uptake oxLDL to induce enhancement of NET formation.

Accelerated NET formation induced by subsequent treatment with oxLDL was associated with enhanced release of MPO into the culture media. Native-PAGE analysis demonstrated that extracellular MPO interacted with apoB, corresponding with the results of previous studies which revealed that MPO binds to LDL and oxLDL (43, 44). MPO may contribute to high electronegative affinity with oxLDL because MPO is a highly cationic protein (45). MPO mediates the oxidative modification on the surface of LDL via ROS formation and/or MPO-catalyzed nitration and chlorination of proteins (46). Presumably, increased NET formation by oxLDL also promotes oxLDL production. Elevated plasma MPO levels were associated with acute myocardial infarction (47). In accordance, elevated level of MPO-DNA complex, the marker of NETosis, correlated with the severity of coronary artery diseases (26). A significant positive correlation between oxLDL and MPO in plasma from patients with ST-elevation myocardial infarction has been reported (7). MPO mediates the conversion of hydrogen peroxide to hypochlorous acid (HOCl)—a compound which induces the production of chlorinated lipid. Recently, 2-chlorofatty acid was reported as one of the lipid mediators that induce NET formation (48). MPO has also been implicated in endothelial dysfunction (49) and atherosclerosis (50). Our observation and other reports collectively suggest a possibility that circulating oxLDL acts as a carrier of MPO derived from neutrophils and contributes to the development of vascular inflammation.

As many studies, including ours (51), have previously reported, oxLDL contains various lipids including oxidized phosphatidylcholines, lysoPCs, and fatty acids. Neutrophils are susceptible to the toxic action of free fatty acids which causes cell death accompanied by direct membrane perturbation (52). Recent studies have extensively reported that lipid mediators are involved not only in NETosis but also in other types of programmed neutrophil death. Oxidized phospholipids and lysoPCs are mediators of NET formation (40). Ceramides are involved in NET induction (19, 53). Some studies demonstrated that various sphingolipid signaling mediated by sphingolipids and their analogs play roles in programmed neutrophil death, such as apoptosis and necroptosis (54, 55). Lipidomic analysis would be necessary to investigate the mechanism underlying oxLDL-mediated enhancement of NET formation.

Our data demonstrate that the culture medium of HL-60-derived neutrophils, which was treated with PMA and subsequently with oxLDL induced marked alteration of HAECs. Under suitable conditions, MMP-1 protein expression was induced in HAECs. MMP-1, also known as interstitial collagenase, is involved in the degradation of vascular extracellular matrix including collagen type I, II, and III. Elevated level of MMP-1 expression has been reported not only in rheumatoid arthritis (RA) (56) but also in atherosclerotic plaques (57, 58). Increased MMP-1 expression is also implicated in plaque rupture (59) and in *in vitro* endothelial cell senescence (60). A previous study demonstrated that oxLDL but not native LDL upregulated MMP-1 protein expression in human coronary endothelial cells (61). Our present study demonstrated that MMP-1 protein expression was dramatically induced in HAECs by culture medium containing NETs components with subsequent coincubation with oxLDL; whereas, notably, the coincubation of NETs components with native LDL also induced MMP-1 expression. One possible explanation is that the coincubation of NETs components and native LDL for 24 h may cause the oxidation of LDL to produce oxLDL which acts together with NETs on HAECs.

It is noteworthy that oxLDL alone does not induce NET formation but have an ability to enhance PMA-induced NET formation associated with exaggerated inflammatory responses in HAECs. Stimulation of HL-60-derived neutrophils with PMA alone induces NET formation to a certain extent. The cell shape of HAECs changed to elongated form with decreased VE-cadherin staining, induced by the culture medium of HL-60-derived neutrophils containing NETs components. This observation is consistent with that in a recent study by Meegan et al. who demonstrated that citrullinated histone H3 causes barrier dysfunction of human umbilical vein endothelial cells characterized by the disruption of cell–cell adherent junction and cytoskeleton reorganization with increased F-actin stress fibers, leading to a decrease in intercellular barrier strength and microvascular leakage as evaluated by mouse mesenteric microvessels (62). The elongation of HAECs elicited by oxLDL causes the alteration of structural and/or mechanical integrity of the endothelial barrier (63). Notably, we found that enhanced elongation in HAECs was induced by supernatant of culture medium comprising NETs from HL-60-derived neutrophils treated with either native LDL or oxLDL. This strongly indicates that accelerated NET formation induced by oxLDL and possibly native LDL-derived oxLDL generated during incubation with NETs components, may contribute to exacerbated endothelial barrier dysfunction, thereby causing vulnerability of atherosclerotic plaque.

NETs also comprise various proteases including cathepsin G, proteinase 3, MMP-9, and elastase. Cleavage of VE-cadherin mediated by elastase, one of the proteases associated with NETs, abolishes cell–cell contacts and increases cell permeability (64). Recent evidence demonstrated that cathepsin

G, which is also a NETs-associating protease, mediates the degradation of apoB in native LDL to cause LDL fusion and enhanced binding of LDL to isolated human aortic proteoglycans and human atherosclerotic lesions *ex vivo*, thereby contributing to atherogenesis (65, 66). Interestingly, it is presumable that cooperative pathophysiological relevance of proteases on NETs and lipoproteins causes the progression of atherosclerosis.

The limitations of the present study include the lack of mechanistic analysis of oxLDL-mediated enhancement of NET formation including exploring receptors involved in oxLDL-mediated NET formation. It is also important to explore oxLDL-mediated NET formation under physiologically relevant stimulation, such as inflammatory cytokines (67, 68). IL-8 is a relatively weak NET inducer compared to PMA (67), and hence, human neutrophils may utilize oxLDL for enhanced NET formation under physiological stimuli, therefore, human neutrophils should be utilized to explore the contribution of oxLDL to NET formation. Future research is desired to investigate the mechanisms behind oxLDL-mediated acceleration of NET formation by human neutrophils.

In conclusion, we demonstrated in this study that oxLDL has the ability to enhance NET formation. In addition, oxLDL and NETs components have synergistic effects on morphological changes and inflammatory responses in HAECs. NET formation may possibly amplify the production of oxidatively and proteolytically modified LDL which can augment further production of oxLDL and NET formation. Our study provides information on the novel possibility that circular oxLDL and vascular NETs act together as risk factors for the initiation and progression of atherosclerosis and other vascular diseases.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the **Supplementary Files**.

ETHICS STATEMENT

Written informed consent in accordance with the Declaration of Helsinki was prepared and all human subjects voluntarily gave their signatures for participation in this study. The protocol was approved by the Ethical Committee of Showa University School of Pharmacy (No. 231).

AUTHOR CONTRIBUTIONS

TO designed the research projects. TO and HO performed the experiments and analyzed the data. TO and SI performed the isolation and oxidation of lipoproteins. TO and TT performed the electron microscopic analysis. NS, TA, and RK participated in the interpretation of the data and the discussion. HI supervised the study. TO and HI prepared the figures and wrote the manuscript. All authors reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01899/full#supplementary-material

REFERENCES

1. Itabe H, Obama T, Kato R. The dynamics of oxidized LDL during atherogenesis. *J Lipids.* (2011) 2011:418313. doi: 10.1155/2011/418313
2. Ehara S, Ueda M, Naruko T, Haze K, Itoh A, Otsuka M, et al. Elevated levels of oxidized low density lipoprotein show a positive relationship with the severity of acute coronary syndromes. *Circulation.* (2001) 103:1955–60. doi: 10.1161/01.CIR.103.15.1955
3. Glass CK, Witztum JL. Atherosclerosis. The road ahead. *Cell.* (2001) 104:503–16. doi: 10.1016/S0092-8674(01)00238-0
4. Moore KJ, Tabas I. Macrophages in the pathogenesis of atherosclerosis. *Cell.* (2011) 145:341–55. doi: 10.1016/j.cell.2011.04.005
5. Tabas I, Garcia-Cardena G, Owens GK. Recent insights into the cellular biology of atherosclerosis. *J Cell Biol.* (2015) 209:13–22. doi: 10.1083/jcb.201412052
6. Naruko T, Ueda M, Haze K, van der Wal AC, van der Loos CM, Itoh A, et al. Neutrophil infiltration of culprit lesions in acute coronary syndromes. *Circulation.* (2002) 106:2894–900. doi: 10.1161/01.CIR.0000042674.89762.20
7. Yunoki K, Naruko T, Sugioka K, Inaba M, Iwasa Y, Komatsu R, et al. Erythrocyte-rich thrombus aspirated from patients with ST-elevation myocardial infarction: association with oxidative stress and its impact on myocardial reperfusion. *Eur Heart J.* (2012) 33:1480–90. doi: 10.1093/eurheartj/ehr486
8. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, et al. Neutrophil extracellular traps kill bacteria. *Science.* (2004) 303:1532–5. doi: 10.1126/science.1092385
9. Wang Y, Wang W, Wang N, Tall AR, Tabas I. Mitochondrial oxidative stress promotes atherosclerosis and neutrophil extracellular traps in aged mice. *Arterioscler Thromb Vasc Biol.* (2017) 37:e99–107. doi: 10.1161/ATVBAHA.117.309580
10. Jorch SK, Kubes P. An emerging role for neutrophil extracellular traps in noninfectious disease. *Nat Med.* (2017) 23: 279–87. doi: 10.1038/nm.4294
11. Erpenbeck L, Schon MP. Neutrophil extracellular traps: protagonists of cancer progression? *Oncogene.* (2017) 36:2483–90. doi: 10.1038/onc.2016.406

12. Pietronigro EC, Della Bianca V, Zenaro E, Constantin G. NETosis in Alzheimer's disease. *Front Immunol.* (2017) 8:211. doi: 10.3389/fimmu.2017.00211
13. Wong SL, Demers M, Martinod K, Gallant M, Wang Y, Goldfine AB, et al. Diabetes primes neutrophils to undergo NETosis, which impairs wound healing. *Nat Med.* (2015) 21:815–9. doi: 10.1038/nm.3887
14. Eliason JL, Hannawa KK, Ailawadi G, Sinha I, Ford JW, Deogracias MP, et al. Neutrophil depletion inhibits experimental abdominal aortic aneurysm formation. *Circulation.* (2005) 112:232–40. doi: 10.1161/CIRCULATIONAHA.104.517391
15. Houard X, Touat Z, Ollivier V, Louedec L, Philippe M, Sebbag U, et al. Mediators of neutrophil recruitment in human abdominal aortic aneurysms. *Cardiovasc Res.* (2009) 82:532–41. doi: 10.1093/cvr/cvp048
16. Doring Y, Soehnlein O, Weber C. Neutrophil extracellular traps in atherosclerosis and atherothrombosis. *Circ Res.* (2017) 120:736–43. doi: 10.1161/CIRCRESAHA.116.309692
17. Quillard T, Araujo HA, Franck G, Shvartz E, Sukhova G, Libby P. TLR2 and neutrophils potentiate endothelial stress, apoptosis and detachment: implications for superficial erosion. *Eur Heart J.* (2015) 36:1394–404. doi: 10.1093/eurheartj/ehv044
18. Yan H, Zhou HF, Akk A, Hu Y, Springer LE, Ennis TL, et al. Neutrophil proteases promote experimental abdominal aortic aneurysm via extracellular trap release and plasmacytoid dendritic cell activation. *Arterioscler Thromb Vasc Biol.* (2016) 36:1660–9. doi: 10.1161/ATVBAHA.116.307786
19. Meher AK, Spinosa M, Davis JP, Pope N, Laubach VE, Su G, et al. Novel role of IL (interleukin)-1beta in neutrophil extracellular trap formation and abdominal aortic aneurysms. *Arterioscler Thromb Vasc Biol.* (2018) 38:843–53. doi: 10.1161/ATVBAHA.117.309897
20. von Bruhl ML, Stark K, Steinhart A, Chandraratne S, Konrad I, Lorenz M, et al. Monocytes, neutrophils, and platelets cooperate to initiate and propagate venous thrombosis in mice *in vivo*. *J Exp Med.* (2012) 209:819–35. doi: 10.1084/jem.20112322
21. Brill A, Fuchs TA, Savchenko AS, Thomas GM, Martinod K, De Meyer SF, et al. Neutrophil extracellular traps promote deep vein thrombosis in mice. *J Thromb Haemost.* (2012) 10:136–44. doi: 10.1111/j.1538-7836.2011.04544.x
22. Kapoor S, Opneja A, Nayak L. The role of neutrophils in thrombosis. *Thromb Res.* (2018) 170:87–96. doi: 10.1016/j.thromres.2018.08.005
23. Drechsler M, Megens RT, van Zandvoort M, Weber C, Soehnlein O. Hyperlipidemia-triggered neutrophilia promotes early atherosclerosis. *Circulation.* (2010) 122:1837–45. doi: 10.1161/CIRCULATIONAHA.110.961714
24. Soehnlein O. Multiple roles for neutrophils in atherosclerosis. *Circ Res.* (2012) 110:875–88. doi: 10.1161/CIRCRESAHA.111.257535
25. Liu Y, Carmona-Rivera C, Moore E, Seto NL, Knight JS, Pryor M, et al. Myeloid-specific deletion of peptidylarginine deiminase 4 mitigates atherosclerosis. *Front Immunol.* (2018) 9:1680. doi: 10.3389/fimmu.2018.01680
26. Borissoff JI, Joosen IA, Versteylen MO, Brill A, Fuchs TA, Savchenko AS, et al. Elevated levels of circulating DNA and chromatin are independently associated with severe coronary atherosclerosis and a prothrombotic state. *Arterioscler Thromb Vasc Biol.* (2013) 33:2032–40. doi: 10.1161/ATVBAHA.113.301627
27. Knight JS, Luo W, O'Dell AA, Yalavarthi S, Zhao W, Subramanian V, et al. Peptidylarginine deiminase inhibition reduces vascular damage and modulates innate immune responses in murine models of atherosclerosis. *Circ Res.* (2014) 114:947–56. doi: 10.1161/CIRCRESAHA.114.303312
28. Kato R, Mori C, Kitazato K, Arata S, Obama T, Mori M, et al. Transient increase in plasma oxidized LDL during the progression of atherosclerosis in apolipoprotein E knockout mice. *Arterioscler Thromb Vasc Biol.* (2009) 29:33–9. doi: 10.1161/ATVBAHA.108.164723
29. Kato R, Hayashi M, Aiuchi T, Sawada N, Obama T, Itabe H. Temporal and spatial changes of peroxiredoxin 2 levels in aortic media at very early stages of atherosclerotic lesion formation in apoE-knockout mice. *Free Radic Biol Med.* (2019) 130:348–60. doi: 10.1016/j.freeradbiomed.2018.10.458
30. Nose F, Yamaguchi T, Kato R, Aiuchi T, Obama T, Hara S, et al. Crucial role of perilipin-3 (TIP47) in formation of lipid droplets and PGE2 production in HL-60-derived neutrophils. *PLoS ONE.* (2013) 8:e71542. doi: 10.1371/journal.pone.0071542
31. Morita C, Sumioka R, Nakata M, Okahashi N, Wada S, Yamashiro T, et al. Cell wall-anchored nuclease of Streptococcus sanguinis contributes to escape from neutrophil extracellular trap-mediated bacteriocidal activity. *PLoS ONE.* (2014) 9:e103125. doi: 10.1371/journal.pone.0103125
32. Obama T, Kato R, Masuda Y, Takahashi K, Aiuchi T, Itabe H. Analysis of modified apolipoprotein B-100 structures formed in oxidized low-density lipoprotein using LC-MS/MS. *Proteomics.* (2007) 7:2132–41. doi: 10.1002/pmic.200700111
33. Palmer LJ, Cooper PR, Ling MR, Wright HJ, Huissoon A, Chapple IL. Hypochlorous acid regulates neutrophil extracellular trap release in humans. *Clin Exp Immunol.* (2012) 167:261–8. doi: 10.1111/j.1365-2249.2011.04518.x
34. Kimura S, Ikeda-Saito M. Human myeloperoxidase and thyroid peroxidase, two enzymes with separate and distinct physiological functions, are evolutionarily related members of the same gene family. *Proteins.* (1988) 3:113–20. doi: 10.1002/prot.340030206
35. Mendonca MC, Koles N, Sellitti DF. Protein kinase C-delta (PKC-delta) and PKC-alpha mediate Ca(2+)-dependent increases in CNP mRNA in human vascular cells. *Vascul Pharmacol.* (2012) 57:98–104. doi: 10.1016/j.vph.2012.05.002
36. Zhu DD, Tang RN, Lv LL, Wen Y, Liu H, Zhang XL, et al. Interleukin-1beta mediates high glucose induced phenotypic transition in human aortic endothelial cells. *Cardiovasc Diabetol.* (2016) 15:42. doi: 10.1186/s12933-016-0358-9
37. Folco EJ, Mawson TL, Vromman A, Bernardes-Souza B, Franck G, Persson O, et al. Neutrophil extracellular traps induce endothelial cell activation and tissue factor production through interleukin-1alpha and cathepsin G. *Arterioscler Thromb Vasc Biol.* (2018) 38:1901–12. doi: 10.1161/ATVBAHA.118.311150
38. Aldabbous L, Abdul-Salam V, McKinnon T, Duluc L, Pepke-Zaba J, Southwood M, et al. Neutrophil extracellular traps promote angiogenesis: evidence from vascular pathology in pulmonary hypertension. *Arterioscler Thromb Vasc Biol.* (2016) 36:2078–87. doi: 10.1161/ATVBAHA.116.307634
39. Wang H, Wang Q, Venugopal J, Wang J, Kleiman K, Guo C, et al. Obesity-induced endothelial dysfunction is prevented by neutrophil extracellular trap inhibition. *Sci Rep.* (2018) 8:4881. doi: 10.1038/s41598-018-23256-y
40. Awasthi D, Nagarkoti S, Kumar A, Dubey M, Singh AK, Pathak P, et al. Oxidized LDL induced extracellular trap formation in human neutrophils via TLR-PKC-IRAK-MAPK and NADPH-oxidase activation. *Free Radic Biol Med.* (2016) 93:190–203. doi: 10.1016/j.freeradbiomed.2016.01.004
41. Byfield FJ, Tikku S, Rothblat GH, Gooch KJ, Levitan I. OxLDL increases endothelial stiffness, force generation, and network formation. *J Lipid Res.* (2006) 47:715–23. doi: 10.1194/jlr.M500439-JLR200
42. Holvoet P, Mertens A, Verhamme P, Bogaerts K, Beyens G, Verhaeghe R, et al. Circulating oxidized LDL is a useful marker for identifying patients with coronary artery disease. *Arterioscler Thromb Vasc Biol.* (2001) 21:844–8. doi: 10.1161/01.ATV.21.5.844
43. Carr AC, Myzak MC, Stocker R, McCall MR, Frei B. Myeloperoxidase binds to low-density lipoprotein: potential implications for atherosclerosis. *FEBS Lett.* (2000) 487:176–80. doi: 10.1016/S0014-5793(00)02227-4
44. Sokolov AV, Kostevich VA, Runova OL, Gorudko IV, Vasilyev VB, Cherenkevich SN, et al. Proatherogenic modification of LDL by surface-bound myeloperoxidase. *Chem Phys Lipids.* (2014) 180:72–80. doi: 10.1016/j.chemphyslip.2014.02.006
45. Vargunam M, Adu D, Taylor CM, Michael J, Richards N, Neuberger J, et al. Endothelium myeloperoxidase-antimyeloperoxidase interaction in vasculitis. *Nephrol Dial Transplant.* (1992) 7:1077–81.
46. Delporte C, Boudjeltia KZ, Noyon C, Furtmuller PG, Nuyens V, Slomianny MC, et al. Impact of myeloperoxidase-LDL interactions on enzyme activity and subsequent posttranslational oxidative modifications of apoB-100. *J Lipid Res.* (2014) 55:747–57. doi: 10.1194/jlr.M047449
47. Yunoki K, Naruko T, Komatsu R, Shirai N, Nakagawa M, Sugioka K, et al. Relation of elevated levels of plasma myeloperoxidase to impaired myocardial microcirculation after reperfusion in patients with acute myocardial infarction. *Am J Cardiol.* (2010) 105:922–9. doi: 10.1016/j.amjcard.2009.11.013

48. Palladino END, Katunga LA, Kolar GR, Ford DA. 2-Chlorofatty acids: lipid mediators of neutrophil extracellular trap formation. *J Lipid Res*. (2018) 59:1424–32. doi: 10.1194/jlr.M084731
49. Vita JA, Brennan ML, Gokce N, Mann SA, Goormastic M, Shishehbor MH, et al. Serum myeloperoxidase levels independently predict endothelial dysfunction in humans. *Circulation*. (2004) 110:1134–9. doi: 10.1161/01.CIR.0000140262.20831.8F
50. Teng N, Maghzal GJ, Talib J, Rashid I, Lau AK, Stocker R. The roles of myeloperoxidase in coronary artery disease and its potential implication in plaque rupture. *Redox Rep*. (2017) 22:51–73. doi: 10.1080/13510002.2016.1256119
51. Sasabe N, Keyamura Y, Obama T, Inoue N, Masuko Y, Igarashi Y, et al. Time course-changes in phosphatidylcholine profile during oxidative modification of low-density lipoprotein. *Lipids Health Dis.* (2014) 13:48. doi: 10.1186/1476-511X-13-48
52. Lux CA, Koschinski A, Dersch K, Husmann M, Bhakdi S. Hypersusceptibility of neutrophil granulocytes towards lethal action of free fatty acids contained in enzyme-modified atherogenic low density lipoprotein. *Atherosclerosis*. (2009) 207:116–22. doi: 10.1016/j.atherosclerosis.2009.04.004
53. Corriden R, Hollands A, Olson J, Derieux J, Lopez J, Chang JT, et al. Tamoxifen augments the innate immune function of neutrophils through modulation of intracellular ceramide. *Nat Commun*. (2015) 6:8369. doi: 10.1038/ncomms9369
54. Skrzeczynska-Moncznik J, Bzowska M, Nogiec A, Sroka A, Zarebski M, Vallieres L, et al. Rapid externalization of 27-kDa heat shock protein (HSP27) and atypical cell death in neutrophils treated with the sphingolipid analog drug FTY720. *J Leukoc Biol*. (2015) 98:591–9. doi: 10.1189/jlb.3VMA1114-522RR
55. Mizugishi K, Yamashita K. Neutrophil extracellular traps are critical for pregnancy loss in sphingosine kinase-deficient mice on 129Sv/C57BL/6 background. *FASEB J*. (2017) 31:5577–91. doi: 10.1096/fj.201700399RR
56. Yoshihara Y, Nakamura H, Obata K, Yamada H, Hayakawa T, Fujikawa K, et al. Matrix metalloproteinases and tissue inhibitors of metalloproteinases in synovial fluids from patients with rheumatoid arthritis or osteoarthritis. *Ann Rheum Dis*. (2000) 59:455–61. doi: 10.1136/ard.59.6.455
57. Nikkari ST, O'Brien KD, Ferguson M, Hatsukami T, Welgus HG, Alpers CE, et al. Interstitial collagenase (MMP-1) expression in human carotid atherosclerosis. *Circulation*. (1995) 92:1393–8. doi: 10.1161/01.CIR.92.6.1393
58. Galis ZS, Sukhova GK, Lark MW, Libby P. Increased expression of matrix metalloproteinases and matrix degrading activity in vulnerable regions of human atherosclerotic plaques. *J Clin Invest*. (1994) 94:2493–503. doi: 10.1172/JCI117619
59. Lee RT, Schoen FJ, Loree HM, Lark MW, Libby P. Circumferential stress and matrix metalloproteinase 1 in human coronary atherosclerosis. Implications for plaque rupture. *Arterioscler Thromb Vasc Biol*. (1996) 16:1070–73. doi: 10.1161/01.ATV.16.8.1070
60. Struewing IT, Durham SN, Barnett CD, Mao CD. Enhanced endothelial cell senescence by lithium-induced matrix metalloproteinase-1 expression. *J Biol Chem*. (2009) 284:17595–606. doi: 10.1074/jbc.M109.001735
61. Li D, Liu L, Chen H, Sawamura T, Ranganathan S, Mehta JL. LOX-1 mediates oxidized low-density lipoprotein-induced expression of matrix metalloproteinases in human coronary artery endothelial cells. *Circulation*. (2003) 107:612–7. doi: 10.1161/01.CIR.0000047276.52039.FB
62. Meegan JE, Yang X, Beard RS Jr, Jannaway M, Chatterjee V, Taylor-Clark TE, et al. Citrullinated histone 3 causes endothelial barrier dysfunction. *Biochem Biophys Res Commun*. (2018) 503:1498–502. doi: 10.1016/j.bbrc.2018.07.069
63. Chow SE, Lee RS, Shih SH, Chen JK. Oxidized LDL promotes vascular endothelial cell pinocytosis via a prooxidation mechanism. *FASEB J*. (1998) 12:823–30. doi: 10.1096/fasebj.12.10.823
64. Pieterse E, Rother N, Garsen M, Hofstra JM, Satchell SC, Hoffmann M, et al. Neutrophil extracellular traps drive endothelial-to-mesenchymal transition. *Arterioscler Thromb Vasc Biol*. (2017) 37:1371–1379. doi: 10.1161/ATVBAHA.117.309002
65. Wang J, Sjoberg S, Tang TT, Oorni K, Wu W, Liu C, et al. Cathepsin G activity lowers plasma LDL and reduces atherosclerosis. *Biochim Biophys Acta*. (2014) 1842:2174–83. doi: 10.1016/j.bbadis.2014.07.026
66. Maaninka K, Nguyen SD, Mayranpaa MI, Plihtari R, Rajamaki K, Lindsberg PJ, et al. Human mast cell neutral proteases generate modified LDL particles with increased proteoglycan binding. *Atherosclerosis*. (2018) 275:390–9. doi: 10.1016/j.atherosclerosis.2018.04.016
67. Marin Oyarzun CP, Carestia A, Lev PR, Glembotsky AC, Castro Rios MA, Moiraghi B, et al. Neutrophil extracellular trap formation and circulating nucleosomes in patients with chronic myeloproliferative neoplasms. *Sci Rep*. (2016) 6:38738. doi: 10.1038/srep38738
68. Silvestre-Roig C, Braster Q, Wichapong K, Lee EY, Teulon JM, Berrebeh N, et al. Externalized histone H4 orchestrates chronic inflammation by inducing lytic cell death. *Nature*. (2019) 569:236–40. doi: 10.1038/s41586-019-1167-6

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REVIEW
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Neutrophils in Psoriasis

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Neutrophils are the most abundant innate immune cells. The pathogenic roles of neutrophils are related to chronic inflammation and autoimmune diseases. Psoriasis is a chronic systemic inflammatory disease affecting ∼2–3% of the world population. The abundant presence of neutrophils in the psoriatic skin lesions serves as a typical histopathologic hallmark of psoriasis. Recent reports indicated that oxidative stress, granular components, and neutrophil extracellular traps from psoriatic neutrophils are related to the initial and maintenance phases of psoriasis. This review provides an overview on the recent (up to 2019) advances in understanding the role of neutrophils in the pathophysiology of psoriasis, including the effects of respiratory burst, degranulation, and neutrophil extracellular trap formation on psoriatic immunity and the clinical relationships.

Keywords: neutrophils, psoriasis, immunity, respiratory burst, degranulation, neutrophil extracellular traps

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INTRODUCTION

Neutrophils are the most abundant cells in innate immunity. The main offensive functions of neutrophils include respiratory burst accompanied by reactive oxygen species (ROS) generation, degranulation (release of granules), and the formation of neutrophil extracellular traps (NETs) (**Figure 1**) (1, 2). Neutrophils shape adaptive immunity because they communicate and interact with the antigen-presenting cells and lymphocytes at the sites of inflammation (3, 4). Recently great attention was brought to the role of neutrophils in the development and progression of autoimmune diseases such as psoriasis.

Psoriatic neutrophils produce augmented respiratory burst with an overt accumulation of oxidative stress involving complicated inflammatory pathways. Proteases released in the degranulation step by neutrophils, such as myeloperoxidase (MPO), neutrophil elastase (NE), proteinase 3, and cathepsin G, participate in the generation of ROS, proteolytical activation of inflammatory mediators, and formation of autoantigens in psoriasis. The complexity and prevalence of psoriasis among the population since antiquity encouraged scientists to study the etiology of the disease and its relation to the immune system and inflammatory responses. Elegant reviews described the role of inflammation and immune system in the development of psoriasis (5, 6), the importance of biologic therapies targeting systemic inflammation (7), the significance of

utilizing specific antibodies (8), the contribution of platelets to regulation of NET formation (9), the antimicrobial and pathogenetic roles of neutrophils in autoimmune, autoinflammatory, metabolic (10) and cardiovascular diseases (11). Also, recent books summarized the causes, symptoms, and treatment options of psoriasis (12–15).

However, the role of neutrophils in psoriasis was not deeply analyzed and summarized based on recent literature. Thus, in this work, we focus on summarizing recent findings on the main offensive features of neutrophils including respiratory burst, degranulation and NETs and their direct connection with development and progression of psoriasis (**Figure 2**). We hope that our work would provide a foundation for further studies to attenuate overstimulation of neutrophils in psoriasis and aid patients with a debilitating disease.

PSORIASIS

Psoriasis affects ∼2–3% of the world population (>125 million people). Psoriasis is a common, chronic, immune-mediated disease that is manifested mainly as skin lesions and extracutaneous comorbidities (16, 17). It is associated with systemic inflammation, similar to that observed in obesity, malignancy, psoriatic arthritis, cardiovascular disorders, chronic obstructive pulmonary diseases, type 2 diabetes mellitus, liver and renal diseases, and inflammatory bowel diseases (18–20). Psoriasis affects men and women equally and usually starts to be manifested at the age of 20 to 30, but children and teenagers can be also affected (21). Clinical types of psoriasis include psoriasis vulgaris, guttate psoriasis, inverse psoriasis, pustular psoriasis, and erythrodermic psoriasis (22). Typical skin manifestations of psoriasis include erythematous, indurated, and scaling plaques that are painful, itchy, and have a burning sensation (16, 23). Psoriasis decreases patients' quality of life due to unpleasant symptoms and related public stigma (24, 25). The unpleasant skin appearance contributes to reduced employment levels and thus, affects the financial status of patients (26). Depression and suicidal tendencies are also increased in patients with psoriasis (27, 28). Therefore, psoriasis results in long-term physical, psychological, and economic burden at both the individual and societal levels.

NEUTROPHILS IN PSORIASIS

Psoriasis is an immunogenetic disease that is associated with the interactions between the innate and adaptive immune systems (29). The immunology disturbance in psoriasis is related to overstimulation of neutrophils, dendritic cells, T cells, keratinocytes, fibroblasts (6, 30), mast cells (31), and melanocytes (30, 32). Munro's microabscesses filled with neutrophils, which were first described in 1898, are considered as one of the major histopathological hallmarks of psoriasis (33). Neutrophils are now thought to be regulators between the innate and adaptive immune systems (34, 35).

There is no cure for psoriasis, however the symptoms can be reduced either by avoiding triggers or by medications. Currently, the available treatments such as phototherapy, topical therapy (corticosteroids, vitamin D analogs), systemic therapy (methotrexate, apremilast, cyclosporin), and biological treatments offer a relieve for patients with different severity of psoriasis. However, the risk-benefit ratio must be well-considered on individual basis, particularly, considering the chronic course of the disease and limitations of the long-term use of certain drugs (16). Although T cell immunology-related treatments have emerged as attractive options for psoriasis, according to a systematic review of adherence and satisfaction to current treatment covering studies conducted in 2002 (36) or between beginning of 2009 and end of 2014 in European Union (37), psoriatic patients expressed only moderate satisfaction with the available treatments as evidenced by the poor adherence rates, in particular to topical treatments (36, 37). That might be the biggest motivating factor for the use of alternative treatment methods such as traditional Chinese medicine and herbs (*Scutellaria baicalensis*, *Zingiber officinale*, *Indigo naturalis*, *Mahonia aquifolium, Aloe vera*) (38), dietary supplements (fish oil, vitamin D) or other (39). Furthermore, the combination of certain alternative medicines with conventional drug therapies has been shown to improve the treatment efficacy, which points to the importance of evaluation of safety of combined treatments, education of doctors but also improvements in patient-doctor interactions (40). Indeed, modern biological therapies demonstrate improved safety and efficacy, as well as better satisfaction in patients, but belong to an expensive class of drugs, with limited availability (41). Biological therapies of psoriasis include monoclonal antibodies or inhibitors targeting tumor necrosis factor (TNF)α (infliximab, adalimumab; etanercept) (42–44), interleukin (IL)-23/IL-12 (ustekinumab) (42), IL-23 (guselkumab, tildrakizumab, risankizumab) (45, 46), or IL-17 (secukinumab, ixekizumab, brodalumab) (11, 47, 48). The available therapies indirectly affect the function and numbers of neutrophils. According to previous studies, the neutrophil-to-lymphocyte ratio (NLR) and platelet-to-lymphocyte ratio (PLR) are significantly increased in patients with psoriasis (49). While the elevated NLR and PLR values are particularly associated with psoriasis, they do not indicate the severity of the condition (50). The importance of NLR level in the progression of the disease was demonstrated by the reduction in NLR level following psoriasis treatment (51). That brings in question the effectiveness of certain treatments such as narrow-band ultraviolet B phototherapy which does not affect the increased level of NLR (52). Increased NLR and PLR levels emerged as unrecognized predictors of subclinical atherosclerosis in patients with psoriasis (53). The presence of excessive amount of neutrophils is characteristic for the generalized pustular psoriasis. Recent reports indicated that the depletion of neutrophils significantly relieves the symptoms of pustular psoriasis in patients that did not respond well to conventional treatments (54). Understanding the role of

Abbreviations: CCL, CC-chemokine ligand; CXCL, chemokine (C-X-C motif) ligand; IL, interleukin; IFN, interfenon; LPS, lipopolysaccharide; mDCs, myeloid dendritic cells; MPO, myeloperoxidase; NET, neutrophil extracellular trap; NOX, NADPH oxidase; NE, neutrophil elastase; NLR, neutrophil-to-lymphocyte ratio, pDCs, plasmacytoid dendritic cells; PLR, platelet-to-lymphocyte ratio; Th, T helper cell; TLR, Toll-like receptor; TNF, tumor necrosis factor.

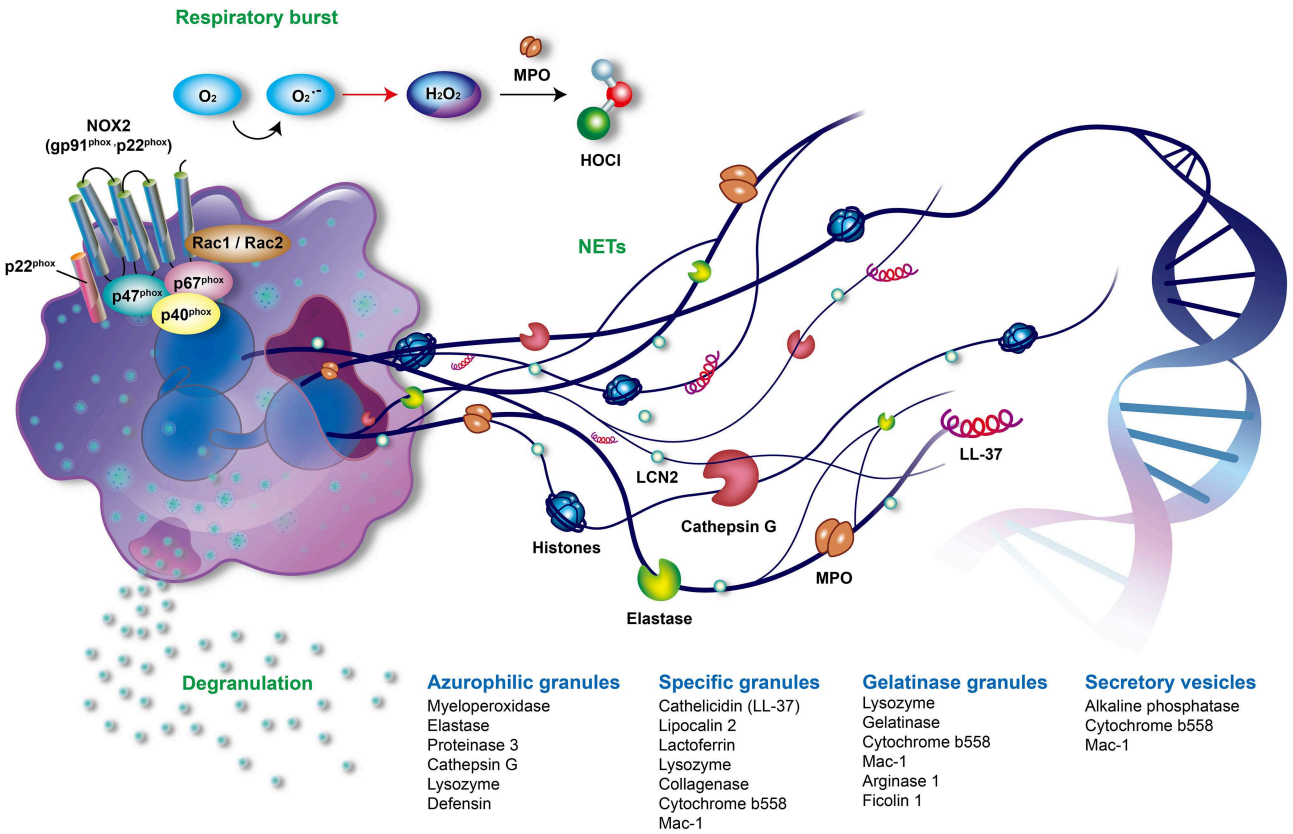

FIGURE 1 | The offensive mechanisms of neutrophils in inflammation. In the activated neutrophils, respiratory burst, degranulation, and the formation of neutrophil extracellular traps (NETs) are the main mechanisms responsible for inflammation. During the respiratory burst, neutrophils utilize oxygen to produce reactive oxygen species (ROS) such as superoxide (O_2^-), peroxide H_2O_2 or hypochlorous acid (HOCl) by activating NADPH oxidase (NOX2) and myeloperoxidase (MPO). As activation processes are triggered by diverse stimuli, neutrophils mobilize different granules including azurophilic granules, specific granules, gelatinase granules, and secretory vesicles. Subsequently, neutrophils degranulate and release various granular compounds of which neutrophil elastase (NE), MPO, proteinase 3, LL37, and lipocalin 2 play an important role in the pathogenesis of psoriasis. NETs are novel and pivotal components of neutrophils composed of extruded sticky and decondensed chromatin decorated with many antimicrobial compounds, such as histones, MPO, NE, and cathepsin G. The formation of NETs is involved in complicated inflammatory reactions and contributes to the pathogenesis of psoriasis. MPO, myeloperoxidase; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NE, neutrophil elastase; NET, neutrophil extracellular trap; NOX2, NADPH oxidase.

neutrophils in psoriasis attracted the attention of scientific community aiming to develop treatment protocols focusing on attenuating neutrophil overstimulation in this disease (55).

RESPIRATORY BURST AND PSORIASIS

Circulating neutrophils are recruited to inflammatory sites following inflammatory signals. They are then activated to generate and release large amounts of ROS in a phenomenon known as respiratory burst. NADPH oxidase (NOX2) and MPO are two key enzymes that contribute to the respiratory burst (56, 57). NOX2 is composed of transmembrane cytochrome b558 ($p22^{phox}$ and $gp91^{phox}$) and cytosolic subunits ($p40^{phox}$, $p47^{phox}$, $p67^{phox}$, and Rac1/2). The assembled NOX2 complex, at the phagosomal and plasma membranes, is fully activated to generate superoxide anion ($O_2^{\bullet-}$), which is the origin of various ROS produced by neutrophils. Superoxide ($O_2^{\bullet-}$) is rapidly converted to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD). MPO, a heme peroxidase enzyme which is released in a process known as degranulation, utilizes H_2O_2 to produce many secondary reactive products. These products include hypochlorous acid (HOCl), chloramines (R-NHCl), and hypothiocyanite (OSCN–), as well as organic radicals such as products of lipid peroxidation (**Figure 1**) (58, 59).

ROS production is an integral part of the antimicrobial activity of neutrophils. However, the overproduction or inadequate clearance of ROS can cause various oxidative stress-related dysfunctions. These include cell and tissue damage; peroxidation and modification of DNA, lipids, and proteins; autoimmune NET formation; and autoantibody generation (58, 60). Neutrophils obtained from patients with psoriasis were shown to possess increased MPO and NOX2 activities, and release more ROS compared with neutrophils from healthy individuals (61, 62). Keratinocytes and T cells in psoriatic lesions produce priming agents of neutrophils, which results in an augmented respiratory burst by neutrophils with the overproduction of ROS (63–66). Accumulation of oxidative radicals also contributes to the pathogenesis of psoriasis. In response to the overproduction of

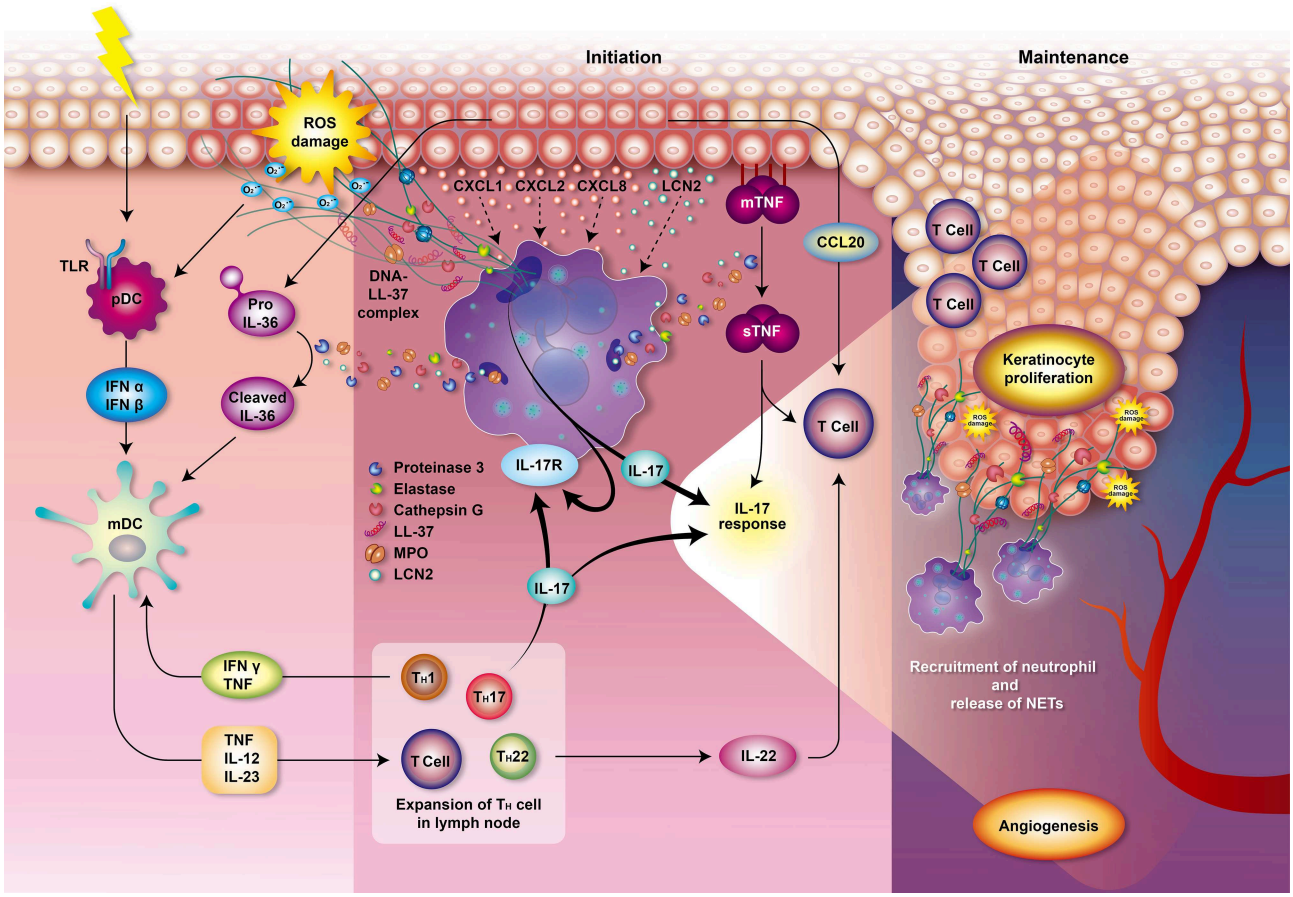

FIGURE 2 | Role of neutrophils in psoriasis. Various and diverse endogenous and exogenous impulses such as antigens, trauma, infection, or emotional stress can trigger the complex immune reactions leading to psoriasis. The interplay of neutrophils, dendritic cells, and T cells bridges the innate immune and adaptive immune systems. T cells and keratinocytes release chemokines, such as IL-17, CXCL1, CXCL2, and CXCL8, that mediate the recruitment of neutrophils. Circulating neutrophils migrate to the psoriatic lesions and induce respiratory burst, degranulation, and formation of NETs, thereby contributing to the immunopathogenesis of psoriasis which involves T cell imbalance, keratinocyte proliferation, angiogenesis, and auto-antigen formation. Neutrophils from patients with psoriasis have enhanced NOX2 and MPO activity and augmented respiratory burst. MPO also participates in generating oxidative stress and upregulating degranulation. The accumulated oxidative stress produced by neutrophils could stimulate the antigen-presenting cells pDCs via TLR receptors 7 and 9 which stimulate antigen-specific CD8+ T cells (memory T cells in dermis and naive T cells in lymph node) to release cytokines, chemokines, and other innate immune mediators. These T cells may also migrate to epidermis and trigger local inflammation and keratinocyte proliferation via MHC I receptor of keratinocytes. Production of IFN-α and IFN-β by pDCs then stimulates mDCs to secrete pro-inflammatory mediators such as TNF, IL-12 and IL-23. Proteinase 3 released from neutrophils cleaves pro-IL-36 to activated IL-36 cytokine amplifying the response of mDCs. TNF, IL-12, and IL-23 play an important role in the initiation of the Th1, Th17, and Th22 cells immune response in lymph node, leading to a secretion of various cytokines and chemokines. IL-1 is further amplifying Th17 response while TNF and IFN-γ is creating a back loop to mDCs activation. Th17 activation then leads to the production of IL-17 activating neutrophils and keratinocytes via IL-17 receptors which generates profound IL-17 response. Keratinocytes produce TNF and CCL20, a chemotactic for T cell and DCs. Neutrophils degranulate and release MPO, NE, proteinase 3, cathepsin G and lipocalin. Proteinase 3 cleaves and converts the resting TNFα located in membrane of epithelial cells (mTNFα) to an activated state called soluble TNFα (sTNFα). Proteinase 3 also contributes to the formation of the LL-37 which serves as autoantigen. The chromatin of NETs (DNA) combined with LL-37 have a profound role in the initiation and maintenance of immune response in psoriasis. NETs further supply IL-17 and induce Th17 cells to release more IL-17, which plays a decisive role in the maintenance of psoriasis. These processes participate in the psoriasis complex inflammatory reactions and lead to the escalation of local psoriatic tissue inflammation. IL-22 contributes to the formation of characteristic psoriatic skin lesions including epidermal hyperplasia and acanthosis (thickening of skin). The activation of the following transcription factors promote TNF and IL-17 production and formation of downstream amplification loops in psoriasis: the Janus kinase (JAK)–signal transducer and activator of transcription (STAT) family, nuclear factor-κB (NF-κB) and cyclic AMP. Furthermore, the activation of endothelial cells induces vascular proliferation, angiogenesis and the expression of adhesion molecules in the endothelium to recruit additional inflammatory cells into the skin such as mast cells and macrophages contributing to the pathogenesis of psoriasis. CCL, CC-chemokine ligand; CXCL, chemokine (C-X-C motif) ligand; IL, interleukin; IFN, interfenon; MHC I, major histocompatibility complex class I, MPO, myeloperoxidase; NET, neutrophil extracellular trap; NOX, NADPH oxidase; NE, neutrophil elastase; pDCs, plasmacytoid dendritic cells; mDCs, myeloid dendritic cells; TLR, Toll-like receptor; TNF, tumor necrosis factor.

ROS, dendritic cells are stimulated to present antigens to the T cells which results in an imbalance of T helper cell (Th)1 and Th2 cells, stimulation of keratinocytes proliferation, and promotion of angiogenesis (**Figure 2**). ROS then serve as the second messenger to activate mitogen-activated protein kinase (MAPK), nuclear factor-kappa B (NF-κB), or the Janus kinase-signal transducer

and activator of transcription proteins (JAK-STAT)-related inflammatory pathways (67, 68). Suppression of respiratory burst of neutrophils emerged as a plausible pathway of attenuating overly immune response associated with psoriatic symptoms.

DEGRANULATION AND PSORIASIS

Neutrophils possess a multi-lobed nucleus, few mitochondria, and many specific storage organelles called granules. Granules are classified into azurophilic granules, specific granules, gelatinase granules, and secretory vesicles depending on their size, reaction with peroxidase-reactive dye, staining with 3,3'-diaminobenzidine, protein content, and tendency to mobilize (**Figure 1**) (69, 70). Azurophilic (peroxidase-positive or primary) granules are packed with MPO, bactericidal/permeability-increasing protein, defensins, lysozyme, and serine proteases, such as neutrophil elastase (NE), proteinase 3, and cathepsin G. Lysozyme is also found in specific and gelatinase granules (55). Specific (secondary) granules contain distinctive iron-binding glycoprotein lactoferrin, neutrophil gelatinase-associated lipocalin (NGAL, also called serum lipocalin-2, LCN2), collagenase, cytochrome b558, MAC-1 (CD11/CD18), and importantly, cathelicidins such as LL-37 (71). Cytochrome b558 and MAC-1 are also present in gelatinase granules and secretory vesicles (55). Gelatinase (tertiary) granules store gelatinase, lysozyme, arginase 1, and ficolin 1 (72). The secretory vesicles contain a characteristic alkaline phosphatase (57).

As activation processes of neutrophils are triggered by diverse stimuli such as bacterial or proinflammatory lipid mediators, neutrophils mobilize different granules and release the aforementioned granular components in a process known as degranulation or exocytosis (57). Degranulation is regulated by complicated control mechanisms, such as calcium signaling and actin remodeling (72–74). Azurophilic granules discharge toxic components into phagosomes and at inflammation sites. The secretion of specific and gelatinase granules promotes migration of neutrophils and the antimicrobial activity. The main purpose of releasing secretory vesicles is to facilitate neutrophils recruitment. Therefore, the degranulation process promotes firm adhesion, migration, respiratory burst, and successive NET formation of activated neutrophils (75). However, the dysregulation of neutrophil degranulation could damage tissues as observed in various diseases, such as hypoxia-related airway injury (76), severe pneumonia and chronic obstructive pulmonary diseases (77), atherosclerosis (78, 79), acute inflammatory liver injury (80), and rheumatoid arthritis (81, 82).

In psoriasis, MPO is significantly increased in skin plaques and is positively correlated with the severity of psoriasis (83). Serum MPO is also increased in patients with psoriasis, which may be related to recruited leukocytes in psoriatic skin lesions (84). MPO, the major enzymatic content of neutrophil granules, accounts for ∼5% of the dry weight of the cell and represents the most toxic enzyme expressed by neutrophils (85). MPO is involved in the respiratory burst and can bind to CD11b/CD18 integrins, thereby contributing to the upregulation and augmentation of neutrophil degranulation in psoriasis (86). Furthermore, the neutrophil granule-derived serine proteinases, such as NE, proteinase 3, and cathepsin G, can activate interleukin (IL)-36 cytokine and lead to the escalation of local psoriatic tissue inflammation (87, 88). Proteinase 3 cleaves and converts the resting TNFα located in membrane of epithelial cells (mTNFα) to an activated state called soluble TNFα (sTNFα), which participates in the psoriasis complex inflammatory reactions (**Figure 2**). Proteinase 3 also contributes to the formation of the LL-37, an antimicrobial peptide belonging to cathelicidin family of polypeptides (89, 90), which serves as autoantigen mediating immune response in psoriasis (33). Antimicrobial peptides, synthesized by various leukocytes and epithelial cells, act via DNA/RNA complexes binding Toll-like receptors (TLR) 7, 8, and 9 to facilitate skin inflammation (91, 92). In addition, NE proteolytically activates the epidermal growth factor receptor (EGFR) signaling pathway resulting in excessive keratinocyte proliferation in psoriasis (93). Thus, the inhibition of neutrophils degranulation process or some of the enzymes contributing to psoriasis (NE, MPO, proteinase 3) are feasible targets for alleviating psoriatic symptoms.

NETS AND PSORIASIS

The process of forming neutrophil extracellular traps (NETs) was first reported in 2004 (94). NETs are composed of extruded sticky chromatin ornamented with many antimicrobial components including histones, MPO, NE, cathepsin G, high mobility group protein B1 (HMGB1) and antimicrobial peptides, such as LL-37 (**Figure 1**) (95). NETs can catch and destroy pathogens in order to prevent microbes from spreading (96). However, the dysregulated formation and clearance of NETs can result in many diseases. These include autoimmune diseases, such as systemic lupus erythematosus, anti-neutrophil cytoplasmic autoantibody (ANCA)-associated vasculitis, rheumatoid arthritis, gout (97), and autoimmune hepatitis (98); cardiovascular diseases, such as atherosclerosis, thrombosis, and abdominal aortic aneurysm (99–101); respiratory disorders inclusive of asthma, chronic obstructive pulmonary disease, cystic fibrosis, tuberculosis, bacterial and viral pneumonia, and transfusion-related acute lung injury (102, 103); digestive diseases, such as inflammatory bowel diseases, primary sclerosing cholangitis, primary biliary cholangitis (98); and cancer-related organ damage, metastasis, and thrombosis (9). Recently, a role of NETs in awakening of dormant cancer cells was discovered (104).

The process of NET formation is termed NETosis, which is subdivided into lytic NETosis and non-lytic NETosis. In lytic NETosis (also called suicidal NETosis), activated neutrophils generate NETs (it takes 2–4 h) and undergo a programmed cell death, which differs from necrosis, necroptosis, and apoptosis (105). Lytic NET formation is triggered by various stimuli undergoing different pathways. For instance, phorbol myristate acetate (PMA) increases cytosol calcium, activates protein kinase C (PKC)/Raf/MEK/ERK pathway, and induces NOX2 to generate ROS. ROS then acts as the second messenger to disintegrate the nuclear membrane and stimulate MPO to translocate NE to the nucleus where it causes proteolysis of histones and decondensation of chromatin. Afterward, peptidyl

arginine deiminase 4 (PAD4)-mediated hypercitrullination of histones allows decondensed chromatin, the main component of NETs, to be readily expelled from the cell nucleus. Finally, as the plasma membrane dissolves, the chromatin decorated with granular components is released as extracellular traps (106). Other pathways of lytic NETosis include stimulation by fungi (such as *Aspergillus* spp.) through Dectin 2 and complement receptor 3 (CR3) (107), LPS (lipopolysaccharide) under special conditions described later in detail (108) or by monosodium urate crystals via receptor-interacting serine/threonine-protein kinase 1 (RIPK1) and RIPK3 pathway (109). All of these pathways involve NOX2, MPO, and NE activation (105). Nevertheless, there are other stimulators of NETosis acting independently of NOX2 such as ionomycin, or immune complexes (110). Ionomycin induces NETs via small conductance calcium-activated potassium channel protein 3 (SK3) and protein kinase C ζ (PKCζ), mitochondrial ROS (mitoROS), NE, and protein-arginine deiminase type 4 (PAD4) (111). Immune complexes related NETosis through FcγRIIIb are highly dependent on mitoROS (110, 112). The lytic-NETosis inducers such as PMA, ionomycin, or living bacteria were confirmed using a live imaging confocal microscopy, however, dead bacteria, LPS, glucose, or activated platelets alone failed to induce NETosis in the *in vitro* experiment (113). Such discrepancy might be due to variations in the experimental design of various studies.

On the other hand, non-lytic NETosis (also called vital NETosis) does not require neutrophils lysis or even the breach of the plasma membrane. Following the release of NETs, neutrophils are alive and keep their functions, such as chemotactic movement, phagocytotic ability, and respiratory burst power (98). This form of NETosis usually occurs early in infection by Gram-positive bacteria in human and mice. The process is very rapid (5–60 min to form NETs), requires both TLR 2 and complement-mediated opsonization, and is independent of NOX2 (114). Non-lytic NETosis can be induced by *Staphylococcus aureus* via a unique mechanism where the inner and outer nuclear membranes are separated, and the vesicles filled with nuclear DNA are extruded intact into the extracellular space where they rupture and release chromatins. Despite that this type of NETs keep a limited amount of proteolytic activity it is still able to kill *S. aureus* (115). Non-lytic NETosis can also be stimulated by *Candida albicans* via interaction with CR3 and fibronectin (116). Moreover, a special type of non-lytic NETosis, which releases mitochondrial DNA and is dependent on ROS, is stimulated by the granulocyte-macrophage colony-stimulating factor (GM-CSF) and LPS (10). Interestingly, Leishmania parasites induce both lytic and non-lytic NETosis (117). In that case, the chromatin decondensed by PAD4 is mixed with granular proteins and subsequently excreted with a minor nuclear envelope disruption and without cell membrane disorganization (10, 98). Delgado-Rizo et al. previously summarized the microbial inducers of NETs (10) but we would like to clarify the effect of LPS. Lipopolysaccharide (LPS) is an important component of the outer membrane of gram-negative bacteria known to trigger immune response (118). For a long time, it was unclear whether the direct interaction between LPS and neutrophils causes NETs release, because several reports showed LPS-induced lytic NET formation (94, 119) while other not (113, 120). Recently, it has been shown that only species- and serotype-specific LPS is able to induce NETs by direct interaction with neutrophils. It was demonstrated that LPS has to be derived from specific bacterial strain of *Escherichia coli* (O128:B12) and *Pseudomonas aeruginosa* (serotype 10) and must be present at sufficient concentration (8 pg per neutrophil). The neutrophils then undergo a lytic-NETosis independent of TLR4. However, non-lytic NETosis is triggered when sufficient amount of LPS regardless of bacterial origin interacts with TLR4 of platelets (108). The process is followed by binding of platelets to the P-selectin glycoprotein ligand-1 (PSGL-1) of neutrophils, and the release of HMGB1 by platelets (9, 95). Moreover, there is a growing evidence of crucial role of the other endogenous and immune factors in the process of NET formation, such as presence of platelets (95, 120), glucose (10), or other effectors (121). To orchestrate inflammatory response, NETs in combination with LPS were shown to induce the production of IL-1β by J774 macrophages via the caspase-1 and caspase-8 pathways (122).

In patients with psoriasis, neutrophils are pre-activated and form NETs in psoriatic skin lesions (55, 123). NETs are increased in blood samples and correlate with the severity of psoriasis (124, 125). They create an extremely immunogenic environment and participate in the initial and maintenance phases of psoriasis (126, 127). NETs stimulate epidermis to release inflammatory cytokines via TLR4 and IL-36 receptor crosstalk (123). Various exogenous and endogenous stimuli and ROS generated by neutrophils initiate immune reaction leading to psoriasis which involves T cell imbalance, keratinocyte proliferation, angiogenesis, and auto-antigen formation (**Figure 2**). The chromatin of NETs in psoriasis plaques is accompanied with antimicrobial peptide LL-37 released by keratinocytes to stimulate the synthesis of inflammatory mediators including IFN-α and IFN-β in plasmacytoid dendritic cells (pDCs) (16). Myeloid DCs (mDCs) are then activated to release many pro-inflammatory mediators, such as IL-6, IL-12, IL-23, and TNFα (91, 92), which play an important role in the initiation of the Th1, Th17, and Th22 cells immune response (16). Proteinase 3 released from neutrophils cleaves pro-IL-36 to activated IL-36 cytokine which is together with TNF and IFN-γ amplifying the response of mDCs. Th17 activation then leads to the production of IL-17 activating neutrophils and keratinocytes via IL-17 receptors which generates profound IL-17 response (16). Secretory leukocyte protease inhibitor (SLPI), a component of NETs with an inhibitory function on NET formation, may bind to DNA and NE in psoriatic skin lesions and activate the pDCs to produce type 1 interferons (IFN-α, IFN-β, etc.) which regulates autoimmunity in psoriasis (128–130). In addition, NETs allow the mDCs to readily sense the neutrophilic antigens and allow the T cells to be primed directly (45, 131, 132). Thus, NETs play an important role in the pathophysiology of psoriasis due to their link between the innate and adaptive immune systems. Psoriasis is accompanied with increased serum levels of TNF-α, interferon (IFN)-γ, IL-1, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-17, IL-18 (133), IL-22 (134), chemerin, resistin (135), lipocalin-2 (LCN2) (123), soluble E-selectin (sE-selectin) (136), complement 3 (137), and decreased levels of transforming growth factor-beta (TGF-β) and adiponectin (133). These cytokines may therefore serve as

potential biomarkers for psoriasis and treatment response in patients. According to a cross-sectional study, psoriasis patients had increased proinflammatory macrophage type 1 (IL-1, IL-6, TNF-α), Th1 (IL-2, IL-12, IFN-γ), Th17 (IL-6, IL-17) but also anti-inflammatory Th2/T regulatory (Treg) (IL-4, IL-10) profiles which may be correlated to the severity of psoriasis (133). Among the important mediators in psoriasis, LCN2 acts as an antimicrobial protein as well as adipokine associated with obesity, insulin resistance, and atherosclerotic disease, and is also responsible for the activation of the immune system in response to inflammatory and toxic stimuli. Importantly, serum LCN2 levels are elevated in psoriatic patients (138) and correlate with the severity of itching and thus might be used as a clinical marker for itching in psoriasis (139). Not only granulocytes but also keratinocytes of epidermis secrete LCN2, which drives the chemotaxis of neutrophils and sustains NET formation, and thereby in turn maintains the psoriatic inflammation (123). The increased LCN2 blood levels were observed in patients with palmoplantar pustular psoriasis (140) as well as other chronic inflammatory skin diseases such as acne inversa (141) or atopic dermatitis (142). Wolk et al. reported a positive correlation between the LCN2 production and IL-1β levels in the epidermis, which was further enhanced by IL-17 and TNF-α, but not by IL-22. The contribution of LCN2 on skin neutrophil infiltration is apparent (141). In the clinical setting, tissue LCN2 was found to be also significantly higher in psoriasis, regardless of dyslipidemia, or metabolic disturbance in patients. But the LCN2 levels together with psoriasis area and severity index (PASI) score significantly dropped after NBUVB treatment (143). The *in vivo* effects of LCN2 on topical imiquimod (IMQ)-induced psoriasis-like skin in BALB/c mice were evaluated by Hau group (138). In addition to markedly exacerbated erythema and scaling in IMQ-treated murine skin, LCN2 increased the mRNA expression of interleukin (IL)-17A, IL-17F, IL-22, IL-23, CC-chemokine ligand (CCL)20, TNF-α, chemokine (C-X-C motif) ligand (CXCL)1, CXCL2, LCN2, and S100A7 while it did not affect the mRNA levels of IFN-γ, or CXCL10 in the skin. Similar effects were observed *in vitro* on human keratinocytes (138). The data suggest a link between NETs-related cytokines and Th-17 activation in psoriasis.

NET-TH17 AXIS AND PSORIASIS

Psoriasis has been considered as a T-helper (Th)1/Th17-mediated, chronic inflammatory dermatosis with relation to metabolic syndromes (144). Apart from keratinocytes and T lymphocytes (145), neutrophils are one of the major cellular sources of IL-17 via NET formation in psoriasis (146), and also mast cells were reported to generate extracellular traps (147). NETs activation has been linked with Th17 responses in psoriasis and has drawn particular attention recently (148). In an experimental model, NETs induced the generation of $CD3^+CD4^+IL\text{-}17^+$ (Th17) cells from peripheral blood mononuclear cells, which requires monocyte and cell-to-cell contact. Th17 induction was enhanced by a psoriasis risk-associated variant in the TRAF3IP2 gene encoding the D10N variant of Act1 which serves as a key mediator of IL-17 signal transduction. That provides an evidence of genetic basis for the enhanced IL-17 expression in psoriasis. IL-17-expressing T cells and neutrophils were suggested to have a cross-talk because IL-17-expressing T cells produce cytokines which promote the development, recruitment, and lifespan of neutrophils (149).

Although many immune diseases including psoriasis or atopic dermatitis have been traditionally classified as Th1/Th2 biphasic disorders, there is a growing evidence supporting a rather systemic activation of other multiple Th-cell subsets, such as Th17 cells producing IL-17 and IL-22 (**Figure 2**). Interestingly, in comparison with psoriasis, atopic dermatitis showed reduced genomic expression of IL-23, IL-17, IFN-γ, and other innate defense genes (hBD2, elafin, LL-37) (150). Elevated IL-17 is detected in psoriatic skin lesions and in the blood (151). IL-17 mainly stimulates keratinocytes to produce neutrophil-tropic chemokines CXCL-1, CXCL-2, CXCL-8 (IL-8), and antimicrobial peptides, such as LL-37. IL-17 serves as an autocrine-amplifying mediator that is simultaneously involved in the recruitment, activation, and survival of neutrophils (6). There are several subtypes of IL-17 family cytokines binding to IL-17 receptors, namely IL-17A, IL-17B, IL-17C, IL-17D, IL-17E (also known as IL-25) and IL-17F (145). IL-17A from neutrophils stimulates keratinocytes to express CCL20, attracting Th17 cells with CCR6 expression to release IL-17A, and finally resulting in positive feedback and the development of the psoriatic lesions (8). IL-17C is a unique cytokine that is produced by keratinocytes and that is involved in such synergistic loops that may be responsible for amplifying the inflammation in both psoriasis and atopic dermatitis. This may ultimately lead to induction of S100As and other molecules that accompany epidermal hyperplasia. Thus, antagonism of IL-17C may be beneficial for psoriasis and atopic dermatitis treatment (152). IL-17E then recruits neutrophils by activating macrophages and contributes to the infiltration of psoriatic neutrophils (153). Besides other innate immune cells, neutrophils significantly contribute to IL-17-related immune regulations in psoriasis by employing several mechanisms including the formation of NETs (45). Moreover, IL-17 released by NETs leads to endothelial dysfunction in atherosclerosis and keratinocyte proliferation in psoriasis, which may explain why patients with psoriasis also suffer from increased risk of atherosclerosis (11). Anti-IL-17 drugs, such as secukinumab, clear the neutrophils in the epidermis and can be used to effectively treat psoriasis (48). The targeted biologic therapies are of great importance with regards to an increasing number of comorbidities associated with psoriasis together with its systemic inflammation nature indicating that these diseases are sharing some common pathological mechanisms (7). In summary, NETs were demonstrated as potential upstream drug targets for the treatment of psoriasis.

CONCLUSION

Neutrophils in psoriasis are of interest, particularly, because of their crucial roles in the innate and adaptive immune system. The respiratory burst with ROS generation, degranulation, and formation of NETs are the main offensive functions of neutrophils and contribute to the immunopathogenesis of psoriasis. Recently, great attention was brought to the role of NETs in psoriasis because activated neutrophils producing

NETs are abundant in psoriatic skin plaques and pustules, as well as in the serum of patients with psoriasis. Overexpression of NETs leads to the activation of other cells releasing IL-17, which stimulates the synthesis of inflammatory mediators and in turn leads to auto-amplification of neutrophils. Therefore, further development of inhibitors and biologic drugs targeting overexpressed offensive features of neutrophils, i.e., respiratory burst, degranulation, and NET formation, is of great importance. We believe that the consideration of the role of neutrophil defense mechanisms in the pathogenesis of psoriasis offered in this review highlights the need to further investigate neutrophils for possible improvements of available treatments in the future.

AUTHOR CONTRIBUTIONS

C-CC and W-JC wrote and revised the manuscript. MK consulted and revised the manuscript. C-YL drew the figures. T-LH initiated the concept and supervised the writing. All authors read and approved the final manuscript.

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REFERENCES

1. Tecchio C, Cassatella MA. Neutrophil-derived chemokines on the road to immunity. *Semin Immunol*. (2016) 28:119–28. doi: 10.1016/j.smim.2016.04.003
2. Nauseef WM, Borregaard N. Neutrophils at work. *Nat Immunol*. (2014) 15:602–11. doi: 10.1038/ni.2921
3. Leliefeld PH, Koenderman L, Pillay J. How Neutrophils Shape Adaptive Immune Responses. *Front Immunol*. (2015) 6:471. doi: 10.3389/fimmu.2015.00471
4. Rosales C, Lowell CA, Schnoor M, Uribe-Querol E. Neutrophils: Their Role in Innate and Adaptive Immunity 2017. *J Immunol Res*. (2017) 2017:9748345. doi: 10.1155/2017/9748345
5. Georgescu SR, Tampa M, Caruntu C, Sarbu MI, Mitran CI, Mitran MI, et al. Advances in understanding the immunological pathways in psoriasis. *Int J Mol Sci*. (2019) 20:739. doi: 10.3390/ijms20030739
6. Chiricozzi A, Romanelli P, Volpe E, Borsellino G, Romanelli M. Scanning the Immunopathogenesis of Psoriasis. *Int J Mol Sci*. (2018) 19:E179. doi: 10.3390/ijms19010179
7. Korman NJ. Management of psoriasis as a systemic disease: What is the evidence? *Br J Dermatol*. (2019). doi: 10.1111/bjd.18245. [Epub ahead of print].
8. Katayama H. Development of psoriasis by continuous neutrophil infiltration into the epidermis. *Exp Dermatol*. (2018) 27:1084–91. doi: 10.1111/exd.13746
9. Cedervall J, Hamidi A, Olsson AK. Platelets, NETs and cancer. *Thromb Res*. (2018) 164(Suppl 1):S148–52. doi: 10.1016/j.thromres.2018.01.049
10. Delgado-Rizo V, Martínez-Guzmán MA, Iñiguez-Gutierrez L, García-Orozco A, Alvarado-Navarro A, Fafutis-Morris M. Neutrophil extracellular traps and its implications in inflammation: an overview. *Front Immunol*. (2017) 8:81. doi: 10.3389/fimmu.2017.00081
11. Sanda GE, Belur AD, Teague HL, Mehta NN. Emerging Associations Between Neutrophils, Atherosclerosis, and Psoriasis. *Curr Atheroscler Rep*. (2017) 19:53. doi: 10.1007/s11883-017-0692-8
12. Bagchi D, Kundu-Raychaudhuri SK, Raychaudhuri SP. *Psoriasis and Psoriatic Arthritis: Pathophysiology, Therapeutic Intervention, and Complementary Medicine*. Portland, OR: CRC Press/Taylor & Francis Group (2018). p. 413.
13. Menter A. RC. *Psoriasis*. 2nd ed. Portland, OR: CRC Press/Taylor & Francis Group (2017). p. 268
14. Warren R. MA. *Handbook of Psoriasis and Psoriatic Arthritis*. Springer International Publishing (2016). p. 116.
15. Adebajo A, Gladman DD, Mease PJ. *Psoriatic Arthritis and Psoriasis: Pathology and Clinical Aspects*. Springer International Publishing (2016). p. 316.
16. Greb JE, Goldminz AM, Elder JT, Lebwohl MG, Gladman DD, Wu JJ, et al. Psoriasis. *Nat Rev Dis Primers*. (2016) 2:16082. doi: 10.1038/nrdp.2016.83
17. Sticherling M. Psoriasis and autoimmunity. *Autoimmun Rev*. (2016) 15:1167–70. doi: 10.1016/j.autrev.2016.09.004
18. Kaushik SB, Lebwohl MG. CME Part I Psoriasis: which therapy for which patient psoriasis comorbidities and preferred systemic agents. *J Am Acad Dermatol*. (2019) 80:27–40. doi: 10.1016/j.jaad.2018.06.057
19. Geller S, Xu H, Lebwohl M, Nardone B, Lacouture ME, Kheterpal M. Malignancy risk and recurrence with psoriasis and its treatments: a concise update. *Am J Clin Dermatol*. (2018) 19:363–75. doi: 10.1007/s40257-017-0337-2
20. Gelfand JM. Psoriasis, type 2 diabetes mellitus, and obesity: weighing the evidence. *JAMA Dermatol*. (2016) 152:753–4. doi: 10.1001/jamadermatol.2016.0670
21. Armstrong AW. Psoriasis. *JAMA Dermatol*. (2017) 153:956. doi: 10.1001/jamadermatol.2017.2103
22. Raychaudhuri SK, Maverakis E, Raychaudhuri SP. Diagnosis and classification of psoriasis. *Autoimmun Rev*. (2014) 13:490–5. doi: 10.1016/j.autrev.2014.01.008
23. Nestle FO, Kaplan DH, Barker J. Psoriasis. *N Engl J Med*. (2009) 361:496–509. doi: 10.1056/NEJMra0804595
24. Vanderpuye-Orgle J, Zhao Y, Lu J, Shrestha A, Sexton A, Seabury S, et al. Evaluating the economic burden of psoriasis in the United States. *J Am Acad Dermatol*. (2015) 72:961–7.e5. doi: 10.1016/j.jaad.2015.02.1099
25. Brezinski EA, Dhillon JS, Armstrong AW. Economic burden of psoriasis in the United States: a systematic review. *JAMA Dermatol*. (2015) 151:651–8. doi: 10.1001/jamadermatol.2014.3593
26. Hawro T, Zalewska A, Hawro M, Kaszuba A, Krolikowska M, Maurer M. Impact of psoriasis severity on family income and quality of life. *J Eur Acad Dermatol Venereol*. (2015) 29:438–43. doi: 10.1111/jdv.12572
27. Singh S, Taylor C, Kornmehl H, Armstrong AW. Psoriasis and suicidality: a systematic review and meta-analysis. *J Am Acad Dermatol*. (2017) 77:425–40.e2. doi: 10.1016/j.jaad.2017.05.019
28. Dowlatshahi EA, Wakkee M, Arends LR, Nijsten T. The prevalence and odds of depressive symptoms and clinical depression in psoriasis patients: a systematic review and meta-analysis. *J Invest Dermatol*. (2014) 134:1542–51. doi: 10.1038/jid.2013.508

29. Prinz JC. Autoimmune aspects of psoriasis: heritability and autoantigens. *Autoimmun Rev*. (2017) 16:970–9. doi: 10.1016/j.autrev.2017.07.011
30. Hwang ST, Nijsten T, Elder JT. Recent highlights in psoriasis research. *J Invest Dermatol*. (2017) 137:550–6. doi: 10.1016/j.jid.2016.11.007
31. Harvima IT, Nilsson G, Suttle MM, Naukkarinen A. Is there a role for mast cells in psoriasis? *Arch Dermatol Res*. (2008) 300:461–78. doi: 10.1007/s00403-008-0874-x
32. Prinz JC. Melanocytes: target cells of an HLA-C*06:02-restricted autoimmune response in psoriasis. *J Invest Dermatol*. (2017) 137:2053–8. doi: 10.1016/j.jid.2017.05.023
33. Mrowietz U. Neutrophils' sexiness is independent of trendy fashion. *Exp Dermatol*. (2017) 26:312–3. doi: 10.1111/exd.13102
34. Mantovani A, Cassatella MA, Costantini C, Jaillon S. Neutrophils in the activation and regulation of innate and adaptive immunity. *Nat Rev Immunol*. (2011) 11:519–31. doi: 10.1038/nri3024
35. Jaillon S, Galdiero MR, Del Prete D, Cassatella MA, Garlanda C, Mantovani A. Neutrophils in innate and adaptive immunity. *Semin Immunopathol*. (2013) 35:377–94. doi: 10.1007/s00281-013-0374-8
36. Dubertret L, Mrowietz U, Ranki A, van de Kerkhof PC, Chimenti S, Lotti T, et al. European patient perspectives on the impact of psoriasis: the EUROPSO patient membership survey. *Br J Dermatol*. (2006) 155:729–36. doi: 10.1111/j.1365-2133.2006.07405.x
37. Belinchón I, Rivera R, Blanch C, Comellas M, Lizán L. Adherence, satisfaction and preferences for treatment in patients with psoriasis in the European Union: a systematic review of the literature. *Patient Prefer Adherence*. (2016) 10:2357–67. doi: 10.2147/PPA.S117006
38. Meng S, Lin Z, Wang Y, Wang Z, Li P, Zheng Y. Psoriasis therapy by Chinese medicine and modern agents. *Chin Med*. (2018) 13:16. doi: 10.1186/s13020-018-0174-0
39. Damevska K, Franca K, Lotti T, Nikolovska S, Pollozhani N. Complementary and integrative therapies for psoriasis: looking forward. *Dermatol Ther*. (2018) 31:e12627. doi: 10.1111/dth.12627
40. Talbott W, Duffy N. Complementary and alternative medicine for psoriasis: what the dermatologist needs to know. *Am J Clin Dermatol*. (2015) 16:147–65. doi: 10.1007/s40257-015-0128-6
41. Costanzo A, Malara G, Pelucchi C, Fatiga F, Barbera G, Franchi A, et al. Effectiveness end points in real-world studies on biological therapies in psoriasis: systematic review with focus on drug survival. *Dermatology*. (2018) 234:1–12. doi: 10.1159/000488586
42. Yamanaka K, Umezawa Y, Yamagiwa A, Saeki H, Kondo M, Gabazza EC, et al. Biologic therapy improves psoriasis by decreasing the activity of monocytes and neutrophils. *J Dermatol*. (2014) 41:679–85. doi: 10.1111/1346-8138.12560
43. de Groot M, Picavet DI, van Kuijk AW, Tak PP, Bos JD, de Rie MA, et al. A prospective, randomized, placebo-controlled study to identify biomarkers associated with active treatment in psoriatic arthritis: effects of adalimumab treatment on lesional and nonlesional skin. *Dermatology*. (2012) 225:298–303. doi: 10.1159/000343290
44. Tan JK, Aphale A, Malaviya R, Sun Y, Gottlieb AB. Mechanisms of action of etanercept in psoriasis. *J Investig Dermatol Symp Proc*. (2007) 12:38–45. doi: 10.1038/sj.jidsymp.5650037
45. Schon MP, Erpenbeck L. The Interleukin-23/Interleukin-17 Axis Links Adaptive and Innate Immunity in Psoriasis. *Front Immunol*. (2018) 9:1323. doi: 10.3389/fimmu.2018.01323
46. Chen F, Cao A, Yao S, Evans-Marin HL, Liu H, Wu W, et al. mTOR Mediates IL-23 Induction of Neutrophil IL-17 and IL-22 Production. *J Immunol*. (2016) 196:4390–9. doi: 10.1097/01.ccm.0000508877.60312.7c
47. Liu L, Lu J, Allan BW, Tang Y, Tetreault J, Chow CK, et al. Generation and characterization of ixekizumab, a humanized monoclonal antibody that neutralizes interleukin-17A. *J Inflamm Res*. (2016) 9:39–50. doi: 10.2147/JIR.S100940
48. Reich K, Papp KA, Matheson RT, Tu JH, Bissonnette R, Bourcier M, et al. Evidence that a neutrophil-keratinocyte crosstalk is an early target of IL-17A inhibition in psoriasis. *Exp Dermatol*. (2015) 24:529–35. doi: 10.1111/exd.12710
49. Polat M, Bugdayci G, Kaya H, Oguzman H. Evaluation of neutrophil-to-lymphocyte ratio and platelet-to-lymphocyte ratio in Turkish patients with chronic plaque psoriasis. *Acta Dermatovenerol Alp Pannonica Adriat*. (2017) 26:97–100. doi: 10.15570/actaapa.2017.28
50. Paliogiannis P, Satta R, Deligia G, Farina G, Bassu S, Mangoni AA, et al. Associations between the neutrophil-to-lymphocyte and the platelet-to-lymphocyte ratios and the presence and severity of psoriasis: a systematic review and meta-analysis. *Clin Exp Med*. (2019) 19:37–45. doi: 10.1007/s10238-018-0538-x
51. Balevi A, Olmuscelik O, Ustuner P, Ozdemir M. Is there any correlation between red cell distribution width, mean platelet volume neutrophil count, lymphocyte count, and psoriasis area severity index in patients under treatment for psoriasis? *Acta Dermatovenerol Croat*. (2018) 26:199–205.
52. Erek Toprak A, Ozlu E, Uzuncakmak TK, Yalcinkaya E, Sogut S, Karadag AS. Neutrophil/lymphocyte ratio, serum endocan, and nesfatin-1 levels in patients with psoriasis vulgaris undergoing phototherapy treatment. *Med Sci Monit*. (2016) 22:1232–7. doi: 10.12659/MSM.898240
53. Yurtdas M, Yaylali YT, Kaya Y, Ozdemir M, Ozkan I, Aladag N. Neutrophil-to-lymphocyte ratio may predict subclinical atherosclerosis in patients with psoriasis. *Echocardiography*. (2014) 31:1095–104. doi: 10.1111/echo.12511
54. Ikeda S, Takahashi H, Suga Y, Eto H, Etoh T, Okuma K, et al. Therapeutic depletion of myeloid lineage leukocytes in patients with generalized pustular psoriasis indicates a major role for neutrophils in the immunopathogenesis of psoriasis. *J Am Acad Dermatol*. (2013) 68:609–17. doi: 10.1016/j.jaad.2012.09.037
55. Schon MP, Broekaert SM, Erpenbeck L. Sexy again: the renaissance of neutrophils in psoriasis. *Exp Dermatol*. (2017) 26:305–11. doi: 10.1111/exd.13067
56. Belambri SA, Rolas L, Raad H, Hurtado-Nedelec M, Dang PM, El-Benna J. NADPH oxidase activation in neutrophils: role of the phosphorylation of its subunits. *Eur J Clin Invest*. (2018) 2:e12951. doi: 10.1111/eci.12951
57. Amulic B, Cazalet C, Hayes GL, Metzler KD, Zychlinsky A. Neutrophil function: from mechanisms to disease. *Annu Rev Immunol*. (2012) 30:459–89. doi: 10.1146/annurev-immunol-020711-074942
58. Glennon-Alty L, Hackett AP, Chapman EA, Wright HL. Neutrophils and redox stress in the pathogenesis of autoimmune disease. *Free Radic Biol Med*. (2018) 125:25–35. doi: 10.1016/j.freeradbiomed.2018.03.049
59. Winterbourn CC, Kettle AJ, Hampton MB. Reactive oxygen species and neutrophil function. *Annu Rev Biochem*. (2016) 85:765–92. doi: 10.1146/annurev-biochem-060815-014442
60. Hoffmann MH, Griffiths HR. The dual role of ROS in autoimmune and inflammatory diseases: Evidence from preclinical models. *Free Radic Biol Med*. (2018) 125:62–71. doi: 10.1016/j.freeradbiomed.2018.03.016
61. Bloomfield FJ, Young MM. Enhanced release of inflammatory mediators from lithium-stimulated neutrophils in psoriasis. *Br J Dermatol*. (1983) 109:9–13. doi: 10.1111/j.1365-2133.1983.tb03985.x
62. Bloomfield FJ, Young MM. Enhanced chemiluminescence production by phagocytosing neutrophils in psoriasis. *Inflammation*. (1988) 12:153–9. doi: 10.1007/BF00916398
63. Martin G, Guerard S, Fortin MM, Rusu D, Soucy J, Poubelle PE, et al. Pathological crosstalk *in vitro* between T lymphocytes and lesional keratinocytes in psoriasis: necessity of direct cell-to-cell contact. *Lab Invest*. (2012) 92:1058–70. doi: 10.1038/labinvest.2012.69
64. Vogt KL, Summers C, Chilvers ER, Condliffe AM. Priming and de-priming of neutrophil responses *in vitro* and *in vivo*. *Eur J Clin Invest*. (2018) 47:e12967. doi: 10.1111/eci.12967
65. Boehncke WH, Schon MP. Psoriasis. *Lancet*. (2015) 386:983–94. doi: 10.1016/S0140-6736(14)61909-7
66. Guerard S, Allaeys I, Martin G, Pouliot R, Poubelle PE. Psoriatic keratinocytes prime neutrophils for an overproduction of superoxide anions. *Arch Dermatol Res*. (2013) 305:879–89. doi: 10.1007/s00403-013-1404-z
67. Lin X, Huang T. Oxidative stress in psoriasis and potential therapeutic use of antioxidants. *Free Radic Res*. (2016) 50:585–95. doi: 10.3109/10715762.2016.1162301
68. Lai R, Xian D, Xiong X, Yang L, Song J, Zhong J. Proanthocyanidins: novel treatment for psoriasis that reduces oxidative stress and modulates Th17 and Treg cells. *Redox Rep*. (2018) 23:130–5. doi: 10.1080/13510002.2018.1462027
69. Lacy P. Mechanisms of degranulation in neutrophils. *Allergy Asthma Clin Immunol*. (2006) 2:98–108. doi: 10.1186/1710-1492-2-3-98

70. Sheshachalam A, Srivastava N, Mitchell T, Lacy P, Eitzen G. Granule protein processing and regulated secretion in neutrophils. *Front Immunol*. (2014) 5:448. doi: 10.3389/fimmu.2014.00448
71. Kahlenberg JM, Kaplan MJ. Little peptide, big effects: the role of LL-37 in inflammation and autoimmune disease. *J Immunol*. (2013) 191:4895–901. doi: 10.4049/jimmunol.1302005
72. Cowland JB, Borregaard N. Granulopoiesis and granules of human neutrophils. *Immunol Rev*. (2016) 273:11–28. doi: 10.1111/imr.12440
73. Mitchell T, Lo A, Logan MR, Lacy P, Eitzen G. Primary granule exocytosis in human neutrophils is regulated by Rac-dependent actin remodeling. *Am J Physiol Cell Physiol*. (2008) 295:C1354–65. doi: 10.1152/ajpcell.00239.2008
74. Jog NR, Rane MJ, Lominadze G, Luerman GC, Ward RA, McLeish KR. The actin cytoskeleton regulates exocytosis of all neutrophil granule subsets. *Am J Physiol Cell Physiol*. (2007) 292:C1690–700. doi: 10.1152/ajpcell.00384.2006
75. Yin C, Heit B. Armed for destruction: formation, function and trafficking of neutrophil granules. *Cell Tissue Res*. (2018) 371:455–71. doi: 10.1007/s00441-017-2731-8
76. Hoenderdos K, Lodge KM, Hirst RA, Chen C, Palazzo SG, Emerenciana A, et al. Hypoxia upregulates neutrophil degranulation and potential for tissue injury. *Thorax*. (2016) 71:1030–8. doi: 10.1136/thoraxjnl-2015-207604
77. Polverino E, Rosales-Mayor E, Dale GE, Dembowsky K, Torres A. The role of neutrophil elastase inhibitors in lung diseases. *Chest*. (2017) 152:249–62. doi: 10.1016/j.chest.2017.03.056
78. Chistiakov DA, Bobryshev YV, Orekhov AN. Neutrophil's weapons in atherosclerosis. *Exp Mol Pathol*. (2015) 99:663–71. doi: 10.1016/j.yexmp.2015.11.011
79. Sivalingam Z, Larsen SB, Grove EL, Hvas AM, Kristensen SD, Magnusson NE. Neutrophil gelatinase-associated lipocalin as a risk marker in cardiovascular disease. *Clin Chem Lab Med*. (2017) 56:5–18. doi: 10.1515/cclm-2017-0120
80. Ramaiah SK, Jaeschke H. Role of neutrophils in the pathogenesis of acute inflammatory liver injury. *Toxicol Pathol*. (2007) 35:757–66. doi: 10.1080/01926230701584163
81. Wright HL, Moots RJ, Bucknall RC, Edwards SW. Neutrophil function in inflammation and inflammatory diseases. *Rheumatology*. (2010) 49:1618–31. doi: 10.1093/rheumatology/keq045
82. Wright HL, Moots RJ, Edwards SW. The multifactorial role of neutrophils in rheumatoid arthritis. *Nat Rev Rheumatol*. (2014) 10:593–601. doi: 10.1038/nrrheum.2014.80
83. Dilek N, Dilek AR, Taskin Y, Erkinuresin T, Yalcin O, Saral Y. Contribution of myeloperoxidase and inducible nitric oxide synthase to pathogenesis of psoriasis. *Postepy Dermatol Alergol*. (2016) 33:435–9. doi: 10.5114/ada.2016.63882
84. Cao LY, Soler DC, Debanne SM, Grozdev I, Rodriguez ME, Feig RL, et al. Psoriasis and cardiovascular risk factors: increased serum myeloperoxidase and corresponding immunocellular overexpression by Cd11b. (+) CD68. (+) macrophages in skin lesions. *Am J Transl Res*. (2013) 6:16–27.
85. Strzepa A, Pritchard KA, Dittel BN. Myeloperoxidase: a new player in autoimmunity. *Cell Immunol*. (2017) 317:1–8. doi: 10.1016/j.cellimm.2017.05.002
86. Lau D, Mollnau H, Eiserich JP, Freeman BA, Daiber A, Gehling UM, et al. Myeloperoxidase mediates neutrophil activation by association with CD11b/CD18 integrins. *Proc Natl Acad Sci USA*. (2005) 102:431–6. doi: 10.1073/pnas.0405193102
87. Guo J, Tu J, Hu Y, Song G, Yin Z. Cathepsin G cleaves and activates IL-36γ and promotes the inflammation of psoriasis. *Drug Des Devel Ther*. (2019) 13:581–8. doi: 10.2147/DDDT.S194765
88. Henry CM, Sullivan GP, Clancy DM, Afonina IS, Kulms D, Martin SJ. Neutrophil-derived proteases escalate inflammation through activation of IL-36 family cytokines. *Cell Rep*. (2016) 14:708–22. doi: 10.1016/j.celrep.2015.12.072
89. Xhindoli D, Pacor S, Benincasa M, Scocchi M, Gennaro R, Tossi A. The human cathelicidin LL-37–A pore-forming antibacterial peptide and host-cell modulator. *Biochim Biophys Acta*. (2016) 1858:546–66. doi: 10.1016/j.bbamem.2015.11.003
90. Durr UH, Sudheendra US, Ramamoorthy A. LL-37, the only human member of the cathelicidin family of antimicrobial peptides. *Biochim Biophys Acta*. (2006) 1758:1408–25. doi: 10.1016/j.bbamem.2006.03.030
91. Ganguly D, Chamilos G, Lande R, Gregorio J, Meller S, Facchinetti V, et al. Self-RNA-antimicrobial peptide complexes activate human dendritic cells through TLR7 and TLR8. *J Exp Med*. (2009) 206:1983–94. doi: 10.1084/jem.20090480
92. Lande R, Gregorio J, Facchinetti V, Chatterjee B, Wang YH, Homey B, et al. Plasmacytoid dendritic cells sense self-DNA coupled with antimicrobial peptide. *Nature*. (2007) 449:564–9. doi: 10.1038/nature06116
93. Meyer-Hoffert U, Wingertszahn J, Wiedow O. Human leukocyte elastase induces keratinocyte proliferation by epidermal growth factor receptor activation. *J Invest Dermatol*. (2004) 123:338–45. doi: 10.1111/j.0022-202X.2004.23202.x
94. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, et al. Neutrophil extracellular traps kill bacteria. *Science*. (2004) 303:1532–5. doi: 10.1126/science.1092385
95. Kim SJ, Jenne CN. Role of platelets in neutrophil extracellular trap. (NET) production and tissue injury. *Semin Immunol*. (2016) 28:546–54. doi: 10.1016/j.smim.2016.10.013
96. Branzk N, Lubojemska A, Hardison SE, Wang Q, Gutierrez MG, Brown GD, et al. Neutrophils sense microbe size and selectively release neutrophil extracellular traps in response to large pathogens. *Nat Immunol*. (2014) 15:1017–25. doi: 10.1038/ni.2987
97. Apel F, Zychlinsky A, Kenny EF. The role of neutrophil extracellular traps in rheumatic diseases. *Nat Rev Rheumatol*. (2018) 14:467–75. doi: 10.1038/s41584-018-0039-z
98. Honda M, Kubes P. Neutrophils and neutrophil extracellular traps in the liver and gastrointestinal system. *Nat Rev Gastroenterol Hepatol*. (2018) 15:206–21. doi: 10.1038/nrgastro.2017.183
99. Fernandez-Ruiz I. Inflammation: NETs are involved in AAA. *Nat Rev Cardiol*. (2018) 15:257. doi: 10.1038/nrcardio.2018.28
100. Doring Y, Soehnlein O, Weber C. Neutrophil extracellular traps in atherosclerosis and atherothrombosis. *Circ Res*. (2017) 120:736–43. doi: 10.1161/CIRCRESAHA.116.309692
101. Dabrowska D, Jablonska E, Garley M, Sawicka-Powierza J, Nowak K. The Phenomenon of Neutrophil Extracellular Traps in Vascular Diseases. *Arch Immunol Ther Exp*. (2018) 66:273–81. doi: 10.1007/s00005-018-0505-y
102. Porto BN, Stein RT. Neutrophil extracellular traps in pulmonary diseases: too much of a good thing? *Front Immunol*. (2016) 7:311. doi: 10.3389/fimmu.2016.00311
103. Liu T, Wang FP, Wang G, Mao H. Role of neutrophil extracellular traps in asthma and chronic obstructive pulmonary disease. *Chin Med J*. (2017) 130:730–6. doi: 10.4103/0366-6999.201608
104. Albrengues J, Shields MA, Ng D, Park CG, Ambrico A, Poindexter ME, et al. Neutrophil extracellular traps produced during inflammation awaken dormant cancer cells in mice. *Science*. (2018) 361:eaao4227. doi: 10.1126/science.aao4227
105. Kenny EF, Herzig A, Kruger R, Muth A, Mondal S, Thompson PR, et al. Diverse stimuli engage different neutrophil extracellular trap pathways. *Elife*. (2017) 6:e24437. doi: 10.7554/eLife.24437
106. Papayannopoulos V, Metzler KD, Hakkim A, Zychlinsky A. Neutrophil elastase and myeloperoxidase regulate the formation of neutrophil extracellular traps. *J Cell Biol*. (2010) 191:677–91. doi: 10.1083/jcb.201006052
107. Loures FV, Rohm M, Lee CK, Santos E, Wang JP, Specht CA, et al. Recognition of Aspergillus fumigatus hyphae by human plasmacytoid dendritic cells is mediated by dectin-2 and results in formation of extracellular traps. *PLoS Pathog*. (2015) 11:e1004643. doi: 10.1371/journal.ppat.1004643
108. Pieterse E, Rother N, Yanginlar C, Hilbrands LB, van der Vlag J. Neutrophils discriminate between lipopolysaccharides of different bacterial sources and selectively release neutrophil extracellular traps. *Front Immunol*. (2016) 7:484. doi: 10.3389/fimmu.2016.00484
109. Desai J, Kumar SV, Mulay SR, Konrad L, Romoli S, Schauer C, et al. PMA and crystal-induced neutrophil extracellular trap formation involves RIPK1-RIPK3-MLKL signaling. *Eur J Immunol*. (2016) 46:223–9. doi: 10.1002/eji.201545605
110. Papayannopoulos V. Neutrophil extracellular traps in immunity and disease. *Nat Rev Immunol*. (2018) 18:134–47. doi: 10.1038/nri.2017.105
111. Douda DN, Khan MA, Grasemann H, Palaniyar N. SK3 channel and mitochondrial ROS mediate NADPH oxidase-independent NETosis

induced by calcium influx. *Proc Natl Acad Sci USA*. (2015) 112:2817–22. doi: 10.1073/pnas.1414055112
112. Behnen M, Leschczyk C, Moller S, Batel T, Klinger M, Solbach W, et al. Immobilized immune complexes induce neutrophil extracellular trap release by human neutrophil granulocytes via FcgammaRIIIB and Mac-1. *J Immunol*. (2014) 193:1954–65. doi: 10.4049/jimmunol.1400478
113. Hoppenbrouwers T, Autar ASA, Sultan AR, Abraham TE, van Cappellen WA, Houtsmuller AB, et al. *In vitro* induction of NETosis: Comprehensive live imaging comparison and systematic review. *PLoS ONE*. (2017) 12:e0176472. doi: 10.1371/journal.pone.0176472
114. Yipp BG, Petri B, Salina D, Jenne CN, Scott BN, Zbytnuik LD, et al. Infection-induced NETosis is a dynamic process involving neutrophil multitasking *in vivo*. *Nat Med*. (2012) 18:1386–93. doi: 10.1038/nm.2847
115. Pilsczek FH, Salina D, Poon KK, Fahey C, Yipp BG, Sibley CD, et al. A novel mechanism of rapid nuclear neutrophil extracellular trap formation in response to *Staphylococcus aureus*. *J Immunol*. (2010) 185:7413–25. doi: 10.4049/jimmunol.1000675
116. Byrd AS, O'Brien XM, Johnson CM, Lavigne LM, Reichner JS. An extracellular matrix-based mechanism of rapid neutrophil extracellular trap formation in response to Candida albicans. *J Immunol*. (2013) 190:4136–48. doi: 10.4049/jimmunol.1202671
117. Rochael NC, Guimaraes-Costa AB, Nascimento MT, DeSouza-Vieira TS, Oliveira MP, Garcia e Souza LF, et al. Classical ROS-dependent and early/rapid ROS-independent release of neutrophil extracellular traps triggered by Leishmania parasites. *Sci Rep*. (2015) 5:18302. doi: 10.1038/srep18302
118. Chandler CE, Ernst RK. Bacterial lipids: powerful modifiers of the innate immune response. *F1000Res*. (2017) 6:F1000. doi: 10.12688/f1000research.11388.1
119. Petretto A, Bruschi M, Pratesi F, Croia C, Candiano G, Ghiggeri G, et al. Neutrophil extracellular traps. (NET) induced by different stimuli: a comparative proteomic analysis. *PLoS ONE*. (2019) 14:e0218946. doi: 10.1371/journal.pone.0218946
120. Clark SR, Ma AC, Tavener SA, McDonald B, Goodarzi Z, Kelly MM, et al. Platelet TLR4 activates neutrophil extracellular traps to ensnare bacteria in septic blood. *Nat Med*. (2007) 13:463–9. doi: 10.1038/nm1565
121. Liu S, Su X, Pan P, Zhang L, Hu Y, Tan H, et al. Neutrophil extracellular traps are indirectly triggered by lipopolysaccharide and contribute to acute lung injury. *Sci Rep*. (2016) 6:37252. doi: 10.1038/srep37252
122. Hu Z, Murakami T, Tamura H, Reich J, Kuwahara-Arai K, Iba T, et al. Neutrophil extracellular traps induce IL-1β production by macrophages in combination with lipopolysaccharide. *Int J Mol Med*. (2017) 39:549–58. doi: 10.3892/ijmm.2017.2870
123. Shao S, Fang H, Dang E, Xue K, Zhang J, Li B, et al. Neutrophil extracellular traps promote inflammatory responses in psoriasis via activating epidermal TLR4/IL-36R crosstalk. *Front Immunol*. (2019) 10:746. doi: 10.3389/fimmu.2019.00746
124. Hu SC, Yu HS, Yen FL, Lin CL, Chen GS, Lan CC. Neutrophil extracellular trap formation is increased in psoriasis and induces human β-defensin-2 production in epidermal keratinocytes. *Sci Rep*. (2016) 6:31119. doi: 10.1038/srep31119
125. Lee KH, Kronbichler A, Park DD, Park Y, Moon H, Kim H, et al. Neutrophil extracellular traps (NETs) in autoimmune diseases: a comprehensive review. *Autoimmun Rev*. (2017) 16:1160–73. doi: 10.1016/j.autrev.2017.09.012
126. Lowes MA, Suárez-Fariñas M, Krueger JG. Immunology of psoriasis. *Annu Rev Immunol*. (2014) 32:227–55. doi: 10.1146/annurev-immunol-032713-120225
127. Pinegin B, Vorobjeva N, Pinegin V. Neutrophil extracellular traps and their role in the development of chronic inflammation and autoimmunity. *Autoimmun Rev*. (2015) 14:633–40. doi: 10.1016/j.autrev.2015.03.002
128. Skrzeczynska-Moncznik J, Wlodarczyk A, Zabieglo K, Kapinska-Mrowiecka M, Marewicz E, Dubin A, et al. Secretory leukocyte proteinase inhibitor-competent DNA deposits are potent stimulators of plasmacytoid dendritic cells: implication for psoriasis. *J Immunol*. (2012) 189:1611–7. doi: 10.4049/jimmunol.1103293
129. Zabieglo K, Majewski P, Majchrzak-Gorecka M, Wlodarczyk A, Grygier B, Zegar A, et al. The inhibitory effect of secretory leukocyte protease inhibitor. (SLPI) on formation of neutrophil extracellular traps. *J Leukoc Biol*. (2015) 98:99–106. doi: 10.1189/jlb.4AB1114-543R
130. Majewski P, Majchrzak-Gorecka M, Grygier B, Skrzeczynska-Moncznik J, Osiecka O, Cichy J. Inhibitors of serine proteases in regulating the production and function of neutrophil extracellular traps. *Front Immunol*. (2016) 7:261. doi: 10.3389/fimmu.2016.00261
131. Tillack K, Breiden P, Martin R, Sospedra M. T lymphocyte priming by neutrophil extracellular traps links innate and adaptive immune responses. *J Immunol*. (2012) 188:3150–9. doi: 10.4049/jimmunol.1103414
132. Sangaletti S, Tripodo C, Chiodoni C, Guarnotta C, Cappetti B, Casalini P, et al. Neutrophil extracellular traps mediate transfer of cytoplasmic neutrophil antigens to myeloid dendritic cells toward ANCA induction and associated autoimmunity. *Blood*. (2012) 120:3007–18. doi: 10.1182/blood-2012-03-416156
133. Cataldi C, Mari NL, Lozovoy MAB, Martins LMM, Reiche EMV, Maes M, et al. Proinflammatory and anti-inflammatory cytokine profiles in psoriasis: use as laboratory biomarkers and disease predictors. *Inflamm Res*. (2019) 68:557–67. doi: 10.1007/s00011-019-01238-8
134. Murakami M, Hagforsen E, Morhenn V, Ishida-Yamamoto A, Iizuka H. Patients with palmoplantar pustulosis have increased IL-17 and IL-22 levels both in the lesion and serum. *Exp Dermatol*. (2011) 20:845–7. doi: 10.1111/j.1600-0625.2011.01325.x
135. Gisondi P, Lora V, Bonauguri C, Russo A, Lippi G, Girolomoni G. Serum chemerin is increased in patients with chronic plaque psoriasis and normalizes following treatment with infliximab. *Br J Dermatol*. (2013) 168:749–55. doi: 10.1111/bjd.12118
136. Shveta, Agarwal K, Ranchander, Agarwal S. Serum sE-selectin levels in newly diagnosed psoriasis patients and correlation with psoriasis area and severity index score. *J Clin Diagnost Res*. (2017) 11:EC09–12. doi: 10.7860/JCDR/2017/29957.10831
137. Torres T, Bettencourt N, Mendonca D, Vasconcelos C, Silva BM, Selores M. Complement C3 as a marker of cardiometabolic risk in psoriasis. *Arch Dermatol Res*. (2014) 306:653–60. doi: 10.1007/s00403-014-1467-5
138. Hau CS, Kanda N, Tada Y, Shibata S, Uozaki H, Fukusato T, et al. Lipocalin-2 exacerbates psoriasiform skin inflammation by augmenting T-helper 17 response. *J Dermatol*. (2016) 43:785–94. doi: 10.1111/1346-8138.13227
139. Aizawa N, Ishiuji Y, Tominaga M, Sakata S, Takahashi N, Yanaba K, et al. Relationship between the degrees of itch and serum lipocalin-2 levels in patients with psoriasis. *J Immunol Res*. (2019) 2019:8171373. doi: 10.1155/2019/8171373
140. Wolk K, Frambach Y, Jacobi A, Wilsmann-Theis D, Phillipp S, Witte-Handel E, et al. Increased levels of lipocalin 2 in palmoplantar pustular psoriasis. *J Dermatol Sci*. (2018) 90:68–74. doi: 10.1016/j.jdermsci.2017.12.018
141. Wolk K, Wenzel J, Tsaousi A, Witte-Handel E, Babel N, Zelenak C, et al. Lipocalin-2 is expressed by activated granulocytes and keratinocytes in affected skin and reflects disease activity in acne inversa/hidradenitis suppurativa. *Br J Dermatol*. (2017) 177:1385–93. doi: 10.1111/bjd.15424
142. Esaki H, Brunner PM, Renert-Yuval Y, Czarnowicki T, Huynh T, Tran G, et al. Early-onset pediatric atopic dermatitis is TH2 but also TH17 polarized in skin. *J Allergy Clin Immunol*. (2016) 138:1639–51. doi: 10.1016/j.jid.2016.02.064
143. Abdel Hay R, Samir N, Safwat M, Rashed L, Soliman M. Tissue lipocalin-2 in psoriasis: is it a marker of metabolic disturbance or a possible marker of therapeutic efficacy after narrow band ultraviolet B? *J Dermatolog Treat*. (2019) 17:1–5. doi: 10.1080/09546634.2019.1605141
144. Cai Y, Fleming C, Yan J. New insights of T cells in the pathogenesis of psoriasis. *Cell Mol Immunol*. (2012) 9:302–9. doi: 10.1038/cmi.2012.15
145. Brembilla NC, Senra L, Boehncke WH. The IL-17 family of cytokines in psoriasis: IL-17A and beyond. *Front Immunol*. (2018) 9:1682. doi: 10.3389/fimmu.2018.01682
146. Blauvelt A, Chiricozzi A. The immunologic role of IL-17 in psoriasis and psoriatic arthritis pathogenesis. *Clin Rev Allergy Immunol*. (2018) 55:379–90. doi: 10.1007/s12016-018-8702-3

147. Lin AM. Mast cells and neutrophils release IL-17 through extracellular trap formation in psoriasis. *J Immunol*. (2011) 187:490–500. doi: 10.4049/jimmunol.1100123
148. Di Domizio J, Gilliet M. Psoriasis caught in the NET. *J Invest Dermatol*. (2019) 139:1426–9. doi: 10.1016/j.jid.2019.04.020
149. Lambert S, Hambro CA, Johnston A, Stuart PE, Tsoi LC, Nair RP, et al. Neutrophil extracellular traps induce human Th17 cells: effect of psoriasis-associated TRAF3IP2 genotype. *J Invest Dermatol*. (2019) 139:1245–53. doi: 10.1016/j.jid.2018.11.021
150. Guttman-Yassky E, Lowes MA, Fuentes-Duculan J, Zaba LC, Cardinale I, Nograles KE, et al. Low expression of the IL-23/Th17 pathway in atopic dermatitis compared to psoriasis. *J Immunol*. (2008) 181:7420–7. doi: 10.4049/jimmunol.181.10.7420
151. Chen K, Kolls JK. Interluekin-17A (IL17A). *Gene*. (2017) 614:8–14. doi: 10.1016/j.gene.2017.01.016
152. Guttman-Yassky E, Krueger JG. IL-17C: a unique epithelial cytokine with potential for targeting across the spectrum of atopic dermatitis and psoriasis. *J Invest Dermatol*. (2018) 138:1467–9. doi: 10.1016/j.jid.2018.02.037
153. Senra L, Mylonas A, Kavanagh RD, Fallon PG, Conrad C, Borowczyk-Michalowska J, et al. IL-17E (IL-25) Enhances innate immune responses during skin inflammation. *J Invest Dermatol*. (2019) 139:1732–42.e17. doi: 10.1016/j.jid.2019.01.021

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Short-Term Fever-Range Hyperthermia Accelerates NETosis and Reduces Pro-inflammatory Cytokine Secretion by Human Neutrophils

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Fever is a hallmark of infections and inflammatory diseases, represented by an increase of 1–4°C in core body temperature. Fever-range hyperthermia (FRH) has been shown to increase neutrophil recruitment to local sites of infection. Here, we evaluated the impact of a short period (1 h) of FRH (STFRH) on pro-inflammatory and bactericidal human neutrophil functions. STFRH did not affect neutrophil spontaneous apoptosis but reverted the lipopolysaccharide (LPS)-induced anti-apoptotic effect compared with that under normothermic conditions. Furthermore, STFRH accelerated phorbol myristate acetate (PMA)-induced NETosis evaluated either by the nuclear DNA decondensation at 2 h post-stimulation or by the increase in extracellular DNA that colocalized with myeloperoxidase (MPO) at 4 h post-stimulation. Increased NETosis upon STFRH was associated with an increase in reactive oxygen species (ROS) production but not in autophagy levels. STFRH also increased NETosis in response to *Pseudomonas aeruginosa* challenge but moderately reduced its phagocytosis. However, these STFRH-induced effects did not influence the ability of neutrophils to kill bacteria after 4 h of co-culture. STFRH also significantly reduced neutrophil capacity to release the pro-inflammatory cytokines chemokine (C-X-C motif) ligand 8/interleukin 8 (CXCL8/IL-8) and IL-1β in response to LPS and *P. aeruginosa* challenge. Altogether, these results indicate that a short and mild hyperthermal period is enough to modulate neutrophil responses to bacterial encounter. They also suggest that fever spikes during bacterial infections might lead neutrophils to trigger an emergency response promoting neutrophil extracellular trap (NET) formation to ensnare bacteria in order to wall off the infection and to reduce their release of pro-inflammatory cytokines in order to limit the inflammatory response.

Keywords: neutrophils, hyperthermia, NETosis, NETs, cytokines

INTRODUCTION

Neutrophils are the most numerous leukocytes in human circulation. These cells are rapidly and massively recruited to tissues in response to multiple microbial and sterile challenges (1). Their localization to the site of inflammation is essential for the clearance of infections (2). When arriving at the site of infection, they recognize the insulting pathogens and deploy an extensive repertoire of antimicrobial weapons that includes oxidants, proteases, and antimicrobial proteins (3). Neutrophils mediate the killing of microbes through phagocytosis, degranulation, and neutrophil extracellular trap (NET) generation (3).

NETs are fibrous structures that usually consist of a nuclear DNA scaffold with associated histones and nuclear, cytoplasmic, and granular proteins. They are released by neutrophils in response to microbial and sterile agents and play an important role in the defense against bacteria, fungi, and viruses (4–6). NETs ensnare microorganisms to prevent their dissemination and wall off the infection (7). Their associated antimicrobial compounds, such as proteases, antimicrobial peptides, and histones, can directly kill pathogens (6).

Although neutrophils play an indisputable role as microbicidal cells, evidence from recent years indicates that their biological functions transcend the killing of pathogens (8). In fact, human neutrophils produce potent pro-inflammatory cytokines and chemokines like chemokine (C-X-C motif) ligand 8/interleukin 8 (CXCL8/IL-8), interleukin 1 beta (IL-1β), and tumor necrosis factor alpha (TNF-α). The huge daily generation of neutrophils ($\sim 10^{11}$), which dramatically increases upon infection/inflammation (9), together with their massive recruitment to inflammatory foci suggests that neutrophil-derived cytokines might be of foremost importance.

Fever is a cardinal response to infection. Given that it has been conserved throughout evolution, it is thought that it confers a survival advantage. Indeed, substantial evidence has accumulated suggesting that febrile temperatures are associated with improvement in survival and the resolution of infections (10). Previous studies showed that fever-range hyperthermia (FRH) increases neutrophil pulmonary recruitment in experimental Gram-negative bacterial pneumonia (11). Even though an augmented expression of CXC chemokines was involved in the hyperthermia-mediated enhancement of neutrophil recruitment to the lungs (12), additional evidence indicated that FRH also exerts direct effects on both neutrophils and the endothelium that augment their extravasation (13). Other studies showed that both spontaneous and lipopolysaccharide (LPS)-induced reactive oxygen species (ROS) production by adherent human neutrophils are enhanced at febrile temperatures (14). Earlier studies found contrasting findings regarding the impact of hyperthermia on the neutrophil capacity to kill pneumococci and *Escherichia coli in vitro* (15). Besides, a slight although significant increase in neutrophil bactericidal capacity against *E. coli*, *Salmonella typhimurium*, and *Listeria monocytogenes* at 40°C was reported to ensue at 1 h but was not detectable at 2 h. This effect was not observed with *Staphylococcus aureus* (16).

In this study, we evaluated the impact of a short period (1 h) of FRH (STFRH; 39.5°C) on microbicidal and pro-inflammatory functions of human neutrophils and on its capacity to fight against *Pseudomonas aeruginosa* infections.

MATERIALS AND METHODS

The experimental protocols performed were approved by the Biosafety and Research Review Board of IMEX-CONICET-ANM and the Ethical Committee of the Institutos de la Academia Nacional de Medicina. The methods were carried out in accordance with the approved guidelines.

Reagents and Materials

Roswell Park Memorial Institute (RPMI) 1640 culture media, Pierce lactate dehydrogenase (LDH) Cytotoxicity Assay Kit, TO-PRO-3, and TMB substrate were purchased from Thermo Fisher Scientific (Massachusetts, MA, USA). Fetal bovine serum (FBS) was purchased from Internegocios (Buenos Aires, Argentina). Luria broth (LB) medium was purchased from Acumedia (Michigan, USA), bacteriological agar was purchased from Britania (Buenos Aires, Argentina). Ficoll was purchased from GE Healthcare (Munich, Germany). DNase (Dornasae alpha) was from Roche, Argentina. Anti-myeloperoxidase (MPO)–fluorescein isothiocyanate (FITC) antibody was purchased from Biolegend (San Diego, USA); rabbit gamma globulin, anti-rabbit Alexa 647, and Alexa Fluor 488 $F(ab')_2$ fragment goat anti-rabbit IgG cat. #111-546-144 were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Rabbit polyclonal antibody anti-LC3B cat. #sc28266 was from Santa Cruz Biotechnology (Dallas, TX, USA). SYBR Gold and Sytox Green were from Life Technologies (Carlsbad, CA, USA). Phycoerythrin-conjugated anti-CD14 antibody; the OptEIA human IL-1β, CXCL8/IL-8, and TNF-α enzyme-linked immunosorbent assay (ELISA) sets; and substrate reagents A and B were purchased from BD Biosciences (Franklin Lakes, NJ, USA). Aqua-Poly/Mount coverslipping medium was purchased from Polysciences (Warrington, PA, USA). Lab-Tek chambers were purchased from Nalge Nunc International, New York, NY, USA. NucSpot live 488 was from Biotium (Fremont, CA, USA). Anti-green fluorescent protein (GFP) antibody was purchased from GenScript (Piscataway, NJ, USA). Unless otherwise stated, all the chemicals employed were from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany).

Human Neutrophil Isolation

Neutrophils were isolated from heparinized human blood from healthy donors who gave written informed consent, by centrifugation on Ficoll-Paque, dextran sedimentation, and hypotonic lysis (17). Cells were suspended at 5×10^6/mL in RPMI 1640 supplemented with 10% FBS previously heated at 65°C for 30 min for nuclease inactivation, and with or without penicillin (100 U/mL) and Streptomycin (100 μg/mL). After isolation, neutrophil preparations were stained with an anti-CD14-PE antibody and analyzed with a FACSCalibur (Beckton Dickinson, San Jose, CA, USA) or a CyFlow cytometer (Sysmex

Partec, Germany) to guarantee that monocyte contamination was <0.5%. Cells were used immediately after isolation.

Bacterial Strains

Pseudomonas aeruginosa PAO-1 strain was kindly provided by Prof. Barbara Iglewski (Department of Microbiology and Immunology, University of Rochester, Rochester, NY). GFP-tagged PAO-1 strain was kindly provided by Prof. Tim Tolker-Nielsen (Centre for BioScience and Technology, Technical University of Denmark, Lyngby, Denmark).

P. aeruginosa was grown on Luria broth agar (LA) plates and kept at 4°C. For individual experiments, the organism was grown overnight in LB medium at 37°C and then was diluted in fresh LB medium and grown at 37°C with agitation until an OD600 of 0.6. After that, it was washed twice by centrifugation and suspended in RPMI medium without phenol red. The desired concentration was attained by monitoring spectrophotometric absorbance at 600 nm, using an appropriate optical density/colony-forming units (CFUs) curve.

Neutrophil Stimulation

Except otherwise stated, neutrophils were cultured for 1 h at 37 or 39.5°C in the presence or absence of phorbol myristate acetate (PMA) (25 ng/mL) or LPS from *E. coli* O111:B4 (250 ng/mL) and then were cultured for one or three additional hours at 37°C (PMA) or 4 h (LPS). In some experiments, 2 h after stimulation with LPS, cells were treated with ATP (2.5 mM). In another set of experiments, neutrophils were first cultured for 1 h at 37 or 39.5°C and then were challenged with *P. aeruginosa* PAO-1 and cultured for three or four additional hours. When indicated, Ac-YVAD-cmk (50 μM) was added 20 min before PMA stimulation. After culture, supernatants were collected and employed to measure DNA or MPO, or were frozen at −20°C until cytokines concentrations were determined by ELISA, following the manufacturer's instructions. In cell pellets, viability was determined by annexin V-FITC/propidium iodide (PI) or VivaFix dye staining and flow cytometry analysis.

Microscopic Assessment of NET Formation

At the end of the experiment, neutrophils were fixed with 4% paraformaldehyde (PFA), permeabilized with acetone in PBS for 7 min, rehydrated for 7 min, and blocked with 5% goat serum for 60 min at 37°C. Then, they were incubated with a FITC-conjugated anti-MPO antibody, or the corresponding isotype control for 1 h. Then DNA was stained with PI (1 μg/mL) for 10 min, and cells were mounted using Aqua-Poly/Mount coverslip medium. Images were acquired by using a FluoView FV1000 confocal microscope (Olympus, Tokyo, Japan) equipped with a Plapon 60×/NA1.42 objective and then analyzed with FIJI software.

NET Quantification

NETs were quantified by determining the concentrations of DNA and MPO in culture supernatants. DNA was quantified by SYBR Gold or Sytox Green (1:2,000) staining and fluorometry detection after interpolation from a standard DNA concentration curve as previously described (18). MPO concentration was quantified by reaction of supernatants containing NETs with BD substrate reagent (A + B) for 10 min. The change in optical density at 450–570 nm was measured by spectrophotometry, and concentration was calculated by interpolation from a standard MPO curve. Alternatively, NETosis was evaluated by determining the number of cells with nuclear DNA decondensation after fixing with 4% PFA, PI staining, and confocal laser scanning microscopy (CLSM).

LDH Assay

After culture in the presence or absence of PAO-1 (multiplicity of infection (MOI) 0.1), supernatants were harvested and LDH levels were measured by using a Pierce LDH Cytotoxicity Assay Kit. The same number of cells was lysed with 0.5% Triton X-100 and used as a positive control.

Intracellular LC3 Immunostaining, CLSM Acquisition, and Automated Image Analysis

At the end of the experiment, after fixation with PFA 4% for 30 min, cells were blocked with PBS–glycine (0.1 M) for 15 min, permeabilized with chilled acetone (−20°C) for 7 min, rehydrated with PBS, and blocked with PBS supplemented with 5% goat serum overnight at 4°C. Then, neutrophils were incubated with an anti-LC3B antibody for 1 h at room temperature, washed, and then incubated with the corresponding secondary Alexa 488-conjugated antibody for 1 h at room temperature. Then, PI was added for nuclei staining for 10 min. Afterwards, cells were washed, mounted with Aqua-Poly/Mount mounting medium, and stored at 4°C until confocal microscopy examination. Images were analyzed, and the fluorescence was quantitated with a specific macro with Fiji software (National Institutes of Health, NIH) as we previously described (17).

Dihydrorhodamine 123 (DHR123) Flow Cytometry Assay

Neutrophils were loaded with DHR123 (10 μM) for 5 min at 37°C. Then, they were cultured for 1 h at 37 or 39.5°C and 15 min before the end of the experiment and were stimulated or not with PMA (25 ng/mL). Then, fluorescence was determined by flow cytometry.

Phagocytosis Assay

Phagocytosis assays were conducted as previously described with minor modifications (19). Briefly, neutrophils were cultured for 1 h at 37 or 39.5°C and then were incubated for 10 min at 37°C in the presence or absence of NaN_3 (100 μM). Then GFP-tagged *P. aeruginosa* PAO-1 (MOI 10) or vehicle was added, and cells were co-cultured with shaking for 15 min at 37°C. Afterwards, non-ingested bacteria were removed from neutrophils by centrifugation, and media were replaced. Neutrophils were incubated at 37°C for an additional 45 min. After fixation with PFA 4%, neutrophil fluorescence was determined by flow cytometry. In some experiments, cells were immediately stained at 4°C with an anti-GFP rabbit antibody followed by an Alexa 647 anti-rabbit secondary antibody. Alternatively, cells

were cytospinned, mounted with Aqua-Poly/Mount mounting medium, and examined by CLSM.

Killing Assay

P. aeruginosa (PAO-1) was cultured for 2 h in LB medium and then was washed and suspended in RPMI medium without phenol red. Neutrophils were cultured for 1 h at 37 or 39.5°C and then were challenged with PAO-1 at MOI 1 or 0.1 and co-cultured for four additional hours at 37°C and 5% of CO_2. Then, DNase (10 U/mL) was added for 15 min to release entrapped bacteria. Samples were centrifuged, the supernatants were collected, and the cell pellets were treated with distilled water for neutrophil lysis and mixed with the supernatant fractions. These preparations were plated in duplicates in LB medium. Plates were incubated at 37°C for 18 h, and then the number of CFUs was counted.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism v6.00 for Windows, GraphPad Software, La Jolla, CA, USA. Comparisons between groups were performed by two-way analysis of variance (ANOVA) with repeated measures followed by Bonferroni's multiple-comparisons test. Otherwise, the Mann–Whitney *U*-test was used for the analysis of two unpaired groups of confocal images. Statistical significance was defined as $p < 0.05$.

RESULTS

Previous studies have evaluated the effects of hyperthermia on neutrophil functions; however, these works mainly analyzed the impact of prolonged periods of high temperatures (3 h or longer), and in most cases, temperatures evaluated were outside the physiological range. Thus, this study was aimed to determine the effects of a short-term (1 h) FRH on neutrophil functions, to mimic the impact of fever spikes that could take place during infections.

In previous studies, we and others determined that prolonged febrile range hyperthermia (8 h) accelerated neutrophil apoptosis (20, 21). By contrast, a shorter period of hyperthermia (1 h) did not affect spontaneous apoptosis and slightly reverted the anti-apoptotic effect of LPS treatment after 20 h of culture (22). Thus, to determine whether STFRH affects apoptosis rates at early time points, we first evaluated its impact on cells that were stimulated or not with LPS for 1 h at 37 or 39.5°C and then cultured for 4 h at 37°C. Results indicated that STFRH did not significantly modulate neutrophil spontaneous apoptosis. However, similarly to what has been previously observed after 20 h, STFRH prevented the LPS-anti-apoptotic effect after the 5 h of culture (**Figures 1A,B**).

Then we analyzed the impact of STFRH on NETosis induced by PMA. NET formation was first evaluated by analyzing the nuclear expansion that characterizes PMA-induced NETosis. To this end, neutrophils were stimulated or not with 25 ng/mL PMA for 1 h at 37 or at 39.5°C and then cultured for one additional hour at 37°C. Afterwards, cells were stained with PI and analyzed by confocal microscopy. As shown in **Figure 1C**, and as was confirmed by their quantitation (**Figure 1D**), STFRH markedly increased the number of cells exhibiting morphologic features of cells undergoing NETosis characterized by nuclear DNA decondensation. This effect was also evident when cells were stimulated with 100 nM (62 ng/mL) of PMA (**Figure S1**). At this concentration after 2 h post-stimulation (p.s.), some neutrophils have already released NETs when exposed to 39.5°C, but only an incipient NETosis was observed at 37°C. The enhancement effects of STFRH on PMA-induced NETosis were also detected when it was evaluated by DNA and MPO quantitation in culture supernatants of neutrophils that were stimulated with PMA for 1 h at 37 or 39.5°C and then for three additional hours at 37°C (**Figures 1E,F**). We confirmed that DNA found in supernatants corresponded to NETs and not to DNA released by other cell death pathways, by detection of colocalization of MPO and DNA by confocal microscopy (**Figure 1G**).

Additional evidence that STFRH was promoting a NETosis program was obtained by differential staining with NucSpot live 488, a cell-permeable DNA dye, and PI, which only penetrates cells with a compromised integrity of their plasma membranes, and by time-lapse live microscopy. As shown in **Video S1**, since the beginning of the culture, the whole population of neutrophils was stained with NucSpot live 488 (green fluorescence) but not with PI, and it was characterized by brightly condensed lobulated nuclei. As time went on and plasma membrane disintegrated, DNA was released and brightly stained with the extracellular PI, fluorescing yellow by the overlapping of green and red fluorescence signals, which is in agreement with the characteristic steps reported for neutrophils undergoing NETosis under normothermic conditions (5, 23). As shown in **Figure S2**, nuclei of unstimulated neutrophils subjected to STFRH mainly remained morphologically lobulated at the end of the experiment (3 h 40 min) sharply contrasting with the appearance of PMA-stimulated cells subjected to STFRH.

Additionally, the caspase-1 inhibitor Ac-YVAD-cmk did not significantly modulate DNA and MPO concentrations in culture supernatants regarded as NETosis parameters, at either 37 or 39.5°C (**Figure S3**). These results further support that hyperthermia promotes a canonical NETosis program, which, as former studies indicated, is a caspase-1-independent process (24).

Previous studies showed that autophagy and ROS production are required for PMA-induced NET formation in human neutrophils (23). Therefore, to obtain insights into the mechanism that leads to NETosis acceleration by STFRH, we then evaluated autophagy levels in neutrophils that were stimulated with PMA for 1 h at 37 or at 39.5°C. As in cells undergoing autophagy, the cytoplasmic protein LC3B (LC3B-I) conjugates with phosphatidylethanolamine in the membrane of autophagosomes, and this leads to the appearance of LC3B+ vesicles that can be detected by confocal microscopy. Thus, the intensity of vesicular LC3B/cell serves as a readout of autophagy levels. Quantification of multiple images like those shown in **Figure 2A**, acquired from three independent experiments, indicated that a small but significant decrease in autophagy levels was detected in PMA-stimulated neutrophils at 39.5°C. These findings ruled out that an enhancement of autophagy

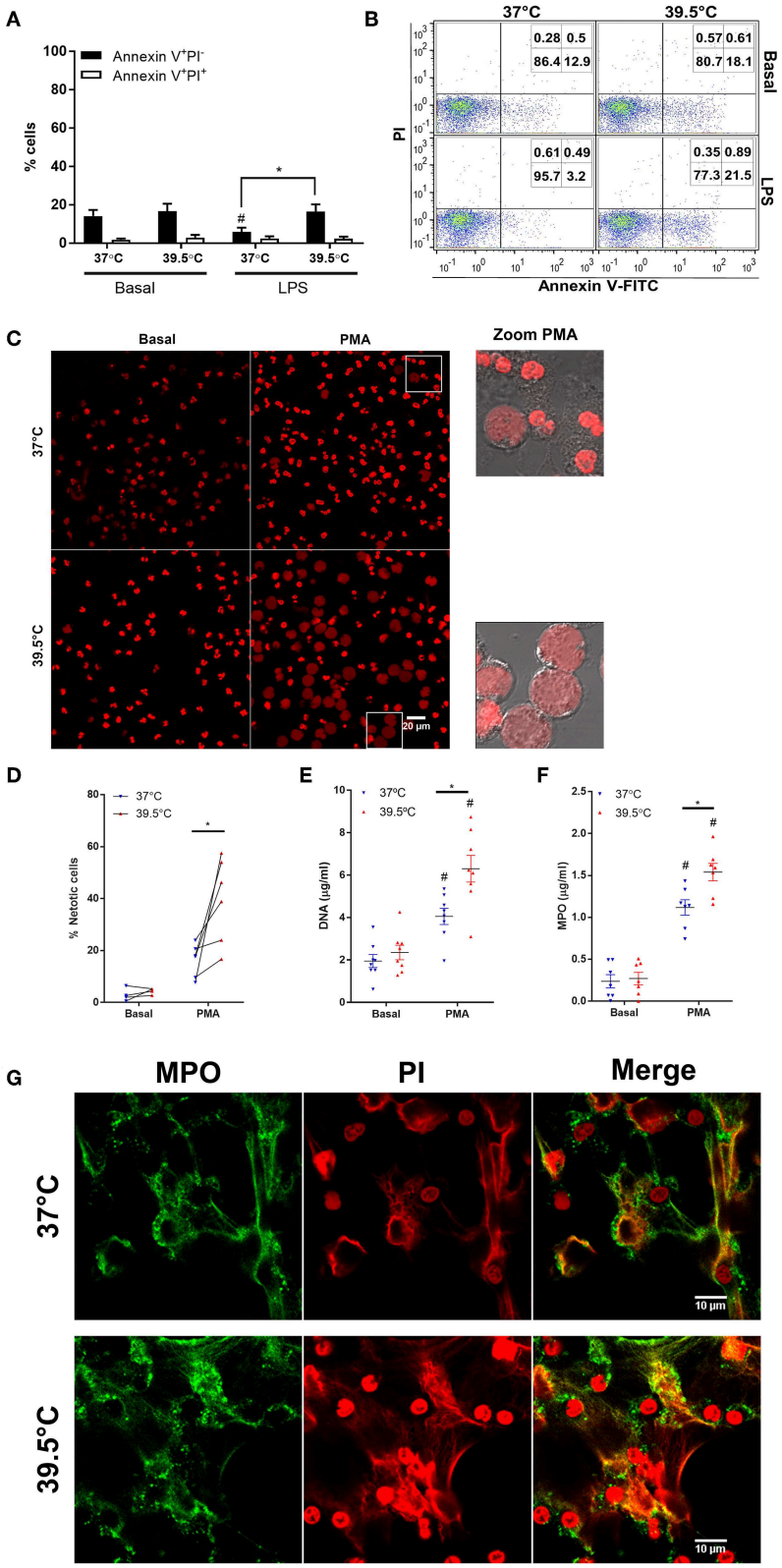

FIGURE 1 | Short-term fever-range hyperthermia accelerates PMA-induced NETosis. Neutrophils were cultured for 1 h at 37 or 39.5°C in the absence (basal) or presence of LPS (250 ng/mL) and then cultured at 37°C for four additional hours **(A,B)** or were cultured for 1 h at 37 or 39.5°C in the absence (basal) or presence of PMA (25 ng/mL) and then cultured at 37°C for one additional hour **(C,D)** or three additional hours **(E–G)**. **(A,B)** After culture, the percentages of apoptotic

(Continued)

FIGURE 1 | (annexin V^+PI^-) and necrotic (annexin V^+PI^+) cells were then evaluated by annexin-V FITC/propidium iodide staining and flow cytometry. **(A)** Data are depicted as the mean ± SEM of four experiments. **(B)** A representative experiment is shown. **(C,D)** Alternatively, after culture, cells were stained with PI and visualized by confocal microscopy. **(C)** Left and center panels, PI fluorescence; zoomed PMA panels, DIC, and PI merge of cells inside the square gate in PMA panels. **(D)** Quantification of cells undergoing NETosis distinguished by their expanded nuclear areas in experiments depicted in **(C)**. At least 300 cells were scored for each treatment per donor. Data correspond to the percentage of cells with expanded nuclear areas like those depicted in zoomed areas in C per donor; individual donors are linked by a connecting line. **(E)** DNA and **(F)** MPO concentrations in culture supernatants collected at 4 h post-stimulation. Bars represent the mean ± SEM of the independent experiments depicted. **(E)** Data are depicted as the mean DNA value of assays performed in triplicate per donor. **(G)** Representative confocal microscopy images of NETs of eight donors identified by MPO and PI staining at 4 h post-stimulation. $^*p < 0.01$ PMA 37°C vs. PMA 39.5°C and $^{\#}p < 0.05$ PMA vs. basal at their respective temperatures; two-way ANOVA with Bonferroni's multiple-comparisons test. PMA, phorbol myristate acetate; LPS, lipopolysaccharide; PI, propidium iodide; DIC, differential interference contrast; NETs, neutrophil extracellular traps; MPO, myeloperoxidase; ANOVA, analysis of variance.

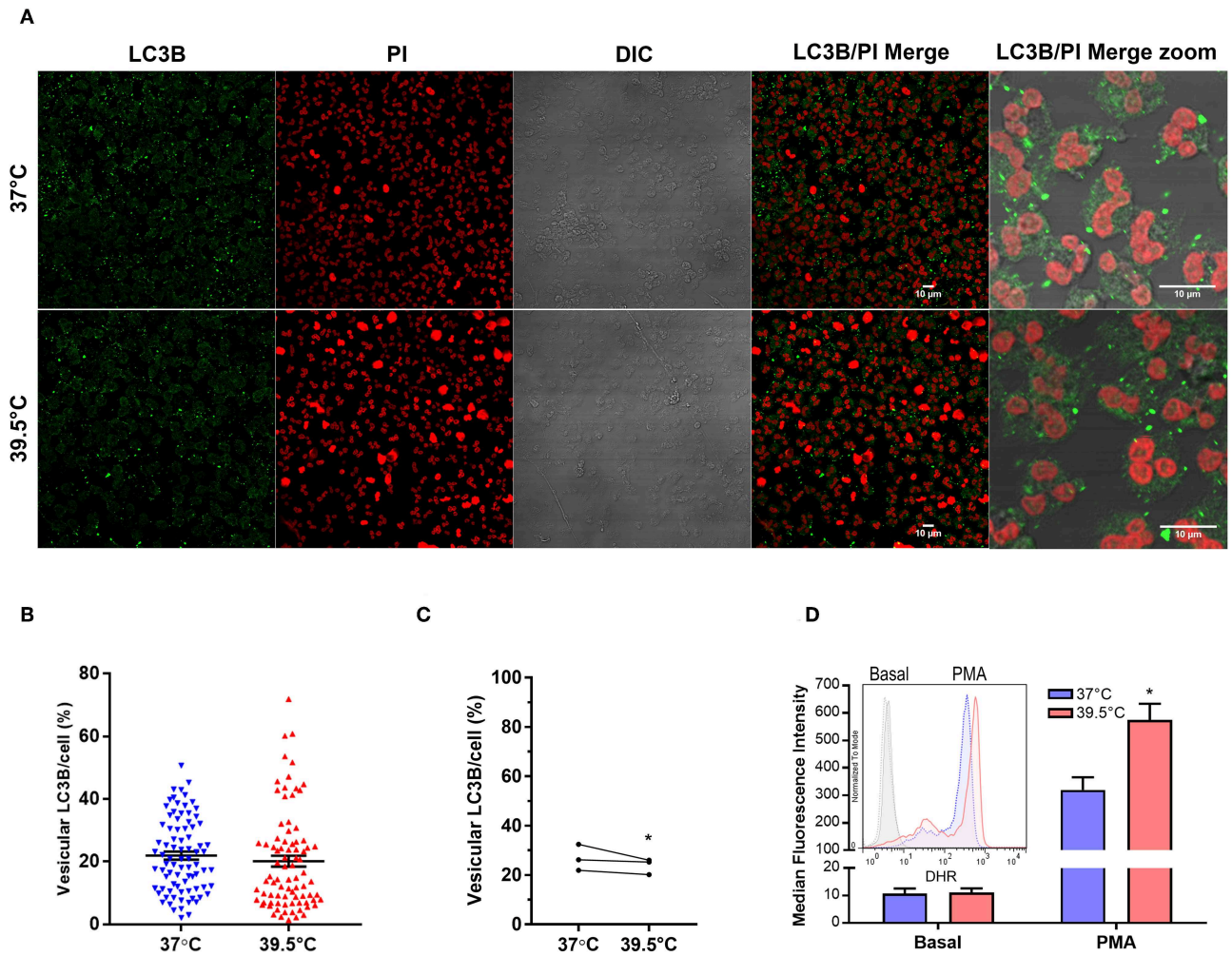

FIGURE 2 | Short-term fever-range hyperthermia increases PMA-induced ROS production but not autophagy levels. **(A)** Representative confocal microscopy images of PMA-stimulated neutrophils cultured for 1 h at 37 or 39.5°C and then stained with a specific antibody anti-LC3B and PI. Image quantifications **(B,C)** were performed by using a specific macro with Fiji software. **(B)** Scatter plot depicts the percentage of vesicular LC3B intensity/cell from a representative experiment of three. Black bars indicate the mean ± SEM values of 83 (37°C) and 84 (39.5°C) cells analyzed. **(C)** Data represent the mean value of vesicular LC3B intensity/cell from experiments like that depicted in **(B)**, performed with three different donors in which at least 69 cells were analyzed for each temperature. $^*p < 0.05$; Mann–Whitney *U*-test analysis. **(D)** Effect of STFRH on neutrophil ROS production induced by PMA. Neutrophils were stained with DHR for 5 min at 37°C and then were split off and stimulated or not with PMA (25 ng/mL) and incubated for 1 h at 37 or 39.5°C, and fluorescence was determined by flow cytometry. Bar graph depicts the median fluorescence intensity of six experiments. Inset shows the histograms of a representative experiment. $^*p < 0.01$ vs. 37°C; two-way ANOVA with Bonferroni's multiple-comparisons test. PMA, phorbol myristate acetate; ROS, reactive oxygen species; STFRH, short period (1 h) of fever-range hyperthermia; DHR, dihydrorhodamine; ANOVA, analysis of variance; PI, propidium iodide.

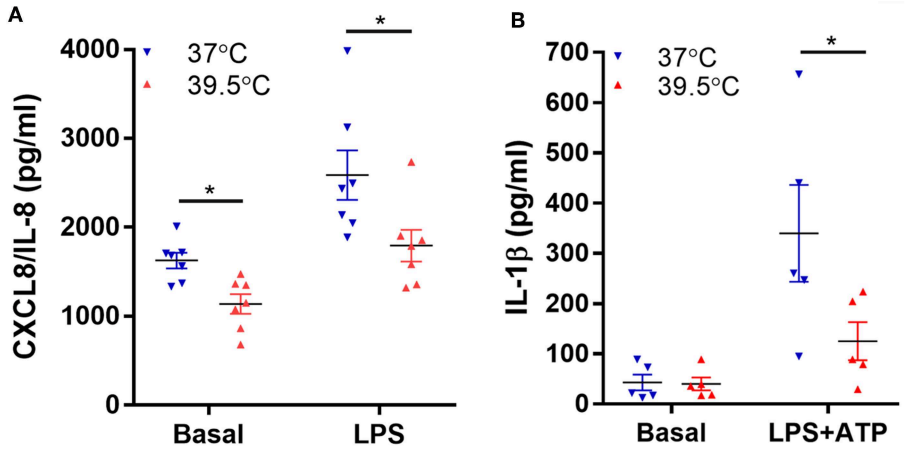

FIGURE 3 | Short-term fever-range hyperthermia reduces pro-inflammatory cytokines secretion induced by LPS. Neutrophils were cultured for 1 h at 37 or 39.5°C in the absence (basal) or presence of LPS (250 ng/mL) and 2 h later **(B)** were stimulated with ATP (2.5 mM), and at 5 h post-LPS stimulation, cells were centrifuged, supernatants were collected, and the concentrations of CXCL8/IL-8 **(A)** and IL-1β **(B)** were determined by ELISA. Data depicted correspond to the mean value of cytokine concentrations secreted by 10^6 neutrophils of assays performed in triplicate per donor. Bars represent the mean ± SEM of the independent experiments performed. *$p < 0.01$; two-way ANOVA with Bonferroni's multiple-comparisons test. LPS, lipopolysaccharide; CXCL8/IL-8, chemokine (C-X-C motif) ligand 8/interleukin 8; ELISA, enzyme-linked immunosorbent assay; ANOVA, analysis of variance.

is responsible for NETosis acceleration by hyperthermia (**Figures 2B,C**).

We then analyzed if STFRH modulates ROS production. As shown in **Figure 2D**, STFRH significantly increased PMA-induced ROS production, suggesting that this effect could contribute to the accelerated NETosis rates observed upon STFRH.

Previous studies also showed that neutrophil exposure to 42°C for 1 h inhibits NF-κB activation induced by LPS as well as TNF-α mRNA expression, an effect that was also observed when neutrophils were exposed to 40°C (22). Thus, we then determined if a lower hyperthermic condition (39.5°C) is able to modulate neutrophil capacity to synthesize NF-κB-dependent pro-inflammatory cytokines (25). We evaluated both spontaneous and LPS-induced neutrophil CXCL8/IL-8 and IL-1β secretion after 1 h exposure to either 37 or 39.5°C and four additional hours at 37°C. For IL-1β determinations, ATP was added 2 h after LPS stimulation in order to induce the inflammasome activation and consequently IL-1β secretion (17). Our results indicated that STFRH significantly reduced both spontaneous and LPS-triggered CXCL8/IL-8 production (**Figure 3A**). By contrast, STFRH did not affect basal IL-1β release but significantly reduced LPS-induced IL-1β secretion (**Figure 3B**). Of mention, LPS is a weak NETosis inducer and, as we showed in **Figure 1A**, exerts anti-apoptotic effects that can be prevented by STFRH. Thus, to evaluate the potential contribution of a loss of viability to the effects induced by hyperthermia on cytokine production, we analyzed cell viability under each of the experimental conditions assessed. As shown in **Figure S4**, although STFRH significantly prevented the anti-apoptotic effect induced by LPS, the reduced magnitude of this effect at this time point suggests that the inhibition in cytokine secretion upon neutrophil febrile temperature exposure was not merely due to a differential cell death rate. This conjecture is supported by the fact that STFRH reduced CXCL8/IL-8 basal secretion (**Figure 3A**) even though it did not affect the viability of unstimulated neutrophils (**Figure S4**).

To determine if the impact of STFRH could be also observed when neutrophils were challenged with bacteria, we exposed neutrophils to either 37 or 39.5°C for 1 h and then co-incubated them with *P. aeruginosa* at MOI 1 for one, two, three, or four additional hours and then analyzed their ability to generate NETs. This experimental setting was chosen in order to avoid the contribution of the impact of hyperthermia on bacteria. As shown in **Figures 4A,B**, at 4 h p.s. STFRH significantly increased NETosis levels compared with those attained at 37°C, as indicated by increased concentrations of DNA and MPO detected in culture supernatants. Moreover, we confirmed that DNA detected in culture supernatants corresponded to NETs, as MPO was found colocalizing with DNA by confocal microscopy (**Figure 4C**). Noteworthy, bacteria were found ensnared in NETs.

We performed additional experiments to determine whether STFRH also modulates bacterial phagocytosis. To this end, we exposed neutrophils to either 37 or 39.5°C for 1 h and then co-incubated them with GFP-tagged *P. aeruginosa* for 1 h at 37°C, a time point at which NETs had not yet been detected in culture supernatants. As shown in **Figures 4D–G**, STFRH significantly reduced either the percentage of cells containing GFP-fluorescent bacteria or the median fluorescence intensity associated with neutrophils. However, considering that previous studies indicated that HOCl produced in the phagosome by the MPO–H_2O_2-chloride system can quench GFP fluorescence, we performed additional experiments in the presence of NaN_3, a compound capable to inhibit this effect (19), to rule out a contribution of HOCl to the results obtained. Nevertheless, we did not find significant differences in the percentage of

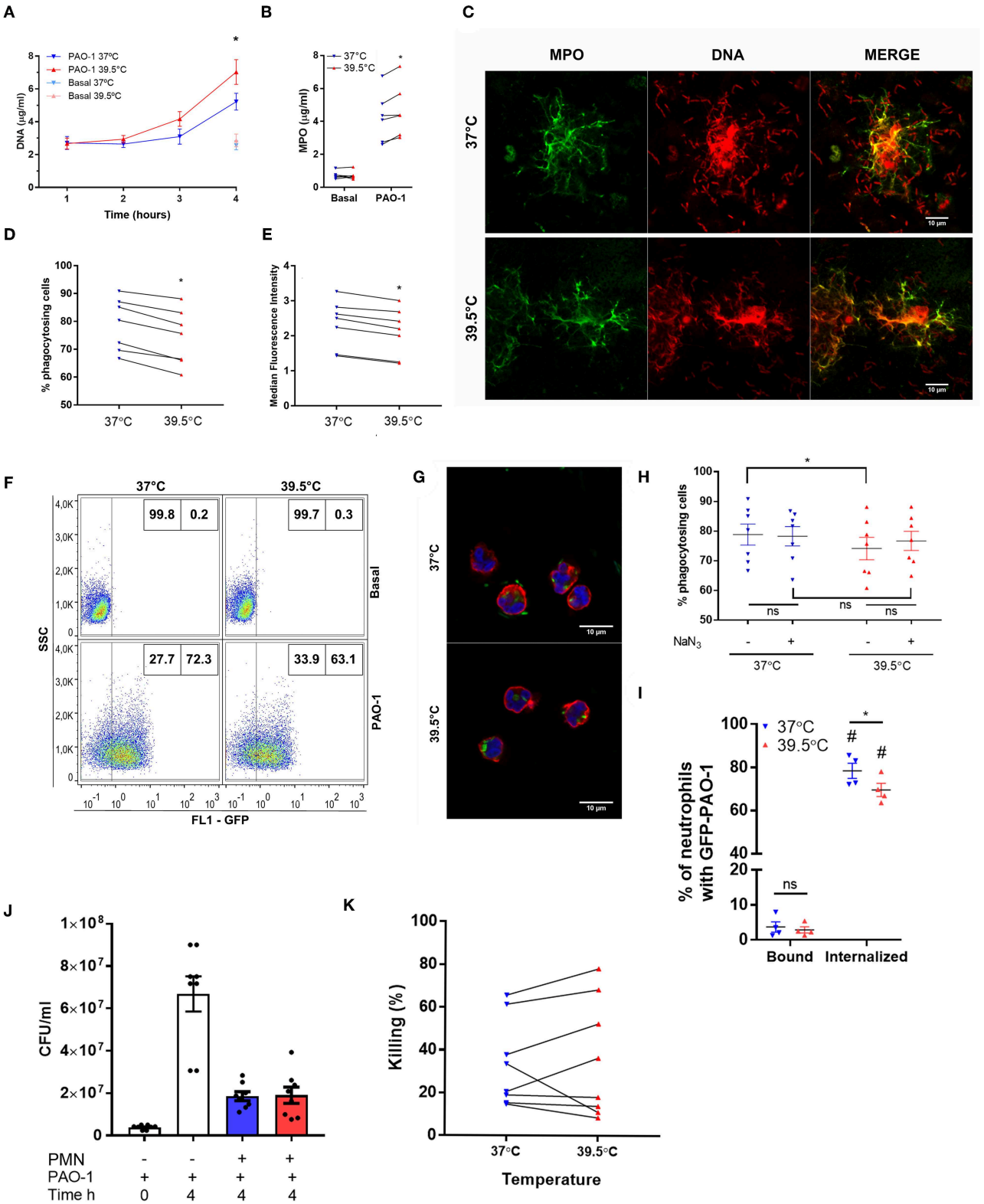

FIGURE 4 | Short-term fever-range hyperthermia reduces bacterial phagocytosis and increases NET production induced by *Pseudomonas aeruginosa*. Neutrophils were cultured for 1 h at 37 or 39.5°C and then challenged with *P. aeruginosa* at MOI 1 **(A–C,J,K)** or MOI 10 **(D–I)** and cultured at 37°C for 1, 2, 3, or 4 h **(A)**, 4 h **(B,C,J,K)**, or 1 h (**D–I**; GFP-tagged *P. aeruginosa* PAO-1). After culture, **(A)** DNA and **(B)** MPO were evaluated as a readout of the presence of NETs. Data are

(Continued)

FIGURE 4 | depicted as the mean ± SEM of four **(A)** or six **(B)** independent experiments. *$p < 0.01$ 39.5 vs. 37°C at 4 h. **(C)** cells were fixed and permeabilized, MPO was stained with a FITC-conjugated specific monoclonal antibody, and DNA was stained with PI. Representative confocal microscopy images of two experiments. **(D–F)** GFP-PAO-1 phagocytosis was evaluated by flow cytometry. **(D)** Percentage of GFP-positive cells (% of phagocytosing cells) and **(E)** GFP-median fluorescence intensity of neutrophils challenged with GFP-PAO-1; *$p < 0.01$ 39.5 vs. 37°C. **(F)** Representatives dot plots of one of the experiments depicted in **(D)**. **(G)** Representative confocal microscopy images of neutrophils challenged with GFP-PAO-1 like in **(D)**, which at the end of the experiments were fixed and stained with TRITC–phalloidin (red) to delimitate cell contours, and TO-PRO-3 to stain DNA (blue). **(H)** Percentage of GFP-positive cells after treatment or not with NaN_3 (100 μM) added 10 min before bacterial challenge; *$p < 0.01$, ns: non-significant. **(I)** After co-culture with GFP-PAO-1, cells were stained with an Alexa 647 anti-GFP antibody to discriminate extracellular (bound) bacteria from those internalized (unable to stain with anti-GFP antibody). #$p < 0.0001$ vs. bound at their respective temperatures; *$p = 0.05$, 39.5 vs. 37°C, ns: non-significant. **(J)** After co-culture, samples were centrifuged, and neutrophils were lysed and mixed with their respective supernatants previously treated with DNase to release bacteria entrapped in NETs and then were plated in LB agar. After culture for 20 h at 37°C, the CFUs were counted. PAO-1 time 0 h represents the original inoculum, and PAO-1 time 4 h represents CFU of bacteria cultured in the absence of neutrophils throughout the experiment. PAO-1 with neutrophils (PMN) previously cultured for 1 h at 37°C (blue bar) or 39.5°C (red bar). **(K)** Percentage of *P. aeruginosa* killing by neutrophils that were cultured at the indicated temperatures. *$p < 0.01$; two-way ANOVA with Bonferroni's multiple-comparisons test. NET, neutrophil extracellular trap; MOI, multiplicity of infection; GFP, green fluorescent protein; MPO, myeloperoxidase; FITC, fluorescein isothiocyanate; PI, propidium iodide; TRITC, tetramethylrhodamine; LB, Luria broth; CFUs, colony-forming units; PMA, phorbol myristate acetate; ANOVA, analysis of variance.

neutrophils with GFP fluorescence in the presence or absence of NaN_3 at either 37 or 39.5°C (**Figure 4H**). Furthermore, we evaluated the percentage of GFP-fluorescent neutrophils, which, without being permeabilized, were also able to bind an anti-GFP antibody, as a readout of the cells with surface-attached bacteria (**Figure 4I**). Less than 5% of neutrophils exhibited GFP bacteria bound to the cell surface in contrast to ∼70% that had ingested bacteria, but more importantly, no differences were found in the attached bacteria between both temperatures. Moreover, internalized bacteria could be visualized in a three-dimensional reconstruction from Z-stack images acquired by confocal microscopy of PAO-1-challenged neutrophils (**Videos S2, S3**). Collectively, these findings suggest that STFRH moderately reduces *P. aeruginosa* phagocytosis.

As STFRH inhibited *P. aeruginosa* phagocytosis but significantly increased NETosis, we then analyzed if STFRH modulates neutrophil microbicidal capacity against *P. aeruginosa*. To this end, we evaluated bacterial viability after co-culture for 4 h at 37°C with neutrophils that had been exposed for 1 h at either 37 or 39.5°C. As expected, co-culture with neutrophils markedly reduced the number of CFU than did bacteria that were cultured in the absence of neutrophils for 4 h (**Figure 4J**). However, STFRH did not significantly modulate neutrophil capacity to kill *P. aeruginosa* (**Figures 4J,K**), because when NETs were dismantled by DNase treatment, the number of CFU recovered from co-cultures of neutrophils subjected to each temperature was not significantly different. These findings suggest that hyperthermia might increase the capacity of neutrophils to wall off the infection but does not increase their microbicidal capacity, at least at this time point evaluated.

In another set of experiments, we analyzed whether STFRH also modulates neutrophils to secrete cytokines when challenged with bacteria. Thus, to avoid a potential contribution of a loss of viability to cytokine extracellular concentrations, we evaluated neutrophil response to *P. aeruginosa* at a lower MOI (0.1). To this end, we exposed neutrophils to either 37 or 39.5°C for 1 h and then challenged them with *P. aeruginosa* for three or four additional hours. Then, we determined the concentrations of CXCL8/IL-8, IL-1β, and TNF-α in culture supernatants. Results indicated that hyperthermia significantly reduced the secretion induced by *P. aeruginosa* of CXCL8/IL-8 at 4 h of culture and IL-1β at either 3 or 4 h of culture (**Figures 5A,B**). By contrast, TNF-α secretion was not significantly modulated by hyperthermia (**Figure 5C**). In order to rule out that the reduction in cytokine secretion observed was due to differential cell death, we also evaluated neutrophil viability in the pellets of the same samples by analyzing the number of cells that had lost plasma membrane integrity by Vivafix staining and flow cytometry. We did not detect significant differences in the percentages of dead cells at both temperatures when evaluated at either 3 or 4 h of culture (**Figure S5**). We obtained similar results when we evaluated cell viability by annexin V-FITC and PI staining (**Figure S6A**) and lytic cell death by analyzing the presence of LDH in cell supernatants (**Figure S6B**), even though *P. aeruginosa* was still able to induce some NETosis at this low MOI (0.1) (**Figure S7**). Altogether, these results indicated that STFRH reduces pro-inflammatory cytokine secretion upon challenge with *P. aeruginosa* and this effect is not associated with a decreased cell viability.

DISCUSSION

In this study, we determined that STFRH accelerates NETosis. Our findings indicated that PMA-induced NETs generation was increased when cells were exposed to hyperthermia for only 1 h. Moreover, we observed that ROS production triggered by PMA was also increased by STFRH, suggesting that this effect could contribute to the accelerated NETosis rates. By contrast, autophagy levels induced by PMA were not increased by STFRH, ruling out a causal relationship with NETosis acceleration. We also determined that STFRH accelerated NETosis induced by *P. aeruginosa* but reduced the capacity of neutrophils to phagocytose the bacteria. However, STFRH did not affect the neutrophil capacity to kill *P. aeruginosa* after 4 h of co-culture, suggesting that hyperthermia might contribute to bacterial ensnarement to avoid its dissemination.

Studies performed by Kettritz et al. indicated that short exposure (1 h) of neutrophils to 42°C modulated neither their spontaneous apoptosis nor the anti-apoptotic effect of GM-CSF, IL-8, and dexamethasone but prevented the anti-apoptotic effect triggered by LPS evaluated 20 h later (22). However, the authors showed that a short exposure of neutrophils to either 39 or 40°C

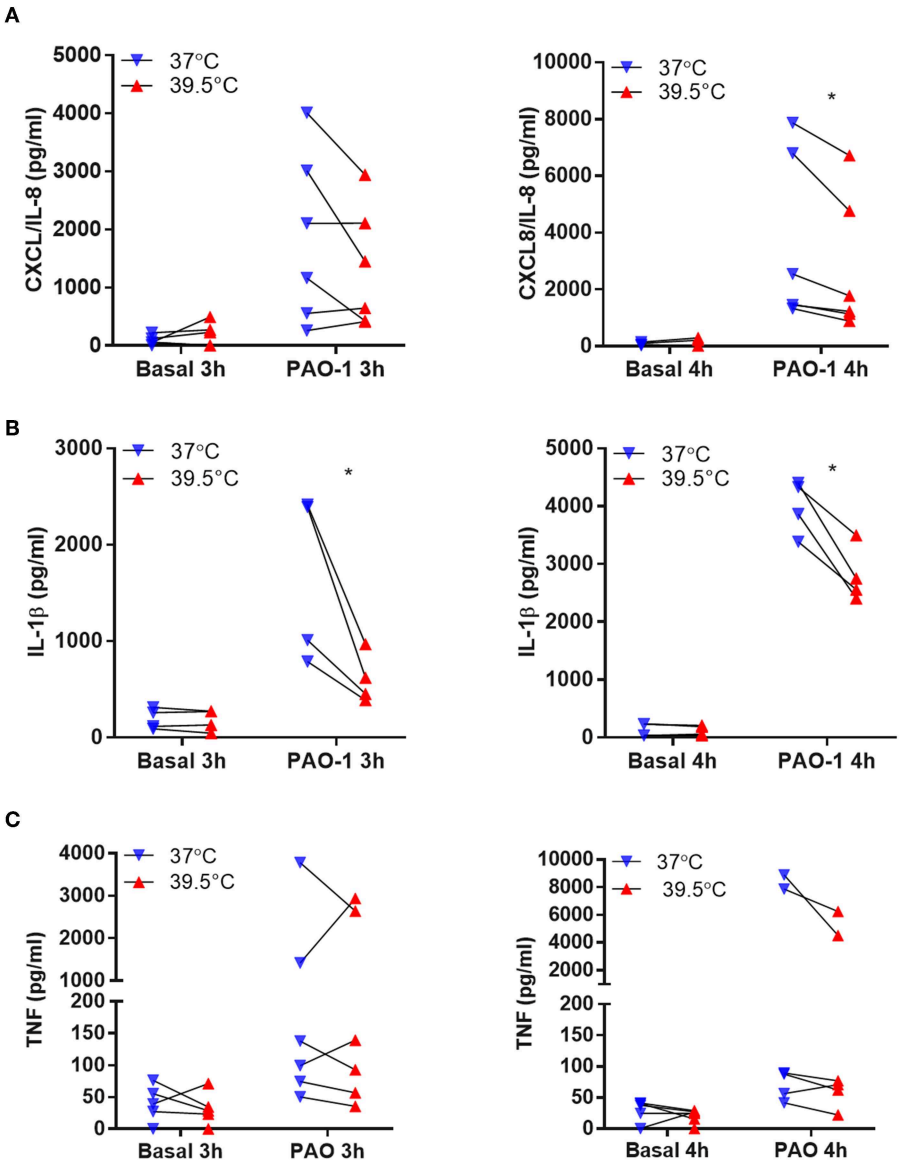

FIGURE 5 | Short-term fever-range hyperthermia reduces the pro-inflammatory cytokines response to *Pseudomonas aeruginosa*. Neutrophils were cultured for 1 h at 37 or 39.5°C and then were challenged with *P. aeruginosa* MOI 0.1 for 3 or 4 h. Afterwards, cells were centrifuged, supernatants were collected, and the concentrations of CXCL8/IL-8 **(A),** IL-1β **(B)**, and TNF-α **(C)** were determined by ELISA. Data depicted correspond to the mean value of cytokine concentrations secreted by 5×10^5 neutrophils of assays performed in triplicate per donor. *$p < 0.01$; two-way ANOVA with Bonferroni's multiple-comparisons test. MOI, multiplicity of infection; IL, interleukin; TNF-α, tumor necrosis factor alpha; ELISA, enzyme-linked immunosorbent assay; ANOVA, analysis of variance.

did not modulate their apoptosis levels after 20 h of being left unstimulated and, at this time point, slightly counteracted the LPS anti-apoptotic effects only upon short exposure at 40°C. In this study, we confirmed that STFRH does not modulate neutrophil spontaneous apoptosis and, in line with Kettritz et al.'s findings, determined that the ability of STFRH to prevent the LPS anti-apoptotic effect is already evident at early stages. Other studies showed that 90 min at 39 or 41°C inhibited the spontaneous apoptotic DNA fragmentation after 24 h of culture and preserved the membrane asymmetry characteristic of viable cells, even though it surprisingly decreased CD16 expression (26). Whether this hyperthermia-triggered behavior of unstimulated cells increases their propensity to undergo NETosis upon agonist stimulation remains to be determined. Other studies demonstrated that long-term FRH (8 h or more) accelerates caspase-dependent apoptosis (21), in contrast to the effects exerted by short-term exposure to fever-range temperatures. Thus, it appears that both the time extent to FRH and the temperature to which cells are exposed differentially modulate either spontaneous or induced cell death.

On the other hand, in studies addressing the effects of anti-inflammatory drugs on NET formation, similar to what we found here after 1 h of FRH, the authors showed an increased NETs release after 3 h of exposure of neutrophils at 40°C in the presence of PMA. However, in this study, only an ELISA to detect nucleosomes was employed as a readout of the presence of NETs (27).

We also found that in contrast to the impact on NET production, STFRH reduced *P. aeruginosa* phagocytosis by neutrophils. Moreover, our findings indicated that although neutrophils were able to kill *P. aeruginosa*, STFRH did not modulate this function, at least after 4 h of co-culture. However, we cannot rule out that longer exposure of bacteria to the network represented by NETs, which were increased by STFRH, can lead to differential killing at later time points. Our results let us speculate that the reduced capacity to kill the bacteria by phagocytosis observed upon STFRH conditions is counterbalanced by an increased NETosis.

Our studies with the caspase-1 inhibitor Ac-YVAD-cmk indicated that STFRH promotes the canonical NETosis mechanism that is a caspase-1-independent process (5). Recent studies indicated that *P. aeruginosa* PAO-1 after engulfment by macrophages is able to escape the phagosome to the cytosol (28). *Citrobacter rodentium* (a Gram-negative bacterium that is well-established to escape the phagosome) was shown to induce caspase-4-dependent NETosis of human neutrophils by non-canonical inflammasome activation (29). In the light of these new findings, we cannot rule out that STFRH is also enhancing NETosis that might be induced by PAO-1 through this pathway. Further studies are required to address this possibility.

Kettritz et al. also found that short neutrophil exposure (1 h) to 40°C inhibited NF-κB activation as it decreased LPS-induced IκBα degradation and IκBα mRNA expression (22). Results of our study indicating that STFRH inhibits the secretion of CXCL8/IL-8 and IL-1β, two cytokines that are synthesized in an NF-κB-dependent manner (25), are in line with these findings. These authors also determined that a short neutrophil exposure (1 h) to 40°C reduced TNF-α mRNA expression induced by LPS. However, in our study, STFRH did not significantly modulate neutrophil TNF-α secretion induced by *P. aeruginosa*, although a reduction in this cytokine secretion was observed in five out of six donors analyzed after 4 h of PAO-1 challenge.

Interestingly, in a mice model of experimental peritonitis induced by *Klebsiella pneumoniae*, FRH reduced by 100,000-fold the intraperitoneal bacterial burden and increased mice survival from 0 to 50% in comparison with that in normothermic infected animals (30). The authors showed that FRH did not affect the bacterial *in vitro* proliferation rates, which suggested that enhanced host defense mechanisms accounted for the reduction in pathogen load. However, in another study aimed to analyze FRH impact on pneumonia caused by the same pathogen, although FRH (core temperature ∼39°C) diminished in 400-fold intrapulmonary bacterial burden, unlike the peritonitis model, it conferred no survival advantage (11). Moreover, the authors found that hyperthermia also accounted for a marked increase in mortality either in the *K. pneumoniae*-induced pneumonia treated with antibiotics or in the intra-tracheal LPS challenge, which were both non-lethal in euthermic mice (11). Altogether, these findings lead the authors to suggest that the host response in the hyperthermic mice rather than the pathogens themselves might contribute to death. These findings suggested that differences in mice survival in the peritonitis and pneumonia models under hyperthermic conditions were a consequence of a balance between an expedited bacterial clearance and increased collateral tissue damage (31). In line with this possibility, FRH greatly accelerated lethal lung injury in a mice model of non-infectious injury caused by oxygen toxicity (12). Noteworthy, an increased neutrophil infiltration upon exposure to FRH was observed in both LPS and hyperoxia-induced acute lung injury models (11, 12). In previous studies, we demonstrated that NETs promote a pro-inflammatory response by stimulating the production of higher levels of CXCL8/IL-8 and IL-6 by airway epithelial cells and macrophages (18). Thus, regardless of the beneficial antimicrobial properties of NETs, their exacerbated production by STFRH might contribute to increased inflammation in response to Gram-negative bacterial infection.

Studies performed by Schauer et al. showed that neutrophil stimulation with monosodium urate crystals at high cellular density induces the formation of aggregated NETs structures, which proteolytically degrade cytokines and chemokines produced by mouse neutrophils and consequently reduce inflammation (32). Thus, it is tempting to speculate that the increased NET production promoted by STFRH might also contribute to the reduced cytokine levels observed under hyperthermic conditions. If this were the case, it might represent an additional mechanism to restrain excessive inflammation.

Studies performed by Bzowska et al. described that exposure of human neutrophils to STFRH in the absence of stimulation results in a non-phlogistic recognition by macrophages (26). Thus, additional mechanisms might also act in concert to lessen inflammation incited by hyperthermia not only by amplifying NET production but also by acting on other cell types (11, 31).

Collectively, the results of our study suggest that fever spikes could lead neutrophils to trigger an emergency response promoting NET formation to ensnare bacteria in order to wall off the infection. However, taking into account the pro-inflammatory properties ascribed to NETs (18, 33–35), we speculate that the ability of STFRH to prevent the LPS-induced anti-apoptotic effect together with its capacity to reduce neutrophil pro-inflammatory cytokine secretion might represent feedback mechanisms to limit an increased inflammatory response that might be detrimental for the host (**Figure S8**).

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethical Committee of the Institutos de la Academia Nacional de Medicina. The participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

IK and FS designed the experiments, carried out most of them, analyzed and interpreted the data. CS, DR, MM, and MG conducted some experiments. FF programmed the image quantification macro, and performed microscopy acquisitions together with IK and FS. CG and CL contributed to bacterial assays. JG, JRG, and CJ provided scientific expertise and contributed analysis and data interpretation. MIG and AT designed experiments and analyzed and interpreted the data. AT conceived the research and wrote the manuscript. All authors reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02374/full#supplementary-material

REFERENCES

1. Ng LG, Ostuni R, Hidalgo A. Heterogeneity of neutrophils. *Nat Rev Immunol*. (2019) 19:255–65. doi: 10.1038/s41577-019-0141-8
2. Kolaczkowska E, Kubes P. Neutrophil recruitment and function in health and inflammation. *Nat Rev Immunol*. (2013) 13:159–75. doi: 10.1038/nri3399
3. Amulic B, Cazalet C, Hayes GL, Metzler KD, Zychlinsky A. Neutrophil function: from mechanisms to disease. *Annu Rev Immunol*. (2012) 30:459–89. doi: 10.1146/annurev-immunol-020711-074942
4. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, et al. Neutrophil extracellular traps kill bacteria. *Science*. (2004) 303:1532–5. doi: 10.1126/science.1092385
5. Fuchs TA, Abed U, Goosmann C, Hurwitz R, Schulze I, Wahn V, et al. Novel cell death program leads to neutrophil extracellular traps. *J Cell Biol*. (2007) 176:231–41. doi: 10.1083/jcb.200606027
6. Boeltz S, Amini P, Anders HJ, Andrade F, Bilyy R, Chatfield S, et al. To NET or not to NET:current opinions and state of the science regarding the formation of neutrophil extracellular traps. *Cell Death Differ*. (2019) 26:395–408. doi: 10.1038/s41418-018-0261-x
7. Menegazzi R, Decleva E, Dri P. Killing by neutrophil extracellular traps: Fact or folklore? *Blood*. (2012) 119:1214–6. doi: 10.1182/blood-2011-07-364604
8. Tamassia N, Bianchetto-Aguilera F, Arruda-Silva F, Gardiman E, Gasperini S, Calzetti F, et al. Cytokine production by human neutrophils: revisiting the "dark side of the moon." *Eur J Clin Invest*. (2018) 48(Suppl. 2):e12952. doi: 10.1111/eci.12952
9. Mitroulis I, Kalafati L, Hajishengallis G, Chavakis T. Myelopoiesis in the context of innate immunity. *J Innate Immun*. (2018) 10:365–72. doi: 10.1159/000489406
10. Evans SS, Repasky EA, Fisher DT. Fever and the thermal regulation of immunity: the immune system feels the heat. *Nat Rev Immunol*. (2015) 15:335–49. doi: 10.1038/nri3843
11. Rice P, Martin E, He JR, Frank M, DeTolla L, Hester L, et al. Febrile-range hyperthermia augments neutrophil accumulation and enhances lung injury in experimental gram-negative bacterial pneumonia. *J Immunol*. (2005) 174:3676–85. doi: 10.4049/jimmunol.174.6.3676
12. Hasday JD, Garrison A, Singh IS, Standiford T, Ellis GS, Rao S, et al. Febrile-range hyperthermia augments pulmonary neutrophil recruitment and amplifies pulmonary oxygen toxicity. *Am J Pathol*. (2003) 162:2005–17. doi: 10.1016/S0002-9440(10)64333-7
13. Tulapurkar ME, Almutairy EA, Shah NG, He J, Puche AC, Shapiro P, et al. Febrile-range hyperthermia modifies endothelial and neutrophilic functions to promote extravasation. *Am J Respir Cell Mol Biol*. (2012) 46:807–14. doi: 10.1165/rcmb.2011-0378OC
14. Rosenspire AJ, Kindzelskii AL, Petty HR. Cutting edge: fever-associated temperatures enhance neutrophil responses to lipopolysaccharide: a potential mechanism involving cell metabolism. *J Immunol*. (2014) 169:5396–400. doi: 10.4049/jimmunol.169.10.5396
15. Sebag J, Reed WP, Williams RC. Effect of temperature on bacterial killing by serum and by polymorphonuclear leukocytes. *Infect Immun*. (1977) 16:947–54.
16. Roberts NJ, Steigbigel RT. Hyperthermia and human leukocyte functions: effects on response of lymphocytes to mitogen and antigen and bactericidal capacity of monocytes and neutrophils. *Infect Immun*. (1977) 18:673–9.
17. Iula L, Keitelman IA, Sabbione F, Fuentes F, Guzman M, Galletti JG, et al. Autophagy mediates interleukin-1β secretion in human neutrophils. *Front Immunol*. (2018) 9:269. doi: 10.3389/fimmu.2018.00269
18. Sabbione F, Keitelman IA, Iula L, Ferrero M, Giordano MN, Baldi P, et al. Neutrophil extracellular traps stimulate proinflammatory responses in human airway epithelial cells. *J Innate Immun*. (2017) 9:387–402. doi: 10.1159/000460293
19. Schwartz J, Leidal KG, Femling JK, Weiss JP, Nauseef WM. Neutrophil bleaching of GFP-expressing staphylococci: probing the intraphagosomal fate of individual bacteria. *J Immunol*. (2009) 183:2632–41. doi: 10.4049/jimmunol.0804110
20. Díaz FE, Dantas E, Cabrera M, Benítez CA, Delpino MV, Duette G, et al. Fever-range hyperthermia improves the anti-apoptotic effect induced by low pH on human neutrophils promoting a proangiogenic profile. *Cell Death Dis*. (2016) 7:e2437. doi: 10.1038/cddis.2016.337
21. Nagarsekar A, Greenberg RS, Shah NG, Singh IS, Hasday JD, Singh IS, et al. Febrile-range hyperthermia accelerates caspase-dependent apoptosis in human neutrophils. *J Immunol*. (2014) 181:2636–43. doi: 10.4049/jimmunol.181.4.2636
22. Kettritz R, Choi M, Salanova B, Wellner M, Rolle S, Luft FC. Fever-like temperatures affect neutrophil NF-kappaB signaling, apoptosis, and ANCA-antigen expression. *J Am Soc Nephrol*. (2006) 17:1345–53. doi: 10.1681/ASN.2005090948
23. Remijsen Q, Vanden Berghe T, Wirawan E, Asselbergh B, Parthoens E, De Rycke R, et al. Neutrophil extracellular trap cell death requires both autophagy and superoxide generation. *Cell Res*. (2011) 21:290–304. doi: 10.1038/cr.2010.150
24. Sollberger G, Choidas A, Burn GL, Habenberger P, Di Lucrezia R, Kordes S, et al. Gasdermin D plays a vital role in the

generation of neutrophil extracellular traps. *Sci Immunol*. (2018) 3:eaar6689. doi: 10.1126/sciimmunol.aar6689
25. Abraham E. Nuclear factor—κB and its role in sepsis-associated organ failure. *J Infect Dis*. (2003) 187(Suppl. 2):S364–9. doi: 10.1086/374750
26. Bzowska M, Hamczyk M, Skalniak A, Guzik K. Rapid decrease of CD16 (FcγRIII) expression on heat-shocked neutrophils and their recognition by macrophages. *J Biomed Biotechnol*. (2011) 2011:1–14. doi: 10.1155/2011/284759
27. Lapponi MJ, Carestia A, Landoni VI, Rivadeneyra L, Etulain J, Negrotto S, et al. Regulation of neutrophil extracellular trap formation by anti-inflammatory drugs. *J Pharmacol Exp Ther*. (2013) 345:430–7. doi: 10.1124/jpet.112.202879
28. Garai P, Berry L, Moussouni M, Bleves S, Blanc-Potard AB. Killing from the inside: intracellular role of T3SS in the fate of *Pseudomonas aeruginosa* within macrophages revealed by mgtC and oprF mutants. *PLoS Pathog*. (2019) 15:e1007812. doi: 10.1371/journal.ppat.1007812
29. Chen KW, Monteleone M, Boucher D, Sollberger G, Ramnath D, Condon ND, et al. Noncanonical inflammasome signaling elicits gasdermin D–dependent neutrophil extracellular traps. *Sci Immunol*. (2018) 3:eaar6676. doi: 10.1126/sciimmunol.aar6676
30. Jiang Q, Hasday JD, Viscardi RM, Singh IS, Chen TT, Cross AS. Febrile core temperature is essential for optimal host defense in bacterial peritonitis. *Infect Immun*. (2002) 68:1265–70. doi: 10.1128/iai.68.3.1265-1270.2000
31. Hasday JD, Mackowiak PA, Tulapurkar M, Nagarsekar A, Singh I. Fever, hyperthermia and the lung. *Trans Am Clin Climatol Assoc*. (2011) 122:34–47.
32. Schauer C, Janko C, Munoz LE, Zhao Y, Kienhöfer D, Frey B, et al. Aggregated neutrophil extracellular traps limit inflammation by degrading cytokines and chemokines. *Nat Med*. (2014) 20:511–7. doi: 10.1038/nm.3547
33. Saffarzadeh M, Juenemann C, Queisser MA, Lochnit G, Galuska SP, Lohmeyer J, et al. Neutrophil extracellular traps directly induce epithelial and endothelial cell death: a predominant role of histones. *PLoS ONE*. (2012) 7:e32366. doi: 10.1371/journal.pone.0032366
34. Narasaraju T, Yang E, Samy RP, Ng HH, Poh WP, Liew AA, et al. Excessive neutrophils and neutrophil extracellular traps contribute to acute lung injury of influenza pneumonitis. *Am J Pathol*. (2011) 179:199–210. doi: 10.1016/j.ajpath.2011.03.013
35. Thomas GM, Carbo C, Curtis BR, Martinod K, Mazo IB, Schatzberg D, et al. Extracellular DNA traps are associated with the pathogenesis of TRALI in humans and mice. *Blood*. (2012) 119:6335–43. doi: 10.1182/blood-2012-01-405183

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REVIEW
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The Regulatory Effects of Interleukin-4 Receptor Signaling on Neutrophils in Type 2 Immune Responses

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Interleukin-4 (IL-4) receptor (IL-4R) signaling plays a pivotal role in type 2 immune responses. Type 2 immunity ensures several host-protective processes such as defense against helminth parasites and wound repair, however, type 2 immune responses also drive the pathogenesis of allergic diseases. Neutrophil granulocytes (neutrophils) have not traditionally been considered a part of type 2 immunity. While neutrophils might be beneficial in initiating a type 2 immune response, their involvement and activation is rather unwanted at later stages. This is evidenced by examples of type 2 immune responses where increased neutrophil responses are able to enhance immunity, however, at the cost of increased tissue damage. Recent studies have linked the type 2 cytokines IL-4 and IL-13 and their signaling via type I and type II IL-4Rs on neutrophils to inhibition of several neutrophil effector functions. This mechanism directly curtails neutrophil chemotaxis toward potent intermediary chemoattractants, inhibits the formation of neutrophil extracellular traps, and antagonizes the effects of granulocyte colony-stimulating factor on neutrophils. These effects are observed in both mouse and human neutrophils. Thus, we propose for type 2 immune responses that neutrophils are, as in other immune responses, the first non-resident cells to arrive at a site of inflammation or infection, thereby guiding and attracting other innate and adaptive immune cells; however, as soon as the type 2 cytokines IL-4 and IL-13 predominate, neutrophil recruitment, chemotaxis, and effector functions are rapidly shut off by IL-4/IL-13-mediated IL-4R signaling in neutrophils to prevent them from damaging healthy tissues. Insight into this neutrophil checkpoint pathway will help understand regulation of neutrophilic type 2 inflammation and guide the design of targeted therapeutic approaches for modulating neutrophils during inflammation and neutropenia.

Keywords: neutrophil, type 2 immunity, interleukin-4, interleukin-13, interleukin-4 receptor, inflammation, helminth, neutropenia

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INTRODUCTION

Neutrophil granulocytes (neutrophils) are the most abundant leukocytes in human blood accounting for ∼60–70% of immune cells in circulation at steady state (1). With a very short life span of approximately 5 days, there is a constant need for replenishment of this vast pool (2). Neutrophils are generated in the bone marrow at a rate of 1–2 × 10^{11} cells per day (3). Their release

from the bone marrow is regulated by the signaling of C-X-C chemokine receptors (CXCR) 2 and 4. CXCR2-binding chemokines, CXCL8 in humans and CXCL1 and CXCL2 in mice, promote the mobilization of neutrophils and their egress into the blood stream (4, 5). Conversely, the engagement of CXCR4 with its ligand CXCL12 presented on bone marrow stromal cells keeps the newly-formed granulocytes in their bone marrow niche (6). At steady state, this delicate interplay ensures the maintenance of a stable peripheral blood pool of neutrophils. In case of inflammation or infection, pro-inflammatory mediators, such as granulocyte colony-stimulating factor (G-CSF), are produced by the affected tissue and shift the balance toward increased neutrophil generation and mobilization, for example by increasing CXCR2 and decreasing CXCR4 surface expression on neutrophils (7, 8).

Neutrophils are typically the first non-resident immune cells that arrive at a site of inflammation (9, 10). Upon local activation of the vasculature, endothelial cells present chemokines and cell adhesion molecules at their luminal side (11). These ligands are recognized by their counterparts on the surface of bypassing neutrophils, leading to deceleration and eventually firm adhesion of the leukocytes to the endothelium. The neutrophils then crawl along the vessel wall following fixed gradients of so-called intermediary chemoattractants, particularly CXCR2-binding chemokines, before they transmigrate through the endothelium into the interstitial space. There, they advance to their destination by tracking gradients of other chemotactic stimuli such as N-formylmethionine-leucyl-phenylalanine (fMLP) released by bacteria (12, 13). These end-target chemoattractants override the signals emanating from intermediary chemokines (14). Once they reach the site of infection, neutrophils employ various mechanisms to kill or inactivate pathogens, including phagocytosis, degranulation, production of reactive oxygen species (ROS), and neutrophils extracellular trap (NET) formation (**Figure 1**; discussed in section Neutrophil Effector Functions).

Given the abundance and readiness of these heavily-armed immune cells, overshooting neutrophil activity can lead to detrimental tissue damage. Thus, mechanisms need to be in place, which keep neutrophil responses in check but at the same time allow efficient clearance of pathogens. Although neutrophils have traditionally been associated with type 1 and type 3 immunity, they have recently been found to contribute to type 2 immune responses, thus also aiding in the removal of helminth parasites (15, 16). Conversely, neutrophils are often conspicuously absent from tissues where the type 2 cytokines interleukin (IL)-4 and IL-13 dominate (17, 18). In this review, we will propose a mechanism that connects these seemingly contradictory findings. We will first summarize beneficial and harmful effects of neutrophils in type 1, type 2, and type 3 immune responses. We will then focus on a growing body of evidence that suggests involvement of neutrophils in type 2 immunity. Finally, we will discuss recent results from mouse and human neutrophil research that have demonstrated how IL-4 and IL-13 receptor signaling inhibits several neutrophil effector functions (19–21), and we will elaborate on a temporal model unifying the reported findings.

NEUTROPHIL EFFECTOR FUNCTIONS

Phagocytosis

The most prominent neutrophil effector function is phagocytosis, the process during which pathogens are recognized, encapsulated and internalized in membranous vesicles, and digested by fusion of the vesicles with lysosomes and granules containing enzymes and antimicrobial peptides. The recognition step usually depends on pathogen- and damage-associated molecular patterns (PAMPs and DAMPs, respectively) as well as opsonization of the pathogen by antibodies or complement factors that bind to Fc or complement receptors on the neutrophil, respectively (22–24). Engagement of these receptors leads to the active engulfment of the pathogen. Subsequently, lysosomal hydrolases and granular enzymes are released into the vesicle by fusion with the respective organelles and start the destruction of the phagocytosed material (25). Additionally, the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex is assembled on the phagolysosome membrane, leading to the formation and discharge of superoxide anions (O_2^-) into the lumen. These harm pathogens directly or react further into other ROS, all of which have antimicrobial activity (see below) (26).

Degranulation

Neutrophils possess a large pool of intracellular vesicles (granules) whose contents can be released into the extracellular space or into phagosomes in a process called degranulation. Not all neutrophil granules are the same: There are four very distinct types of secretory organelles that can be distinguished by their content, membrane proteins, function, and time point of mobilization. Primary or azurophilic granules contain the most potent cargo: myeloperoxidase (MPO), defensins, neutrophil elastase (NE), and cathepsins. These proteins are powerful antimicrobial agents and are released only after several checkpoints have been passed and the neutrophil is in closest proximity to the pathogen. Secondary or specific granules also accommodate antimicrobial factors (e.g., lactoferrin, neutrophil-gelatinase associated lipocalin) together with matrix metalloproteases (MMPs) neutrophil collagenase (MMP-8) and leukolysin (MMP-25). The antimicrobial factors provide protection against pathogens while the MMPs can degrade extracellular matrix and thus enable more efficient neutrophil migration through tissues. Tertiary or gelatinase granules predominantly contain their namesake gelatinase (MMP-9) which also facilitates migration. The fourth type are secretory vesicles, which serve as reservoirs of membrane proteins that are needed on the cell surface upon priming, such as the CD11b–CD18 heterodimer ($\alpha_M\beta_2$-integrin, Mac-1, complement receptor 3), CD35 (complement receptor 1), and the fMLP receptor. They do not contain notable soluble cargo. Besides this multitude of proteins working *in situ*, neutrophils are also capable of releasing several chemokines, cytokines, and growth factors that recruit other immune cells to a site of inflammation (27–32).

Despite being an essential means of combating infection, degranulation can also cause serious damage if not kept in check. While MMPs may be beneficial for cell migration, they can also destroy the connective tissue, and several constituents of

the azurophilic granules not only kill pathogens, but are also cytotoxic to host cells (33) (**Figure 1**). Moreover, uncontrolled recruitment and activation of immune cells leads to excessive inflammation. It is thus not surprising that over-shooting degranulation of neutrophils has been associated with several inflammatory disorders such as septic shock, severe lung injury, rheumatoid arthritis, and severe asthma (34, 35).

Reactive Oxygen Species

ROS are a group of small, unstable oxygen-based molecules. Owing to their chemical instability, they are highly reactive and can denature or damage proteins, lipids, and DNA (36). In neutrophils, ROS are produced in a process called respiratory or oxidative burst by the multi-unit enzyme complex NADPH oxidase (37). The different subunits of the enzyme are stored in separate compartments of the cell to prevent accidental generation of ROS. Upon activation, the NADPH oxidase assembles on the phagosomal or plasma membrane and produces and releases superoxide anions into the phagosome or extracellular space, respectively (38). Superoxide can react further with protons to hydrogen peroxide (H_2O_2), which is used by MPO to create hypochlorous acid (HClO) (39). All these types of ROS have microbicidal activity, but also consume protons in their chain of reactions, thereby neutralizing the acidic content from lysosomes and granules (40). This in turn facilitates the liberation of NE and cathepsins, which at low pH are tightly bound to proteoglycans and thus less active (41). In fact, ROS likely participate less in direct pathogen killing, but they rather facilitate proper activation of azurophilic granule enzymes, which cause pathogen killing in the phagosome (42).

Whether ROS are directly or indirectly responsible for pathogen killing, they are a vital part of innate immunity. In fact, bacterial strains that are able to disarm ROS by producing superoxide dismutase (SOD), which catalyzes the reaction of O_2^- into O_2 and H_2O_2, and catalase, which in turn catalyzes the decomposition of H_2O_2 into O_2 and H_2O, are much more virulent than their SOD- or catalase-negative counterparts (43). Another example is chronic granulomatous disease (CGD), a genetic disorder affecting the NADPH oxidase, which renders patients incapable of producing ROS. These patients suffer from frequent and recurrent infections, also with opportunistic pathogens (44). Despite their importance in combatting infection, unchecked production of extracellular ROS leads to tissue damage by virtue of their lack of pathogen specificity (45) (**Figure 1**).

Neutrophil Extracellular Traps

NETs are meshes of DNA decorated with antimicrobial peptides that can be released by neutrophils in response to various stimuli. They were first described by Brinkmann et al. as a novel mechanism of how neutrophils can combat infection (46). Pathogens, mainly yeast and bacteria, stick to the DNA fibrils, which prevents them from spreading in the tissue, and they are degraded by the granule proteins that are attached to the chromatin network (47). Since 2004, numerous stimuli have been described to induce NET formation, of which large pathogens seem to be the main trigger (48). The exact process of how NETs form remains an active area of research. The most widely accepted model involves chromatin decondensation including histone citrullination, disintegration of nuclear, and granule membranes, intracellular mixing of the components and, finally, release into the extracellular space (49). Some reports provided evidence that the NADPH oxidase was necessary for NET formation. Interestingly, however, despite their lack of a functional NADPH oxidase CGD patients have been shown to form NETs by using mitochondrial ROS (50). It seems that depending on signal type and strength, NET formation can be fast and non-lytic, leaving behind an intact anuclear cytoplast (51), or slow and lytic, spilling the cell contents while the NET breaks free from the cell membrane (52). Some studies also present the possibility of NET release by living cells using mitochondrial in lieu of nuclear DNA (50, 53).

NETs have been shown to have several beneficial properties. Their main use is in immobilizing and degrading bacteria, fungi, and viruses (54–56). Another less prominent function is the shielding of damaged tissues that might otherwise elicit an unwanted inflammation (57). As helpful these mechanisms may be, NETs have also been implicated as players in a multitude of different diseases. Firstly, the release of nuclear material into the extracellular space provides access to otherwise shielded antigens and may result in the formation of autoantibodies (**Figure 1**), as suggested for rheumatoid arthritis (RA), systemic lupus erythematodes, anti-phospholipid syndrome, and anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (58–61). Secondly, the massive release of proteins can be a double threat. On the one hand, cytokines may drive inflammation leading to tissue damage or atherosclerosis (62). On the other hand, the proteases associated with NETs may degrade cytokines and chemokines, resulting in a possibly unwanted anti-inflammatory effect (63). Moreover, the giant and sticky structures of NETs can occlude blood vessels, leading to thrombosis or sepsis (14). Finally, NETs have also been proposed as players in cancer dissemination and metastasis formation (64, 65) (**Figure 1**).

ROLE OF NEUTROPHILS IN DIFFERENT TYPES OF IMMUNE RESPONSES

The immune system has evolved different types of effector immune responses to counter the various pathogens. These are commonly referred to as type 1, type 2, and type 3 immunity, and each engage different subtypes of innate lymphoid cells (ILCs) and other innate immune cells, $CD4^+$ helper T (T_H) and $CD8^+$ cytotoxic T cells, as well as $CD4^+$ follicular helper T (T_{FH}) cells and antibody responses by B cells, as discussed below (**Figure 2**).

Type 1 Immunity

Type 1 immunity serves to protect against intracellular pathogens by the production of the effector cytokine interferon-γ (IFNγ) and engagement of type 1 ILCs (ILC1), natural killer (NK) cells, type 1 T_H (T_H1) cells, $CD8^+$ cytotoxic T cells, type 1 T_{FH} cells and immunoglobulin (Ig) G1 and IgG3 antibody responses. Together with these immune cells, also macrophages

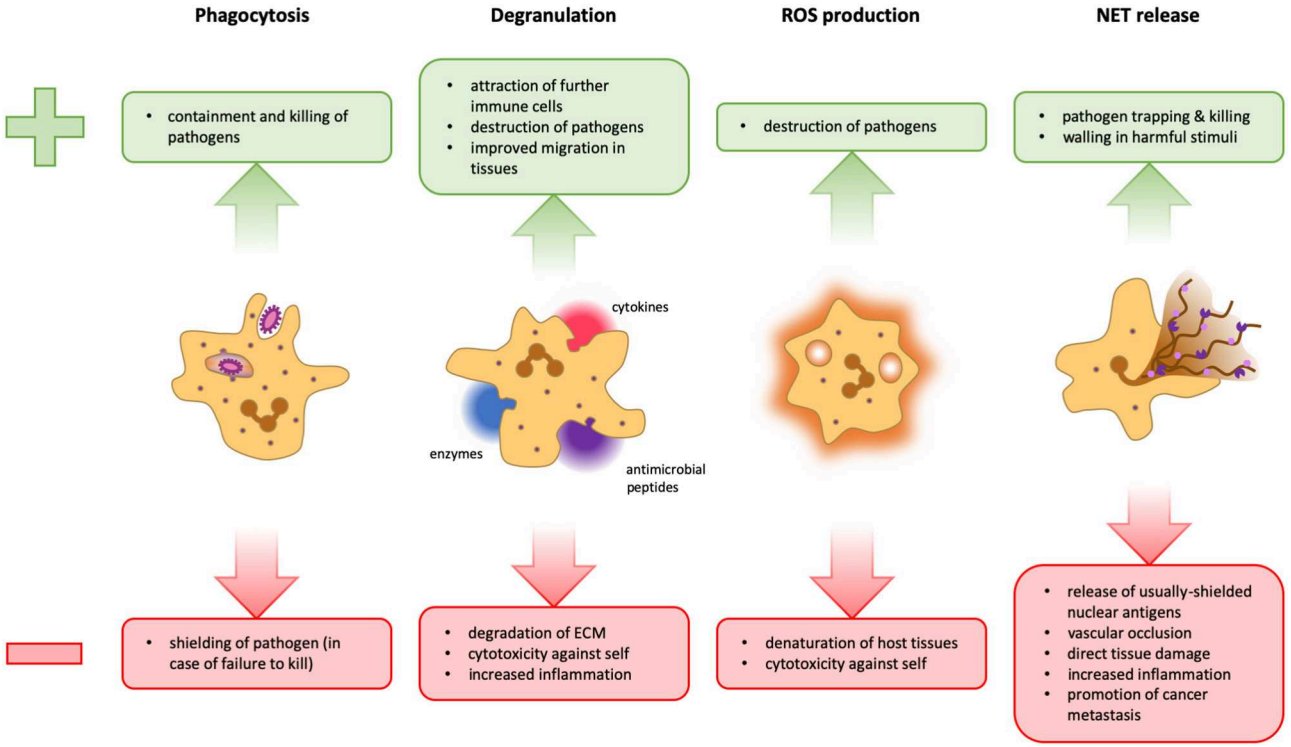

FIGURE 1 | Advantages and disadvantages of neutrophil effector functions. During phagocytosis, microbes are engulfed and degraded in specialized organelles called phagolysosomes. This is a very powerful and clean method to dispose of pathogens because it takes place in a contained space, thus preventing widespread inflammation. However, should a pathogen manage to survive intracellular degradation, it is protected from extracellular factors and other immune cells that could potentially contain it **(Left)**. Neutrophils can release several different cytokines, antimicrobial peptides, and granular enzymes into their surroundings, a response termed degranulation. This mechanism facilitates migration within the tissue, activates and attracts other immune cells and can help fight pathogens that cannot be phagocytosed. However, the release of too many cytokines can lead to an overshooting immune activation. Tissue degradation could aid the spreading of pathogens and destroy the structural basis of organs, and several granular proteins are also toxic for host cells **(Middle Left)**. Neutrophils can release reactive oxygen species (ROS) into phagolysosomes as well as into the extracellular space. These molecules can potentiate pathogen killing, but are not selective and can therefore also damage host cells **(Middle Right)**. The formation of NETs can be an efficient means to trap and kill pathogens and potentially walling off harmful stimuli. However, the release of cytoplasmic and nuclear proteins can cause the formation of autoantibodies, favoring autoimmunity. Furthermore, NETs can obstruct blood vessels and glandular ducts leading to inflammation. Granular proteins attached to the chromatin fibrils damage host tissue and the release of pro-inflammatory mediators may result in overshooting inflammation. Moreover, NETs have also been implicated in the facilitation of tumor metastasis **(Right)**.

and neutrophils play a central role in type 1 immunity. These innate cells can directly detect foreign microbes via PAMPs, which they recognize by pattern recognition receptors (PRRs) (66). During infection with the intracellular pathogen *Listeria monocytogenes*, neutrophils were shown to have an important protective role. This was particularly seen in the liver, which is one of the primary target organs of this bacteria. Here, the early phagocytosis mediated by recruited neutrophils was essential for limiting bacterial spread and controlling infection (67–69). It has also been suggested that IFNγ has a direct effect on neutrophil activation and can induce MHC class II expression, at least *ex vivo* (70). Although macrophages have a protective role against intracellular pathogens, they are also very susceptible to becoming infected themselves. In the case of *Mycobacterium tuberculosis* infection of macrophages, it has been reported that neutrophil granules can be transferred to macrophages and facilitate the clearance of chronically infected cells (71).

Type 2 Immunity

Type 2 immunity has evolved to efficiently induce resistance and tolerance to parasitic infections, especially helminthic infestations. Type 2 immunity is typically initiated by the activation of epithelial cells and PRR-expressing myeloid cells that secrete IL-25, IL-33, and thymic stromal lymphopoietin (TSLP). In response to these cytokines, type 2 ILCs (ILC2) begin to produce IL-5 and IL-13, which induce the differentiation of $CD4^+$ T cells to type 2 T_H (T_H2) cells, which in turn secrete the characteristic type 2 cytokines IL-4, IL-5, IL-9, and IL-13 (72–74). This cytokine milieu fosters the development and proliferation of other cells involved in type 2 immunity, including basophils, eosinophils, mast cells, and NKT cells, and drives the differentiation of type 2 T_{FH} cells and IgE antibody responses. Especially IL-4 and IL-13 are central cytokines in type 2 immunity, and they both signal via the IL-4 receptor (IL-4R) system (75). Two types of IL-4Rs exist (**Figure 3**): type I IL-4Rs consist of IL-4Rα and the common gamma chain

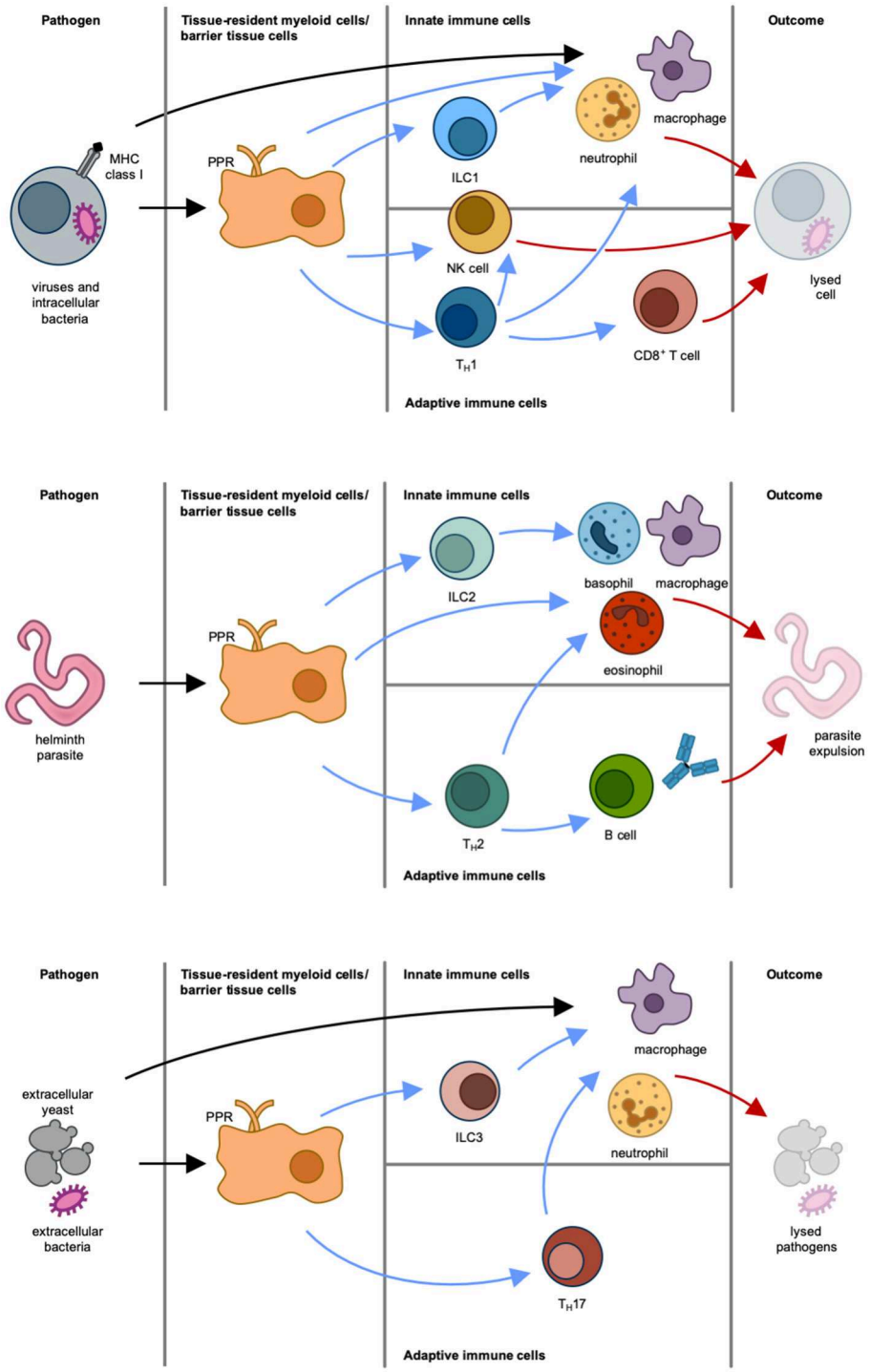

FIGURE 2 | Different types of immune responses. Type 1 immune response **(Top):** Intracellular pathogens induce differentiation of naïve T cells into T_H1 cells via antigen-presenting cells and IL-12. IFNγ is the main effector cytokine that stimulates and actives NK cells, type 1 innate lymphoid cells (ILC1), and T_H1 cells, which in turn activate $CD8^+$ cytotoxic T cells, macrophages and neutrophils. NK cells, macrophages and neutrophils can also be directly activated via pattern recognition receptor (PRR)-mediated recognition of pathogen-associated molecular patterns (PAMPs). The result of this immune response is lysis of cell and pathogen. Type 2 immune response **(Middle):** Parasites induce epithelial damage, which leads to the release of thymic stromal lymphopoietin (TSLP), IL-25, and IL-33 from

(Continued)

FIGURE 2 | epithelial cells. This in turn causes both differentiation of naïve T cells into T_H2 cells via antigen-presenting cells and stimulation of ILC2, mast cells, basophils and eosinophils. In response to this, the activated immune cells produce IL-4, IL-5, IL-9, and IL-13. These effector cytokines stimulate B cells and induce isotype switching to immunoglobulin E and differentiation of macrophages to alternatively-activated macrophages (also termed M2 macrophages). The result of this immune response is parasite expulsion. Type 3 immune response **(Bottom)**: Extracellular pathogens induce differentiation of naïve T cells into T_H17 cells via antigen-presenting cells and IL-23. Production of IL-17 and IL-22 by ILC3 and T_H17 cells leads to the activation of macrophages and neutrophils. The result of this immune response is pathogen killing. Initiation of immune response is indicated by black arrows. Blue arrows mark stimulatory signals by cytokines produced by immune cells. Effector responses are indicated by red arrows.

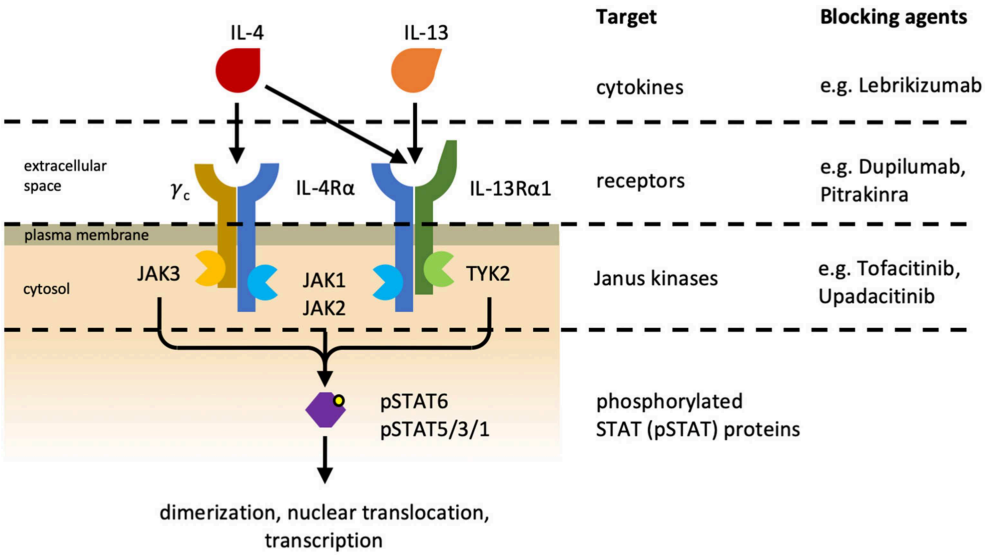

FIGURE 3 | Interleukin-4 receptors. Different heterodimeric IL-4 receptors (IL-4Rs) exist and they share the IL-4Rα subunit. The type I IL-4R **(Left)** consists of IL-4Rα and the common gamma chain cytokine receptor (γ_C), and the type II IL-4R **(Right)** is made of IL-4Rα and IL-13Rα1. IL-4 can associate with and signal via both IL-4Rs, whereas IL-13 can only bind to and signal via the type II IL-4R. Additionally, IL-13 can bind to IL-13Rα2 (not depicted), which is referred to as non-signaling decoy receptor. Cytokine-mediated receptor dimerization leads to the activation of receptor-associated Janus kinases (JAK) and consequently to the phosphorylation and activation of signal transducer and activator of transcription (STAT) proteins. Phosphorylated STAT (pSTAT) proteins subsequently dimerize and translocate to the nucleus where they initiate transcription of their target genes. Different treatment strategies have been developed to inhibit IL-4R signaling. Lebrikizumab is a neutralizing anti-IL-13 antibody. Pitrakinra, an IL-4 antagonist, and Dupilumab, an IL-4Rα-blocking antibody, are both preventing cytokine–receptor interaction. JAKs can also be inhibited pharmaceutically by small molecules. Upadacitinib is a selective JAK1 inhibitor currently being investigated in clinical trials, whereas Tofacitinib inhibits both JAK1 and JAK3 and is approved for the treatment of rheumatoid arthritis and psoriatic arthritis (76).

cytokine receptor, whereas type II IL-4Rs are made of IL-4Rα and IL-13Rα1. IL-4 can signal via both type I and type II IL-4Rs, whereas IL-13 only signals via the type II IL-4R. Additionally, IL-13 can bind to IL-13Rα2, which is thought to be a non-signaling decoy receptor of very high affinity to IL-13. Currently, there are several treatment strategies in clinical use that interfere with IL-4R signaling. For targeting both IL-4 and IL-13 signaling Dupilumab, a monoclonal antibody blocking IL-4Rα, and Pitrakinra, an IL-4Rα antagonist, exist. For more selectively blocking of IL-13, Lebrikizumab, a monoclonal antibody targeting IL-13, is of use. Downstream of the IL-4R, the Janus kinases (JAKs) JAK1, JAK2, and JAK3 can the inhibited by the use of the small molecule JAK inhibitors Upadacitinib and Tofacitinib (see also section Biologics and Small Molecules Targeting the IL-4R Signaling Axis).

Upon the establishment of a type 2 cytokine environment a positive feed-back loop is initiated where more naïve T cells differentiate into T_H2 cells and stimulation of eosinophils, basophils, and ILC2 takes place. B cells respond to type 2 cytokines by isotype switching to IgE and production of antibodies. Macrophages develop under the influence of type 2 cytokines into alternatively-activated macrophages (also termed M2 macrophages).

At barrier organs, such as the skin, lungs, and intestine, ILC2 are more numerous than in internal organs without barrier function. As ILC2 likely serve to amplify type 2 immune responses initiated by tissue and tissue-resident myeloid cells carrying PRRs, it is conceivable that type 2 immune responses at non-barrier sites differ from those at external and internal barriers. In this context, cobalt chromium microparticles injected intraperitoneally caused a type 2 inflammation dependent on IL-33 release by macrophages, followed by recruitment of neutrophils and production of IL-4, IL-5, IL-13, arginase-1, chitinase-like protein 3 (Chil3 or Ym1), and resistin-like molecule (RELM)-α (77).

In the lung, type 2 immune responses induce goblet cell hyperplasia. These cells produce mucins and anti-nematode protein RELM-β (78, 79). RELM-β together with RELM-α and arginase-1, produced by epithelial cells and fibroblasts, respectively, are also involved in tissue repair and deposition

of extracellular matrix, which in the context of helminth infections can serve to encapsulate and trap the parasite. Both IgG and IgE antibodies, produced by B cells, help to limit the motion and fecundity of the worms (80). Antibodies are also crucial for surface labeling of pathogens, which favors opsonization and antibody-dependent cell-mediated cytotoxicity (ADCC). ADCC is mediated via Fc receptors expressed on many innate immune cells. Altogether, these Fc receptor-bearing cells contribute to the reduction of worm fitness, lower transmission, and expulsion, which all serve to protect the body from nematode infections.

Whether and how neutrophils are involved in type 2 immunity and which role the affected barrier vs. non-barrier tissue plays, remains unclear. In mouse models of helminth infection, neutrophils have been reported to have a beneficial role during the early pulmonary stages (15), however this also comes at the price of increased tissue damage (81, 82). Conversely, in human type 2 inflammatory disorders neutrophils have been reported to be absent (17). The role of neutrophils in type 2 immune responses will be further discussed later.

Uncontrolled type 2 immune responses can lead to the development of allergic diseases, including allergic conjunctivitis, allergic rhinitis, allergic asthma, allergic gastrointestinal disorders, and atopic dermatitis (AD). These diseases are characterized by elevated levels of type 2 cytokines and accumulation of the above-mentioned immune cells (83). Dupilumab, a monoclonal antibody blocking IL-4Rα (**Figure 3**), has been shown to be effective as a treatment of moderate-to-severe AD and moderate-to-severe asthma (84, 85). Thus, interference with this central type 2 cytokine receptor subunit can control some of these allergic diseases.

Type 3 Immunity

Type 3 immunity is typically directed against extracellular bacterial and fungal infections, and it is characterized by the presence of the effector cytokines IL-17 and IL-22, which are prominently synthesized by type 3 ILCs (ILC3) and IL-17-producing T_H (T_H17) cells (86). Moreover, IL-26, TNF, and granulocyte-macrophage colony-stimulating factor (GM-CSF) are also produced during type 3 immunity. Fibroblasts, epithelial cells, macrophages and, particularly, neutrophils become activated in response to these cytokines. IL-17 and GM-CSF induce extensive recruitment, activation and survival of neutrophils (87). IL-22 promotes epithelial cell homeostasis and has also been found to be important in antimicrobial defense (88).

Type 3 immunity is characterized by extensive neutrophil infiltration. Neutrophils are crucial for protection against and clearance of fungi (89). Also, immunity to certain encapsulated extracellular bacteria (e.g., *Staphylococcus aureus*) depends on efficient recruitment and activation of neutrophils. The importance of neutrophils in anti-fungal and anti-bacterial immunity is also apparent in subjects suffering from CGD and in patients receiving IL-17-targeting biologic agents (biologics), as these individuals are more susceptible to fungal and staphylococcal infections (90, 91).

A typical pathologic manifestation characterized by a type 3 immune response is the chronic-inflammatory skin disease psoriasis (92). Notably, neutrophil-rich microabscesses (termed Munro's microabscesses) in the uppermost layers of the epidermis are a characteristic hallmark of the skin lesions in plaque-type psoriasis. Inhibition of IL-17 by the use of IL-17-targeting biologics is very effective in psoriasis.

NEUTROPHILS IN TYPE 2 IMMUNE RESPONSES

The Importance of Neutrophils During Helminth Infections

Several groups have shown that neutrophils are important for limiting parasite survival and spreading in mouse models of helminth infections (15, 81, 82). Upon inoculation of mice with infective third-stage larvae of *Nippostrongylus brasiliensis*, the larvae migrate via the lungs to the intestine. Around the same time as the larvae arrive to the lungs, an increase of lung-infiltrating neutrophils is observed, which most likely represents a rapid mechanism to contain spreading. Thus, both mouse and human neutrophils have been shown to kill helminth larvae *in vitro*, when collaborating with macrophages (15). In fact, there are a number of studies showing that neutrophils and macrophages collaborate in immobilization and killing of these parasites. In different nematode infectious models, neutrophils and macrophages have been described to cluster together around the pathogen, however, neither of the two cell types was able to kill the larvae efficiently by itself (93–95).

As previously described, neutrophils are professional phagocytes, which is important in situations where the microbe is small. On the contrary, helminths are macropathogens and due to their size impossible for a neutrophil to ingest. Instead, neutrophils take advantage of other effector functions, namely degranulation and NET formation. Guided by virulence factors and chemokines neutrophils and other granulocytes arrive to the site of infection. Interaction between antibodies covering the helminth and Fc receptors on the cells causes ADCC-mediated degranulation (96). Complement factors serve a similar function as antibodies and can lead to complement-dependent cytotoxicity (15). Although eosinophils are acknowledged as the main effector granulocytes in helminth infestations, basophils and neutrophils are also recognized for their importance (97, 98). Both neutrophil granule proteins and NETs are efficient effector mechanisms to trap helminth larvae (99). Exposure of human and mouse neutrophils to *Strongyloides stercoralis* induces an even faster formation of NETs than the extremely potent NET stimulator phorbol 12-myristate 13-acetate (PMA).

Even though neutrophils have a very short life span, they are capable of initiating immune responses that persist long after they die. One example of this is priming of macrophages. M2 macrophages have been shown to rapidly surround nematodes and facilitate killing and clearance during helminth infection, particularly, during secondary infection (100). This priming of macrophages to become M2 macrophages during an initial infection was, however, only possible in the presence of neutrophils. Without neutrophils, macrophages could not

efficiently adapt an M2-like transcriptional phenotype, which also resulted in impaired helminth expulsion.

All these studies support the importance of neutrophils especially in the early stages of a type 2 immune response against helminths. However, with activated neutrophils comes unavoidable tissue damage (82), and it is therefore important that activated neutrophils are kept in check.

Type 2 Cytokines Dampen Neutrophil Functions

Evidence From Mouse Models

In contrast to the apparent importance of neutrophils in helminth infections, there is accumulating evidence that IL-4R signaling by IL-4 and IL-13 inhibits neutrophil effector functions (21). Woytschak et al. found that treatment of mice with IL-4 during cutaneous and systemic bacterial infections increased bacterial and disease burden, while blood neutrophil counts, neutrophil migration, and overall survival of animals decreased (19). The same trend was observed during sterile inflammation induced by using G-CSF, IL-1β, or monosodium urate crystals. Peripheral neutrophils of mice treated with IL-4 were found to have lower CXCR2 expression levels, which fits neatly with their impaired migratory ability. Conversely, bone marrow neutrophils of IL-4-injected animals expressed higher levels of CXCR4, offering an explanation for their decreased egress into the blood stream. All these effects were not observed in IL-4Rα-deficient mice as well as animals treated with a blocking anti-IL-4 monoclonal antibody. Moreover, mice lacking the IL-4Rα were shown to survive a systemic infection with *Listeria monocytogenes* that was lethal for wild-type control animals (19). Throughout all of the experiments performed in this work, the neutrophils behaved homogenously and there was no observation of duality that could hint at the presence of distinct *bona fide* neutrophil subpopulations.

Chen and colleagues found that neutrophil infiltration into the lungs upon infection of mice with the helminth *Nippostrongylus brasiliensis* was associated with IL-4R signaling (82). In this model, IL-4Rα-deficient mice presented with increased pulmonary neutrophil infiltration and worse disease scores, while neutrophil depletion led to a decrease of acute lung injury. In a mouse model of RA, a disease where neutrophils are believed to play a major role in the pathogenesis (101), Schmid et al. demonstrated that treatment of mice with IL-4 protected from joint inflammation (102). Other groups made similar observations treating RA with IL-4 and identified neutrophils and macrophages as the main targets of the therapy (103, 104). In a study combining helminth infection and RA, elicitation of a type 2 immune response by the parasite improved joint inflammation (105). In a recent publication, Harris and colleagues showed that IL-4R signaling under hypoxic conditions directly inhibited the survival of human neutrophils (106). These results highlight that IL-4R signaling during hypoxic acute respiratory distress syndrome, protects against neutrophil-induced lung injury. Under normoxic conditions, IL-4 or IL-13 did not appear to increase neutrophil apoptosis (20). Thus, IL-4R signaling seems to have yet another way of limiting neutrophil actions and protect against the harmful consequences of uncontrolled neutrophils.

As discussed earlier, micro- and nanoparticles are potent inducers of type 2 immunity. Since the body cannot degrade them, microparticle-elicited reactions often do not resolve, but lead to repeated waves of inflammation and immune cell influx. Here, IL-4R-mediated dampening of neutrophil responses again may serve as a preservation mechanism because neutrophils will not be able to clear the microparticles, but will only potentiate tissue damage while trying to do so.

Using a unique intravital imaging method, Wang et al. studied the migration behavior of neutrophils after a focal thermal injury in the liver. They found that after a short extensive influx, neutrophils proceeded to reenter the vasculature 12 h after injury and were finally recruited to the bone marrow in a CXCR4-dependent manner (107). Here, neutrophils were found to be responsible for the creation of paths through the tissue which facilitated vascular regrowth and access for other immune cells. Woytschak et al. found that treatment of neutrophils with IL-4 reduced their ability to migrate toward CXCL2 and resulted in downregulation of its receptor CXCR2, but not CXCR4 (19). Therefore, IL-4R engagement on neutrophils may not only dampen their migration toward CXCL2, but also promote reverse migration by shifting the balance toward signaling via CXCR4.

Ma and colleagues showed that the local phenotype of neutrophils change over time after myocardial infarction in mice (108). They found that proinflammatory N1 neutrophils dominated at day 1, whereas anti-inflammatory N2 neutrophils prevailed at days 5 and 7 after injury while they were not detected in peripheral blood. Tissue repair and fibrosis is part of the remodeling taking place after myocardial infarction. It has been shown that IL-4 and IL-13 are key drivers of these processes (109). It is therefore possible that the emerging tissue repair environment with type 2 cytokine milieu shifts the neutrophils from a pro-inflammatory N1 to an anti-inflammatory N2 phenotype. Although neutrophils seem to lose their pro-inflammatory phenotype in a type 2 immune milieu and may even contribute to the resolution of inflammation, they are distinct from conventional myeloid-derived suppressor cells (MDSCs) since the former are mature cells that change their phenotype in response to type 2 cytokines while the latter form newly from myeloid precursors in response to G-CSF/GM-CSF, IL-6, and a variety of other cytokines (110). There have, however, been reports of IL-4R signaling being important for the immunosuppressive nature of MDSCs (111, 112).

Translation to the Human Setting

Evidence of IL-4R signaling inhibiting neutrophils not only covers preclinical studies in mice, but there is also clinical data suggesting that this mechanism is evolutionarily conserved. Impellizzieri et al. recently demonstrated that incubation of human neutrophils with IL-4 or IL-13 significantly reduced their ability to migrate toward CXCL8 and produce NETs (20). These findings were mirrored when using freshly-isolated neutrophils from allergic patients with acute symptoms. Moreover, IL-4- or IL-13-treated neutrophils from healthy donors and neutrophils from allergic individuals showed lower surface expression of

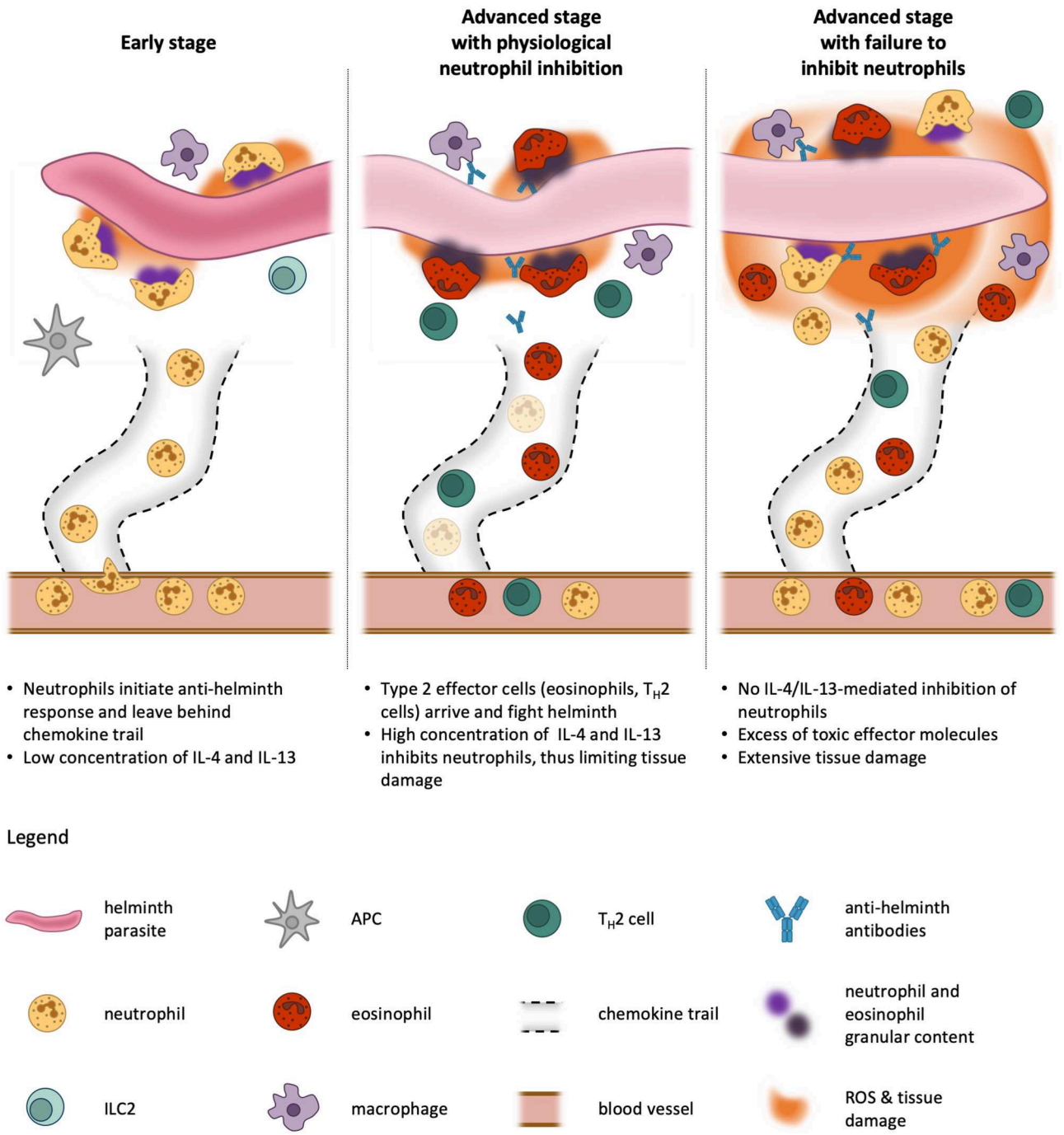

FIGURE 4 | Kinetics of neutrophil activation and inhibition during type 2 immune responses. In an early phase of helminth infestation, neutrophils are quick to be recruited to the site of infection because they are abundant in the blood stream and are primed rapidly. On their way through the tissue, they leave behind channels and trails for other cells to migrate more efficiently toward the parasite. Once they encounter the helminth, the neutrophils release pro-inflammatory cytokines and exert effector functions against the invader. This facilitates the activation of neighboring antigen-presenting cells (APCs). At this stage, only little IL-4 and or IL-13 is produced by tissue-resident cells, such as type 2 innate lymphoid cells (ILC2), and thus neutrophil functions are unblunted **(Left)**. At later stages, APCs migrate to the lymph nodes where they initiate differentiation of T_H2 cells, which in turn home to the infested tissue and produce their signature cytokines IL-4, IL-5, and IL-13. These cytokines cause recruitment and activation of professional type 2 effector cells, eosinophils and alternatively-activated macrophages, which use the preformed neutrophil channels to efficiently reach the pathogen. Our model proposes that, in a physiological condition, the presence of IL-4 and IL-13 dampens neutrophil activity and thus prevents excessive tissue damage, as these first responders are not needed anymore at this stage and would do more harm than good **(Middle)**. If this inhibitory mechanism fails, neutrophils continue to fight the helminth simultaneously with eosinophils and macrophages, resulting in profound tissue damage far beyond the necessary immune response to expulse the parasite **(Right)**. The size of the helminth parasite is not to scale, which would be larger in reality.

CXCR2 and its functional twin CXCR1, compared to untreated neutrophils from healthy donors, whereas CXCR4 expression was unaltered (20). The decrease in migratory ability *in vitro* upon IL-4 stimulation was confirmed and extended in a humanized mouse model, where an air pouch was induced in the back of NOD-*Prkdc*^{scid}-*Il2rg*^{null} (NSG) mice, followed by triggering of local inflammation by injection of CXCL8 and lipopolysaccharide in the air pouch and intravenous adoptive transfer of human neutrophils. Flow cytometric analysis of the air pouch content revealed significantly lower counts of human neutrophils when the cells were pretreated with IL-4 as opposed to controls, indicating that IL-4R signaling in human neutrophils leads to impaired migration also *in vivo*. In accordance with the findings of Woytschak et al. with mouse neutrophils, Impellizzieri et al. did not find a duality in neutrophil functionality in their experiments that would hint at different *bona fide* subpopulations.

Biologics and Small Molecules Targeting the IL-4R Signaling Axis

In a small clinical trial with patients suffering from plaque-type psoriasis, an autoimmune disease characterized by type 3 inflammation and cutaneous neutrophil infiltration, treatment with IL-4 resulted in marked disease improvement (113); this therapeutic effect was likely due to a shift in the cytokine milieu from a type 3 to a type 2 immune response as well as a direct inhibition of neutrophils by IL-4. Conversely, in AD, an inflammatory skin disorder known for its type 2 cytokine signature, afflicted individuals often suffer from recurrent skin infections (18). Here, neutrophils are conspicuously absent in both healthy and lesional skin (17, 114). However, neutrophil chemoattractants were found to be elevated similarly in psoriatic and atopic skin (115), thus the stark difference in skin-infiltrating neutrophils in psoriasis and AD cannot be explained by differences in chemoattractants. Moreover, treatment of moderate-to-severe AD patients with the IL-4Rα-blocking antibody Dupilumab has been shown to not only significantly decrease disease burden, but also lower the incidence of skin infections (84, 116, 117). This protective effect against infections has been observed also for other type 2 immune diseases when treated with biologics targeting the IL-4R complex, such as the anti-IL-13 antibody Lebrikizumab in asthma and AD (118, 119) and the IL-4Rα-blocking agent Pitrakinra in asthma (120). There are also small molecule inhibitors of the IL-4R signaling axis targeting receptor-associated JAKs. Since JAKs are shared between different cytokine receptors, they are less selective for IL-4 or IL-13 signaling, but can also be used to suppress other pathways. Tofacitinib inhibits JAK1 and JAK3 and is approved for the treatment of RA and psoriatic arthritis (76). It has also been investigated for use in AD, both as systemic and topical treatment (121, 122). The selective JAK1 inhibitor Upadacitinib is currently being investigated as an alternative to tofacitinib for treating RA and psoriatic arthritis (76), but it may also be interesting for the treatment of type 2 immune diseases since IL-4Rα also uses JAK1 for signaling (**Figure 3**).

None of the studies involving IL-4 as a therapeutic agent or inhibitors of the IL-4/IL-13-signaling axis mentioned here, however, directly examined neutrophil activity before, after or during treatment. Therefore, we can only speculate that the amelioration of plaque-type psoriasis upon IL-4 treatment and the decrease in infections in type 2 diseases upon IL-4R signaling blockade is, at least in part, the direct result of increased or decreased IL-4R signaling on neutrophils, respectively. Further investigations focusing on neutrophils in these disease and treatment conditions will reveal to what extent direct IL-4R-mediated neutrophil inhibition contributes to the clinical pictures.

Taken together, the growing body of evidence indicates that IL-4 and IL-13 exert an inhibitory effect on neutrophils. Such inhibition affects neutrophil migration and tissue infiltration as well as neutrophil effector functions. These effects limit neutrophil-mediated tissue damage. Although some of these effects could be the result of the classical type 1 and type 3 vs. type 2 immune regulation, the aforementioned studies clearly prove that cell-autonomous IL-4R signaling directly curtails mouse and human neutrophil effector functions.

Hypothesis of Kinetic Involvement and Exclusion of Neutrophils in Type 2 Immune Responses

Having discussed the evidence suggesting an inhibitory role of IL-4R signaling on neutrophils, it seems awkward that neutrophils have been reported to play a significant role in type 2 immunity against helminth infections. Can these two seemingly contradictory paradigms fit together? We propose a hypothesis that involves a temporal separation of the two events and considers biological, physiological, and clinical aspects of type 2 immune diseases (**Figure 4**).

Neutrophils are abundant in the blood stream and very easily primed for attack of pathogens. Hence, as soon as a helminth parasite invades the body and is recognized as foreign, there are countless neutrophils already at the right place to extravasate and serve as a first line of host defense. By creating an inflammatory environment, neutrophils promote the activation of antigen-presenting cells (APCs). Moreover, pathogens killed by neutrophils in these very early stages may be easier for APCs to take up, digest, and present. Furthermore, starting from the blood vessel, neutrophils create a channel decorated with chemokine trails in the tissue, thus facilitating access for other immune cells (123). At this stage, neutrophils constitute the overwhelming majority of leukocytes in the tissue and there are only low concentrations of IL-4 or IL-13 produced by resident immune cells. Thus, the neutrophils are not inhibited. APCs then migrate to the lymph nodes where they initiate differentiation of antigen-specific naïve T cells to T_H2 cells. These in turn home to the infected and inflamed tissue where they produce their signature type 2 cytokines IL-4 and IL-13. Due to clonal expansion, there soon are many activated T_H2 cells present and the resulting cytokine milieu leads to inhibition of neutrophils, but also the recruitment and activation of eosinophils which are now taking over the neutrophils'

job. Eosinophils are better equipped for fighting parasites and helminths, but take longer to be activated and accumulate in sufficient numbers. Simultaneous action of neutrophils and eosinophils would be detrimental for the surrounding host tissues. Thus, as soon as the type 2 immune response is established, the neutrophils step back and become quiescent due to IL-4R signaling.

In summary, the neutrophils bridge the time between pathogen invasion and the arrival of sufficient numbers of type 2 effector cells by initiating defense and help recruiting other immune cells. In doing so, they also facilitate the establishment of an inflammatory microenvironment. Since overshooting neutrophil activity would be destructive for host tissues, neutrophils are inhibited once they are not needed anymore. We thus postulate that the connection between IL-4R signaling and neutrophil inhibition has evolved as a safety mechanism to protect the body from neutrophil-inflicted harm.

AUTHOR CONTRIBUTIONS

CE, LH, and OB set the outline of the manuscript. CE and LH prepared the figures and wrote the first draft of manuscript with input from DI. OB revised and edited the manuscript and figures.

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REFERENCES

1. Amulic B, Cazalet C, Hayes GL, Metzler KD, Zychlinsky A. Neutrophil function: from mechanisms to disease. *Ann Rev Immunol*. (2012) 30:459–89. doi: 10.1146/annurev-immunol-020711-074942
2. Schouten HC. Neutropenia management. *Ann Oncol*. (2006) 17(Suppl. 10):x85–9. doi: 10.1093/annonc/mdl243
3. Borregaard N. Neutrophils, from marrow to microbes. *Immunity*. (2010) 33:657–70. doi: 10.1016/j.immuni.2010.11.011
4. Ronnefarth VM, Erbacher AI, Lamkemeyer T, Madlung J, Nordheim A, Rammensee HG, et al. TLR2/TLR4-independent neutrophil activation and recruitment upon endocytosis of nucleosomes reveals a new pathway of innate immunity in systemic lupus erythematosus. *J Immunol*. (2006) 177:7740–9. doi: 10.4049/jimmunol.177.11.7740
5. Eash KJ, Greenbaum AM, Gopalan PK, Link DC. CXCR2 and CXCR4 antagonistically regulate neutrophil trafficking from murine bone marrow. *J Clin Invest*. (2010) 120:2423–31. doi: 10.1172/JCI41649
6. Hernandez PA, Gorlin RJ, Lukens JN, Taniuchi S, Bohinjec J, Francois F, et al. Mutations in the chemokine receptor gene CXCR4 are associated with WHIM syndrome, a combined immunodeficiency disease. *Nat Genet*. (2003) 34:70–4. doi: 10.1038/ng1149
7. Summers C, Rankin SM, Condliffe AM, Singh N, Peters AM, Chilvers ER. Neutrophil kinetics in health and disease. *Trends Immunol*. (2010) 31:318–24. doi: 10.1016/j.it.2010.05.006
8. Bajrami B, Zhu H, Kwak HJ, Mondal S, Hou Q, Geng G, et al. G-CSF maintains controlled neutrophil mobilization during acute inflammation by negatively regulating CXCR2 signaling. *J Exp Med*. (2016) 213:1999–2018. doi: 10.1084/jem.20160393
9. Deniset JF, Kubes P. Recent advances in understanding neutrophils. *F1000Res*. (2016) 5:2912. doi: 10.12688/f1000research.9691.1
10. Galli SJ, Borregaard N, Wynn TA. Phenotypic and functional plasticity of cells of innate immunity: macrophages, mast cells and neutrophils. *Nat Immunol*. (2011) 12:1035–44. doi: 10.1038/ni.2109
11. Middleton J, Neil S, Wintle J, Clark-Lewis I, Moore H, Lam C, et al. Transcytosis and surface presentation of IL-8 by venular endothelial cells. *Cell*. (1997) 91:385–95. doi: 10.1016/S0092-8674(00)80422-5
12. Sadik CD, Kim ND, Luster AD. Neutrophils cascading their way to inflammation. *Trends Immunol*. (2011) 32:452–60. doi: 10.1016/j.it.2011.06.008
13. Li Jeon N, Baskaran H, Dertinger SK, Whitesides GM, Van de Water L, Toner M. Neutrophil chemotaxis in linear and complex gradients of interleukin-8 formed in a microfabricated device. *Nat Biotechnol*. (2002) 20:826–30. doi: 10.1038/nbt712
14. Phillipson M, Kubes P. The neutrophil in vascular inflammation. *Nat Med*. (2011) 17:1381–90. doi: 10.1038/nm.2514
15. Bonne-Annee S, Kerepesi LA, Hess JA, O'Connell AE, Lok JB, Nolan TJ, et al. Human and mouse macrophages collaborate with neutrophils to kill larval Strongyloides stercoralis. *Infect Immun*. (2013) 81:3346–55. doi: 10.1128/IAI.00625-13
16. Allen JE, Sutherland TE, Ruckerl D. IL-17 and neutrophils: unexpected players in the type 2 immune response. *Curr Opin Immunol*. (2015) 34:99–106. doi: 10.1016/j.coi.2015.03.001
17. De Benedetto A, Agnihothri R, McGirt LY, Bankova LG, Beck LA. Atopic dermatitis: a disease caused by innate immune defects? *J Invest Dermatol*. (2009) 129:14–30. doi: 10.1038/jid.2008.259
18. Boguniewicz M, Leung DY. Atopic dermatitis: a disease of altered skin barrier and immune dysregulation. *Immunol Rev*. (2011) 242:233–46. doi: 10.1111/j.1600-065X.2011.01027.x
19. Woytschak J, Keller N, Krieg C, Impellizzieri D, Thompson RW, Wynn TA, et al. Type 2 interleukin-4 receptor signaling in neutrophils antagonizes their expansion and migration during infection and inflammation. *Immunity*. (2016) 45:172–84. doi: 10.1016/j.immuni.2016.06.025
20. Impellizzieri D, Ridder F, Raeber ME, Egholm C, Woytschak J, Kolios AGA, et al. IL-4 receptor engagement in human neutrophils impairs their migration and extracellular trap formation. *J Allergy Clin Immunol*. (2019) 144:267–79.e4. doi: 10.1016/j.jaci.2019.01.042
21. Heeb LEM, Egholm C, Impellizzieri D, Ridder F, Boyman O. Regulation of neutrophils in type 2 immune responses. *Curr Opin Immunol*. (2018) 54:115–22. doi: 10.1016/j.coi.2018.06.009
22. Kim JS, Kwon HY, Choi WH, Jeon CY, Kim JI, Kim J, et al. Phagocytosis of serum- and IgG-opsonized zymosan particles induces apoptosis through superoxide but not nitric oxide in macrophage J774A.1. *Exp Mol Med*. (2003) 35:211–21. doi: 10.1038/emm.2003.29
23. Kim MK, Huang ZY, Hwang PH, Jones BA, Sato N, Hunter S, et al. Fcγ receptor transmembrane domains: role in cell surface expression, γ chain interaction, and phagocytosis. *Blood*. (2003) 101:4479–84. doi: 10.1182/blood.V101.11.4479
24. Van Kessel KPM, Bestebroer J, Van Strijp JAG. Neutrophil-mediated phagocytosis of *Staphylococcus aureus*. *Front Immunol*. (2014) 5:467. doi: 10.3389/fimmu.2014.00467
25. Mayadas TN, Cullere X, Lowell CA. The multifaceted functions of neutrophils. *Ann Rev Pathol*. (2014) 9:181–218. doi: 10.1146/annurev-pathol-020712-164023
26. Dale DC, Boxer L, Liles WC. The phagocytes: neutrophils and monocytes. *Blood*. (2008) 112:935–45. doi: 10.1182/blood-2007-12-077917
27. Borregaard N, Cowland JB. Granules of the human neutrophilic polymorphonuclear leukocyte. *Blood*. (1997) 89:3503–21.

28. Lacy P. Mechanisms of degranulation in neutrophils. *Allergy Asthma Clin Immunol*. (2006) 2:98–108. doi: 10.1186/1710-1492-2-3-98
29. Simard JC, Girard D, Tessier PA. Induction of neutrophil degranulation by S100A9 via a MAPK-dependent mechanism. *J Leukoc Biol*. (2010) 87:905–14. doi: 10.1189/jlb.1009676
30. Faurschou M, Borregaard N. Neutrophil granules and secretory vesicles in inflammation. *Microbes Infect*. (2003) 5:1317–27. doi: 10.1016/j.micinf.2003.09.008
31. Tecchio C, Micheletti A, Cassatella MA. Neutrophil-derived cytokines: facts beyond expression. *Front Immunol*. (2014) 5:508. doi: 10.3389/fimmu.2014.00508
32. Naegelen I, Beaume N, Plançon S, Schenten V, Tschirhart EJ, Bréchard S. Regulation of neutrophil degranulation and cytokine secretion: a novel model approach based on linear fitting. *J Immunol Res*. (2015) 2015:1–15. doi: 10.1155/2015/817038
33. Kruger P, Saffarzadeh M, Weber ANR, Rieber N, Radsak M, Von Bernuth H, et al. Neutrophils: between host defence, immune modulation, and tissue injury. *PLOS Pathog*. (2015) 11:e1004651. doi: 10.1371/journal.ppat.1004651
34. Wark PAB, Johnston SL, Moric I, Simpson JL, Hensley MJ, Gibson PG. Neutrophil degranulation and cell lysis is associated with clinical severity in virus-induced asthma. *Eur Respir J*. (2002) 19:68–75. doi: 10.1183/09031936.02.00226302
35. Soehnlein O, Oehmcke S, Rothfuchs AG, Frithiof R, Van Rooijen N, Morgelin M, et al. Neutrophil degranulation mediates severe lung damage triggered by streptococcal M1 protein. *Eur Respir J*. (2008) 32:405–12. doi: 10.1183/09031936.00173207
36. Ray PD, Huang B-W, Tsuji Y. Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. *Cell Signal*. (2012) 24:981–90. doi: 10.1016/j.cellsig.2012.01.008
37. Graham DB, Robertson CM, Bautista J, Mascarenhas F, Diacovo MJ, Montgrain V, et al. Neutrophil-mediated oxidative burst and host defense are controlled by a Vav-PLCγ2 signaling axis in mice. *J Clin Investig*. (2007) 117:3445–52. doi: 10.1172/JCI32729
38. Panday A, Sahoo MK, Osorio D, Batra S. NADPH oxidases: an overview from structure to innate immunity-associated pathologies. *Cell Mol Immunol*. (2015) 12:5–23. doi: 10.1038/cmi.2014.89
39. Winterbourn CC. Reconciling the chemistry and biology of reactive oxygen species. *Nat Chem Biol*. (2008) 4:278–86. doi: 10.1038/nchembio.85
40. Murphy R, Decoursey TE. Charge compensation during the phagocyte respiratory burst. *Biochim Biophys Acta Bioenerg*. (2006) 1757:996–1011. doi: 10.1016/j.bbabio.2006.01.005
41. Reeves EP, Lu H, Jacobs HL, Messina CGM, Bolsover S, Gabella G, et al. Killing activity of neutrophils is mediated through activation of proteases by K+ flux. *Nature*. (2002) 416:291–7. doi: 10.1038/416291a
42. Segal AW. How neutrophils kill microbes. *Annu Rev Immunol*. (2005) 23:197–223. doi: 10.1146/annurev.immunol.23.021704.115653
43. Uribe-Querol E, Rosales C. Control of phagocytosis by microbial pathogens. *Front Immunol*. (2017) 8:1368. doi: 10.3389/fimmu.2017.01368
44. Dinauer MC. Inflammatory consequences of inherited disorders affecting neutrophil function. *Blood*. (2019) 133:2130–9. doi: 10.1182/blood-2018-11-844563
45. Bergamini CM, Gambetti S, Dondi A, Cervellati C. Oxygen, reactive oxygen species and tissue damage. *Curr Pharm Des*. (2004) 10:1611–26. doi: 10.2174/1381612043384664
46. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, et al. Neutrophil extracellular traps kill bacteria. *Science*. (2004) 303:1532–5. doi: 10.1126/science.1092385
47. Brinkmann V, Zychlinsky A. Neutrophil extracellular traps: is immunity the second function of chromatin? *J Cell Biol*. (2012) 198:773–83. doi: 10.1083/jcb.201203170
48. Papayannopoulos V. Neutrophil extracellular traps in immunity and disease. *Nat Rev Immunol*. (2018) 18:134–47. doi: 10.1038/nri.2017.105
49. Van Avondt K, Hartl D. Mechanisms and disease relevance of neutrophil extracellular trap formation. *Eur J Clin Invest*. (2018) 48(Suppl. 2):e12919. doi: 10.1111/eci.12919
50. Lood C, Blanco LP, Purmalek MM, Carmona-Rivera C, De Ravin SS, Smith CK, et al. Neutrophil extracellular traps enriched in oxidized mitochondrial DNA are interferogenic and contribute to lupus-like disease. *Nat Med*. (2016) 22:146–53. doi: 10.1038/nm.4027
51. Pilsczek FH, Salina D, Poon KKH, Fahey C, Yipp BG, Sibley CD, et al. A novel mechanism of rapid nuclear neutrophil extracellular trap formation in response to *Staphylococcus aureus*. *J Immunol*. (2010) 185:7413–25. doi: 10.4049/jimmunol.1000675
52. Fuchs TA, Abed U, Goosmann C, Hurwitz R, Schulze I, Wahn V, et al. Novel cell death program leads to neutrophil extracellular traps. *J Cell Biol*. (2007) 176:231–41. doi: 10.1083/jcb.200606027
53. Yousefi S, Mihalache C, Kozlowski E, Schmid I, Simon HU. Viable neutrophils release mitochondrial DNA to form neutrophil extracellular traps. *Cell Death Differ*. (2009) 16:1438–44. doi: 10.1038/cdd.2009.96
54. Schönrich G, Raftery MJ. Neutrophil extracellular traps go viral. *Front Immunol*. (2016) 7:366. doi: 10.3389/fimmu.2016.00366
55. Delgado-Rizo V, Martínez-Guzmán MA, Iñiguez-Gutierrez L, García-Orozco A, Alvarado-Navarro A, Fafutis-Morris M. Neutrophil extracellular traps and its implications in inflammation: an overview. *Front Immunol*. (2017) 8:81. doi: 10.3389/fimmu.2017.00081
56. Mantovani A, Cassatella MA, Costantini C, Jaillon S. Neutrophils in the activation and regulation of innate and adaptive immunity. *Nat Rev Immunol*. (2011) 11:519–31. doi: 10.1038/nri3024
57. Boeltz S, Amini P, Anders H-J, Andrade F, Bilyy R, Chatfield S, et al. To NET or not to NET:current opinions and state of the science regarding the formation of neutrophil extracellular traps. *Cell Death Differ*. (2019) 26:395–408. doi: 10.1038/s41418-018-0261-x
58. Van Der Linden M, Van Den Hoogen LL, Westerlaken GHA, Fritsch-Stork RDE, Van Roon JAG, Radstake TRDJ, et al. Neutrophil extracellular trap release is associated with antinuclear antibodies in systemic lupus erythematosus and anti-phospholipid syndrome. *Rheumatology*. (2018) 57:1228–34. doi: 10.1093/rheumatology/key067
59. Bouts YM, Wolthuis DF, Dirkx MF, Pieterse E, Simons EM, van Boekel AM, et al. Apoptosis and NET formation in the pathogenesis of SLE. *Autoimmunity*. (2012) 45:597–601. doi: 10.3109/08916934.2012.719953
60. Lande R, Ganguly D, Facchinetti V, Frasca L, Conrad C, Gregorio J, et al. Neutrophils activate plasmacytoid dendritic cells by releasing self-DNA-peptide complexes in systemic lupus erythematosus. *Sci Transl Med*. (2011) 3:73ra19. doi: 10.1126/scitranslmed.3001180
61. Nakazawa D, Tomaru U, Ishizu A. Possible implication of disordered neutrophil extracellular traps in the pathogenesis of MPO-ANCA-associated vasculitis. *Clin Exp Nephrol*. (2013) 17:631–3. doi: 10.1007/s10157-012-0738-8
62. Giaglis S, Stoikou M, Grimolizzi F, Subramanian BY, van Breda SV, Hoesli I, et al. Neutrophil migration into the placenta: good, bad or deadly? *Cell Adh Migr*. (2016) 10:208–25. doi: 10.1080/19336918.2016.1148866
63. Schauer C, Janko C, Munoz LE, Zhao Y, Kienhöfer D, Frey B, et al. Aggregated neutrophil extracellular traps limit inflammation by degrading cytokines and chemokines. *Nat Med*. (2014) 20:511–7. doi: 10.1038/nm.3547
64. Albrengues J, Shields MA, Ng D, Park CG, Ambrico A, Poindexter ME, et al. Neutrophil extracellular traps produced during inflammation awaken dormant cancer cells in mice. *Science*. (2018) 361:eaao4227. doi: 10.1126/science.aao4227
65. Sorensen OE, Borregaard N. Neutrophil extracellular traps - the dark side of neutrophils. *J Clin Invest*. (2016) 126:1612–20. doi: 10.1172/JCI84538.
66. Mogensen TH. Pathogen recognition and inflammatory signaling in innate immune defenses. *Clin Microbiol Rev*. (2009) 22:240–73. doi: 10.1128/CMR.00046-08
67. Gregory SH, Sagnimeni AJ, Wing EJ. Bacteria in the bloodstream are trapped in the liver and killed by immigrating neutrophils. *J Immunol*. (1996) 157:2514–20.
68. Carr KD, Sieve AN, Indramohan M, Break TJ, Lee S, Berg RE. Specific depletion reveals a novel role for neutrophil-mediated protection in the liver during Listeria monocytogenes infection. *Eur J Immunol*. (2011) 41:2666–76. doi: 10.1002/eji.201041363

69. Witter AR, Okunnu BM, Berg RE. The essential role of neutrophils during infection with the intracellular bacterial pathogen *Listeria monocytogenes*. *J Immunol*. (2016) 197:1557–65. doi: 10.4049/jimmunol.1600599
70. Ellis TN, Beaman BL. Interferon-γ activation of polymorphonuclear neutrophil function. *Immunology*. (2004) 112:2–12. doi: 10.1111/j.1365-2567.2004.01849.x
71. Tan BH, Meinken C, Bastian M, Bruns H, Legaspi A, Ochoa MT, et al. Macrophages acquire neutrophil granules for antimicrobial activity against intracellular pathogens. *J Immunol*. (2006) 177:1864–71. doi: 10.4049/jimmunol.177.3.1864
72. Lloyd CM, Snelgrove RJ. Type 2 immunity: expanding our view. *Sci Immunol*. (2018) 3:eaat1604. doi: 10.1126/sciimmunol.aat1604
73. Allen JE, Maizels RM. Diversity and dialogue in immunity to helminths. *Nat Rev Immunol*. (2011) 11:375–88. doi: 10.1038/nri2992
74. Boyman O, Kaegi C, Akdis M, Bavbek S, Bossios A, Chatzipetrou A, et al. EAACI IG biologicals task force paper on the use of biologic agents in allergic disorders. *Allergy*. (2015) 70:727–54. doi: 10.1111/all.12616
75. LaPorte SL, Juo ZS, Vaclavikova J, Colf LA, Qi X, Heller NM, et al. Molecular and structural basis of cytokine receptor pleiotropy in the interleukin-4/13 system. *Cell*. (2008) 132:259–72. doi: 10.1016/j.cell.2007.12.030
76. Bechman K, Yates M, Galloway JB. The new entries in the therapeutic armamentarium: the small molecule JAK inhibitors. *Pharmacol Res*. (2019) 147:104392. doi: 10.1016/j.phrs.2019.104392
77. Mishra PK, Palma M, Buechel B, Moore J, Davra V, Chu N, et al. Sterile particle-induced inflammation is mediated by macrophages releasing IL-33 through a Bruton's tyrosine kinase-dependent pathway. *Nat Mater*. (2019) 18:289–97. doi: 10.1038/s41563-018-0271-6
78. Hasnain SZ, Evans CM, Roy M, Gallagher AL, Kindrachuk KN, Barron L, et al. Muc5ac: a critical component mediating the rejection of enteric nematodes. *J Exp Med*. (2011) 208:893–900. doi: 10.1084/jem.20102057
79. Herbert DR, Yang JQ, Hogan SP, Groschwitz K, Khodoun M, Munitz A, et al. Intestinal epithelial cell secretion of RELM-β protects against gastrointestinal worm infection. *J Exp Med*. (2009) 206:2947–57. doi: 10.1084/jem.20091268
80. McCoy KD, Stoel M, Stettler R, Merky P, Fink K, Senn BM, et al. Polyclonal and specific antibodies mediate protective immunity against enteric helminth infection. *Cell Host Microbe*. (2008) 4:362–73. doi: 10.1016/j.chom.2008.08.014
81. Sutherland TE, Logan N, Ruckerl D, Humbles AA, Allan SM, Papayannopoulos V, et al. Chitinase-like proteins promote IL-17-mediated neutrophilia in a tradeoff between nematode killing and host damage. *Nat Immunol*. (2014) 15:1116–25. doi: 10.1038/ni.3023
82. Chen F, Liu Z, Wu W, Rozo C, Bowdridge S, Millman A, et al. An essential role for TH2-type responses in limiting acute tissue damage during experimental helminth infection. *Nat Med*. (2012) 18:260–6. doi: 10.1038/nm.2628
83. Wynn TA. Type 2 cytokines: mechanisms and therapeutic strategies. *Nat Rev Immunol*. (2015) 15:271–82. doi: 10.1038/nri3831
84. Simpson EL, Bieber T, Guttman-Yassky E, Beck LA, Blauvelt A, Cork MJ, et al. Two phase 3 trials of dupilumab versus placebo in atopic dermatitis. *N Engl J Med*. (2016) 375:2335–48. doi: 10.1056/NEJMoa1610020
85. Castro M, Corren J, Pavord ID, Maspero J, Wenzel S, Rabe KF, et al. Dupilumab efficacy and safety in moderate-to-severe uncontrolled asthma. *N Engl J Med*. (2018) 378:2486–96. doi: 10.1056/NEJMoa1804092
86. Annunziato F, Romagnani C, Romagnani S. The 3 major types of innate and adaptive cell-mediated effector immunity. *J Allergy Clin Immunol*. (2015) 135:626–35. doi: 10.1016/j.jaci.2014.11.001
87. Nembrini C, Marsland BJ, Kopf M. IL-17-producing T cells in lung immunity and inflammation. *J Allergy Clin Immunol*. (2009) 123:986–94. doi: 10.1016/j.jaci.2009.03.033
88. Rutz S, Eidenschenk C, Ouyang W. IL-22, not simply a Th17 cytokine. *Immunol Rev*. (2013) 252:116–32. doi: 10.1111/imr.12027
89. Gazendam RP, van de Geer A, Roos D, van den Berg TK, Kuijpers TW. How neutrophils kill fungi. *Immunol Rev*. (2016) 273:299–311. doi: 10.1111/imr.12454
90. Marciano BE, Spalding C, Fitzgerald A, Mann D, Brown T, Osgood S, et al. Common severe infections in chronic granulomatous disease. *Clin Infect Dis*. (2015) 60:1176–83. doi: 10.1093/cid/ciu1154
91. Boyman O, Comte D, Spertini F. Adverse reactions to biologic agents and their medical management. *Nat Rev Rheumatol*. (2014) 10:612–27. doi: 10.1038/nrrheum.2014.123
92. Nestle FO, Kaplan DH, Barker J. Psoriasis. *N Engl J Med*. (2009) 361:496–509. doi: 10.1056/NEJMra0804595
93. Al-Qaoud KM, Pearlman E, Hartung T, Klukowski J, Fleischer B, Hoerauf A. A new mechanism for IL-5-dependent helminth control: neutrophil accumulation and neutrophil-mediated worm encapsulation in murine filariasis are abolished in the absence of IL-5. *Int Immunol*. (2000) 12:899–908. doi: 10.1093/intimm/12.6.899
94. Anthony RM, Urban JF Jr, Alem F, Hamed HA, Rozo CT, Boucher JL, et al. Memory T(H)2 cells induce alternatively activated macrophages to mediate protection against nematode parasites. *Nat Med*. (2006) 12:955–60. doi: 10.1038/nm1451
95. Morimoto M, Morimoto M, Whitmire J, Xiao S, Anthony RM, Mirakami H, et al. Peripheral CD4 T cells rapidly accumulate at the host: parasite interface during an inflammatory Th2 memory response. *J Immunol*. (2004) 172:2424–30. doi: 10.4049/jimmunol.172.4.2424
96. Motran CC, Silvane L, Chiapello LS, Theumer MG, Ambrosio LF, Volpini X, et al. Helminth infections: recognition and modulation of the immune response by innate immune cells. *Front Immunol*. (2018) 9:664. doi: 10.3389/fimmu.2018.00664
97. Bass DA, Szejda P. Eosinophils versus neutrophils in host defense. Killing of newborn larvae of Trichinella spiralis by human granulocytes *in vitro*. *J Clin Invest*. (1979) 64:1415–22. doi: 10.1172/JCI109599
98. Voehringer D. The role of basophils in helminth infection. *Trends Parasitol*. (2009) 25:551–6. doi: 10.1016/j.pt.2009.09.004
99. Bonne-Annee S, Kerepesi LA, Hess JA, Wesolowski J, Paumet F, Lok JB, et al. Extracellular traps are associated with human and mouse neutrophil and macrophage mediated killing of larval *Strongyloides stercoralis*. *Microbes Infect*. (2014) 16:502–11. doi: 10.1016/j.micinf.2014.02.012
100. Chen F, Wu W, Millman A, Craft JF, Chen E, Patel N, et al. Neutrophils prime a long-lived effector macrophage phenotype that mediates accelerated helminth expulsion. *Nat Immunol*. (2014) 15:938–46. doi: 10.1038/ni.2984
101. Spohn G, Arenas-Ramirez N, Bouchaud G, Boyman O. Endogenous polyclonal anti-IL-1 antibody responses potentiate IL-1 activity during pathogenic inflammation. *J Allergy Clin Immunol*. (2017) 139:1957–65 e3. doi: 10.1016/j.jaci.2016.09.033
102. Schmid AS, Hemmerle T, Pretto F, Kipar A, Neri D. Antibody-based targeted delivery of interleukin-4 synergizes with dexamethasone for the reduction of inflammation in arthritis. *Rheumatology*. (2018) 57:748–55. doi: 10.1093/rheumatology/kex447
103. Wermeling F, Anthony RM, Brombacher F, Ravetch JV. Acute inflammation primes myeloid effector cells for anti-inflammatory STAT6 signaling. *Proc Natl Acad Sci USA*. (2013) 110:13487–91. doi: 10.1073/pnas.1312525110
104. Cao Y, Brombacher F, Tunyogi-Csapo M, Glant TT, Finnegan A. Interleukin-4 regulates proteoglycan-induced arthritis by specifically suppressing the innate immune response. *Arthritis Rheum*. (2007) 56:861–70. doi: 10.1002/art.22422
105. Chen Z, Andreev D, Oeser K, Krljanac B, Hueber A, Kleyer A, et al. Th2 and eosinophil responses suppress inflammatory arthritis. *Nat Commun*. (2016) 7:11596. doi: 10.1038/ncomms11596
106. Harris AJ, Mirchandani AS, Lynch RW, Murphy F, Delaney L, Small D, et al. IL4Rα signaling abrogates hypoxic neutrophil survival and limits acute lung injury responses *in vivo*. *Am J Respir Crit Care Med*. (2019) 200:235–46. doi: 10.1164/rccm.201808-1599OC
107. Wang J, Hossain M, Thanabalasuriar A, Gunzer M, Meininger C, Kubes P. Visualizing the function and fate of neutrophils in sterile injury and repair. *Science*. (2017) 358:111–6. doi: 10.1126/science.aam9690
108. Ma Y, Yabluchanskiy A, Iyer RP, Cannon PL, Flynn ER, Jung M, et al. Temporal neutrophil polarization following myocardial infarction. *Cardiovasc Res*. (2016) 110:51–61. doi: 10.1093/cvr/cvw024
109. Gieseck RL III, Wilson MS, Wynn TA. Type 2 immunity in tissue repair and fibrosis. *Nat Rev Immunol*. (2018) 18:62–76. doi: 10.1038/nri.2017.90

110. Mantovani A. The growing diversity and spectrum of action of myeloid-derived suppressor cells. *Eur J Immunol*. (2010) 40:3317–20. doi: 10.1002/eji.201041170
111. Lee CR, Lee W, Cho SK, Park SG. Characterization of multiple cytokine combinations and TGF-β on differentiation and functions of myeloid-derived suppressor cells. *Int J Mol Sci*. (2018) 19:E869. doi: 10.3390/ijms19030869
112. Mandruzzato S, Solito S, Falisi E, Francescato S, Chiarion-Sileni V, Mocellin S, et al. IL4Rα+ myeloid-derived suppressor cell expansion in cancer patients. *J Immunol*. (2009) 182:6562–8. doi: 10.4049/jimmunol.0803831
113. Ghoreschi K, Thomas P, Breit S, Dugas M, Mailhammer R, van Eden W, et al. Interleukin-4 therapy of psoriasis induces Th2 responses and improves human autoimmune disease. *Nat Med*. (2003) 9:40–6. doi: 10.1038/nm804
114. Dhingra N, Suarez-Farinas M, Fuentes-Duculan J, Gittler JK, Shemer A, Raz A, et al. Attenuated neutrophil axis in atopic dermatitis compared to psoriasis reflects TH17 pathway differences between these diseases. *J Allergy Clin Immunol*. (2013) 132:498–501 e3. doi: 10.1016/j.jaci.2013.04.043
115. Choy DF, Hsu DK, Seshasayee D, Fung MA, Modrusan Z, Martin F, et al. Comparative transcriptomic analyses of atopic dermatitis and psoriasis reveal shared neutrophilic inflammation. *J Allergy Clin Immunol*. (2012) 130:1335–43 e5. doi: 10.1016/j.jaci.2012.06.044
116. Fleming P, Drucker AM. Risk of infection in patients with atopic dermatitis treated with dupilumab: a meta-analysis of randomized controlled trials. *J Am Acad Dermatol*. (2018) 78:62–9 e1. doi: 10.1016/j.jaad.2017.09.052
117. Ou Z, Chen C, Chen A, Yang Y, Zhou W. Adverse events of Dupilumab in adults with moderate-to-severe atopic dermatitis: a meta-analysis. *Int Immunopharmacol*. (2018) 54:303–10. doi: 10.1016/j.intimp.2017.11.031
118. Korenblat P, Kerwin E, Leshchenko I, Yen K, Holweg CTJ, Anzures-Cabrera J, et al. Efficacy and safety of lebrikizumab in adult patients with mild-to-moderate asthma not receiving inhaled corticosteroids. *Respir Med*. (2018) 134:143–9. doi: 10.1016/j.rmed.2017.12.006
119. Simpson EL, Flohr C, Eichenfield LF, Bieber T, Sofen H, Taieb A, et al. Efficacy and safety of lebrikizumab (an anti-IL-13 monoclonal antibody) in adults with moderate-to-severe atopic dermatitis inadequately controlled by topical corticosteroids: a randomized, placebo-controlled phase II trial (TREBLE). *J Am Acad Dermatol*. (2018) 78:863–71.e11. doi: 10.1016/j.jaad.2018.01.017
120. Wenzel S, Wilbraham D, Fuller R, Getz EB, Longphre M. Effect of an interleukin-4 variant on late phase asthmatic response to allergen challenge in asthmatic patients: results of two phase 2a studies. *Lancet*. (2007) 370:1422–31. doi: 10.1016/S0140-6736(07)61600-6
121. Levy LL, Urban J, King BA. Treatment of recalcitrant atopic dermatitis with the oral Janus kinase inhibitor tofacitinib citrate. *J Am Acad Dermatol*. (2015) 73:395–9. doi: 10.1016/j.jaad.2015.06.045
122. Wernham AGH, Veitch D, Grindlay DJC, Rogers NK, Harman KE. What's new in atopic eczema? An analysis of systematic reviews published in 2017. Part 1: treatment and prevention. *Clin Exp Dermatol*. (2019) 44:363–69. doi: 10.1111/ced.14044
123. Lim K, Hyun YM, Lambert-Emo K, Capece T, Bae S, Miller R, et al. Neutrophil trails guide influenza-specific CD8(+) T cells in the airways. *Science*. (2015) 349:aaa4352. doi: 10.1126/science.aaa4352

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The Prolyl Isomerase Pin1 Controls Lipopolysaccharide-Induced Priming of NADPH Oxidase in Human Neutrophils

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Production of superoxide anion and other reactive oxygen species (ROS) by neutrophils has a vital role in host defense against microbes. However, over-production can induce cell injury participating to inflammation. Superoxide anion is produced by the phagocyte NADPH oxidase/NOX2, a multicomponent enzyme system consisting of six proteins: two trans-membrane proteins (gp91^{phox} and p22^{phox}) and four soluble cytosolic proteins (p40^{phox}, p67^{phox}, p47^{phox}, and the small G-proteins, Rac1/2). Phosphorylation of p47^{phox} on several serines regulates NADPH oxidase activation. LPS released by gram negative bacteria can enhance or prime neutrophil superoxide production in combination with other agonists such as the bacterial peptide formyl-Met-Leu-Phe (fMLP). Since the pathways involved in LPS-induced priming are not completely understood, we investigated the role of the prolyl *cis/trans* isomerase Pin1 in this process. Two different Pin1 inhibitors, PiB, and Juglone are able to block LPS-induced priming of ROS production by human neutrophils in a concentration dependent manner. PiB and Juglone did not inhibit LPS-induced CD11b translocation neither CD62L shedding. LPS induced an increase of Pin1 activity in neutrophils similar to TNFα and fMLP. Since the phosphorylation of p47^{phox} on Ser345 is critical for NADPH oxidase up-regulation, we investigated the effect of LPS on this process. Results show that LPS induced the phosphorylation of p47^{phox} mainly on serine 345 and induced the activation of p38MAPKinase and ERK1/2. These results suggest that the prolyl *cis/trans* isomerase Pin1 may control LPS-induced priming of superoxide production in human neutrophils. Pharmacological targeting of Pin1 could be a valuable approach in sepsis.

Keywords: neutrophils, LPS, Pin1, NADPH oxidase, NOX2, priming, ROS, p47^{phox}

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INTRODUCTION

Polymorphonuclear neutrophils (PMN) are the most abundant immune cells in human blood (1). PMN have a central role in host defense against pathogens and in inflammation (1, 2). Upon inflammation and infection, PMN are the first circulating cells to reach the inflammatory and infection site (3, 4). They are attracted by a variety of peptides, chemokines and lipids such as

the C5a, N-formyl-methionyl-leucyl-phenylalanine (fMLF or fMLP), interleukin 8 (IL-8), LTB4, and platelet activating factor (PAF). Then they recognize microbes by their TLR receptors, engulf them and release huge number of anti-bacterial agents such as reactive oxygen species (ROS), myeloperoxidase, proteases, glucosidases, and anti-bacterial peptides in order to kill and eliminate microbes (3–6).

The enzyme responsible for ROS production is the nicotinamide adenine dinucleotide phosphate reduced form (NADPH) oxidase, also referred as NOX2 (7, 8); which produces superoxide anion ($O_2^{\bullet -}$), the source of other ROS molecules such as hydrogen peroxide (H_2O_2) which is used by myeloperoxidase to produce hypochlorous acid (HOCl), all of which cause the destruction and death of pathogens in the phagosome (6–9).

The phagocyte NADPH oxidase/NOX2 is a multicomponent enzyme system consisting of six proteins: two transmembrane proteins (gp91^{phox} and p22^{phox}) and four soluble cytosolic proteins (p40^{phox}, p67^{phox}, p47^{phox}, and the small G-proteins, Rac1/2) (7, 8). In resting cells, NOX2 is in a dormant state with spatial separation of the components. After neutrophil stimulation by different agents such as fMLP or phorbol myristate acetate (PMA), the cytosolic subunits p47^{phox}, p67^{phox}, and p40^{phox} are phosphorylated and migrate to associate with gp91^{phox} and p22^{phox} in the membrane to assemble the active NADPH oxidase (10). Phosphorylation of p47^{phox} on several serines (Ser303-379) located in the C-terminal portion of the protein plays an important role in NADPH oxidase activation (10, 11).

In addition to resting and activated state, NOX2 can be found in a primed state, a ready-to-go state which enhances its activation and thus ROS production (8, 12, 13). Neutrophil ROS production is primed by various mediators such as TNFα, GM-CSF, IL-8, and TLR agonists such as Lipopolysaccharides (LPS) and CL097 (8, 12–20). Physiological priming of the neutrophil NOX2 is believed to have many beneficial effects, such as efficient anti-bacterial and anti-fungal elimination (8, 21, 22). However, excessive priming of NOX2 results in excessive ROS production contributing to tissue damage involved in inflammatory diseases (8, 23–28). LPS is a main component of the outer membrane of gram-negative bacteria and it is released during bacterial infection. LPS is one of the most pathogenic molecules inducing immune cell activation and inflammation via TLR4 receptor (21, 29). LPS is known to induce NADPH oxidase priming in neutrophils by inducing NOX2 translocation to the membranes and p47^{phox} phosphorylation (14–17).

The peptidyl-prolyl *cis-trans* isomerase (PPIase), Pin1 is an enzyme which catalyzes the isomerization of prolyl peptide bonds from *cis*-conformation to *trans*-orientation (30). Pin1 recognizes a phosphor-Ser/Thr-Pro sequence and has been demonstrated to be a crucial regulator of many proteins phosphorylated on serine/threonine (31). Pin1 plays significant roles in a range of pathologies, including cancer, cardiovascular disease, and Alzheimer disease (32). We have shown that Pin1 was involved in TNF- and CL097 (a TLR7/8 agonist)-induced priming of NADPH oxidase in human neutrophils (18, 19, 33). However, the role of Pin1 in LPS-induced priming of NOX2 in neutrophils is not known. In this study we show that Pin1 is a key enzyme in LPS-induced priming of NOX2 in human PMN. Targeting Pin1 could be a new approach to treat inflammation and sepsis.

MATERIALS AND METHODS

Reagents

Lipopolysaccaride (LPS) from E. Coli O111:B4 strain, Juglone, PiB, Phosphate Buffered Saline (PBS), Hanks' Balanced Salt Solution (HBSS), protease and phosphatase inhibitors were obtained from Sigma Aldrich (Saint Quentin Fallavier, France). Dextran T500 and Ficoll was from GE healthcare (Orsay, France). Sodium dodecyl-sulfate polyacrylamide (SDS-PAGE) and western blotting reagents were supplied by Bio-Rad (Hercules, CA, USA). The rabbit polyclonal antibodies against phospho-p47^{phox} sites (phospho-Ser345, phospho-Ser320, phospho-Ser304, phospho-Ser315, phospho-Ser328), p67^{phox}, and p47^{phox} were produced by our lab as described elsewhere (18, 33). Anti-phospho(P)-ERK1/2, ERK1/2, P-p38, and p38 were from cell signaling Technology (Boston, MA, USA). HRP-conjugated goat anti-mouse were from Santa Cruz Biotechnology Inc. (Heidelberg, Germany).

Ethics Statement

Neutrophils were isolated from healthy volunteers' venous blood with their signed informed consent. The collection and analyses of data were performed anonymously. All experiments were supported by the Inserm Institutional Review Board and ethics committee.

Isolation of Human PMN

Neutrophils were isolated from blood of healthy volunteers as described previously (18, 33, 34). After hypotonic lysis of erythrocytes, the neutrophil pellets were collected and washed in PBS before cell counting. Viability was tested using Trypan Blue dye exclusion. This isolation method consistently yielded PMN with 96% pure and 99% viable.

Luminol-Enhanced Chemiluminescence

To determine ROS production we used luminol-enhanced chemiluminescence method (33, 34). Neutrophils (2.5×10^5) were resuspended in 0.5 mL of HBSS containing 10 μM of luminol with or without different concentrations of agents (PiB or juglone) for 20 min at 37°C, LPS was added for another 20 min; then fMLP (10^{-7} M) was used to stimulate the cells. Chemiluminescence was recorded using a luminometer (LB937; Berthold-Biolumat).

Determination of CD11b-Expression and CD62L Shedding at the Neutrophil Surface

Neutrophils (10×10^6 /ml) were incubated at 37°C in HBSS alone (control) or in the presence of PiB (50 μM) or Juglone (400 nM) for 30 min at 37°C. Samples were then incubated with LPS (1 μg/ml) or PBS (control) for another 20 min. A total of 100 μL of each sample was then stained with 10 μL of PE-conjugated anti-human CD11b monoclonal antibody (BD Biosciences, San Jose, CA) or 10 μL of fluorescein isothiocyanate

(FITC)-conjugated anti-human CD62L monoclonal antibody for 30 min at room temperature in the dark. Cells were resuspended in 1% paraformaldehyde-PBS and kept on ice until flow cytometry. Non-specific antibody binding was determined on cells incubated with the same concentration of an irrelevant antibody of the same isotype. Forward and side scatter were used to identify the neutrophil population and to gate out other cells and debris in a FACS CantoII (BD Biosciences). The purity of the gated cells was assessed by using monoclonal anti-CD15 antibodies (BD Biosciences). The mean fluorescent intensity of ethidium, CD11b-positive cells and CD62L-positive cells was then determined in the neutrophil populations. Five thousand events per sample were analyzed, and all results were obtained with a constant photomultiplier gain value. Results were expressed as mean fluorescence intensity (MFI).

Pin 1 Activity Assay

Pin1 activity was determined as previously described (18, 30, 33) with some modifications. In short, neutrophils were resuspended in an ice cold lysis buffer containing 50 mM HEPES pH 7.5, 0.25% CHAPS, 100 mM NaCl, 1 mM β-glycerophosphate, 5 mM NaF and 1 mM EGTA, at 10^7/ml, and lysed with several a 2 ml-syringe pressures. The assay mixture contains 369 µL HEPES buffer (50 mM HEPES (pH 7.8), 25 µL (60 mg/ml) chymotrypsin solution (Sigma-Aldrich), 6 µL (6 mM) of the peptide Suc-Ala-Glu-Pro-phe-pNA (BACHEM), and 50 µL cell lysate (10^6 cell equivalent). The absorbance change due to pNA release was monitored at 410 nm at 4°C by spectrophotometer (18, 30, 33) and the results were expressed as OD/min/1 million cells.

SDS-PAGE and Western Blotting

Neutrophils (10^7 cells in 500 µl of HBSS) were incubated with or without increasing LPS concentrations for 20 min at 37°C. The reaction was stopped by adding 125 µl of concentrated modified Laemmli sample buffer (5X) (35) containing 50 µg/mL pepstatin, 50 µg/mL leupeptin, 25 mM NaF, 12.5 mM Na3VO4, 12.5 mM EDTA, 12.5 mM EGTA, 6.25 mM p-NPP, and 50 µg/mL aprotinin. Samples were denatured in boiling water (100°C) for 3 min and stored at −80°C until use. Samples were thawed and sonicated for 10 s before use and then they were

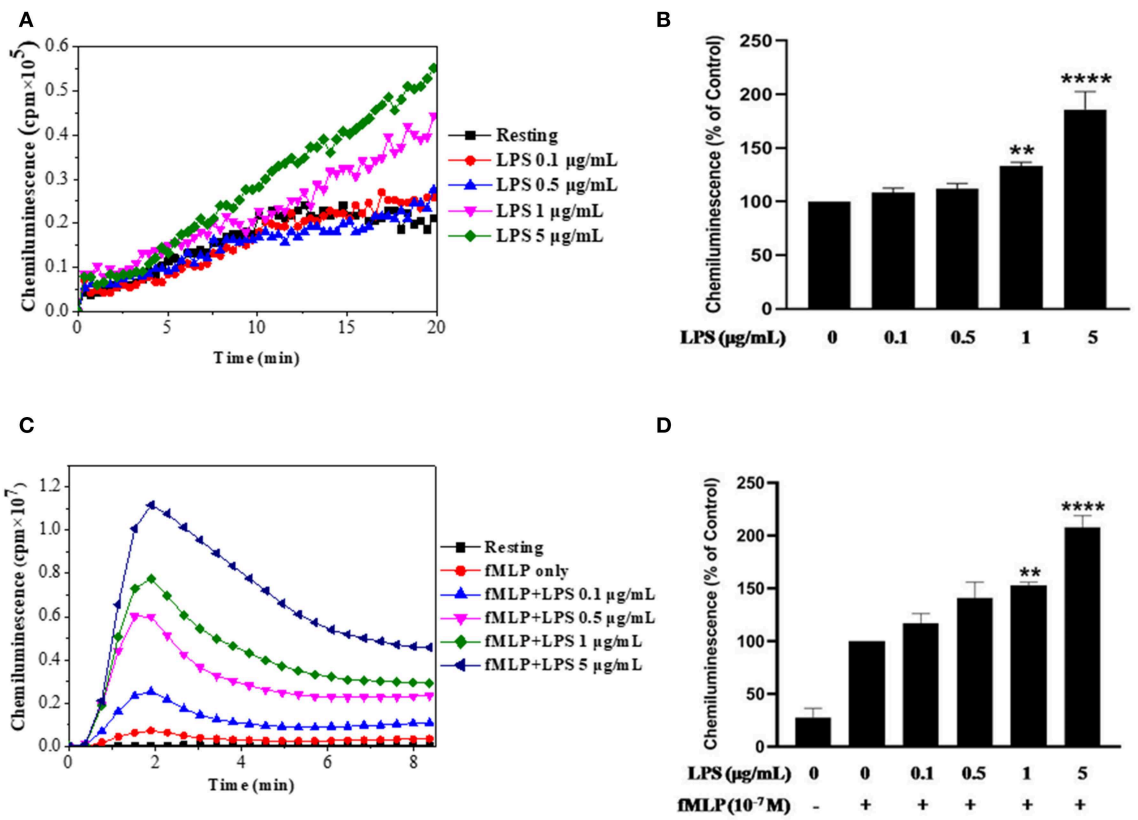

FIGURE 1 | Effect of LPS alone or in combination with fMLP on ROS production by human neutrophils. **(A)** Human neutrophils (1 × 10^6/ml) were incubated in HBSS without or with different concentrations of LPS for 20 min at 37°C. ROS production was measured using a luminol-amplified chemiluminescence technique. **(B)** Total chemiluminescence in each experimental condition is expressed as mean plus or minus SEM of 3 different experiments as compared to untreated cells (control 100%). **(C)** Human neutrophils (1 × 10^6/ml) were incubated in the presence or absence of increasing concentrations of LPS for 20 min at 37°C, then stimulated with fMLP (10^{-7}M). **(D)** Data are mean plus or minus SEM of 5 experiments as compared to fMLP only (control 100%) (**$p < 0.05$, ****$p < 0.01$).

subjected to 10% SDS–PAGE with classical techniques (35). The separated proteins were transferred to nitrocellulose membranes (35). Nitrocellulose membranes were blocked with 5% non-fat dry milk in a mixture of tris-bufferd saline and tween-20. The membranes were incubated overnight at 4°C in solution containing specific relevant primary antibodies; anti-phospho-S345 (1:10,000), anti-phospho-S328 (1:2,500), anti-phospho-S304 (1:2,500), anti-phospho-S315 (1:2,500), anti-phospho-S320 (1:2,500), anti-phospho-ERK1/2 (1/2,000), anti-phospho-p38 (1:2,000), and p47^{phox}(1:5,000) following by incubation in secondary antibodies (Santa Cruz, Heidelberg, Germany). Blots were visualized by using ECL Western blotting reagents (Amersham Pharmacia).

Statistical Analysis

Experiments were repeated at least three times. All results are reported as means ± SEM. Significant differences ($p < 0.05$) were evaluated with Student's *t* tests and one-way ANOVA followed by Tukey's *post-hoc* test using Prism 8.0 software (GraphPad).

RESULTS

LPS-Induced Priming of fMLP-Induced ROS Production in Human Neutrophils Is Impaired by Two Different Pin1 Inhibitors PiB and Juglone

In this study we used LPS from E. Coli O111:B4 strain, we wanted first to check its effect on ROS production by using the luminol-enhanced chemiluminescence assay, a very sensitive technique. Results show that LPS alone had no effect on ROS production by neutrophils at low concentrations and we found a weak but significant increase in ROS production starting from 1 µg/ml of LPS (**Figures 1A,B**). However, when neutrophils were treated by LPS and stimulated with fMLP (10^{-7} M), ROS production was markedly enhanced compared to neutrophils stimulated with fMLP alone (**Figures 1C,D**). The priming effect of LPS on fMLP-stimulated neutrophils is dose-dependent, and starts to be significant at 1 µg/ml in our conditions.

After confirming the priming effect of LPS on fMLP-induced ROS production in our experimental conditions, we next

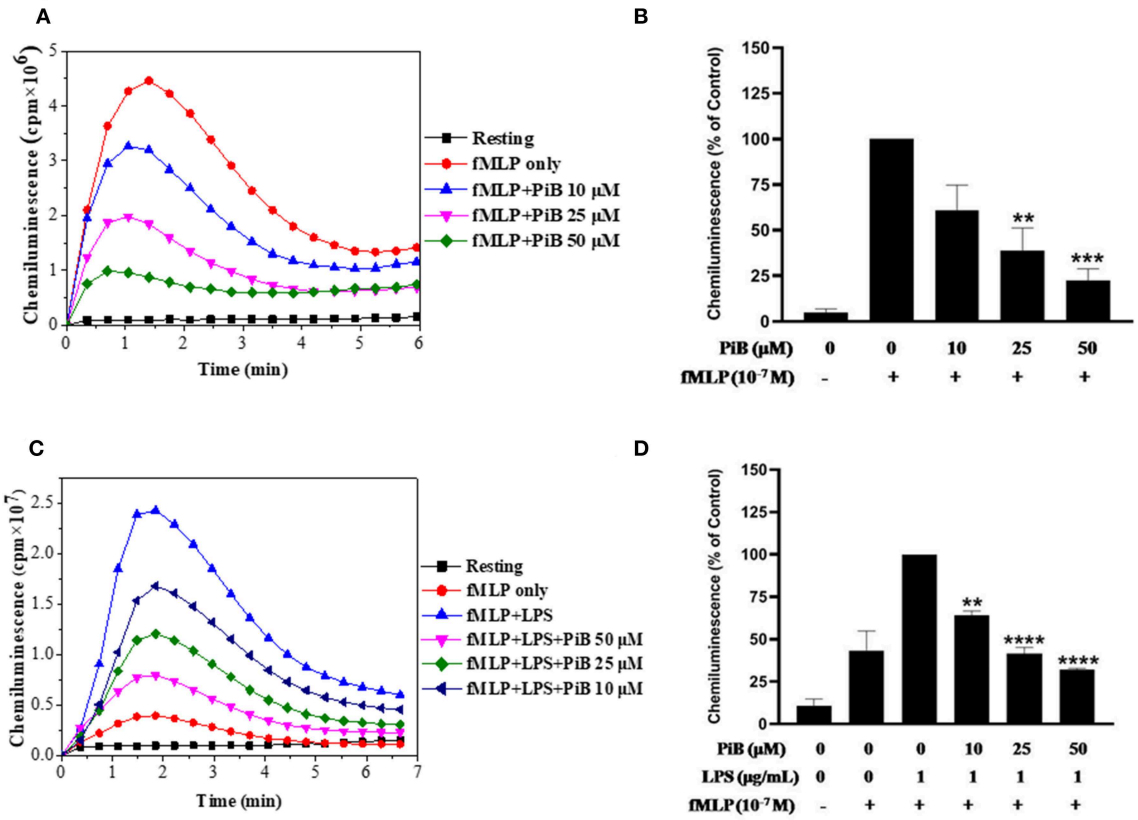

FIGURE 2 | Effect of PiB, a Pin1 inhibitor on fMLP-induced and LPS-primed ROS production by human neutrophils. **(A)** Human neutrophils (1 × 10^6/ml) were incubated in the absence or presence of increasing concentrations of PiB for 20 min at 37°C, then stimulated with fMLP (10^{-7} M). ROS production was measured with a luminol-amplified chemiluminescence technique. **(B)** Total chemiluminescence in each experimental condition is expressed as mean plus or minus SEM of 3 experiments. $^{**}p < 0.05$ and $^{***}p < 0.01$ as compared with fMLP alone (control 100%). **(C)** Human neutrophils (1 × 10^6/ml) were incubated in the absence or presence of increasing concentrations of PiB for 20 min at 37°C, then LPS (1 µg/mL) was added for another 20 min before stimulation with fMLP (10^{-7} M). ROS production was measured with a luminol-amplified chemiluminescence technique. **(D)** Total chemiluminescence in each experimental condition is expressed as mean plus or minus SEM of 3 experiments. $^{**}p < 0.05$, $^{****}p < 0.001$ as compared with control (100%).

investigated the role of Pin 1 in LPS-induced priming of ROS production. In order to do so, we used two known Pin 1 selective inhibitors: PiB (36) and Juglone (37). Neutrophils were incubated with PiB (10–50 μM) or Juglone (100–400 nM) for 20 min, treated with LPS (0.1–5 μg/ml) for another 20 min, then stimulated with fMLP (10^{-7}μM). ROS production was measured with luminol-enhanced chemiluminescence assay. Results show that PiB exhibited a significant inhibition of fMLP-induced ROS production in unprimed neutrophils (**Figures 2A,B**). In LPS primed cells, the inhibition was obtained at lower PiB concentrations (**Figures 2C,D**). Indeed at 25 μM, PiB was able to completely abolish the priming effect of LPS but did not completely inhibit fMLP-stimulated neutrophils. Juglone at 100–400 nM, showed a similar inhibition pattern as PiB on fMLP- and LPS-primed neutrophils (**Figure 3**). These results suggest a potential role of Pin 1 in fMLP-induced and LPS- primed ROS production in human neutrophils.

Pin1 Inhibitors PiB and Juglone Do Not Inhibit LPS-Induced CD11b Plasma Membrane Translocation and CD62L Shedding

LPS is known to induce neutrophil degranulation, an other key neutrophil function (15, 16). We thus wanted to investigate the effect of Pin1 inhibitors on this function using flow cytometry to detect CD11b (a marker of specific and gelatinase marker) expression at the plasma membrane and CD62L plasma membrane shedding. Results show that LPS induced a clear CD11b expression at the plasma membrane and a clear shedding of CD62L compared to control untreated neutrophils (**Figure 4**). PiB did not affect CD11b expression nether CD62L shedding (**Figure 4**). However, Juglone did not inhibit these processes, rather it has an unexpected stimulatory effect. These results suggest that Pin1 is not involved in LPS-induced neutrophil degranulation.

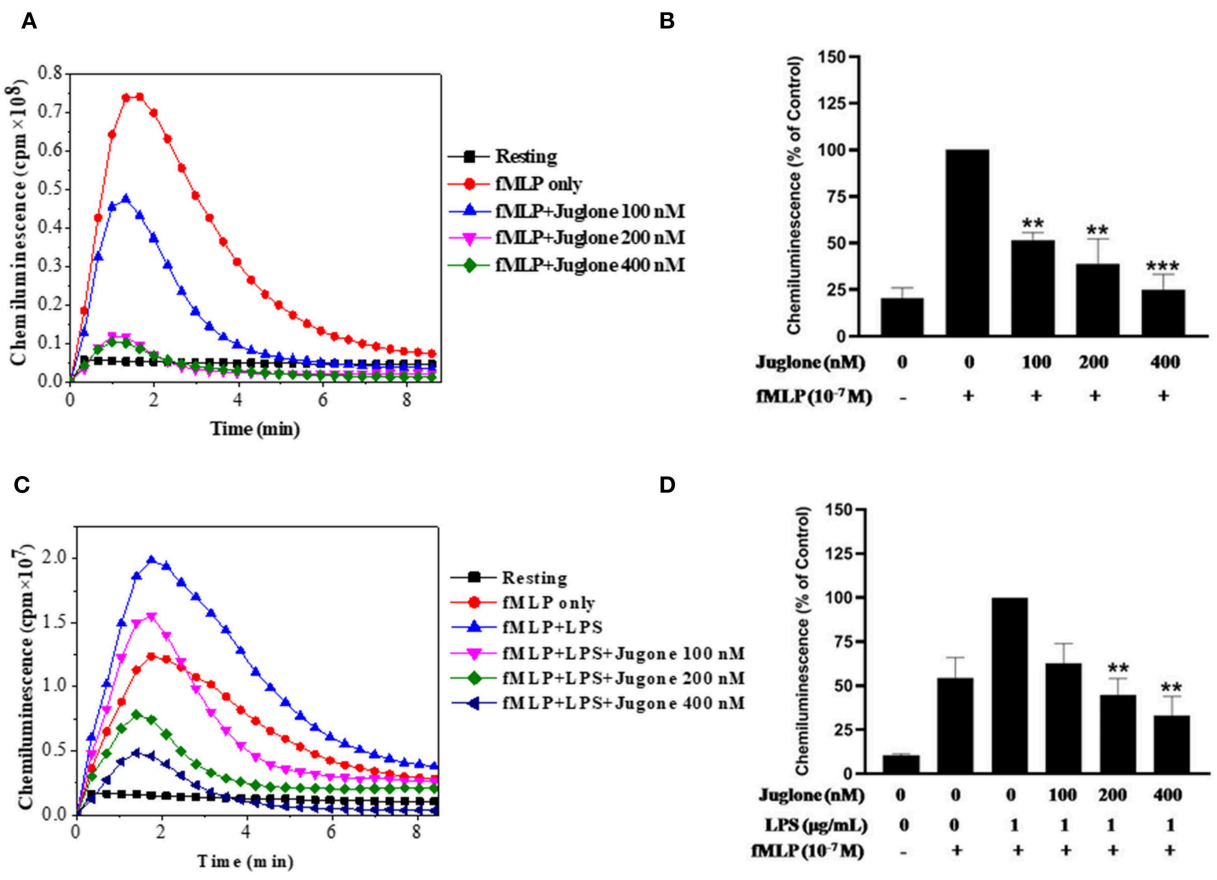

FIGURE 3 | Effect of Juglone, a Pin1 inhibitor on fMLP-induced and LPS-primed ROS production by human neutrophils. **(A)** Human neutrophils (1 × 10^6/ml) were incubated in the absence or presence of increasing concentrations of Juglone for 20 min at 37°C, then stimulated with fMLP (10^{-7} M). ROS production was measured with a luminol-amplified chemiluminescence technique. **(B)** Total chemiluminescence in each experimental condition is expressed as mean plus or minus SEM of 3 experiments. $^{**}p < 0.05$ and $^{***}p < 0.001$ as compared with fMLP alone (control 100%). **(C)** Human neutrophils (1 × 10^6/ml) were incubated in the absence or presence of increasing concentrations of Juglone for 20 min at 37°C, then LPS (1 μg/mL) was added for another 20 min before stimulation with fMLP (10^{-7} M). ROS production was measured with a luminol-amplified chemiluminescence technique. **(D)** Total chemiluminescence in each experimental condition is expressed as mean plus or minus SEM of 3 experiments. $^{**}p < 0.01$ as compared with control (100%).

LPS Is Able to Induce Pin1 Activation in Human Neutrophils

To further investigate the implication of Pin1 in the priming effect of LPS on neutrophil ROS production, we investigated the effect of LPS on Pin1 activation. Neutrophils were incubated with LPS for 20 min, in the absence and presence of Juglone, then lysed. TNFα and fMLP were used as positive controls (18, 33). The activity of Pin 1 was determined by measuring the absorbance of free pNA resulted from the cleavage of Suc-Ala-Glu-Pro-Phe-pNA after it *cis to trans* conformational changes. Results presented in **Figure 5** show that LPS strongly increased Pin1 activity ($P < 0.0001$ compared to resting cells). The activation effect of LPS on Pin1 was very similar to the one exhibited by fMLP (**Figure 5**) and TNFα (data not shown) which are strong enhancers of Pin 1 activity (33). Treatment of cells with Juglone markedly reduced LPS-induced activation of Pin 1, showing that this assay is specific for Pin1 ($P < 0.001$ compared to cells treated with LPS alone). Interestingly, Pin1 activity in neutrophils treated with both LPS + fMLP is higher than the activity in neutrophils treated with LPS alone or fMLP alone and Juglone inhibited this process. The results obtained suggest a key role of Pin 1 in LPS-induced priming of ROS production by human neutrophils in response to fMLP.

LPS Induces Phosphorylation of p47^{phox} Mainly on Serine 345, a Pin1 Binding Site

Priming of the NADPH oxidase in neutrophils is controlled by different pathways, mainly the phosphorylation of p47^{phox} and the translocation of NOX2 from granules to the plasma membrane (8, 33). To further understand the mechanisms of LPS induced priming of ROS production by neutrophil, we studied the phosphorylation of different sites of p47^{phox}. Results show that stimulation of neutrophils with LPS (0.1, 0.5, 1 and 5 μg/mL) induced a significant ($p < 0.05$) dose-dependent phosphorylation of p47^{phox} mainly on Ser345 and at lower level on Ser328 (**Figures 6A,B**). However, no phosphorylation effect was exerted on Ser304, Ser315, and Ser320 of p47^{phox}. Kinetic

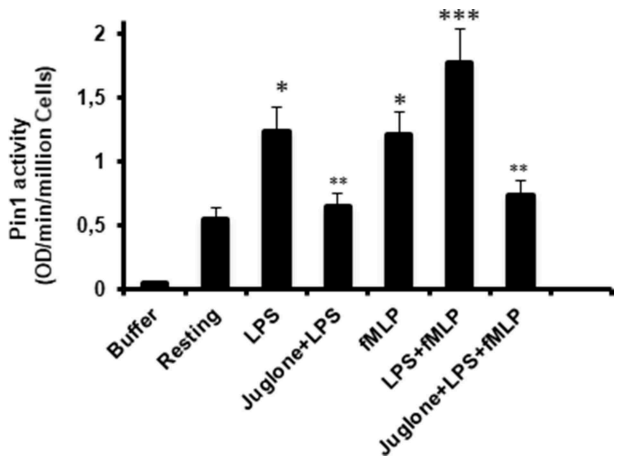

FIGURE 5 | Pin1 is activated by LPS, fMLP, and LPS + fMLP in neutrophils and Juglone markedly decreased Pin 1 activity. Neutrophils were incubated in the absence and presence of Juglone (400 nM for 20 min) and then treated with LPS (5 μg/mL), fMLP (10^{-7} M), or LPS + fMLP before lysis. Pin1 activity was determined by measuring the absorbance of free pNA cleaved from Trp-Phe-Tyr-Ser (PO_3H_2)-Pro-Arg-pNA Suc at 410 nm. Results are expressed as Optical Density (OD)/min/million cells. Data are mean plus or minus SEM of 8 experiments. $p < 0.01$ as LPS and fMLP compared to resting cells (*); Cells treated with juglone compared to untreated cells (**); and LPS+fMLP as compared to LPS alone or fMLP alone (***).

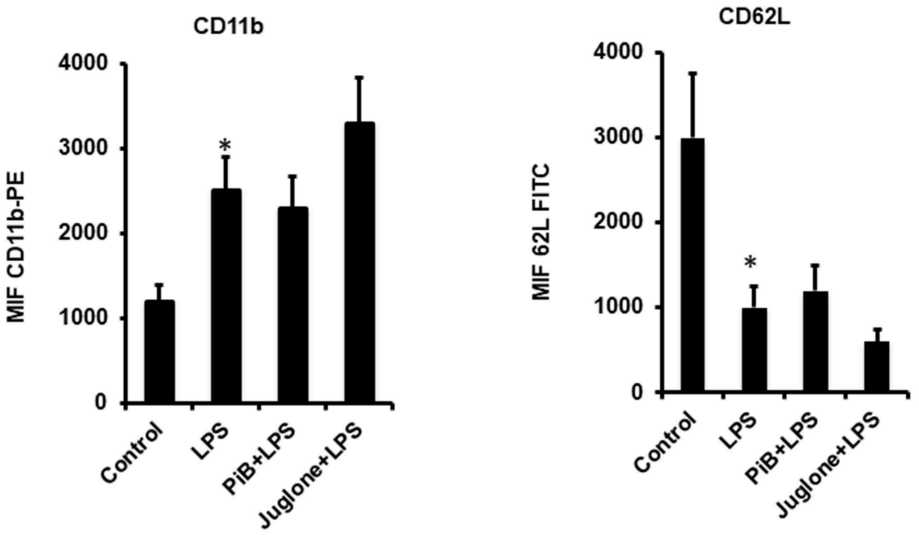

FIGURE 4 | Effect of PiB and Juglone on LPS-induced CD11b expression on neutrophil plasma membrane and CD62L shedding. Human neutrophils (10×10^6/ml) were incubated in the absence or presence of PiB (50 μM) or Juglone (400 nM) for 20 min at 37°C, then treated by LPS (1 μg/ml) for another 20 min. CD11b **(Left)** and CD62L **(Right)** expression at the plasma membrane was determined by flow cytometry using specific antibodies. Data are mean ± SEM of three experiments. *$p < 0.005$ when LPS treated cells were compared to control untreated cells.

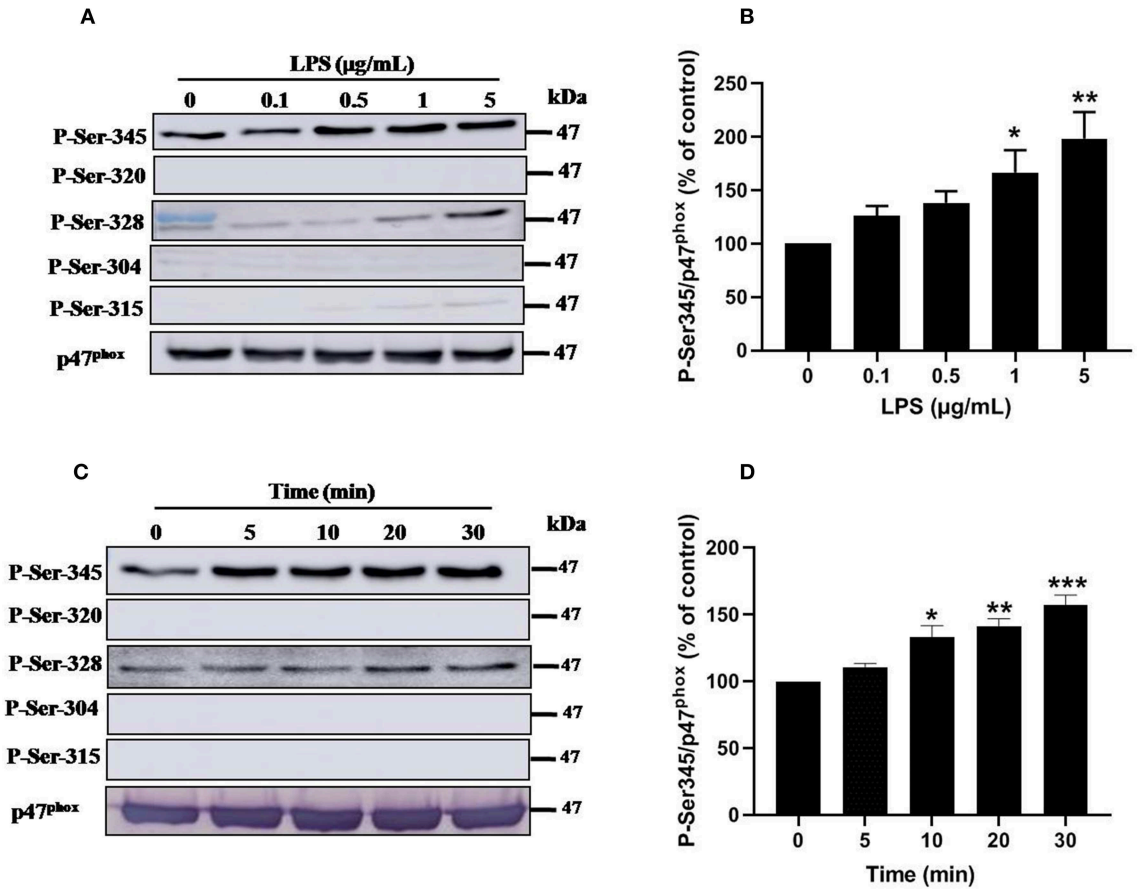

FIGURE 6 | Effect of LPS on p47^{phox} phosphorylation in neutrophils. **(A)** Neutrophils (10 × 10^6 cells/mL) were incubated with various concentrations of LPS (0, 0.1, 0.5, 1, 5 µg/mL) for 20 min at 37°C and the cells were lyzed. Proteins were analyzed by SDS-PAGE and western blot using anti-phospho-Ser-345, anti-phospho-Ser-320, anti-phospho-Ser-328, anti-phospho-Ser-304, anti-phospho-Ser-315, and anti-p47^{phox} antibodies. **(B)** Western blots from different experiments were scanned and quantified. The intensity of bands was expressed relative to total p47^{phox} amount. The cumulated data of phospho-Ser-345 is shown in the histogram as percentage to control (Resting 100%). **(C)** Neutrophils (10 × 10^6 cells/mL) were incubated with LPS (1 µg/mL) for indicated times (0, 5, 10, 20, 30 min). Cells were analyzed by SDS-PAGE and immunoblotting with anti-phospho-Ser-345, anti-phospho-Ser-320, anti-phospho-Ser-328, anti-phospho-Ser-304, anti-phospho-Ser-315, and anti-p47^{phox} antibodies. **(D)** Western blots from different experiments for the kinetic effect were scanned and quantified. The intensity of bands was expressed relative to total p47^{phox} amount. Data are mean ± SEM of three or more separate experiments. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ as compared to control (100%).

study of LPS induced phophosrylation of p47^{phox} showed that LPS (1 µg/mL) rapidly induced phosphorylation Ser345 up to 30 min (**Figures 6C,D**). The phosphorylation of Ser328 was also weakly induced but not the phosphorylation of Ser304, 315, and 320 even at 30 min of incubation. These results clearly show that LPS induced p47^{phox} phosphorylation on Ser345, a binding site for Pin1 in human neutrophils (33, 38).

LPS Induces Activation of p38MAPK and ERK1/2 Signaling Pathways in Human Neutrophils

Ser345 of p47^{phox} is located in a MAPKinase phosphorylated site (-PGPQS(345)PG-) and is phosphorylated *in vitro* by p38MAPK and ERK1/2 (38). To investigate the kinase(s) involved in LPS-induced phosphorylation of Ser345 we first studied the phosphorylation (which reflects the activation) of different MAPKinases in LPS stimulated neutrophils. Our results show that neutrophils treatment with different concentrations of LPS induced a significant phosphorylation of p38MAPK in a dose-dependent manner. This phosphorylation was 6–8 folds higher than the basal one (**Figures 7A,B**). The kinetic study shows a retarded effect of LPS on p38MAPK (later than 10 min), with a maximum effect at 30 min (**Figures 7C,D**). Results also showed that LPS induced ERK1/2 phosphorylation in neutrophils in a concentration-dependent manner (**Figures 8A,B**). Likely to p38MAPK, kinetic study showed a retarded effect of LPS on ERK1/2 activation, starting at 10 min and which is potentiated till 30 min of treatment (**Figures 8C,D**). We also studied the effect of LPS on JNK1/2 phosphorylation in human neutrophils, results showed that LPS did not induce JNK phosphorylation (data

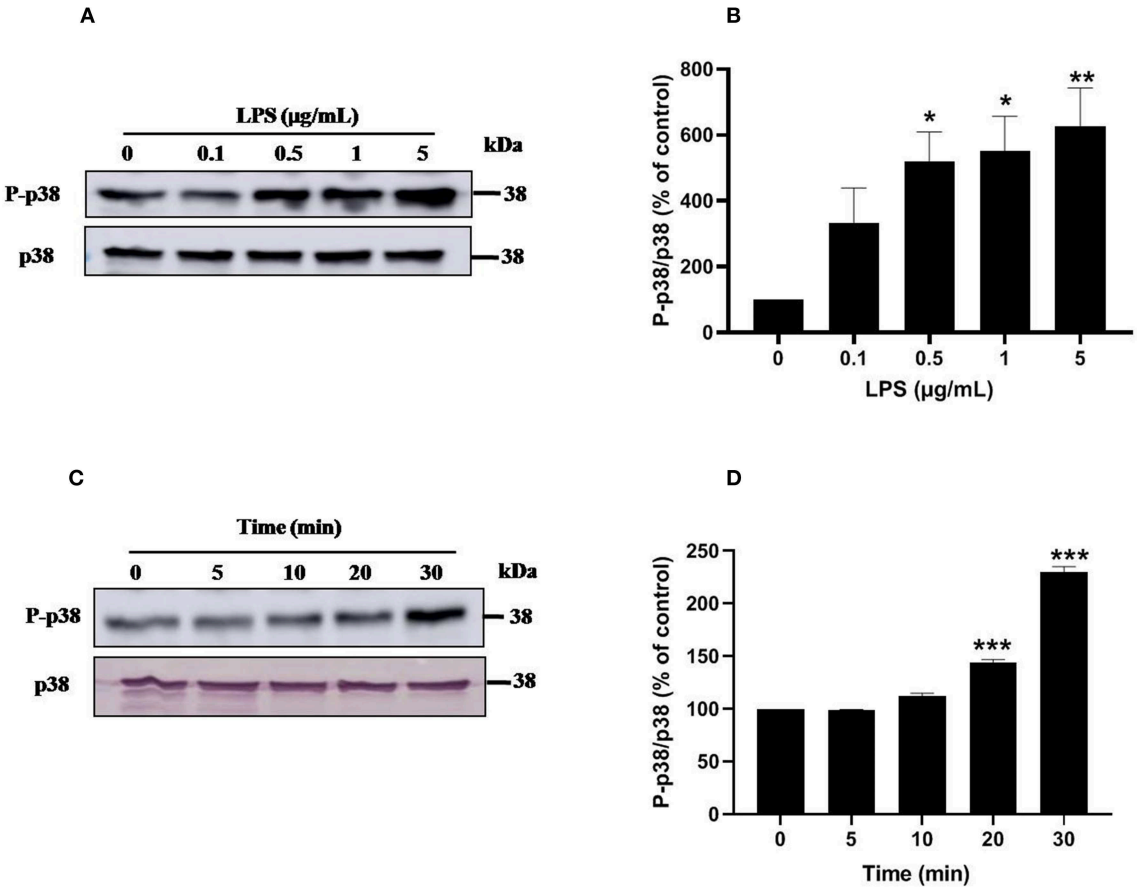

FIGURE 7 | LPS induces p38 MAPK activation in human neutrophils. **(A)** Neutrophils (10×10^6 cells/mL) were incubated with various concentration of LPS (0, 0.1, 0.5, 1, 5 µg/mL) for 20 min at 37°C. Cells were analyzed by SDS-PAGE and western blot using anti-phospho-p38 and anti-p38 antibodies. **(B)** Western blots from different experiments were scanned and quantified, total p38 were quantified by densitometry, and the intensity of phosphorylated p38 was corrected for the protein amount of p38. **(C)** Neutrophils (10×10^6 cells/mL) were incubated with LPS (1 µg/mL) for indicated times (0, 5, 10, 20, 30 min). Cells were analyzed by SDS-PAGE and immunoblotting with anti-phospho-p38 and anti-p38 antibodies. **(D)** Western blots from different experiments for the kinetic effect were scanned and quantified, total p38 were quantified by densitometry, and the intensity of phosphorylated p38 was corrected for the protein amount of p38. Data are mean ± SEM of three or more separate experiments. $p < 0.005$ as compared to control (100%). $^*p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$.

not shown). Taken together, these results confirmed data from the literature (39, 40) and suggest that p38MAPK and ERK1/2 signaling pathways might be important effectors in LPS mediated priming of NOX2 in human PMN.

DISCUSSION

LPS or endotoxin is released by gram negative bacteria at sites of infection. It induced several neutrophil functions such as priming of superoxide production in combination to other stimuli such as the bacterial peptide fMLP. This LPS-induced priming of superoxide production was known since several years but the pathways involved in this process are unknown. Here we show that the peptidylprolyl cis/trans isomerase is a key enzyme of the LPS-induced NADPH oxidase priming.

This study, confirmed that LPS alone was not able to induce superoxide production as measured by luminol-amplified chemiluminescence as shown in **Figure 1**. At higher concentrations of LPS (>5 µg/ml) a weak superoxide production was observed but was not detected by an other specific technique such as cytochrome C reduction assay (data not shown). However, LPS even at low concentrations was able to enhance fMLP-induced superoxide production confirming its ability to prime this function.

To study the role of Pin1 in LPS-induced priming of superoxide production by neutrophils, we used two Pin1 inhibitors, PiB and Juglone as neutrophils are resistant to transfection. Both molecules inhibited LPS-induced priming of superoxide production and higher concentrations they inhibited also fMLP-induced activation. To check if Pin1 is also involved in LPS-induced degranulation and shedding to other neutrophil function, we tested the effect of PiB and Juglone on LPS-induced CD11b externalization from specific and gelatinase granules at the plasma membrane and the release of CD62L from the plasma membrane. PiB at high concentration (50 µM) did not have any effect, however Juglone stimulated this function. The

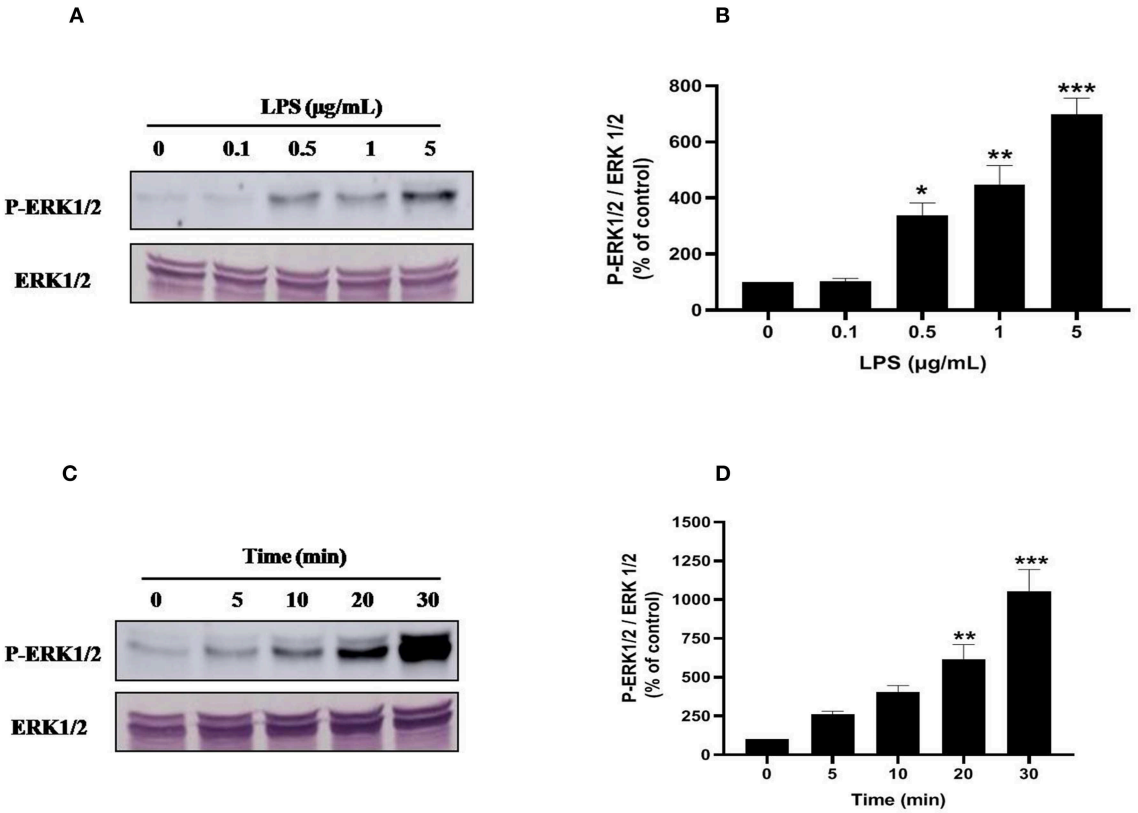

FIGURE 8 | LPS induces ERK1/2 (p44/42 MAPK) activation in human neutrophils. **(A)** Neutrophils (10×10^6 cells/mL) were incubated with various concentration of LPS (0, 0.1, 0.5, 1, 5 µg/mL) for 20 min at 37°C. Cells were analyzed by SDS-PAGE and western blot using anti-phospho-ERK1/2 and anti-ERK1/2 antibodies. **(B)** Western blots from different experiments were scanned, total ERK1/2 were quantified by densitometry, and the intensity of phosphorylated ERK 1/2 was corrected for the protein amount of ERK1/2. **(C)** Neutrophils (10×10^6 cells/mL) were incubated with LPS (1 µg/mL) for indicated times (0, 5, 10, 20, 30 min). Cells were analyzed by SDS-PAGE and immunoblotting with anti-phospho-ERK1/2 and anti-ERK1/2 antibodies. **(D)** Western blots from different experiments for the kinetic effect were scanned and phosphorylated, total ERK1/2 were quantified by densitometry, and the intensity of phosphorylated ERK1/2 was corrected for the protein amount of ERK1/2. Data are mean ± SEM of three or more separate experiments. $^*p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ as compared to control (100%).

reasons for these contrasting results are unknown and should be more investigated in the future. Juglone was shown to have some other effects and PiB is believed to be more selective for Pin1 (36, 41). CD11b is localized at the membrane of the same granules as gp91^{phox}/NOX2 and p22^{phox} (15, 42–45), thus these results suggest also that PiB does not inhibit NOX2 translocation at the plasma membranes and Pin1 is not involved in this process.

To further investigate the role of Pin1 in LPS-treated neutrophils, we showed that LPS is able to increase Pin1 activation. Pin1 is constitutively active in resting cells in agreement with other reports and LPS was able to increase this activation in a comparable manner of TNF and fMLP (33). The pathways involved in the stimulation of Pin1 activity by TLR4 activation are unknown. It was shown that Pin1 is phosphorylated *in vitro* and in cells by protein kinase A (PKA) (46) and death-associated protein kinase 1 (DAPKinase 1) (47) and these phosphorylations inhibited its activity. To check if LPS is able to induce Pin1 dephosphorylation in human neutrophils, we tested different anti-phospho-Pin1 antibodies from different sources but the results were not conclusive. New anti-phospho-Pin1 antibodies are needed to study this pathway.

Upon activation Pin1 binds to phosphorylated Ser or Thr near a Pro. The NADPH oxidase component p47^{phox} is phosphorylated on Ser345 which is a Pin1 recognition motif (33, 38). We thus investigated the effect of LPS on p47^{phox} phosphorylation. Interestingly, LPS induced mainly the phosphorylation of Ser345 and at lesser extent the phosphorylation of Ser328. It is noteworthy that LPS alone did not induce the phosphorylation of Ser304, Ser315, and Ser320 required for NADPH oxidase activation explaining the lack of superoxide production with LPS alone. LPS also induced the phosphorylation of p38MAPKinase and ERK1/2 that are able to phosphorylate p47^{phox} on Ser345. We tested the effect of p38MAPK and ERK1/2 inhibitors which both inhibited p47phox phosphorylation on Ser345 (data not shown), suggesting that these MAPKinases converge to phosphorylate this site. In our previous work (33), we showed that Pin1 facilitates p47^{phox} phosphorylation on Ser328 and other serines upon fMLP

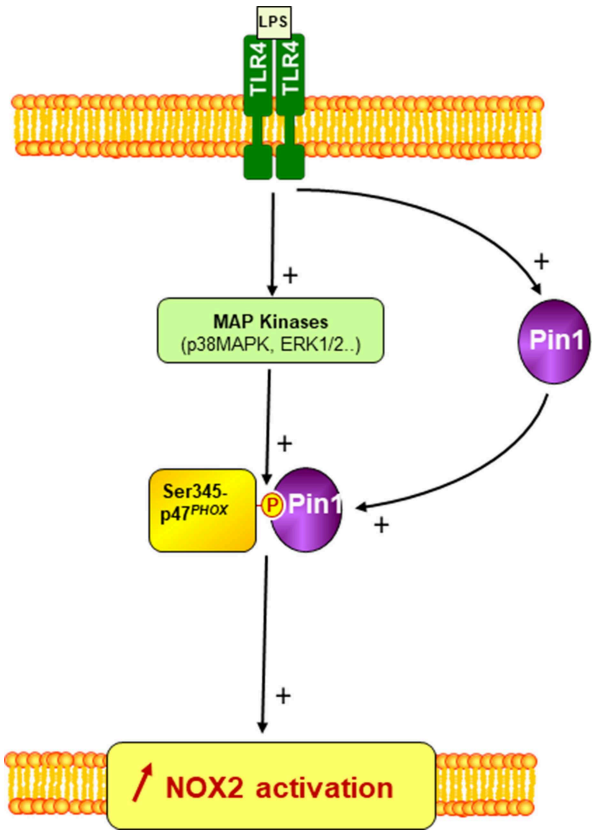

FIGURE 9 | A scheme of the involvement of Pin1 and p47^{phox} phosphorylation in LPS-induced priming of NOX2. LPS via TLR4 induces Pin1 and MAP Kinases (p38MAPK and ERK1/2) activation in neutrophil cytosol. Active MAP Kinases phosphorylate p47^{phox} on serine 345 which is a binding site for activated Pin1. Pin1 binds to phosphorylated Ser345 and induces conformational changes of p47^{phox} to facilitate its phosphorylation by other Ser/Thr kinases and thus enhances NOX2 activation by a secondary agonist such as bacteria or fMLP.

stimulation. To check if in LPS-primed neutrophils, Pin1 is able to facilitate the phosphorylation of Ser328 we tested Juglone and PiB on this process. Results showed that Juglone and PiB were able to inhibit phosphorylation of p47^{phox} on Ser328 (data not shown), confirming our previous data.

LPS is the natural ligand of TLR4 (29). LPS induces neutrophil NADPH oxidase hyper-activation and activation of other immune cell functions mediating infection-induced inflammation and sepsis (27, 48). The results presented in this manuscript clearly show that Pin1 inhibitors inhibited LPS-induced priming of neutrophil NADPH oxidase activation, LPS induced Pin1 activation in human neutrophils and induced the phosphorylation of p47^{phox} on specific sites (Ser345 and 328) along with the activation of two MAPKinases p38 and ERK1/2. These events were presented in a scheme in **Figure 9**. Inhibitors of Pin1 at low concentrations could inhibit LPS-induced excessive ROS production at inflammatory site and might have anti-inflammatory effects while preserving the physiological ability of the bacterial N-formyl peptides to activate neutrophils.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

ML, SB, MH-N, and CP designed and performed the experiments. PD, SY, and JE-B designed the experiments and analyzed the data. All authors contributed to writing the manuscript.

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REFERENCES

1. Nauseef WM, Borregaard N. Neutrophils at work. *Nat Immunol.* (2014) 15:602–11. doi: 10.1038/ni.2921
2. Malech HL, Deleo FR, Quinn MT. The role of neutrophils in the immune system: an overview. *Methods Mol Biol.* (2014) 1124:3–10. doi: 10.1007/978-1-62703-845-4_1
3. Mócsai A. Diverse novel functions of neutrophils in immunity, inflammation, and beyond. *J Exp Med.* (2013) 210:1283–99. doi: 10.1084/jem.20122220
4. Witko-Sarsat V, Rieu P, Descamps-Latscha B, Lesavre P, Halbwachs-Mecarelli L. Neutrophils: molecules, functions and pathophysiological aspects. *Lab Invest.* (2000) 80:617–53. doi: 10.1038/labinvest.3780067
5. Borregaard N. Neutrophils, from marrow to microbes. *Immunity.* (2010) 33:657–70. doi: 10.1016/j.immuni.2010.11.011
6. Nauseef WM. How human neutrophils kill and degrade microbes: an integrated view. *Immunol Rev.* (2007) 219:88–102. doi: 10.1111/j.1600-065X.2007.00550.x
7. El-Benna J, Dang, PM, Gougerot-Pocidalo MA, Elbim C. Phagocyte NADPH oxidase: a multicomponent enzyme essential for host defenses. *Arch Immunol Ther Exp.* (2005) 3:199–206.
8. El-Benna J, Dang PMC, Hurtado-Nedelec M, Marie JC, Gougerot-Pocidalo MA. Priming of the neutrophil respiratory burst: role in host defense and inflammation. *Imm Rev.* (2016) 273:180–93. doi: 10.1111/imr.12447
9. Hampton MB, Kettle AJ, Winterbourn CC. Inside the neutrophil phagosome: oxidants, myeloperoxidase, and bacterial killing.

Blood. (1998) 12:3007–17. doi: 10.1182/blood.V92.9.3007.421k47_3007_3017
10. Belambri SA, Rolas L, Raad H, Hurtado-Nedelec M, Dang PM, El-Benna J. NADPH oxidase activation in neutrophils: role of the phosphorylation of its subunits. *Eur J Clin Invest*. (2018) 48(Suppl. 2):e12951. doi: 10.1111/eci.12951
11. El-Benna J, Dang PM, Gougerot-Pocidalo MA, Marie JC, Braut-Boucher F. p47phox, the phagocyte NADPH oxidase/NOX2 organizer: structure, phosphorylation and implication in diseases. *Exp Mol Med.* (2009) 41:217–25. doi: 10.3858/emm.2009.41.4.058
12. El-Benna J, Dang PM, Gougerot-Pocidalo MA. Priming of the neutrophil NADPH oxidase activation: role of p47phox phosphorylation and NOX2 mobilization to the plasma membrane. *Semin Immunopathol. (*2008) 30:279–89. doi: 10.1007/s00281-008-0118-3
13. Sheppard FR, Kelher MR, Moore EE, McLaughlin NJ, Banerjee A, Silliman CC. Structural organization of the neutrophil NADPH oxidase: phosphorylation and translocation during priming and activation. *J Leukoc Biol.* (2005) 78:1025–42. doi: 10.1189/jlb.0804442
14. Forehand JR, Pabst MJ, Phillips WA, Johnston RB Jr. Lipopolysaccharide priming of human neutrophils for an enhanced respirator burst. Role of intracellular free calcium. *J Clin Invest.* (1989) 83:74–83. doi: 10.1172/JCI113887
15. Leo FR, Renee J, McCormick S, Nakamura N, Apicella M, Weiss JP, et al. Neutrophils exposed to bacterial lipopolysaccharide upregulate NADPH oxidase assembly. *J Clin Invest.* (1998) 101:455–63. doi: 10.1172/JCI949
16. Almkvist J, Fäldt J, Dahlgren C, Leffler H, Karlsson A. Lipopolysaccharide-induced gelatinase granule mobilization primes neutrophils for activation by galectin-3 and formylmethionyl-Leu-Phe. *Infect Immun.* (2001) 69:832–7. doi: 10.1128/IAI.69.2.832-837.2001
17. Hayashi F, Means TK, Luster AD. Toll-like receptors stimulate human neutrophil function. *Blood.* (2003) 102:2660–9. doi: 10.1182/blood-2003-04-1078
18. Makni-Maalej K, Boussetta T, Hurtado-Nedelec M, Belambri SA, Gougerot-Pocidalo MA, El-Benna J. The TLR7/8 agonist CL097 primes N-formyl-methionyl-leucyl-phenylalanine-stimulated NADPH oxidase activation in human neutrophils: critical role of p47phox phosphorylation and the proline isomerase Pin1. *J Immunol.* (2012) 189:4657–65. doi: 10.4049/jimmunol.1201007
19. Makni-Maalej K, Marzaioli V, Boussetta T, Belambri SA, Gougerot-Pocidalo MA, Hurtado-Nedelec M, et al. TLR8, but not TLR7, induces the priming of the NADPH oxidase activation in human neutrophils. *J Leukoc Biol.* (2015) 97:1081–7. doi: 10.1189/jlb.2A1214-623R
20. Hughes JE, Stewart J, Barclay GR, Govan JR. Priming of neutrophil respiratory burst activity by lipopolysaccharide from *Burkholderia cepacia*. *Infect Immun.* (1997) 65:4281–7.
21. Picard C, Puel A, Bonnet M, Ku CL, Bustamante J, Yang K, et al. Pyogenic bacterial infections in humans with IRAK-4 deficiency. *Science.* (2003) 299:2076–9. doi: 10.1126/science.1081902
22. Karlsson A, Markfjäll M, Strömberg N, Dahlgren C. *Escherichia coli*-induced activation of neutrophil NADPH-oxidase: lipopolysaccharide and formylated peptides act synergistically to induce release of reactive oxygen metabolites. *Infect Immun.* (1995) 63:4606–12.
23. Babior BM. Phagocytes and oxidative stress. *Am J Med.* (2000) 109:33–44. doi: 10.1016/S0002-9343(00)00481-2
24. Condliffe AM, Kitchen E, Chilvers ER. Neutrophil priming: pathophysiological consequences and underlying mechanisms. *Clin Sci.* (1998) 94:461–71. doi: 10.1042/cs0940461
25. Jacobi J, Sela S, Cohen HI, Chezar J, Kristal B. Priming of polymorphonuclear leukocytes: a culprit in the initiation of endothelial cell injury. *Am J Physiol Heart Circ Physiol.* (2006) 290:H2051–8. doi: 10.1152/ajpheart.01040.2005
26. Choi JC, Jung JW, Kwak HW, Song JH, Jeon EJ, Shin JW, et al. Granulocyte macrophage-colony stimulating factor (GM-CSF) augments acute lung injury via its neutrophil priming effects. *J Korean Med Sci.* (2008) 23:288–95. doi: 10.3346/jkms.2008.23.2.288
27. Qian F, Deng J, Cheng N, Welch EJ, Zhang Y, Malik AB, et al. A non-redundant role for MKP5 in limiting ROS production and preventing LPS-induced vascular injury. *EMBO J.* (2009) 28:2896–907. doi: 10.1038/emboj.2009.234
28. Nurcombe HL, Bucknall RC, Edwards SW. Neutrophils isolated from the synovial fluid of patients with rheumatoid arthritis: priming and activation *in vivo*. *Ann Rheum Dis. (*1991) 50:147–53. doi: 10.1136/ard.50.3.147
29. Poltorak A, He X, Smirnova I, Liu MY, Van Huffel C, Du X, et al. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science.* (1998) 282:2085–8. doi: 10.1126/science.282.5396.2085
30. Yaffe MB, Schutkowski M, Shen M, Zhou XZ, Stukenberg PT, Rahfeld JU, et al. Sequence-specific and phosphorylation-dependent proline isomerization: a potential mitotic regulatory mechanism. *Science.* (1997) 278:1957–60. doi: 10.1126/science.278.5345.1957
31. Liou YC, Zhou XZ, Lu KP. Prolyl isomerase Pin1 as a molecular switch to determine the fate of phosphoproteins. *Trends Biochem Sci.* (2011) 36:501–14. doi: 10.1016/j.tibs.2011.07.001
32. Lee TH, Pastorino L, Lu KP. Peptidyl-prolyl cis-trans isomerase Pin1 in ageing, cancer and Alzheimer disease. *Expert Rev Mol Med.* (2011) 13:e21. doi: 10.1017/S1462399411001906
33. Boussetta T, Gougerot-Pocidalo MA, Hayem G, Ciappelloni S, Raad H, Arabi Derkawi R, et al. The prolyl isomerase Pin1 acts as a novel molecular switch for TNF-alpha-induced priming of the NADPH oxidase in human neutrophils. *Blood.* (2010) 116:5795–802. doi: 10.1182/blood-2010-03-273094
34. Hurtado-Nedelec M, Makni-Maalej K, Gougerot-Pocidalo MA, Dang PM, El-Benna J. Assessment of priming of the human neutrophil respiratory burst. *Methods Mol Biol.* (2014) 1124:405–12. doi: 10.1007/978-1-62703-845-4_23
35. Belambri SA, Dang PM, El-Benna J. Evaluation of p47phox phosphorylation in human neutrophils using phospho-specific antibodies. *Methods Mol Biol.* (2014) 1124:427–33. doi: 10.1007/978-1-62703-845-4_25
36. Uchida T, Takamiya M, Takahashi M, Miyashita H, Ikeda H, Terada T, et al. Pin1 and Par14 peptidyl prolyl isomerase inhibitors block cell proliferation. *Chem Biol.* (2003) 10:15–24. doi: 10.1016/S1074-5521(02)00310-1
37. Hennig L, Christner C, Kipping M, Schelbert B, Rücknagel KP, Grabley S, et al. Selective inactivation of parvulin-like peptidyl-prolyl cis/trans isomerases by juglone. *Biochemistry.* (1998) 37:5953–60. doi: 10.1021/bi973162p
38. Dang PM, Stensballe A, Boussetta T, Raad H, Dewas C, Kroviarski Y, et al. A specific p47phox -serine phosphorylated by convergent MAPKs mediates neutrophil NADPH oxidase priming at inflammatory sites. *J Clin Invest.* (2006) 116:2033–43. doi: 10.1172/JCI27544
39. Nick JA, Avdi NJ, Gerwins P, Johnson GL, Worthen GS. Activation of a p38 mitogen-activated protein kinase in human neutrophils by lipopolysaccharide. *J Immunol.* (1996) 156:4867–75.
40. Nolan B, Duffy A, Paquin L, De M, Collette H, Graziano CM, et al. Mitogen-activated protein kinases signal inhibition of apoptosis in lipopolysaccharide-stimulated neutrophils. *Surgery.* (1999) 126:406–12. doi: 10.1016/S0039-6060(99)70185-6
41. Ahmad T, Suzuki YJ. Juglone in Oxidative Stress and Cell Signaling. *Antioxidants.* (2019) 8:E91. doi: 10.3390/antiox8040091
42. Ward RA, Nakamura M, McLeish KR. Priming of the neutrophil respiratory burst involves p38 mitogen-activated protein kinase-dependent exocytosis of flavocytochrome b558-containing granules. *J Biol Chem.* (2000) 275:36713–9. doi: 10.1074/jbc.M003017200
43. Uriarte SM, Rane MJ, Luerman GC, Barati MT, Ward RA, Nauseef WM, et al. Granule exocytosis contributes to priming and activation of the human neutrophil respiratory burst. *J Immunol.* (2011) 187:391–400. doi: 10.4049/jimmunol.1003112
44. Faurschou M, Borregaard N. Neutrophil granules and secretory vesicles in inflammation. *Microbes Infect.* (2003) 5:1317–27. doi: 10.1016/j.micinf.2003.09.008
45. Bai J, Tang L, Lomas-Neira J, Chen Y, McLeish KR, Uriarte SM, et al. TAT-SNAP-23 treatment inhibits the priming of neutrophil functions

contributing to shock and/or sepsis-induced extra-pulmonary acute lung injury. *Innate Immun.* (2015) 21:42–54. doi: 10.1177/1753425913516524

46. Lu PJ, Zhou XZ, Liou YC, Noel JP, Lu KP. Critical role of WW domain phosphorylation in regulating phosphoserine binding activity and Pin1 function. *J Biol Chem.* (2002) 277:2381–4. doi: 10.1074/jbc.C100228200
47. Lee TH, Chen CH, Suizu F, Huang P, Schiene-Fischer C, Daum S, et al. Death-associated protein kinase 1 phosphorylates Pin1 and inhibits its prolyl isomerase activity and cellular function. *Mol Cell*. (2011) 42:147–59. doi: 10.1016/j.molcel.2011.03.005
48. Pillay J, Ramakers BP, Kamp VM, Loi AL, Lam SW, Hietbrink F, et al. Functional heterogeneity and differential priming of circulating neutrophils in human experimental endotoxemia. *J Leukoc Biol.* (2010) 88:211–20. doi: 10.1189/jlb.1209793

Conflict of Interest: ML was employed by the company Yitong Food Industry Co. Ltd, China.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Neutrophil Extracellular Traps in Autoimmunity and Allergy: Immune Complexes at Work

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Neutrophil extracellular traps (NETs) have been initially described as main actors in host defense owing to their ability to immobilize and sometimes kill microorganisms. Subsequent studies have demonstrated their implication in the pathophysiology of various diseases, due to the toxic effects of their main components on surrounding tissues. Several distinct NETosis pathways have been described in response to various triggers. Among these triggers, IgG immune complexes (IC) play an important role since they induce robust NET release upon binding to activating FcγRs on neutrophils. Few *in vitro* studies have documented the mechanisms of IC-induced NET release and evidence about the partners involved is controversial. *In vivo*, animal models and clinical studies have strongly suggested the importance of IgG IC-induced NET release for autoimmunity and anaphylaxis. In this review, we will focus on two autoimmune diseases in which NETs are undoubtedly major players, systemic lupus erythematosus (SLE), and rheumatoid arthritis (RA). We will also discuss anaphylaxis as another example of disease recently associated with IC-induced NET release. Understanding the role of IC-induced NETs in these settings will pave the way for new diagnostic tools and therapeutic strategies.

Keywords: NETs, autoimmunity, anaphylaxis, immune complexes, neutrophils

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INTRODUCTION

Neutrophil extracellular traps (NETs) are extracellular chromatin filaments produced upon cell activation and decorated with many proteins normally confined to neutrophil cytoplasm and granules. This process was first described in 2004 as a new mechanism to catch, immobilize, and potentially kill bacteria (1). Subsequently, NETs have been shown to kill several species of bacteria, and rapidly limit the extent of the infection in some models (2–4). However, NET contribution to infectious diseases is double-edged. On the one hand, they may play a major role in defense against pathogens but on the other hand, collateral damage in infected host tissues can be significant, due to proteolytic enzymes release or histone toxicity (5–7). For instance, a deleterious role for NETs have been described in life-threatening infectious conditions such as sepsis or pneumonia-associated acute respiratory distress syndrome (8–12). Interestingly, besides their major pro-inflammatory role, NETs might also be able to downregulate dendritic cell activation and promote Th2 response, thus participating in the resolution of inflammation (13).

In addition to their role during infection, increasing evidence shows that NETosis also happens in a large number of non-infectious inflammation-associated diseases, including various lung diseases, thrombosis, cancer, and auto-immune diseases (14). In the lung, we and others reported that NETs are found in high concentrations in patients with chronic obstruction pulmonary disease or asthma (15–20). In thrombosis, a major interplay between platelets, components of the coagulation system and NETs have been unraveled, leading to the emergence of a new concept named immunothrombosis (21). Finally, as NETs are both pro-inflammatory and composed of many potential autoimmune targets, it has been hypothesized that NETs could be strong inducers of autoimmunity. Indeed, a crucial role for NETs has been described in various autoimmune diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), vasculitis or diabetes (22–24).

IMMUNE COMPLEXES TRIGGER NET FORMATION

NETosis Mechanism

In the initial studies about NETosis, the authors described a relatively long process (several hours) leading to neutrophil lysis and dependent on NADPH oxidase 2 (NOX2) activation (25–27). Fifteen years later, several distinct pathways in response to various triggers have been described making the definition of NETosis even more complex (28). The lytic or suicidal form of NETosis relies on NOX2-derived reactive oxygen species (ROS) release, allowing the liberation of neutrophil elastase (NE) and myeloperoxidase (MPO) from azurophilic granules. Both histone cleavage by NE and their citrullination by peptidyl deaminase 4 (PAD4) have been initially described as required for chromatin decondensation and extrusion out of the cell. However, several intracellular pathways have been described, and some NET release could be NE- or PAD4-independent (29–31). Autophagy is also probably involved, even if there is yet no consensus as conflicting results were obtained (28, 32–34). Very interestingly, quick non-suicidal pathways of NET release have also been described, where neutrophils remain viable, and can still perform functions such as phagocytosis, chemotaxis or dendritic cell activation (14, 35–37). Some studies have also shown NETs composed of mitochondrial DNA (38) but this mechanism requires more investigation. In addition, NOX2–independent pathways have been described, where calcium influx could triggers the activation of mitochondrial ROS (39, 40). This mechanism has been shown in chronic granulomatous disease patients, who have impaired NOX2 activation (41, 42).

The mechanisms of NET formation and release may vary depending upon the initial trigger (23, 43, 44). A large number of triggers have been described, both artificial and physiological. Among them, IgG and IgA immune complexes (IC) have been shown to trigger NETosis in different situations (38, 45–48).

Immune Complexes Triggering

In this review, we will focus on the role of IC on NET formation. To date, few *in vitro* studies have documented the mechanisms of NET release in response to pre-formed IC in both murine (49, 50) and human neutrophils (49, 51). Moreover, these studies provided conflicting results especially regarding type of FcγR involved or NOX2 requirement. Using soluble bovine serum albumin (BSA)-IgG IC, Chen et al. highlighted some discrepancies between the results obtained in human FcγRIIA transgenic mice and those using blocking antibodies on purified human neutrophils. The first model suggested the importance of human FcγRIIA during *in vivo* NET release whereas the latter supported a role of FcγRIIIB *in vitro*. The differences between the models could explain this discrepancy. Indeed, in transgenic FcγRIIIB^{+}/γ$^{-/-}$ mice which do not express FcγRIIA, FcγRIIIB engagement lead to IC clearance. Moreover, blocking FcγRIIA on human neutrophils does not affect receptor intracellular signaling domain, allowing FcγRIIA to transduce signal mediated by FcγRIIIB engagement. Hence, these results support a cooperative role of FcγRIIA and FcγRIIIB, the two activating receptors constitutively expressed on neutrophils, during IC-induced NET-release (49). However, other studies have pointed out an exclusive role of FcγRIIIB in NET release in response to immobilized ICs (51) or by direct receptor aggregation (52, 53). Similarly, the requirement of NOX2-generated ROS in IC-induced NET release is controversial. Although most studies supported a pivotal role of NOX2 (38, 45, 51, 52), two of them provided opposite results despite similar pharmacological inhibitor [diphenyleneiodonium (DPI)] but different concentrations (49, 54). As DPI inhibits a wide variety of NADP-dependent enzymes as well as mitochondrial flavoenzymes (55), DPI-related NET release inhibition only means that ROS (whatever their cellular origin) are important in this process. Additionally, a role of actin cytoskeleton (49), Syk/Src (49–51), and MAPK (49, 51, 52) have been suggested by a few studies whereas the implication of NE/MPO (49, 51) and PI3K/AKT (49, 51, 52) are debated and need confirmation. All these conflicting results regarding the molecular pathways implicated in IC-induced NET release could be explained at least in part by the heterogeneity of the protocols: type of IC (antigenic system, spatial configuration, antigen, and antibody concentrations), pharmacological inhibitors (type and concentration), blocking antibodies [clone, type (Fab or full Ab)], use of human transgenic mice, and method to quantify NETs. Thus, there is a need for recapitulative studies comparing side by side the different triggers and inhibition strategies to obtain a definitive and clear view of IC-induced NET release pathways.

IMMUNE COMPLEX-INDUCED NETS CAN TRIGGER AND PERPETUATE VARIOUS AUTOIMMUNE DISEASES

As they expose intracellular endogenous components to the immune system, NETs have been very soon suspected to participate in the initiation of the autoimmune response (**Figure 1**). Indeed, autoantibodies against several NET components such as DNA, MPO, elastase, citrullinated histones, or proteinase 3 (PR3) are hallmarks of several systemic autoimmune diseases. It has been speculated for several years that anti-ribonucleoprotein (RNP) and anti-DNA antibodies found

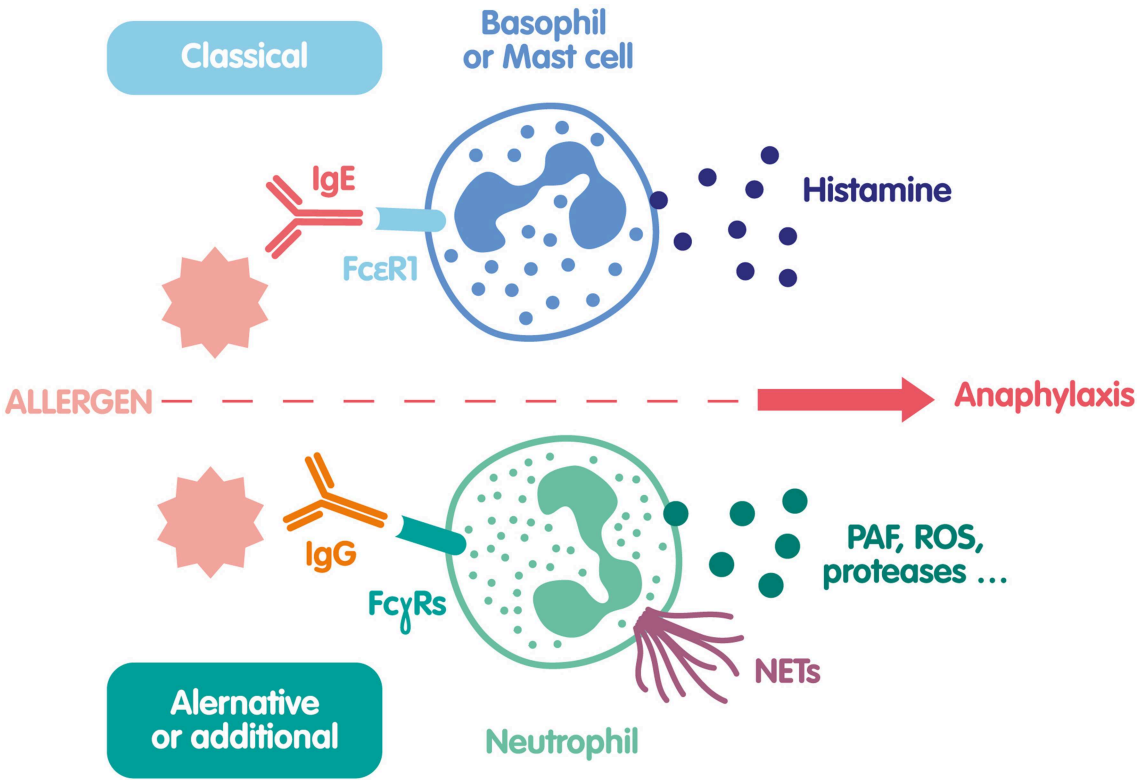

FIGURE 1 | Mechanisms of NET formation in autoimmunity. Autoantigen/IgG IC can bind to several FcγRs expressed at the neutrophil surface and induce their activation. In particular, NOX2 is activated and produce ROS that can in turn activate PAD4 leading to protein citrullination and chromatin decondensation. In parallel, ROS can also help MPO and NE degranulation and translocation to the nucleus contributing to chromatin unfolding. The nuclear membrane breaks down, the decondensed chromatin is released in the cytosol and becomes decorated with various cytosolic and granule-derived proteins. Finally, NETs are released exposing to the immune system a large number of autoantigens that can amplify this mechanism called lytic NETosis. In some conditions, in particular in SLE, these IC can also induce a non-lytic NOX2-independent NETosis via the production of mitochondria-derived ROS and/or DNA; in that case, neutrophils are still alive.

in the serum of patients with SLE could be produced in response to NET constituents and thus participate to the high level of circulating IC in lupus (56–58). ICs containing self-antigens, in particular RNP/anti-RNP ICs, have been shown to induce NET release, creating an amplification loop where NET components induce autoantibodies leading to ICs, which subsequently trigger NET formation and perpetuate the phenomenon. The role of these IC-induced NETs in the pathophysiology of lupus is not obvious, as the high levels of NETs released during infection does not usually lead to autoimmune response. This suggests that some additional mechanisms must lead to a break of tolerance to NETs. Interestingly, RNP-containing ICs were shown to induce mitochondrial hyperpolarization, increased mitochondrial ROS production and extracellular release of oxidized mitochondrial DNA, a potent proinflammatory compound able to activate type 1 interferon pathway (38). Additionally, self-DNA ICs have been shown to activate plasmacytoid dendritic cells via TLR9, also leading to type 1 interferon release, which has been linked to loss of tolerance (56, 57). An impairment of NETs regulatory mechanisms could also favor loss of tolerance. Some patients with active lupus or lupus nephritis have a deficiency in DNase 1 activity and/or anti-NET antibodies that inhibit DNase effect (23, 59, 60), leading to abnormal NET accumulation. This prolonged presence of NETs could favor rupture of tolerance as well as increase tissue damage (61). Another interesting element is that the composition of NETs from SLE patients is different from that of healthy controls. NETs from SLE patients are richer in toxic compounds (e.g., oxidized alpha-enolase) leading to tissue damage, especially in lupus nephritis (62). Recent observations suggest that several PAD4 polymorphisms are associated to SLE and lupus nephritis, reinforcing the link between NETs and SLE pathophysiology (63, 64). Additionally, in lupus nephritis, circulating IC deposit in the glomerular basement membrane, giving an additional pathogenic role for these IC. Taken together these findings emphasize a major role for IC in the different NETosis pathways involved in SLE, particularly in lupus nephritis (65).

RA is another example where IC-induced NETs are of importance (66, 67). Even if the pathogenesis of RA is not fully understood, many studies have shown that in genetically predisposed patients, anti-citrullinated protein antibodies (ACPAs) play a major role. These autoantibodies target citrullinated self-proteins like histones, vimentin, enolase, collagen, filaggrin, fibrinogen, or calreticulin. Citrullination

is a physiological process that occurs in inflammation and during NETosis, due to the activation of PAD4 (66–69). PAD4 activity contributes to RA development, since PAD4-deficient mice have reduced autoantibodies and joint damage in arthritis models (70, 71), and a single nucleotide polymorphism of PAD4 is associated to an increased risk to develop RA in humans (72). Neutrophils from RA patients are activated and produce spontaneously more NETs than healthy donors (73). Additionally, NETosis can be activated by ACPA IgG and IgA ICs (46, 69, 74). Circulating NETs and netting neutrophils in joints are found in patients with RA (69, 75, 76), demonstrating active and widespread NETosis in this context. Thus, IC-induced NETs together with inflammation and synoviocyte activation can enhance the production of citrullinated autoantigens and fuel the autoimmune response, which will in turn produce more ICs (24, 77). Furthermore, synovial fibrocytes can internalize NETs via a RAGE-TLR9 pathway leading to MHC-class II upregulation and presentation to specific T cells of NET-associated citrullinated peptides (78).

The breaking of tolerance to citrullinated proteins is suspected to occur in the airway, in particular in smoker's airway (79–81). Identical citrullinated proteins are present in the joints and in the lung of patients with RA, and high levels of NETs can be found in the sputum of ACPA-positive RA patients, and even in at-risk patients' relatives (positive for HLA-DRB1 allele and ACPA) (82). Nicotine could induce NETs via PAD4 activation (83), and smoking triggered NETosis in several experimental models (84, 85). The link between nicotine, NETs, and loss of tolerance to citrullinated proteins is not fully elucidated yet, but the more recent studies point to an intense lung citrullination process related to high levels of PAD4 and neutrophil activation (22, 86). These NETs can then induce dendritic cell maturation, type 1 IFN release, Th1 expansion, and B cell activation. Furthermore, ectopic lymphoid tissue and high levels of ACPA are observed in the lung of patients with RA reinforcing the idea of a local autoimmune response (82, 87). Finally, the microenvironment, in particular the microbial agents, might themselves play a role in breaking the tolerance; it was for instance recently demonstrated that PAD from *Porphyromonas gingivalis* is able to produce citrullinated proteins and participate to RA pathogenesis (23, 88). Thus, NETs produced in response to infection could constitute in some instances a bridge between infection and autoimmunity. To summarize, NETs are an important source of citrullinated autoantigens in RA, fueling the production of ACPAs in predisposed individuals. They also maintain an inflammatory environment in the lung and in the joints, facilitating neutrophil activation and NET production by the ACPA/citrullinated peptides ICs.

IMMUNE COMPLEX-INDUCED NETS PARTICIPATE TO ANAPHYLAXIS

IgG ICs Formed During Anaphylaxis Induce NET Release

Anaphylaxis is an acute systemic hypersensitivity reaction that can be life-threatening. Because of its extremely fast and unpredictable onset, it is difficult to obtain data on its mechanisms in human, and animal models have been developed to better understand this complex disease (89). The classical pathway is based on the triad IgE/basophil-mastocyte/histamine. During anaphylaxis, cell-surface bound specific IgE on basophils and mast cells react with the allergen and induce the release of preformed mediators such as histamine and proteases, leading to clinical signs of anaphylaxis. However, anaphylaxis can be triggered in mice lacking IgE or their receptor (90, 91), and we reported that up to 30% of patients with neuromuscular blocking agent (NMBA) perioperative anaphylaxis do not have any sign of the IgE pathway (92, 93). An IgE-independent anaphylaxis mechanism has thus been proposed and demonstrated in mice, mediated by neutrophils, specific IgG and FcγRs (94). Specific IgG-IC can bind to various activating FcγRs at the surface of cells such as neutrophils and induce their activation. High circulating levels of several neutrophil-related components and platelet activating factor (PAF) have been described in mice models of anaphylaxis, and in patients experiencing anaphylaxis as markers of neutrophil activation (95–97). The mechanisms of IgG-mediated neutrophil activation during anaphylaxis were first demonstrated in mice models of BSA-induced anaphylaxis. Using depletion and inhibition strategies it was shown that specific IgG-IC binding to neutrophil FcγRIIIA or FcγRIV was sufficient to induce fatal anaphylaxis (94). As human neutrophils do not express these two activating receptors but FcγRIIA, transgenic mice expressing the human FcγRIIA were used to demonstrate a major role for this receptor during anaphylaxis (98, 99). Very recently, these findings were confirmed in an elegant humanized mouse model where the human low-affinity IgG receptor locus, comprising both activating and inhibitory FcγR genes was inserted into the equivalent murine locus (100, 101). The implication of such an IC-mediated anaphylaxis via a new IgG/neutrophil/PAF triad is thus well-demonstrated in animal models and suggested to be relevant in humans by the studies on humanized mice. The existence of this alternative or additional mechanism in humans has been very recently demonstrated in a cohort of 86 patients experiencing NMBA anaphylaxis (93). Blood neutrophils were activated in patients as shown by the upregulation of CD11b, CD18, CD66b, and high levels of circulating elastase. NETosis was also triggered and patients had high levels of circulating NETs remnants (DNA-MPO complexes). Interestingly, a decreased expression of neutrophil FcγRIIA and FCγRIIIB was observed 30 min after anaphylaxis onset. This negative modulation is consistent with the engagement of FcγRs by circulating IC. Moreover, purified anti-rocuronium IgG isolated from a patient could form IC *in vitro* with a rocuronium bioconjugate. These IC were able to activate human neutrophils *in vitro*, and induce NET release (93). Concentration of anti-NMBA IgG and neutrophil activation markers correlated with anaphylaxis severity. This mechanism could be observed in patients lacking any evidence of IgE-dependent anaphylaxis, suggesting that IgG and IgE pathways could be independent, at least in some instances.

Alternative Mechanisms of NET Release During Anaphylaxis

Besides the role of IgG/IC in NET release during anaphylaxis, one could speculate that other mechanisms exist that could

modulate neutrophil activation. Some mediators released both in the acute and the late inflammatory phase of anaphylaxis such as pro-inflammatory cytokines, PAF, or C5a seem able to activate NET release in some conditions, even if there is still conflicting results on the subject (102–105). Recently, a major role of platelets in anaphylaxis has been suggested both in hFcγRIIA transgenic mice model and in humans (106, 107). In the mouse model, the interaction of platelet FcγRIIA with IgG-ICs induced platelet activation/aggregation, whose intensity correlated with the severity of anaphylaxis. Moreover, platelets depletion substantially attenuated symptomatology in mice. As platelets are much more abundant in bloodstream than neutrophils, it seems likely that IgG-ICs interact with platelet FcγRIIA first, before interacting with neutrophils. Activated platelets can aggregate on neutrophils to form platelet-neutrophil complexes detectable *in vivo* during several inflammatory conditions such as sepsis, pulmonary diseases, atherosclerosis (108), and recently allergic shock (106). The formation of these complexes involves the GP1b (glycoprotein 1b)/MAC-1 (macrophage 1 antigen) interaction (108), which is able to induce NET release. Platelets also release several soluble mediators known to activate NETosis (Von Willebrand Factor, platelet factor 4, HMGB1, PAF) (4, 109). Thus, besides direct neutrophil activation by IgG-ICs, other mechanisms involving released mediators or/and activated platelets may contribute to NET release during the acute phase of anaphylaxis.

Contribution of NETs to Anaphylaxis Mechanism

To date, only one study showed NET formation during anaphylaxis in human (93). Therefore, the pathogenic role of NET in this context is still unclear. However, some hypotheses could be raised according to well-established NET component properties. NET cytotoxicity on vascular endothelium and epithelia has already been shown to be responsible for organ failure in mouse models of sepsis and acute lung injury (4, 5, 110) and may therefore contribute to the pulmonary and vascular symptomatology of anaphylaxis. Whether NETs are formed in the lungs during anaphylaxis has not been directly investigated so far, but interstitial accumulation of neutrophil associated with pulmonary congestion has been demonstrated in a model of casein-induced active anaphylaxis (111). It is also possible that circulating NETs reach lung microcirculation and damage the alveolar-capillary interface as observed in mouse models of acute lung injury (112).

Complement activation is one of the mechanisms implicated in alternative routes of anaphylaxis and in worsening classical IgE-mediated anaphylaxis through C3a and C5a production (89). Along with direct toxicity, NETs could amplify mast cell degranulation by activating the alternative complement pathway (113, 114). Similarly, NETs could amplify bradykinin-mediated circulatory complications through their capacity to activate contact coagulation system (115).

To summarize, very recent human studies have shown that allergen/specific IgG IC are able to activate neutrophils and induce NETs. This new anaphylaxis pathway could participate to clinical manifestations of anaphylaxis (**Figure 2**), and should be considered in future investigations of diagnostic markers or therapeutic interventions.

DIAGNOSTIC AND THERAPEUTIC PERSPECTIVES

The implication of NETs in pathology has been prompting several studies investigating its potential as a diagnostic marker or a therapeutic target.

Circulating NETs have been detected in patient's serum in many diseases. Accordingly, NET concentrations have been studied as diagnostic or prognostic markers. Many studies have focused on the concentrations of cell-free DNA or circulating nucleosomes as NET surrogates. However, those are a poor reflect of NETosis since they will be released by any dying cell (116). Some other works focused on citrullinated H3 (H3citr) quantification in serum or tissues. For example, serum H3citr levels predicted the risk of venous thromboembolism in a cohort of 946 cancer patients. Additionally, Jin et al. showed in an interesting study that intratumoral NETs identified by H3citr staining could predict poor survival in post-surgery pancreatic cancer patients (117). The most specific marker of NETs to date are DNA-MPO complexes (116), but fewer studies have investigated their diagnostic relevance. Concentration of DNA-MPO complexes in serum are associated with poor control in asthma (20), severity in anaphylaxis (93), and development of extra-articular nodules in rheumatoid arthritis (118). It could also predict organ dysfunction and 28-day mortality in septic shock (119). High levels of circulating NETs were also associated with poor prognosis in community-acquired pneumonia, though it's not clear exactly which NETs surrogate marker was used in this study (120).

As for therapeutic intervention, two main approaches have been investigated: destruction of NETs or inhibition of their production. NETs can be dismantled by DNAse I treatment or by heparin (121), while their production has been blocked with PAD-4 inhibitors, mostly chloramidine (Cl-Amidine). Disruption of NETs with DNAse I have proven efficient in mouse models of stroke (122), ischemia reperfusion (123, 124), heparin-induced thrombocytopenia (125), and deep vein thrombosis (126). Furthermore, mice treated with DNAse I show less metastasis in mammary tumor models (127, 128). Treatment with chloramidine has shown efficacy in murine models of abdominal aortic aneurysm (122), arterial thrombosis (129), photothrombotic stroke (122), and sepsis (130).

However, studies in pathologies involving IC-induced NETs are scarce. The most relevant works show that pristane-induced lupus is reduced after inhalation of DNAse 1 (131), and arthritis symptoms are reduced by chloramidine treatment in collagen-induced arthritis model (132). Specific inhibition strategies using FcR blocking antibodies represent an interesting possibility, but no study has tested this approach so far, despite the existence of broadly available efficient antibodies.

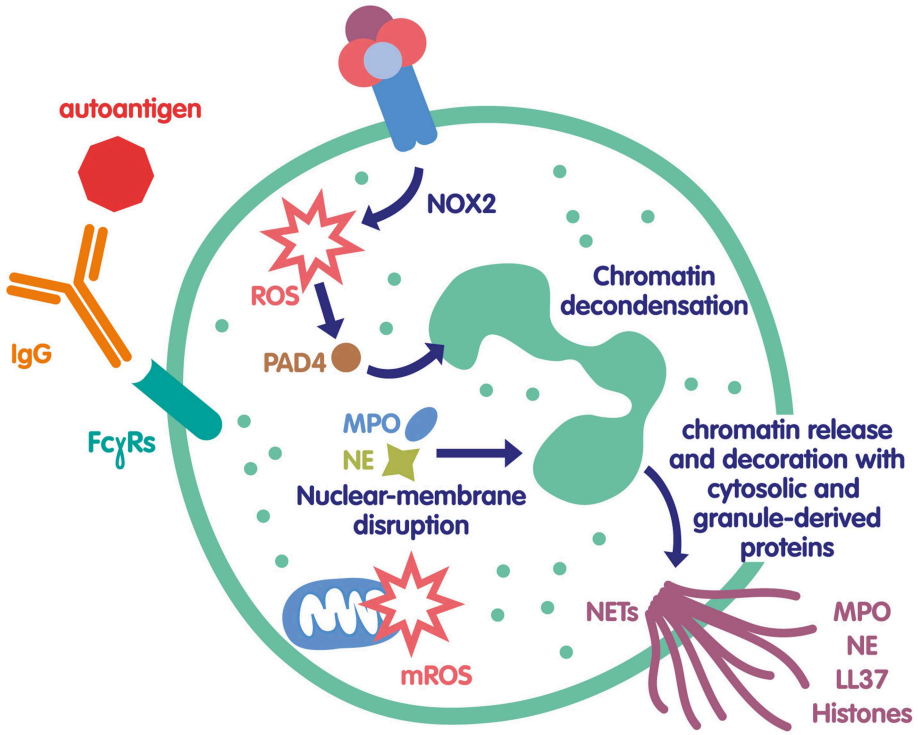

FIGURE 2 | Mechanisms of NET formation during anaphylaxis. The classical pathway of anaphylaxis is based on histamine release by mast cells and basophils activated by the engagement of FcεRI after interaction of specific IgE with an allergen. A second pathway was recently demonstrated both in mice and human. In this pathway the allergen reacts with specific IgG and form an IC that binds to several FcγRs at the neutrophil surface and activate them. In addition to ROS and protease release, neutrophils release PAF and NETs, that could be also involved in anaphylaxis clinical manifestations.

Globally, while several potential clinical uses of NETs have been described, most results come from mouse models, and large scale clinical trials results are missing.

CONCLUSION

ICs can be formed in several clinical conditions. In autoimmunity they can be continuously present in circulation or in tissues, depending on the accessibility of the self-antigen. In contrast, during anaphylaxis they are formed as soon as the allergen enters the body and encounters pre-existing IgGs. As neutrophils express high levels of FcγRs, they can be activated by ICs and release NETs. Beside their tissue toxicity and proinflammatory properties, NETs contain autoantigen and can thus perpetuate autoimmunity. In anaphylaxis, IC-induced NETs release represents a new pathway that may participate in symptoms and severity of the disease. New fundamental and clinical investigations are needed to better elucidate the intracellular mechanisms of IC-induced NET release and evaluate the potential clinical applications of NETs as a biomarker and a therapeutic target.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REFERENCES

1. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, et al. Neutrophil extracellular traps kill bacteria. *Science.* (2004) 303:1532–5. doi: 10.1126/science.1092385
2. Honda M, Kubes P. Neutrophils and neutrophil extracellular traps in the liver and gastrointestinal system. *Nat Rev Gastroenterol Hepatol.* (2018) 15:206–21. doi: 10.1038/nrgastro.2017.183
3. Li P, Li M, Lindberg MR, Kennett MJ, Xiong N, Wang Y. PAD4 is essential for antibacterial innate immunity mediated by neutrophil extracellular traps. *J Exp Med.* (2010) 207:1853–62. doi: 10.1084/jem.20100239
4. Clark SR, Ma AC, Tavener SA, McDonald B, Goodarzi Z, Kelly MM, et al. Platelet TLR4 activates neutrophil extracellular traps to ensnare bacteria in septic blood. *Nat Med.* (2007) 13:463–9. doi: 10.1038/nm1565
5. Saffarzadeh M, Juenemann C, Queisser MA, Lochnit G, Barreto G, Galuska SP, et al. Neutrophil extracellular traps directly induce epithelial and

endothelial cell death: a predominant role of histones. *PLoS ONE.* (2012) 7:e32366. doi: 10.1371/journal.pone.0032366
6. Merza M, Hartman H, Rahman M, Hwaiz R, Zhang E, Renström E, et al. Neutrophil extracellular traps induce trypsin activation, inflammation, and tissue damage in mice with severe acute pancreatitis. *Gastroenterology.* (2015) 149:1920–31.e8. doi: 10.1053/j.gastro.2015.08.026
7. Daniel C, Leppkes M, Muñoz LE, Schley G, Schett G, Herrmann M. Extracellular DNA traps in inflammation, injury and healing. *Nat Rev Nephrol.* (2019) 15:559–75. doi: 10.1038/s41581-019-0163-2
8. Sakurai K, Miyashita T, Okazaki M, Yamaguchi T, Ohbatake Y, Nakanuma S, et al. Role for neutrophil extracellular traps (NETs) and platelet aggregation in early sepsis-induced hepatic dysfunction. *Vivo Athens Greece.* (2017) 31:1051–8. doi: 10.21873/invivo.11169
9. McDonald B, Davis RP, Kim S-J, Tse M, Esmon CT, Kolaczkowska E, et al. Platelets and neutrophil extracellular traps collaborate to promote intravascular coagulation during sepsis in mice. *Blood.* (2017) 129:1357–67. doi: 10.1182/blood-2016-09-741298
10. Lefrançais E, Mallavia B, Zhuo H, Calfee CS, Looney MR. Maladaptive role of neutrophil extracellular traps in pathogen-induced lung injury. *JCI Insight.* (2018) 3:98178. doi: 10.1172/jci.insight.98178
11. Li H, Zhou X, Tan H, Hu Y, Zhang L, Liu S, et al. Neutrophil extracellular traps contribute to the pathogenesis of acid-aspiration-induced ALI/ARDS. *Oncotarget.* (2018) 9:1772–84. doi: 10.18632/oncotarget.22744
12. Liu S, Su X, Pan P, Zhang L, Hu Y, Tan H, et al. Neutrophil extracellular traps are indirectly triggered by lipopolysaccharide and contribute to acute lung injury. *Sci Rep.* (2016) 6:37252. doi: 10.1038/srep37252
13. Barrientos L, Bignon A, Gueguen C, de Chaisemartin L, Gorges R, Sandré C, et al. Neutrophil extracellular traps downregulate lipopolysaccharide-induced activation of monocyte-derived dendritic cells. *J Immunol.* (2014) 193:5689–98. doi: 10.4049/jimmunol.1400586
14. Castanheira FVS, Kubes P. Neutrophils and NETs in modulating acute and chronic inflammation. *Blood.* (2019) 133:2178–85. doi: 10.1182/blood-2018-11-844530
15. Wright TK, Gibson PG, Simpson JL, McDonald VM, Wood LG, Baines KJ. Neutrophil extracellular traps are associated with inflammation in chronic airway disease. *Respirol Carlton Vic.* (2016) 21:467–75. doi: 10.1111/resp.12730
16. Lachowicz-Scroggins ME, Dunican EM, Charbit AR, Raymond W, Looney MR, Peters MC, et al. Extracellular DNA, neutrophil extracellular traps, and inflammasome activation in severe asthma. *Am J Respir Crit Care Med.* (2019) 199:1076–85. doi: 10.1164/rccm.201810-1869OC
17. Dicker AJ, Crichton ML, Pumphrey EG, Cassidy AJ, Suarez-Cuartin G, Sibila O, et al. Neutrophil extracellular traps are associated with disease severity and microbiota diversity in patients with chronic obstructive pulmonary disease. *J Allergy Clin Immunol.* (2017) 141:117–27. doi: 10.1016/j.jaci.2017.04.022
18. Grabcanovic-Musija F, Obermayer A, Stoiber W, Krautgartner W-D, Steinbacher P, Winterberg N, et al. Neutrophil extracellular trap (NET) formation characterises stable and exacerbated COPD and correlates with airflow limitation. *Respir Res.* (2015) 16:59. doi: 10.1186/s12931-015-0221-7
19. Twaddell SH, Baines KJ, Grainge C, Gibson PG. The emerging role of neutrophil extracellular traps in respiratory disease. *Chest.* (2019) 156:774–82. doi: 10.1016/j.chest.2019.06.012
20. Granger V, Taillé C, Roach D, Letuvé S, Dupin C, Hamidi F, et al. Circulating neutrophil and eosinophil extracellular traps are markers of severe asthma. *Allergy.* (2019). doi: 10.1111/all.14059. [Epub ahead of print].
21. Kimball AS, Obi AT, Diaz JA, Henke PK. The emerging role of NETs in venous thrombosis and immunothrombosis. *Front Immunol.* (2016) 7:236. doi: 10.3389/fimmu.2016.00236
22. Skopelja-Gardner S, Jones JD, Rigby WFC. "NETtling" the host: breaking of tolerance in chronic inflammation and chronic infection. *J Autoimmun.* (2018) 88:1–10. doi: 10.1016/j.jaut.2017.10.008
23. Dwivedi N, Radic M. Burning controversies in NETs and autoimmunity: the mysteries of cell death and autoimmune disease. *Autoimmunity.* (2018) 51:267–80. doi: 10.1080/08916934.2018.1523395
24. Lee KH, Kronbichler A, Park DD-Y, Park Y, Moon H, Kim H, et al. Neutrophil extracellular traps (NETs) in autoimmune diseases: a comprehensive review. *Autoimmun Rev.* (2017) 16:1160–73. doi: 10.1016/j.autrev.2017.09.012
25. Fuchs TA, Abed U, Goosmann C, Hurwitz R, Schulze I, Wahn V, et al. Novel cell death program leads to neutrophil extracellular traps. *J Cell Biol.* (2007) 176:231–41. doi: 10.1083/jcb.200606027
26. Kirchner T, Möller S, Klinger M, Solbach W, Laskay T, Behnen M. The impact of various reactive oxygen species on the formation of neutrophil extracellular traps. *Mediators Inflamm.* (2012) 2012:849136. doi: 10.1155/2012/849136
27. Remijsen Q, Vanden Berghe T, Wirawan E, Asselbergh B, Parthoens E, De Rycke R, et al. Neutrophil extracellular trap cell death requires both autophagy and superoxide generation. *Cell Res.* (2011) 21:290–304. doi: 10.1038/cr.2010.150
28. Boeltz S, Amini P, Anders H-J, Andrade F, Bilyy R, Chatfield S, et al. To NET or not to NET:current opinions and state of the science regarding the formation of neutrophil extracellular traps. *Cell Death Differ.* (2019) 26:395–408. doi: 10.1038/s41418-018-0261-x
29. Kenny EF, Herzig A, Krüger R, Muth A, Mondal S, Thompson PR, et al. Diverse stimuli engage different neutrophil extracellular trap pathways. *eLife.* (2017) 6:24437. doi: 10.7554/eLife.24437
30. Silva JC, Rodrigues NC, Thompson-Souza GA, Valdirene de Muniz S, Neves JS, Figueiredo RT. Mac-1 triggers neutrophil DNA extracellular trap formation to *Aspergillus fumigatus* independently of PAD4 histone citrullination. *J Leukoc Biol.* (2019). doi: 10.1002/JLB.4A0119-009RR. [Epub ahead of print].
31. Guiducci E, Lemberg C, Küng N, Schraner E, Theocharides APA, LeibundGut-Landmann S. *Candida albicans*-Induced NETosis Is independent of peptidylarginine deiminase 4. *Front Immunol.* (2018) 9:1573. doi: 10.3389/fimmu.2018.01573
32. Kenno S, Perito S, Mosci P, Vecchiarelli A, Monari C. Autophagy and reactive oxygen species are involved in neutrophil extracellular traps release induced by *C. albicans* morphotypes. *Front Microbiol.* (2016) 7:879. doi: 10.3389/fmicb.2016.00879
33. Xu F, Zhang C, Zou Z, Fan EKY, Chen L, Li Y, et al. Aging-related Atg5 defect impairs neutrophil extracellular traps formation. *Immunology.* (2017) 151:417–32. doi: 10.1111/imm.12740
34. Germic N, Stojkov D, Oberson K, Yousefi S, Simon H-U. Neither eosinophils nor neutrophils require ATG5-dependent autophagy for extracellular DNA trap formation. *Immunology.* (2017) 152:517–25. doi: 10.1111/imm.12790
35. Jorch SK, Kubes P. An emerging role for neutrophil extracellular traps in noninfectious disease. *Nat Med.* (2017) 23:279–87. doi: 10.1038/nm.4294
36. Yipp BG, Petri B, Salina D, Jenne CN, Scott BNV, Zbytnuik LD, et al. Infection-induced NETosis is a dynamic process involving neutrophil multitasking *in vivo*. *Nat Med.* (2012) 18:1386–93. doi: 10.1038/nm.2847
37. Pilsczek FH, Salina D, Poon KKH, Fahey C, Yipp BG, Sibley CD, et al. A novel mechanism of rapid nuclear neutrophil extracellular trap formation in response to *Staphylococcus aureus*. *J Immunol.* (2010) 185:7413–25. doi: 10.4049/jimmunol.1000675
38. Lood C, Blanco LP, Purmalek MM, Carmona-Rivera C, De Ravin SS, Smith CK, et al. Neutrophil extracellular traps enriched in oxidized mitochondrial DNA are interferogenic and contribute to lupus-like disease. *Nat Med.* (2016) 22:146–53. doi: 10.1038/nm.4027
39. Douda DN, Khan MA, Grasemann H, Palaniyar N. SK3 channel and mitochondrial ROS mediate NADPH oxidase-independent NETosis induced by calcium influx. *Proc Natl Acad Sci USA.* (2015) 112:2817–22. doi: 10.1073/pnas.1414055112
40. Naffah de Souza C, Breda LCD, Khan MA, de Almeida SR, Câmara NOS, Sweezey N, et al. Alkaline pH promotes NADPH oxidase-independent neutrophil extracellular trap formation: a matter of mitochondrial reactive oxygen species generation and citrullination and cleavage of histone. *Front Immunol.* (2017) 8:1849. doi: 10.3389/fimmu.2017.01849
41. Flament H, Granger V, Vezinet C, Marzaioli V, Kannengiesser C, de Chaisemartin L, et al. Aspergillus-induced pneumonia in adult without obvious immunodeficiency: test the burst! *Eur Respir J.* (2018) 51:1702711. doi: 10.1183/13993003.02711-2017
42. Dahlgren C, Karlsson A, Bylund J. Intracellular neutrophil oxidants: from laboratory curiosity to clinical reality. *J Immunol.* (2019) 202:3127–34. doi: 10.4049/jimmunol.1900235

43. Petretto A, Bruschi M, Pratesi F, Croia C, Candiano G, Ghiggeri G, et al. Neutrophil extracellular traps (NET) induced by different stimuli: a comparative proteomic analysis. *PLoS ONE.* (2019) 14:e0218946. doi: 10.1371/journal.pone.0218946
44. van der Linden M, Westerlaken GHA, van der Vlist M, van Montfrans J, Meyaard L. Differential signalling and kinetics of neutrophil extracellular trap release revealed by quantitative live imaging. *Sci Rep.* (2017) 7:6529. doi: 10.1038/s41598-017-06901-w
45. Aleyd E, van Hout MWM, Ganzevles SH, Hoeben KA, Everts V, Bakema JE, et al. IgA enhances NETosis and release of neutrophil extracellular traps by polymorphonuclear cells via Fcα receptor I. *J Immunol.* (2014) 192:2374–83. doi: 10.4049/jimmunol.1300261
46. Aleyd E, Al M, Tuk CW, van der Laken CJ, van Egmond M. IgA complexes in plasma and synovial fluid of patients with rheumatoid arthritis induce neutrophil extracellular traps via FcαRI. *J Immunol.* (2016) 197:4552–9. doi: 10.4049/jimmunol.1502353
47. Carmona-Rivera C, Zhao W, Yalavarthi S, Kaplan MJ. Neutrophil extracellular traps induce endothelial dysfunction in systemic lupus erythematosus through the activation of matrix metalloproteinase-2. *Ann Rheum Dis.* (2015) 74:1417–24. doi: 10.1136/annrheumdis-2013-204837
48. Kelley JM, Monach PA, Ji C, Zhou Y, Wu J, Tanaka S, et al. IgA and IgG antineutrophil cytoplasmic antibody engagement of Fc receptor genetic variants influences granulomatosis with polyangiitis. *Proc Natl Acad Sci USA.* (2011) 108:20736–41. doi: 10.1073/pnas.1109227109
49. Chen K, Nishi H, Travers R, Tsuboi N, Martinod K, Wagner DD, et al. Endocytosis of soluble immune complexes leads to their clearance by FcγRIIIB but induces neutrophil extracellular traps via FcγRIIA *in vivo*. *Blood.* (2012) 120:4421–31. doi: 10.1182/blood-2011-12-401133
50. Ozaki N, Suzuki S, Ishida M, Harada Y, Tanaka K, Sato Y, et al. Syk-dependent signaling pathways in neutrophils and macrophages are indispensable in the pathogenesis of anti-collagen antibody-induced arthritis. *Int Immunol.* (2012) 24:539–50. doi: 10.1093/intimm/dxs078
51. Behnen M, Leschczyk C, Möller S, Batel T, Klinger M, Solbach W, et al. Immobilized immune complexes induce neutrophil extracellular trap release by human neutrophil granulocytes via FcγRIIIB and Mac-1. *J Immunol.* (2014) 193:1954–65. doi: 10.4049/jimmunol.1400478
52. Alemán OR, Mora N, Cortes-Vieyra R, Uribe-Querol E, Rosales C. Differential use of human neutrophil Fcγ receptors for inducing neutrophil extracellular trap formation. *J Immunol Res.* (2016) 2016:2908034. doi: 10.1155/2016/2908034
53. Alemán OR, Mora N, Cortes-Vieyra R, Uribe-Querol E, Rosales C. Transforming growth factor-β-activated kinase 1 is required for human FcγRIIIb-induced neutrophil extracellular trap formation. *Front Immunol.* (2016) 7:277. doi: 10.3389/fimmu.2016.00277
54. Kraaij T, Tengström FC, Kamerling SWA, Pusey CD, Scherer HU, Toes REM, et al. A novel method for high-throughput detection and quantification of neutrophil extracellular traps reveals ROS-independent NET release with immune complexes. *Autoimmun Rev.* (2016) 15:577–84. doi: 10.1016/j.autrev.2016.02.018
55. Stoiber W, Obermayer A, Steinbacher P, Krautgartner W-D. The role of reactive oxygen species (ROS) in the formation of extracellular traps (ETs) in humans. *Biomolecules.* (2015) 5:702–23. doi: 10.3390/biom5020702
56. Lande R, Ganguly D, Facchinetti V, Frasca L, Conrad C, Gregorio J, et al. Neutrophils activate plasmacytoid dendritic cells by releasing self-DNA-peptide complexes in systemic lupus erythematosus. *Sci Transl Med.* (2011) 3:73ra19. doi: 10.1126/scitranslmed.3001180
57. Garcia-Romo GS, Caielli S, Vega B, Connolly J, Allantaz F, Xu Z, et al. Netting neutrophils are major inducers of type I IFN production in pediatric systemic lupus erythematosus. *Sci Transl Med.* (2011) 3:73ra20. doi: 10.1126/scitranslmed.3001201
58. Jeremic I, Djuric O, Nikolic M, Vlajnic M, Nikolic A, Radojkovic D, et al. Neutrophil extracellular traps-associated markers are elevated in patients with systemic lupus erythematosus. *Rheumatol Int.* (2019) 39:1849–57. doi: 10.1007/s00296-019-04426-1
59. Leffler J, Martin M, Gullstrand B, Tydén H, Lood C, Truedsson L, et al. Neutrophil extracellular traps that are not degraded in systemic lupus erythematosus activate complement exacerbating the disease. *J Immunol.* (2012) 188:3522–31. doi: 10.4049/jimmunol.1102404
60. Bruschi M, Bonanni A, Petretto A, Vaglio A, Pratesi F, Santucci L, et al. Neutrophil extracellular traps (NETs) profiles in patients with incident SLE and lupus nephritis. *J Rheumatol.* (2019) 46:181232. doi: 10.3899/jrheum.181232
61. Pruchniak MP, Ostafin M, Wachowska M, Jakubaszek M, Kwiatkowska B, Olesinska M, et al. Neutrophil extracellular traps generation and degradation in patients with granulomatosis with polyangiitis and systemic lupus erythematosus. *Autoimmunity.* (2019) 52:126–35. doi: 10.1080/08916934.2019.1631812
62. Bruschi M, Petretto A, Santucci L, Vaglio A, Pratesi F, Migliorini P, et al. Neutrophil Extracellular Traps protein composition is specific for patients with Lupus nephritis and includes methyl-oxidized αenolase (methionine sulfoxide 93). *Sci Rep.* (2019) 9:7934. doi: 10.1038/s41598-019-44379-w
63. Massarenti L, Enevold C, Damgaard D, Ødum N, Nielsen CH, Jacobsen S. Peptidylarginine deiminase-4 gene polymorphisms are associated with systemic lupus erythematosus and lupus nephritis. *Scand J Rheumatol.* (2019) 48:133–40. doi: 10.1080/03009742.2018.1488273
64. Odqvist L, Jevnikar Z, Riise R, Öberg L, Rhedin M, Leonard D, et al. Genetic variations in A20 DUB domain provide a genetic link to citrullination and neutrophil extracellular traps in systemic lupus erythematosus. *Ann Rheum Dis.* (2019) 78:1363–70. doi: 10.1136/annrheumdis-2019-215434
65. Nishi H, Mayadas TN. Neutrophils in lupus nephritis. *Curr Opin Rheumatol.* (2019) 31:193–200. doi: 10.1097/BOR.0000000000000577
66. Apel F, Zychlinsky A, Kenny EF. The role of neutrophil extracellular traps in rheumatic diseases. *Nat Rev Rheumatol.* (2018) 14:467–75. doi: 10.1038/s41584-018-0039-z
67. Podolska MJ, Mahajan A, Knopf J, Hahn J, Boeltz S, Munoz L, et al. Autoimmune, rheumatic, chronic inflammatory diseases: neutrophil extracellular traps on parade. *Autoimmunity.* (2018) 51:281–7. doi: 10.1080/08916934.2018.1519804
68. Pratesi F, Dioni I, Tommasi C, Alcaro MC, Paolini I, Barbetti F, et al. Antibodies from patients with rheumatoid arthritis target citrullinated histone 4 contained in neutrophils extracellular traps. *Ann Rheum Dis.* (2014) 73:1414–22. doi: 10.1136/annrheumdis-2012-202765
69. Khandpur R, Carmona-Rivera C, Vivekanandan-Giri A, Gizinski A, Yalavarthi S, Knight JS, et al. NETs are a source of citrullinated autoantigens and stimulate inflammatory responses in rheumatoid arthritis. *Sci Transl Med.* (2013) 5:178ra40. doi: 10.1126/scitranslmed.3005580
70. Seri Y, Shoda H, Suzuki A, Matsumoto I, Sumida T, Fujio K, et al. Peptidylarginine deiminase type 4 deficiency reduced arthritis severity in a glucose-6-phosphate isomerase-induced arthritis model. *Sci Rep.* (2015) 5:13041. doi: 10.1038/srep13041
71. Suzuki A, Kochi Y, Shoda H, Seri Y, Fujio K, Sawada T, et al. Decreased severity of experimental autoimmune arthritis in peptidylarginine deiminase type 4 knockout mice. *BMC Musculoskelet Disord.* (2016) 17:205. doi: 10.1186/s12891-016-1055-2
72. Mergaert AM, Bawadekar M, Nguyen TQ, Massarenti L, Holmes CL, Rebernick R, et al. Reduced anti-histone antibodies and increased risk of rheumatoid arthritis associated with a single nucleotide polymorphism in PADI4 in North Americans. *Int J Mol Sci.* (2019) 20:E3093. doi: 10.3390/ijms20123093
73. Sur Chowdhury C, Giaglis S, Walker UA, Buser A, Hahn S, Hasler P. Enhanced neutrophil extracellular trap generation in rheumatoid arthritis: analysis of underlying signal transduction pathways and potential diagnostic utility. *Arthritis Res Ther.* (2014) 16:R122. doi: 10.1186/ar4579
74. Ribon M, Seninet S, Mussard J, Sebbag M, Clavel C, Serre G, et al. Neutrophil extracellular traps exert both pro- and anti-inflammatory actions in rheumatoid arthritis that are modulated by C1q and LL-37. *J Autoimmun.* (2019) 98:122–31. doi: 10.1016/j.jaut.2019.01.003
75. Wang W, Peng W, Ning X. Increased levels of neutrophil extracellular trap remnants in the serum of patients with rheumatoid arthritis. *Int J Rheum Dis.* (2017) 21:415–21. doi: 10.1111/1756-185X.13226
76. Pérez-Sánchez C, Ruiz-Limón P, Aguirre MA, Jiménez-Gómez Y, Arias-de la Rosa I, Ábalos-Aguilera MC, et al. Diagnostic potential of NETosis-derived products for disease activity, atherosclerosis and therapeutic effectiveness in rheumatoid arthritis patients. *J Autoimmun.* (2017) 82:31–40. doi: 10.1016/j.jaut.2017.04.007

77. Chen W, Wang Q, Ke Y, Lin J. Neutrophil function in an inflammatory milieu of rheumatoid arthritis. *J Immunol Res.* (2018) 2018:8549329. doi: 10.1155/2018/8549329
78. Carmona-Rivera C, Carlucci PM, Moore E, Lingampalli N, Uchtenhagen H, James E, et al. Synovial fibroblast-neutrophil interactions promote pathogenic adaptive immunity in rheumatoid arthritis. *Sci Immunol.* (2017) 2:eaag3358. doi: 10.1126/sciimmunol.aag3358
79. O'Neil LJ, Kaplan MJ. Neutrophils in rheumatoid arthritis: breaking immune tolerance and fueling disease. *Trends Mol Med.* (2019) 25:215–27. doi: 10.1016/j.molmed.2018.12.008
80. Makrygiannakis D, Hermansson M, Ulfgren A-K, Nicholas AP, Zendman AJW, Eklund A, et al. Smoking increases peptidylarginine deiminase 2 enzyme expression in human lungs and increases citrullination in BAL cells. *Ann Rheum Dis.* (2008) 67:1488–92. doi: 10.1136/ard.2007.075192
81. van Wesemael TJ, Ajeganova S, Humphreys J, Terao C, Muhammad A, Symmons DPM, et al. Smoking is associated with the concurrent presence of multiple autoantibodies in rheumatoid arthritis rather than with anti-citrullinated protein antibodies per se: a multicenter cohort study. *Arthritis Res Ther.* (2016) 18:285. doi: 10.1186/s13075-016-1177-9
82. Demoruelle MK, Harrall KK, Ho L, Purmalek MM, Seto NL, Rothfuss HM, et al. Anti-citrullinated protein antibodies are associated with neutrophil extracellular traps in the sputum in relatives of rheumatoid arthritis patients. *Arthritis Rheumatol.* (2017) 69:1165–75. doi: 10.1002/art.40066
83. Hosseinzadeh A, Thompson PR, Segal BH, Urban CF. Nicotine induces neutrophil extracellular traps. *J Leukoc Biol.* (2016) 100:1105–12. doi: 10.1189/jlb.3AB0815-379RR
84. Lee J, Luria A, Rhodes C, Raghu H, Lingampalli N, Sharpe O, et al. Nicotine drives neutrophil extracellular traps formation and accelerates collagen-induced arthritis. *Rheumatol Oxf Engl.* (2017) 56:644–53. doi: 10.1093/rheumatology/kew449
85. Chrysanthopoulou A, Mitroulis I, Apostolidou E, Arelaki S, Mikroulis D, Konstantinidis T, et al. Neutrophil extracellular traps promote differentiation and function of fibroblasts. *J Pathol.* (2014) 233:294–307. doi: 10.1002/path.4359
86. Corsiero E, Pratesi F, Prediletto E, Bombardieri M, Migliorini P. NETosis as source of autoantigens in rheumatoid arthritis. *Front Immunol.* (2016) 7:485. doi: 10.3389/fimmu.2016.00485
87. Rangel-Moreno J, Hartson L, Navarro C, Gaxiola M, Selman M, Randall TD. Inducible bronchus-associated lymphoid tissue (iBALT) in patients with pulmonary complications of rheumatoid arthritis. *J Clin Invest.* (2006) 116:3183–94. doi: 10.1172/JCI28756
88. Montgomery AB, Kopec J, Shrestha L, Thezenas M-L, Burgess-Brown NA, Fischer R, et al. Crystal structure of *Porphyromonas gingivalis* peptidylarginine deiminase: implications for autoimmunity in rheumatoid arthritis. *Ann Rheum Dis.* (2016) 75:1255–61. doi: 10.1136/annrheumdis-2015-207656
89. Reber LL, Hernandez JD, Galli SJ. The pathophysiology of anaphylaxis. *J Allergy Clin Immunol.* (2017) 140:335–48. doi: 10.1016/j.jaci.2017.06.003
90. Oettgen HC, Martin TR, Wynshaw-Boris A, Deng C, Drazen JM, Leder P. Active anaphylaxis in IgE-deficient mice. *Nature.* (1994) 370:367–70. doi: 10.1038/370367a0
91. Miyajima I, Dombrowicz D, Martin TR, Ravetch JV, Kinet JP, Galli SJ. Systemic anaphylaxis in the mouse can be mediated largely through IgG1 and Fc gammaRIII. Assessment of the cardiopulmonary changes, mast cell degranulation, and death associated with active or IgE- or IgG1-dependent passive anaphylaxis. *J Clin Invest.* (1997) 99:901–14. doi: 10.1172/JCI119255
92. Laroche D, Chollet-Martin S, Léturgie P, Malzac L, Vergnaud M-C, Neukirch C, et al. Evaluation of a new routine diagnostic test for immunoglobulin E sensitization to neuromuscular blocking agents. *Anesthesiology.* (2011) 114:91–7. doi: 10.1097/ALN.0b013e31820164d2
93. Jönsson F, de Chaisemartin L, Granger V, Gouel-Chéron A, Gillis CM, Zhu Q, et al. An IgG-induced neutrophil activation pathway contributes to human drug-induced anaphylaxis. *Sci Transl Med.* (2019) 11:eaat1479. doi: 10.1126/scitranslmed.aat1479
94. Jönsson F, Mancardi DA, Kita Y, Karasuyama H, Iannascoli B, Van Rooijen N, et al. Mouse and human neutrophils induce anaphylaxis. *J Clin Invest.* (2011) 121:1484–96. doi: 10.1172/JCI45232
95. Francis A, Bosio E, Stone SF, Fatovich DM, Arendts G, Nagree Y, et al. Neutrophil activation during acute human anaphylaxis: analysis of MPO and sCD62L. *Clin Exp Allergy J Br Soc Allergy Clin Immunol.* (2017) 47:361–70. doi: 10.1111/cea.12868
96. Vadas P, Gold M, Perelman B, Liss GM, Lack G, Blyth T, et al. Platelet-activating factor, PAF acetylhydrolase, and severe anaphylaxis. *N Engl J Med.* (2008) 358:28–35. doi: 10.1056/NEJMoa070030
97. Francis A, Bosio E, Stone SF, Fatovich DM, Arendts G, MacDonald SPJ, et al. Markers involved in innate immunity and neutrophil activation are elevated during acute human anaphylaxis: validation of a microarray study. *J Innate Immun.* (2019) 11:63–73. doi: 10.1159/000492301
98. Jönsson F, Mancardi DA, Zhao W, Kita Y, Iannascoli B, Khun H, et al. Human FcγRIIA induces anaphylactic and allergic reactions. *Blood.* (2012) 119:2533–44. doi: 10.1182/blood-2011-07-367334
99. Jönsson F, Mancardi DA, Albanesi M, Bruhns P. Neutrophils in local and systemic antibody-dependent inflammatory and anaphylactic reactions. *J Leukoc Biol.* (2013) 94:643–56. doi: 10.1189/jlb.1212623
100. Gillis CM, Jönsson F, Mancardi DA, Tu N, Beutier H, Van Rooijen N, et al. Mechanisms of anaphylaxis in human low-affinity IgG receptor locus knock-in mice. *J Allergy Clin Immunol.* (2017) 139:1253–65.e14. doi: 10.1016/j.jaci.2016.06.058
101. Gillis CM, Zenatti PP, Mancardi DA, Beutier H, Fiette L, Macdonald LE, et al. *In vivo* effector functions of high-affinity mouse IgG receptor FcγRI in disease and therapy models. *J Autoimmun.* (2017) 80:95–102. doi: 10.1016/j.jaut.2016.09.009
102. Keshari RS, Jyoti A, Dubey M, Kothari N, Kohli M, Bogra J, et al. Cytokines induced neutrophil extracellular traps formation: implication for the inflammatory disease condition. *PLoS ONE.* (2012) 7:e48111. doi: 10.1371/journal.pone.0048111
103. Hoppenbrouwers T, Autar ASA, Sultan AR, Abraham TE, van Cappellen WA, Houtsmuller AB, et al. *In vitro* induction of NETosis: Comprehensive live imaging comparison and systematic review. *PLoS ONE.* (2017) 12:e0176472. doi: 10.1371/journal.pone.0176472
104. Guimarães-Costa AB, Nascimento MTC, Wardini AB, Pinto-da-Silva LH, Saraiva EM. ETosis: a microbicidal mechanism beyond cell death. *J Parasitol Res.* (2012) 2012:929743. doi: 10.1155/2012/929743
105. Granger V, Faille D, Marani V, Noël B, Gallais Y, Szely N, et al. Human blood monocytes are able to form extracellular traps. *J Leukoc Biol.* (2017) 102:775–81. doi: 10.1189/jlb.3MA0916-411R
106. Beutier H, Hechler B, Godon O, Wang Y, Gillis CM, de Chaisemartin L, et al. Platelets expressing IgG receptor FcγRIIA/CD32A determine the severity of experimental anaphylaxis. *Sci Immunol.* (2018) 3:eaan5997. doi: 10.1126/sciimmunol.aan5997
107. Peppers BP, Vatsayan A, Dalal J, Bonfield T, Tcheurekdjian H, Hostoffer R. A case series: association of anaphylaxis with a significant decrease in platelet levels and possible secondary risk of thrombosis. *Immun Inflamm Dis.* (2018) 6:377–81. doi: 10.1002/iid3.224
108. Lisman T. Platelet-neutrophil interactions as drivers of inflammatory and thrombotic disease. *Cell Tissue Res.* (2018) 371:567–76. doi: 10.1007/s00441-017-2727-4
109. Carestia A, Kaufman T, Schattner M. Platelets: new bricks in the building of neutrophil extracellular traps. *Front Immunol.* (2016) 7:271. doi: 10.3389/fimmu.2016.00271
110. Caudrillier A, Kessenbrock K, Gilliss BM, Nguyen JX, Marques MB, Monestier M, et al. Platelets induce neutrophil extracellular traps in transfusion-related acute lung injury. *J Clin Invest.* (2012) 122:2661–71. doi: 10.1172/JCI61303
111. Krishnamurthy D, Starkl P, Szalai K, Roth-Walter F, Diesner SC, Mittlboeck M, et al. Monitoring neutrophils and platelets during casein-induced anaphylaxis in an experimental BALB/c mouse model. *Clin Exp Allergy J Br Soc Allergy Clin Immunol.* (2012) 42:1119–28. doi: 10.1111/j.1365-2222.2012.04012.x
112. Cortjens B, van Woensel JBM, Bem RA. Neutrophil extracellular traps in respiratory disease: guided anti-microbial traps or toxic webs? *Paediatr Respir Rev.* (2017) 21:54–61. doi: 10.1016/j.prrv.2016.03.007
113. Yuen J, Pluthero FG, Douda DN, Riedl M, Cherry A, Ulanova M, et al. NETosing neutrophils activate complement both on their own NETs and

bacteria via alternative and non-alternative pathways. *Front Immunol.* (2016) 7:137. doi: 10.3389/fimmu.2016.00137
114. Wang H, Wang C, Zhao M-H, Chen M. Neutrophil extracellular traps can activate alternative complement pathways. *Clin Exp Immunol.* (2015) 181:518–27. doi: 10.1111/cei.12654
115. Oehmcke S, Mörgelin M, Herwald H. Activation of the human contact system on neutrophil extracellular traps. *J Innate Immun.* (2009) 1:225–30. doi: 10.1159/000203700
116. Masuda S, Nakazawa D, Shida H, Miyoshi A, Kusunoki Y, Tomaru U, et al. NETosis markers: quest for specific, objective, and quantitative markers. *Clin Chim Acta.* (2016) 459:89–93. doi: 10.1016/j.cca.2016.05.029
117. Jin W, Xu H-X, Zhang S-R, Li H, Wang W-Q, Gao H-L, et al. Tumor-infiltrating NETs predict postsurgical survival in patients with pancreatic ductal adenocarcinoma. *Ann Surg Oncol.* (2019) 26:635–43. doi: 10.1245/s10434-018-6941-4
118. Bach M, Moon J, Moore R, Pan T, Nelson JL, Lood C. A neutrophil activation biomarker panel in prognosis and monitoring of patients with rheumatoid arthritis. *Arthritis Rheumatol Hoboken NJ.* (2019). doi: 10.1002/art.41062. [Epub ahead of print].
119. Maruchi Y, Tsuda M, Mori H, Takenaka N, Gocho T, Huq MA, et al. Plasma myeloperoxidase-conjugated DNA level predicts outcomes and organ dysfunction in patients with septic shock. *Crit Care Lond Engl.* (2018) 22:176. doi: 10.1186/s13054-018-2109-7
120. Ebrahimi F, Giaglis S, Hahn S, Blum CA, Baumgartner C, Kutz A, et al. Markers of neutrophil extracellular traps predict adverse outcome in community-acquired pneumonia: secondary analysis of a randomised controlled trial. *Eur Respir J.* (2018) 51:1701389. doi: 10.1183/13993003.01389-2017
121. Fuchs TA, Brill A, Duerschmied D, Schatzberg D, Monestier M, Myers DD, et al. Extracellular DNA traps promote thrombosis. *Proc Natl Acad Sci USA.* (2010) 107:15880–5. doi: 10.1073/pnas.1005743107
122. Peña-Martínez C, Durán-Laforet V, García-Culebras A, Ostos F, Hernández-Jiménez M, Bravo-Ferrer I, et al. Pharmacological modulation of neutrophil extracellular traps reverses thrombotic stroke tPA (tissue-type plasminogen activator) resistance. *Stroke.* (2019) 50:3228–37. doi: 10.1161/STROKEAHA.119.026848
123. Wang S, Xie T, Sun S, Wang K, Liu B, Wu X, et al. DNase-1 treatment exerts protective effects in a rat model of intestinal ischemia-reperfusion injury. *Sci Rep.* (2018) 8:17788. doi: 10.1038/s41598-018-36198-2
124. Albadawi H, Oklu R, Raacke Malley RE, O'Keefe RM, Uong TP, Cormier NR, et al. Effect of DNase I treatment and neutrophil depletion on acute limb ischemia-reperfusion injury in mice. *J Vasc Surg.* (2016) 64:484–93. doi: 10.1016/j.jvs.2015.01.031
125. Gollomp K, Kim M, Johnston I, Hayes V, Welsh J, Arepally GM, et al. Neutrophil accumulation and NET release contribute to thrombosis in HIT. *JCI Insight.* (2018) 3:99445. doi: 10.1172/jci.insight.99445
126. Brill A, Fuchs TA, Savchenko AS, Thomas GM, Martinod K, De Meyer SF, et al. Neutrophil extracellular traps promote deep vein thrombosis in mice. *J Thromb Haemost.* (2012) 10:136–44. doi: 10.1111/j.1538-7836.2011.04544.x
127. Park J, Wysocki RW, Amoozgar Z, Maiorino L, Fein MR, Jorns J, et al. Cancer cells induce metastasis-supporting neutrophil extracellular DNA traps. *Sci Transl Med.* (2016) 8:361ra138. doi: 10.1126/scitranslmed.aag1711
128. Rocks N, Vanwinge C, Radermecker C, Blacher S, Gilles C, Marée R, et al. Ozone-primed neutrophils promote early steps of tumour cell metastasis to lungs by enhancing their NET production. *Thorax.* (2019) 74:768–79. doi: 10.1136/thoraxjnl-2018-211990
129. Novotny J, Chandraratne S, Weinberger T, Philippi V, Stark K, Ehrlich A, et al. Histological comparison of arterial thrombi in mice and men and the influence of Cl-amidine on thrombus formation. *PLoS ONE.* (2018) 13:e0190728. doi: 10.1371/journal.pone.0190728
130. Biron BM, Chung C-S, O'Brien XM, Chen Y, Reichner JS, Ayala A. Cl-amidine prevents histone 3 citrullination and neutrophil extracellular trap formation, and improves survival in a murine sepsis model. *J Innate Immun.* (2017) 9:22–32. doi: 10.1159/000448808
131. Jarrot P-A, Tellier E, Plantureux L, Crescence L, Robert S, Chareyre C, et al. Neutrophil extracellular traps are associated with the pathogenesis of diffuse alveolar hemorrhage in murine lupus. *J Autoimmun.* (2019) 100:120–30. doi: 10.1016/j.jaut.2019.03.009
132. Papadaki G, Kambas K, Choulaki C, Vlachou K, Drakos E, Bertsias G, et al. Neutrophil extracellular traps exacerbate Th1-mediated autoimmune responses in rheumatoid arthritis by promoting DC maturation. *Eur J Immunol.* (2016) 46:2542–54. doi: 10.1002/eji.201646542

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IL8 and PMA Trigger the Regulation of Different Biological Processes in Granulocyte Activation

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The molecular mechanisms driving specific regulation of neutrophils are not completely understood to date. In order to characterize fundamental granulocyte features on protein level, we analyzed changes in proteome composition as reaction to stress from cell activation processes. For this purpose, we isolated primary granulocytes from equine whole blood through density gradient centrifugation followed by sodium chloride lysis and stimulated cells for 30 min with interleukin-8 (IL8) due to its role as a chemotactic factor for neutrophils. We additionally used phorbol 12-myristate 13-acetate (PMA) and lipopolysaccharide (LPS), which are primarily associated to neutrophil extracellular trap formation and release of reactive oxygen species. From mass spectrometry analysis, we identified a total of 2,032 proteins describing the whole granulocyte proteome, including 245 proteins (12% of identified proteome) newly associated to *in vivo* expression in primary equine granulocytes (hypothetical proteins). We also found distinct and different changes in protein abundance (ratio $\geq$ 2) after short stimulation of cells with various stimuli, pointing to rapid and differentiated reaction pattern. IL8 stimulation resulted in increased protein abundance of 58 proteins (3% of proteome), whereas PMA induced changed protein abundance of 207 (10 % of proteome) and LPS of 46 proteins (2% of proteome). Enrichment analyses clearly showed fundamental differences between stimuli, with primary association of IL8 stimulation to processes in immune response, receptor signaling and signal transduction. Top enrichment for PMA on the other hand pointed to vesicle mediated transport and exocytosis. Stimulation with LPS did not result in any significant enrichment. Although we detected 43% overlap of enrichment categories for IL8 and PMA stimulation, indicating that activation of neutrophils with different stimuli partly induces some similar biological processes and pathways, hierarchical clustering showed clear differences in distribution and biological relevance of clusters between the chosen stimuli. Our studies provide novel information on the granulocyte proteome and offer insights into early, differentiated granulocyte reaction to stimuli, which contribute to a better understanding of molecular mechanisms involved in activation and recruitment of neutrophils, through inflammatory stimuli.

Keywords: innate immune cell activation, differential proteomics, interleukin 8 (IL8), PMA, LPS, neutrophil, signal transduction, biological process

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INTRODUCTION

Granulocytes have initially been labeled as short-lived, terminally differentiated cells, driving innate immune response through phagocytosis, degranulation, ROS release and, as described more recently, NETosis (1, 2). However, today, neutrophil diversity and plasticity, with many different subpopulations and finely tuned functional features are evident (3–8). Still relatively little is known about specific, differentiated regulation mechanisms in early granulocyte activation involved in subsequent innate immune responses. For this reason, we investigated fundamental granulocyte features by analyzing changes in proteome composition as reaction to cell activation and allocating these changes to different biological processes and pathways in an equine model. In cells from the adaptive immune system, we previously found major differences in regulation of lymphocyte protein expression in autoimmune disease (9–12). Moreover, we detected differences in the granulocyte proteome, with Talin1 as a key player in disease pathogenesis, indicating a role of the innate immune system in lymphocyte-driven autoimmune disease (13, 14). In retrospect, the granulocytes analyzed in these studies most likely represent the subpopulation of low density neutrophils (LDN), which were recently discovered (15). In present study, LDN were excluded from analysis, due to granulocyte isolation protocol. Here, we were especially interested in the impact of initial activation on downstream innate immune response and the pathways switched on in course of activation-induced cell stress in order to provide fundamental knowledge on granulocyte activation mechanisms.

MATERIALS AND METHODS

Sample Processing

The blood used in this study originated from three resident horses of the LMU equine clinic (aged 12, 20, and 21; kept in straw-embedded stalls with daily access to paddocks), which are at the student's disposal for supervised ultrasound- and health assessment training. Health status was assessed by standard clinical routine examinations. No experimental procedures were performed on these horses. Venous whole blood was collected in tubes supplemented with 25.000 I.U. heparin. After rough sedimentation of erythrocytes, PMN were isolated from plasma by density gradient centrifugation (RT, 290 × g, 25 min, brake off) using Ficoll-Paque PLUS separating solution (GE Life Sciences, Freiburg, Germany). Cells were washed gently (4 C, 400 × g, 10 min) in cold PBS (DPBS devoid of $CaCl_2$ and $MgCl_2$; Gibco/ThermoFisher Scientific, Germany) and remaining erythrocytes were removed by sodium chloride lysis (lysis in 0.2% NaCl, after 30 s addition of equal part 1.6% NaCl to restore isotonicity). Cells were washed (4°C, 400 × g, 10 min) and resuspended in PBS with 0.2% Glucose. From each animal used in the experiment, we prepared aliquot portions of 6×10^5 cells/500 µl. These cell aliquots were separately stimulated with recombinant equine interleukin-8 (IL8; Kingfisher Biotec; 1 ng/ml), phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich/Merck, Darmstadt, Germany; 5 µg/ml) and lipopolysaccharide (LPS; Sigma-Aldrich/Merck, Darmstadt, Germany; 5 µg/ml) for 30 min in a CO_2 incubator (37°C, 5% CO_2). Untreated medium control (mc) was incubated under the same conditions but without stimulating agent. After stimulation, each of the stimulated and mc aliquots was topped up to 1 ml with PBS with 0.2% Glucose and pelleted (4°C, 2,300 × g, 10 min). All Samples were stored at −20°C. Shortly before mass spectrometry analysis, cells were thawed and lysed in urea buffer (8 M urea in 0.1 M Tris/HCl pH 8.5), and protein amount was determined with Bradford protein assay (16).

Mass Spectrometry Analysis

From each sample, 10 µg total protein was digested with LysC and trypsin by filter-aided sample preparation (FASP) as previously described (17). Acidified eluted peptides were analyzed in the data-dependent mode on a Q Exactive HF mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) online coupled to a UItimate 3000 RSLC nano-HPLC (Dionex). Samples were automatically injected and loaded onto the C18 trap column, eluted after 5 min and separated on the C18 analytical column (75 µm ID × 15 cm, Acclaim PepMAP 100 C18. 100 Å/size, LC Packings, Thermo Fisher Scientific, Bremen, Germany) by a 90 min non-linear acetonitrile gradient at a flow rate of 250 nl/min. MS spectra were recorded at a resolution of 60,000. After each MS1 cycle, the 10 most abundant peptide ions were selected for fragmentation.

Data Processing

Label-free quantitative analysis was performed using Progenesis QI software (version 2.5, Non-linear Dynamics, Waters, Newcastle upon Tyne, U.K.) as described (18, 19), with raw MS spectral files imported, followed by automatic peak picking and retention time alignment and normalization of total peak intensities across all samples to minimize loading differences. All MS/MS spectra were exported from Progenesis QI software as Mascot generic files (mgf) and searched against Ensembl Horse protein database (version 3.0, http://www.ensembl.org) for peptide identification with Mascot (version 2.5.1). Search parameters used were 10 ppm peptide mass tolerance, 20 mmu fragment mass tolerance, one missed cleavage allowed, carbamidomethylation as fixed modification and methionine oxidation as well as deamidation of asparagine and glutamine as variable modifications. Mascot integrated decoy database search was set to a false discovery rate (FDR) of 1% when searching was performed on the concatenated mgf files with a percolator ion score cut-off of 13 and an appropriate significance threshold *p*. Identifications were re-imported into Progenesis QI and redundancies grouped following the rules of parsimony.

Data Analysis

Differential protein abundance was determined by comparison of the mean normalized peptide abundance from the extracted ion chromatograms. Proteins were considered differentially expressed at stimulating agent/mc ratio $\geq$ 2.0. Bioinformatic analysis was performed on human orthologs of gene names from differentially expressed equine proteins with open source software ShinyGO v0.60: http://bioinformatics.sdstate.edu/go60/

(20) with the following settings: search species human, *P*-value cutoff (FDR) 0.05, number of most significant terms to show 30. *P*-value for enrichment analysis was calculated via hypergeometric distribution, followed by correction using FDR. Venn diagram was made with open source tool: http://bioinformatics.psb.ugent.be/webtools/Venn/.

RESULTS

Two Thousand Thirty-Two Proteins Describing the Granulocyte Proteome

Using mass spectrometry analysis, we identified the equine whole granulocyte proteome, comprising a total of 2,032 proteins. Among the identifications, we found 245 proteins (hypothetical proteins) which have not been associated to the *in vivo* protein expression repertoire of equine granulocytes so far (**Supplemental Table 1**). These proteins represent 12% of the total granulocyte proteome identified here.

Short Stimulation Time of Only 30 min Results in Rapid and Differentiated Reactions of Cells

After stimulation with three different stimulating agents, we found distinct changes in granulocyte protein abundance compared to medium controls (ratio cut-off $\geq$ 2). In detail, cells stimulated with LPS showed higher expression levels of 46 proteins (2% of proteome), whereas PMA induced increased protein abundance of 207 proteins (10% of proteome). IL8 stimulation resulted in increased protein expression levels of 58 proteins (3% of proteome) (**Supplemental Table 2**). All of these differentially abundant proteins summed up to a total of 252, from which only 15 showed higher expression levels in all three stimulating agent groups (**Figure 1**, **Table 1**). Analysis of differentially expressed proteins per stimulation group revealed 12 unique proteins from LPS and 174 from PMA stimulated cells as well as 22 proteins with unique appearance in cells stimulated with IL8 (**Figure 1**, **Table 1**).

Reaction of Innate Immune Cells to Different Stimuli Are Respectively Clustered in Three Distinct Networks

In order to understand the association of the differentially expressed proteins to biological processes and their known role in granulocyte activation pathways, we analyzed the data from the 15 proteins present in all groups (**Figure 1**, **Table 1**, **Supplemental Figure 1**, **Supplemental Table 3**) as well as LPS, PMA, and IL8 groups with open source software ShinyGO. LPS stimulation data did not result in any significant enrichment and clustering of the differentially expressed proteins in these cells. Therefore, we looked into GO category assignment for these proteins and found eight high level categories mainly connected to cell metabolism, intracellular transport and response to stress (**Supplemental Table 4**). Data from IL8 and PMA stimulated cells, however, revealed three distinct clusters. Comparison of these enrichment category clusters showed a 43% overlap between IL8 and PMA stimulation groups, with neutrophil activation and cellular catabolic processes as the two major shared functional categories (**Figure 3**, **Table 3**). The unique clusters for each stimulant, however, showed a clear difference in reaction of cells to stimuli: PMA stimulated innate immune cells reacted with processes involved in intracellular transportation processes, whereas IL8 stimulated cells showed involvement in signal transduction pathways (**Figure 3**, **Table 3**).

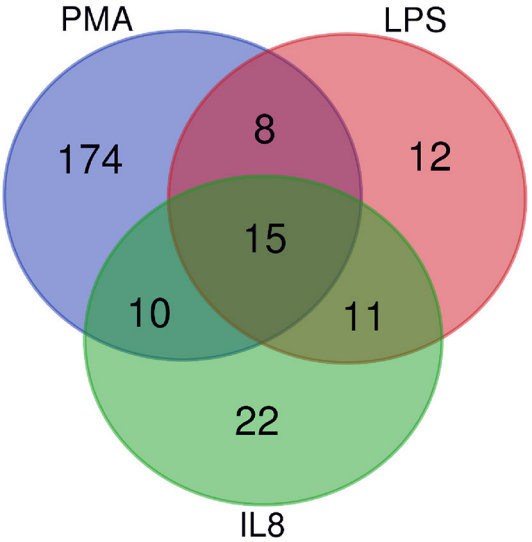

FIGURE 1 | Venn Diagramm of overlapping differentially expressed proteins from IL8, PMA, and LPS stimulated cells. Fifteen proteins are differentially expressed among all stimulation groups.

Unique Reaction of Cells to IL8 Stimulation Associates to Receptor Signaling, Signal Transduction, and Immune Response

For more detailed analysis, we subsequently focused on proteins which were differentially abundant in either IL8 or PMA stimulated cells and therefore described as unique for respective stimulus. Hierarchical clustering of enrichment analysis data from unique proteins expressed after PMA stimulation pointed to primary involvement in vesicle-mediated and intracellular transport as well as exocytosis on the one and metabolic processes on the other hand (**Figure 2**, **Table 2**). Results from IL8 stimulated cells showed primary association of uniquely expressed proteins to receptor signaling, signal transduction, and immune response with top enrichment for Fc-epsilon receptor and Tumor necrosis factor (TNF) mediated signaling pathways (**Figure 2**, **Table 2**). Also, enrichment of PI 3-kinase activity pointed to processes in cytoskeleton dynamics (**Figure 2**, **Table 2**). Interestingly, the protein proteasome 26S subunit, ATPase 6 (PSMC6), was allocated to the majority of functional enrichment categories from the IL8 stimulation group (**Table 2**).

TABLE 1 | Gene names for shared and unique proteins ≥2 from granulocyte-derived mass spectrometry list.

	Shared proteins				Unique proteins							
Stimulating agent	IL8 PMA LPS	IL8 PMA	IL8 LPS	PMA LPS	IL8	PMA						LPS
No. of proteins	15	10	11	8	22	174						12
Gene names	AAMDC	DNM1	ALAD	CALCOCO1	ADAMDEC1	A2M	COMT	GYS1	NT5C1A	RIPOR2	SYT5	ACTBL2
	ATP2B1	EXOSC2	ARID1B	EIF4G2	AIFM1	ABHD14B	COPS6	H2AFV	NUCKS1	RNASEL	SYTL3	DCUN1D1
	DMTN	GMPR2	BAX	IWS1	CARHSP1	ACSL4	COX5A	HARS2	NUDT3	RPL12	TACC3	DCXR
	DNASE1L1	JPT1	CPNE6	PROM1	CASP1	ADAM10	CPSF6	HBE1	NUMB	RPL15	TAF2	DHX58
	EEA1	IMPDH2	ECHDC1	RHEB	CDC37	ADD2	CWF19L1	HIST1H1A	NUP210	RPL18	TAOK3	HIKESHI
	ENSA	KCNA10	GNS	SIRPB1	CNP	ADD3	CXorf58	HIST1H3A	NUP62	RPL9	TAPBP	RPS4X
	FARSB	RAP1A	PPP1R18	TBCC	CREG1	ADPGK	CYP2C19	HSD17B12	OAS3	RPN1	TBC1D13	SDHB
	GLYR1	SRSF4	PSMC1	TMEM128	CRYZ	AGPAT2	DCTN3	HSPE1	PFN	RPS6KA2	TEDC1	SEPT11
	HCFC1	VKORC1	SEC23IP		DDOST	ALDH16A1	DENND3	HUWE1	PGRMC1	RPS8	TM9SF2	SLC47A2
	IPCEF1	ZBTB45	SH2D5		DLAT	ARHGAP10	DES	HVCN1	PI4KA	S100A7	TMED10	UBE2H
	LST1		ZNF207		HDLBP	ARL6IP1	DHCR7	IGSF6	PKP1	SARS	TPD52L2	WASHC2A
	PSIP1				IGHG4	ATP6AP1	DNM1L	ILVBL	PPM1F	SEC24A	TRMT112	WDR44
	RPRD1B				IKBKB	ATP8A1	DOCK10	IMMT	PRPF8	SELENOH	TRPC3	
	RPS4X				NAXD	B3GNT2	DOT1L	IRAK3	PSMA7	SEMA3E	TUFM	
	VPS37C				NPEPL1	BAK1	ECHS1	IRF3	PSMB8	SERBP1	UBE2M	
					PAG1	BCAP29	EIF3H	ISG15	PSMB9	SF3A1	UBR4	
					PSMC6	BIN1	EIF4H	ISG20	PSMD7	SLC17A3	UQCRC2	
					PSMD12	BMX	ENOPH1	JAK3	QSOX1	SLC28A1	USP15	
					SARNP	BPGM	EPB41	KARS	R3HCC1	SMAP2	VARS	
					SH3GLB1	BTBD11	ERH	KLF12	RAB43	SNX27	VDAC3	
					SIK3	CAMKK2	ESYT2	LZIC	RASGRP2	SOD2	VPS11	
					VAV2	CARMIL2	FABP5	MCFD2	RBBP4	SPCS2	VPS26A	
						CASP14	GHDC	MCU	RBBP7	SRSF6	VPS28	
						CASS4	GLOD4	MOGS	RBM8A	SSR1	VTI1A	
						CD109	GM2A	MPDU1	RDH16	STK38	WDR5	
						CD300LF	GMFG	MTCH2	RECQL	STRN	WFIKKN1	
						CES2	GNG12	MYADM	RENBP	SYNE1	YARS	
						CHMP3	GRHPR	MYO1E	REXO2	SYNE2	YIF1B	
						COL4A3BP	GRN	NAF1	RHAG	SYPL1	ZSCAN4	

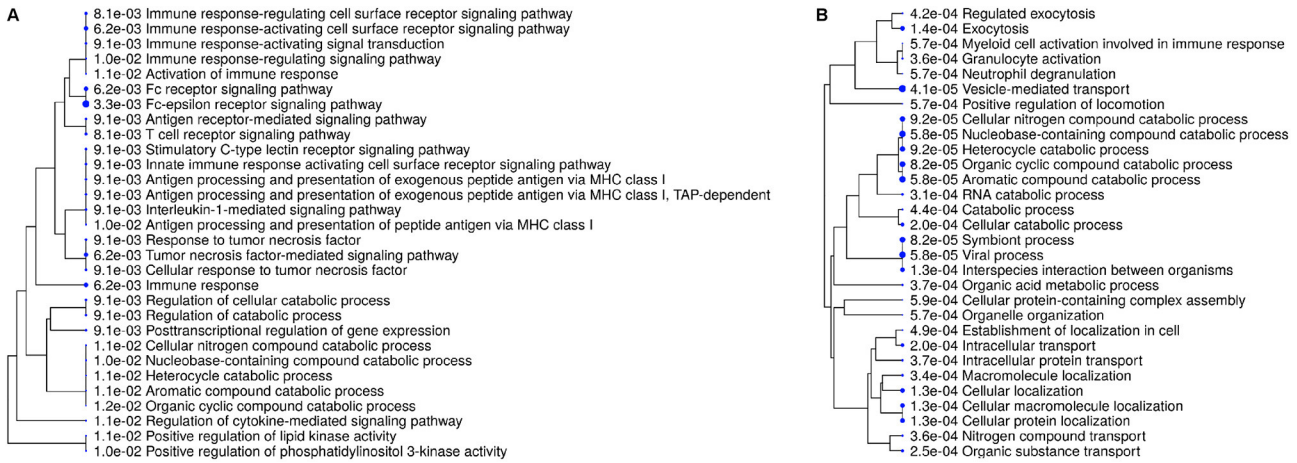

FIGURE 2 | Enrichment tree showing 30 most significant functional categories from biological processes generated from gene names of differentially expressed proteins after stimulation with either IL8 **(A)** or PMA **(B)**. Size of the solid circles corresponds to the enrichment FDR. Proteins used for calculation of enrichment were uniquely present in respective stimulation group.

DISCUSSION

Knowledge about the molecular mechanisms involved in specific granulocyte activation and subsequent choice of pathways depending on different stressors is still incomplete to date. Moreover, in the past, granulocytes have frequently been underestimated in their ability to execute distinct heterogenic reactions rather than uniform response cascades to any, mainly pathogen-induced stimulus. The past decade has yielded more details on granulocyte heterogeneity and function, not only for processes in the innate immune system but also for regulatory involvement in adaptive immune responses (4, 21). Nevertheless, there are still many signaling processes in granulocyte activation, which need clarification. To gain deeper insight into these processes and to find possible downstream reaction differences between initiating stimuli, we performed a short stimulation assay with freshly obtained, primary equine granulocytes. PMA and LPS, were used as universal stimuli. PMA induces exocytosis, ROS release and NET formation through direct activation of protein kinase C (PKC) and subsequent signal transduction cascade (22). LPS triggers similar responses by binding to TLR4 on neutrophils (23). For specific activation of granulocytes, we used IL8. This cytokine is expressed by a variety of cells, such as monocytes, fibroblasts and endothelial cells and acts as a potent chemoattractant for granulocytes, inducing neutrophil recruitment and chemotaxis via chemokine receptors CXCR1 and CXCR2 (24). Granulocyte activation and identification of resulting differences in behavior, gene regulation and protein expression have previously been performed in other uncommon models such as cattle (25) and pigs (26). Predominantly, studies focus on human granulocytes, however, most of these studies concentrate on one particular morphologic (granules, membrane proteins) (27–29) or functional (NET formation) (30, 31) feature of granulocytes. Few studies describe stimulatory experiments and their effect on the whole granulocyte proteome (32, 33). Compared to these studies, we chose a very short stimulation time in order to detect early proteome changes with possibly transient character. Also, we did not separate the proteome via 2D prior to mass spectrometry analysis.

From our initial proteomics experiment, we unraveled the equine granulocyte proteome, detecting 2,032 proteins (**Supplemental Table 1**). Similar proteomic based studies have been performed on human granulocytes (34), granulocytes from other species such as cattle (35) and rats (36), as well as neutrophil-associated BALF proteins in horses (37). However, to our knowledge, the full equine neutrophil proteome has not been described to date. Interestingly, 12% of the identified proteins in our study were classified as "hypothetical proteins," whose existence is predicted, but experimental evidence for *in vivo* expression is lacking. With our studies, we could confirm actual *in vivo* expression of these proteins, associating them to primary granulocyte proteome in horses. We chose equine granulocytes to conduct our experiments, because the equine and human immune system share a wide range of similarities both in granulocyte-lymphocyte ratio, composition and function (38–40). Furthermore, the horse is prone to allergies and autoimmune diseases, which are similarly found in humans (41–45) and adaptive as well as innate immune cells from horses have proven to be valuable tools for studying human diseases (37, 42, 44, 46). Despite certain differences between human and horse neutrophils (47, 48), the horse is still a very promising model, especially for processes and diseases which are not adequately addressed by rodent models. However, more investigations are needed to determine its exact and true translational value, which we provide a basis for with our studies.

Among all identified proteins, we found a total of 252 differentially abundant proteins after cell stimulation with different stimuli (**Supplemental Table 2**). Fifteen of these proteins showed higher expression levels in all three stimulating agent groups (**Figure 1**, **Table 1**, **Supplemental Figure 1**,

TABLE 2 | Enrichment of functional categories describing biological proccesses generated from proteins with differential expression after IL8 and PMA stimulation.

Enrichment FDR	Genes in list	Total genes	Functional category	Genes
Biological proccesses from unique Proteins with ratio IL8/mc ≥ 2				
0.0033	4	133	Fc-epsilon receptor signaling pathway	PSMC6 IKBKB VAV2 PSMD12
0.0062	10	2,602	Immune response	PAG1 CDC37 CASP1 ADAMDEC1 PSMC6 IKBKB CREG1 VAV2 PSMD12 DDOST
0.0062	4	210	Tumor necrosis factor-mediated signaling pathway	IKBKB CASP1 PSMC6 PSMD12
0.0062	4	203	Fc receptor signaling pathway	PSMC6 IKBKB VAV2 PSMD12
0.0062	5	466	Immune response-activating cell surface receptor signaling pathway	PAG1 PSMC6 IKBKB VAV2 PSMD12
0.0081	4	275	T cell receptor signaling pathway	PAG1 PSMC6 IKBKB PSMD12
0.0081	5	520	Immune response-regulating cell surface receptor signaling pathway	PAG1 PSMC6 IKBKB VAV2 PSMD12
0.0091	6	1,050	Regulation of catabolic process	CARHSP1 SH3GLB1 PSMC6 CDC37 AIFM1 PSMD12
0.0091	5	662	Posttranscriptional regulation of gene expression	CARHSP1 CDC37 SARNP PSMC6 PSMD12
0.0091	6	928	Regulation of cellular catabolic process	CARHSP1 SH3GLB1 PSMC6 CDC37 AIFM1 PSMD12
0.0091	3	141	Innate immune response activating cell surface receptor signaling pathway	PSMC6 IKBKB PSMD12
0.0091	3	135	Stimulatory C-type lectin receptor signaling pathway	PSMC6 IKBKB PSMD12
0.0091	4	368	Response to tumor necrosis factor	IKBKB CASP1 PSMC6 PSMD12
0.0091	3	147	Antigen processing and presentation of exogenous peptide antigen via MHC class I	PSMC6 IKBKB PSMD12
0.0091	3	143	Antigen processing and presentation of exogenous peptide antigen via MHC class I, TAP-dependent	PSMC6 IKBKB PSMD12
0.0091	4	323	Antigen receptor-mediated signaling pathway	PAG1 PSMC6 IKBKB PSMD12
0.0091	5	662	Immune response-activating signal transduction	PAG1 PSMC6 IKBKB VAV2 PSMD12
0.0091	3	118	Interleukin-1-mediated signaling pathway	IKBKB PSMC6 PSMD12
0.0091	4	344	Cellular response to tumor necrosis factor	IKBKB CASP1 PSMC6 PSMD12
0.0102	5	712	Nucleobase-containing compound catabolic process	CARHSP1 AIFM1 CNP PSMC6 PSMD12
0.0102	2	34	Positive regulation of phosphatidylinositol 3-kinase activity	SH3GLB1 VAV2
0.0102	5	714	Immune response-regulating signaling pathway	PAG1 PSMC6 IKBKB VAV2 PSMD12
0.0104	3	167	Antigen processing and presentation of peptide antigen via MHC class I	PSMC6 IKBKB PSMD12
0.0110	2	38	Positive regulation of lipid kinase activity	SH3GLB1 VAV2
0.0110	3	182	Regulation of cytokine-mediated signaling pathway	CDC37 CASP1 IKBKB
0.0110	5	763	Activation of immune response	PAG1 PSMC6 IKBKB VAV2 PSMD12
0.0110	5	765	Cellular nitrogen compound catabolic process	CARHSP1 AIFM1 CNP PSMC6 PSMD12
0.0110	5	764	Heterocycle catabolic process	CARHSP1 AIFM1 CNP PSMC6 PSMD12
0.0114	5	778	Aromatic compound catabolic process	CARHSP1 AIFM1 CNP PSMC6 PSMD12
0.0122	5	813	Organic cyclic compound catabolic process	CARHSP1 AIFM1 CNP PSMC6 PSMD12
Biological proccesses from unique proteins with ratio PMA/mc ≥ 2				
4.15E-05	42	2,220	Vesicle-mediated transport	BCAP29 CHMP3 VPS26A HIST1H1A SYT5 SNX27 VTI1A VPS11 VPS28 TMED10 RAB43 SEC24A ATP6AP1 DNM1L DENND3 ESYT2 NUMB BIN1 SYTL3 MCFD2 MYO1E CD300LF TAPBP GRN PKP1 HUWE1 PGRMC1 PSMD7 QSOX1 HVCN1 ATP8A1 UBR4 GMFG DCTN3 ADAM10 S100A7 CD109 FABP5 GHDC AGPAT2 A2M GM2A
5.76E-05	22	778	Aromatic compound catabolic process	COMT RBM8A NUDT3 ISG20 NT5C1A RNASEL ADPGK BPGM PKP1 NAF1 SERBP1 RPL18 PSMA7 PSMD7 NUP210 RPS8 RPL9 RPL15 RPL12 PSMB8 NUP62 PSMB9

(Continued)

TABLE 2 | Continued

Enrichment FDR	Genes in list	Total genes	Functional category	Genes
5.76E-05	21	712	Nucleobase-containing compound catabolic process	RBM8A NUDT3 ISG20 NT5C1A RNASEL ADPGK BPGM PKP1 NAF1 SERBP1 RPL18 PSMA7 PSMD7 NUP210 RPS8 RPL9 RPL15 RPL12 PSMB8 NUP62 PSMB9
5.76E-05	25	951	Viral process	OAS3 CHMP3 RNASEL KARS PSMA7 EIF4H IRF3 UBR4 BIN1 COPS6 ISG15 PSMB8 NUP62 PSMB9 NUCKS1 ISG20 RAB43 PI4KA RPL18 NUP210 RPS8 VPS28 RPL9 RPL15 RPL12
8.17E-05	22	813	Organic cyclic compound catabolic process	COMT RBM8A NUDT3 ISG20 NT5C1A RNASEL ADPGK BPGM PKP1 NAF1 SERBP1 RPL18 PSMA7 PSMD7 NUP210 RPS8 RPL9 RPL15 RPL12 PSMB8 NUP62 PSMB9
8.17E-05	25	1,024	Symbiont process	OAS3 CHMP3 RNASEL KARS PSMA7 EIF4H IRF3 UBR4 BIN1 COPS6 ISG15 PSMB8 NUP62 PSMB9 NUCKS1 ISG20 RAB43 PI4KA RPL18 NUP210 RPS8 VPS28 RPL9 RPL15 RPL12
9.22E-05	21	765	Cellular nitrogen compound catabolic process	RBM8A NUDT3 ISG20 NT5C1A RNASEL ADPGK BPGM PKP1 NAF1 SERBP1 RPL18 PSMA7 PSMD7 NUP210 RPS8 RPL9 RPL15 RPL12 PSMB8 NUP62 PSMB9
9.22E-05	21	764	Heterocycle catabolic process	RBM8A NUDT3 ISG20 NT5C1A RNASEL ADPGK BPGM PKP1 NAF1 SERBP1 RPL18 PSMA7 PSMD7 NUP210 RPS8 RPL9 RPL15 RPL12 PSMB8 NUP62 PSMB9
0.0001	36	1,981	Cellular protein localization	HUWE1 VPS11 BCAP29 TBC1D13 SPCS2 VPS26A TM9SF2 UQCRC2 SNX27 VTI1A VPS28 TMED10 ARL6IP1 RAB43 NUP62 SYNE2 SYNE1 DNM1L RIPOR2 SEC24A ADAM10 SYTL3 PPM1F MTCH2 NUMB EPB41 MYADM RPL18 SRSF6 SSR1 NUP210 RPS8 RPL9 RPL15 RPL12 RBM8A
0.0001	48	3,087	Cellular localization	HUWE1 VPS11 BCAP29 DNM1L TBC1D13 COL4A3BP CHMP3 SPCS2 VPS26A TM9SF2 SYT5 UQCRC2 SNX27 VTI1A VPS28 TMED10 ARL6IP1 RAB43 NUP62 SYNE2 SEC24A SYNE1 TRPC3 BAK1 ATP6AP1 DENND3 RIPOR2 ESYT2 NUMB BIN1 ADAM10 SYTL3 PPM1F MTCH2 CPSF6 EPB41 MYADM RPL18 SRSF6 SSR1 NUP210 DCTN3 RPS8 RPL9 RPL15 MCFD2 RPL12 RBM8A
0.0001	25	1,084	Interspecies interaction between organisms	OAS3 CHMP3 RNASEL KARS PSMA7 EIF4H IRF3 UBR4 BIN1 COPS6 ISG15 PSMB8 NUP62 PSMB9 NUCKS1 ISG20 RAB43 PI4KA RPL18 NUP210 RPS8 VPS28 RPL9 RPL15 RPL12
0.0001	36	1,993	Cellular macromolecule localization	HUWE1 VPS11 BCAP29 TBC1D13 SPCS2 VPS26A TM9SF2 UQCRC2 SNX27 VTI1A VPS28 TMED10 ARL6IP1 RAB43 NUP62 SYNE2 SYNE1 DNM1L RIPOR2 SEC24A ADAM10 SYTL3 PPM1F MTCH2 NUMB EPB41 MYADM RPL18 SRSF6 SSR1 NUP210 RPS8 RPL9 RPL15 RPL12 RBM8A
0.0001	24	1,023	Exocytosis	SYT5 VPS11 ATP6AP1 DNM1L SYTL3 TMED10 GRN PKP1 HUWE1 PGRMC1 PSMD7 QSOX1 HVCN1 ATP8A1 UBR4 GMFG ADAM10 S100A7 CD109 FABP5 GHDC AGPAT2 A2M GM2A
0.0002	35	1,959	Intracellular transport	HUWE1 VPS11 BCAP29 TBC1D13 COL4A3BP CHMP3 SPCS2 VPS26A SYT5 UQCRC2 SNX27 VTI1A VPS28 TMED10 ARL6IP1 RAB43 NUP62 SEC24A SYNE2 DNM1L DENND3 SYTL3 CPSF6 BIN1 RPL18 SRSF6 SSR1 NUP210 DCTN3 RPS8 RPL9 RPL15 MCFD2 RPL12 RBM8A

(Continued)

TABLE 2 | Continued

Enrichment FDR	Genes in list	Total genes	Functional category	Genes
0.0002	41	2,500	Cellular catabolic process	HUWE1 DNM1L COMT PSMA7 PSMD7 UBR4 ECHS1 VPS28 CYP2C19 PSMB8 HBE1 PSMB9 RBM8A NUDT3 CAMKK2 ISG20 RENBP DENND3 NT5C1A USP15 RNASEL ADPGK VPS11 BPGM ISG15 GM2A PKP1 QSOX1 NAF1 VTI1A SERBP1 RPL18 CHMP3 VPS26A NUP210 RPS8 RPL9 FABP5 RPL15 RPL12 NUP62
0.0003	46	3,011	Organic substance transport	HUWE1 VPS11 TBC1D13 COL4A3BP CHMP3 SPCS2 VPS26A ATP8A1 SLC17A3 SYT5 UQCRC2 SNX27 VTI1A SLC28A1 VPS28 TMED10 ARL6IP1 RAB43 NUP62 KARS DNM1L RHAG ACSL4 BCAP29 SEC24A ESYT2 IRF3 NUP210 RNASEL SYTL3 FABP5 MCFD2 GM2A RBM8A PPM1F CPSF6 MCU RPL18 ATP6AP1 VDAC3 SRSF6 SSR1 RPS8 RPL9 RPL15 RPL12
0.0003	15	465	RNA catabolic process	RBM8A ISG20 RNASEL PKP1 NAF1 SERBP1 RPL18 PSMA7 PSMD7 RPS8 RPL9 RPL15 RPL12 PSMB8 PSMB9
0.0003	49	3,354	Macromolecule localization	HUWE1 NAF1 VPS11 BCAP29 TBC1D13 COL4A3BP CHMP3 SPCS2 VPS26A ATP8A1 TM9SF2 UQCRC2 SNX27 VTI1A VPS28 TMED10 ARL6IP1 RAB43 NUP62 SYNE2 KARS DNM1L SYNE1 ACSL4 RIPOR2 SEC24A ESYT2 IRF3 NUP210 ADAM10 SYTL3 MCFD2 GM2A RBM8A PPM1F MTCH2 CPSF6 NUMB MCU EPB41 MYADM RPL18 ATP6AP1 SRSF6 SSR1 RPS8 RPL9 RPL15 RPL12
0.0004	41	2,592	Nitrogen compound transport	HUWE1 VPS11 TBC1D13 RHAG COL4A3BP CHMP3 SPCS2 VPS26A SLC17A3 SYT5 UQCRC2 SNX27 VTI1A SLC28A1 VPS28 TMED10 ARL6IP1 RAB43 NUP62 KARS DNM1L BCAP29 TAPBP SEC24A IRF3 NUP210 SYTL3 MCFD2 RBM8A PPM1F CPSF6 MCU RPL18 ATP6AP1 VDAC3 SRSF6 SSR1 RPS8 RPL9 RPL15 RPL12
0.0004	17	603	Granulocyte activation	KARS GRN PKP1 HUWE1 PGRMC1 PSMD7 QSOX1 HVCN1 ATP8A1 UBR4 GMFG ADAM10 S100A7 FABP5 GHDC AGPAT2 GM2A
0.0004	25	1,194	Intracellular protein transport	HUWE1 VPS11 TBC1D13 SPCS2 VPS26A UQCRC2 SNX27 VTI1A VPS28 TMED10 ARL6IP1 RAB43 NUP62 BCAP29 SEC24A SYTL3 RPL18 SRSF6 SSR1 NUP210 RPS8 RPL9 RPL15 RPL12 RBM8A
0.0004	26	1,276	Organic acid metabolic process	SARS KARS VARS HARS2 ECHS1 YARS ENOPH1 CYP2C19 GRHPR CES2 ACSL4 COMT RENBP HSD17B12 ADPGK FABP5 BPGM SLC17A3 PSMA7 PSMD7 ABHD14B NUP210 B3GNT2 PSMB8 NUP62 PSMB9
0.0004	21	901	Regulated exocytosis	SYT5 DNM1L TMED10 GRN PKP1 HUWE1 PGRMC1 PSMD7 QSOX1 HVCN1 ATP8A1 UBR4 GMFG ADAM10 S100A7 CD109 FABP5 GHDC AGPAT2 A2M GM2A
0.0004	43	2,825	Catabolic process	HUWE1 DNM1L COMT PSMA7 PSMD7 UBR4 ECHS1 VPS28 CYP2C19 PSMB8 HBE1 PSMB9 RBM8A NUDT3 CAMKK2 ISG20 IRAK3 RENBP DENND3 NT5C1A USP15 RNASEL ADPGK VPS11 BPGM ISG15 GM2A PKP1 QSOX1 NAF1 VTI1A SERBP1 RPL18 CHMP3 VPS26A NUP210 RPS8 RPL9 FABP5 CES2 RPL15 RPL12 NUP62

(Continued)

TABLE 2 | Continued

Enrichment FDR	Genes in list	Total genes	Functional category	Genes
0.0005	38	2,364	Establishment of localization in cell	HUWE1 VPS11 BCAP29 TBC1D13 COL4A3BP CHMP3 SPCS2 VPS26A SYT5 UQCRC2 SNX27 VTI1A VPS28 TMED10 ARL6IP1 RAB43 NUP62 SEC24A TRPC3 BAK1 SYNE2 DNM1L DENND3 NUMB SYTL3 CPSF6 BIN1 RPL18 SRSF6 SSR1 NUP210 DCTN3 RPS8 RPL9 RPL15 MCFD2 RPL12 RBM8A
0.0006	16	577	Neutrophil degranulation	GRN PKP1 HUWE1 PGRMC1 PSMD7 QSOX1 HVCN1 ATP8A1 UBR4 GMFG ADAM10 S100A7 FABP5 GHDC AGPAT2 GM2A
0.0006	17	640	Myeloid cell activation involved in immune response	KARS GRN PKP1 HUWE1 PGRMC1 PSMD7 QSOX1 HVCN1 ATP8A1 UBR4 GMFG ADAM10 S100A7 FABP5 GHDC AGPAT2 GM2A
0.0006	16	576	Positive regulation of locomotion	SEMA3E KARS S100A7 CARMIL2 SYNE2 RIPOR2 ATP8A1 PPM1F CHMP3 NUMB GRN CASS4 PFN1 SOD2 ADAM10 MYADM
0.0006	55	4,098	Organelle organization	RBBP4 HUWE1 VPS11 RECQL TACC3 SYNE2 ARHGAP10 ADD2 PKP1 DNM1L CASS4 DOT1L H2AFV PFN1 HIST1H1A GMFG UQCRC2 ADD3 VTI1A EPB41 TMED10 WDR5 RPL12 CAMKK2 BAK1 GRN PPM1F SEC24A SYNE1 USP15 SERBP1 CARMIL2 ARL6IP1 HIST1H3A NUCKS1 RPS6KA2 VDAC3 RBBP7 COL4A3BP BIN1 SEMA3E CHMP3 IMMT NAF1 RAB43 DES MYADM NUP62 SOD2 ZSCAN4 TAPBP DCTN3 VPS28 COPS6 MCFD2
0.0006	25	1,253	Cellular protein-containing complex assembly	ADD2 DNM1L EIF4H PFN1 SRSF6 GMFG NAF1 ADD3 VPS11 PRPF8 RPL12 SF3A1 CPSF6 SEC24A CHMP3 CARMIL2 RBBP4 HIST1H3A HIST1H1A BIN1 EIF3H TAPBP MYADM RBBP7 TMED10

Proteins used were uniquely expressed in each stimulation group.

Supplemental Table 3), indicating onset of some mutual reactions to the different stimuli. A larger number of unique proteins with differential expression per stimulant, however, pointed to predominantly differentiated reactions to the different stimuli (**Figure 1**, **Table 1**). Further assessment of all differentially abundant proteins from PMA and IL8 samples with ShinyGO enrichment analysis revealed 57% unique network clustering for each stimulant, respectively (**Figure 3**, **Table 3**). This shows the ability of granulocytes to distinguish between stimuli and regulate specific pathways in response to selective cell-stressors, although partial immune response is executed independent of stimulation type. Subsequent analysis of solely those proteins that changed abundance uniquely after either IL8 or PMA stimulation highlighted their association to stimulant-characteristic reactions, such as exocytosis and degranulation after PMA stimulation (22), and cytoskeleton dynamics after stimulation with IL8 (49, 50) (**Figure 2**, **Table 2**).

IL8 stimulation yielded the identification of proteasome 26S subunit, ATPase 6 (PSMC6), which showed higher abundance unique to this stimulant (IL8/mc ratio 2.1; $p < 0.001$). PSMC6 is an ATP-dependent proteolytic complex responsible for ubiquitin-dependent protein degradation (51, 52), which is an important regulator of the majority of cellular activity and homeostasis (53). Divergent levels of proteasome activity have a strong impact on disease pathogenesis of several diseases and are used as drug targets in disease treatment (51, 54–56). Thus, the higher abundance of PSMC6 in IL8 stimulated cells might indicate activation of the proteasome in granulocytes with functional importance in downstream regulation of immune response to stress. Subsequent analysis of proteomic data from IL8 stimulated cells revealed that PSMC6 was present in the majority of functional enrichment clusters from biological processes, including the top enriched functional categories tumor necrosis factor (TNF) mediated signaling and Fc-epsilon receptor pathways (**Table 2**). These two pathways are essential for signal transduction in cells, with a wide functional variety of downstream responses such as apoptosis but also immune and inflammatory responses as well as cell survival, activation and differentiation (57, 58). Interestingly, occurrence of Fc receptors on granulocytes have initially been described as a marker of neutrophil heterogeneity rather than a necessity for optimal neutrophil aggregation and adhesion (59). Especially Fc-epsilon receptor signaling is only present in neutrophils under certain conditions and their exact role is still

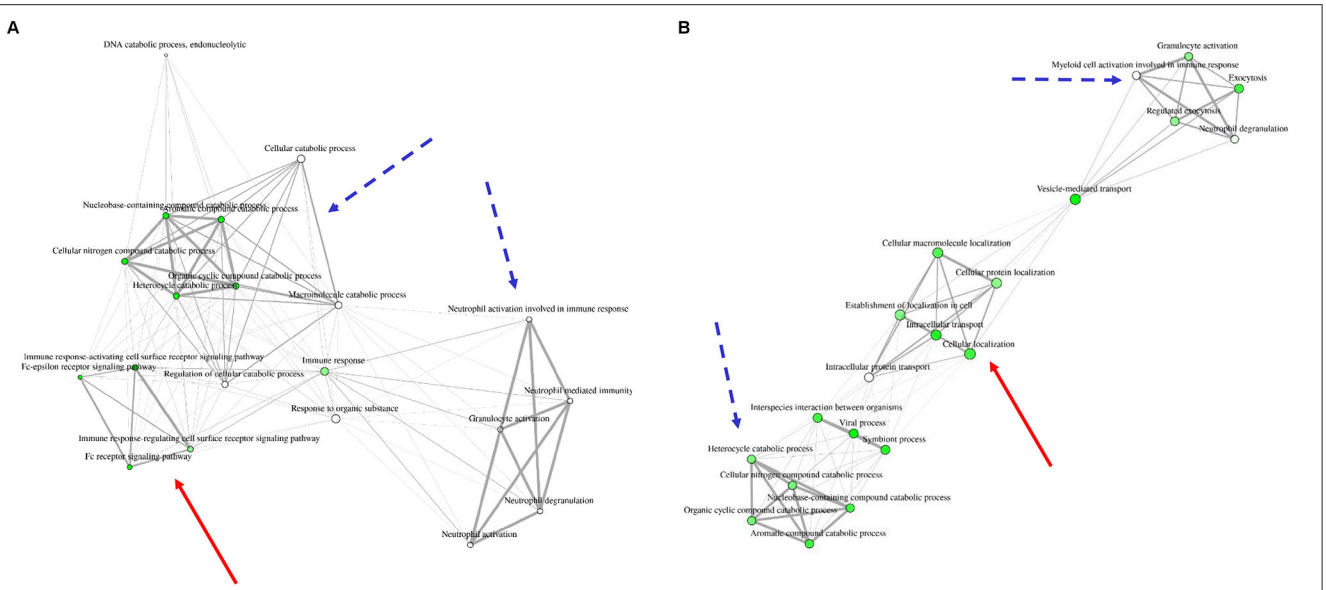

FIGURE 3 | Network clustering for biological processes to which differentially expressed proteins from IL8 **(A)** and PMA **(B)** stimulation were appointed. Three distinct clusters are visible in each stimulation group. Two clusters show similarities between stimulants (dotted blue arrow: granulocyte activation and metabolic processes) whereas one cluster is unique for each group [red arrow: immune response signaling pathways in IL8 stimulated cells **(A)** and cellular protein localization in PMA stimulated cells **(B)**]. For a more clear presentation of clusters, we searched 20 most significant categories instead of 30.

TABLE 3 | Shared and unique functional categories generated from differentially expressed proteins after IL8 and PMA stimulation.

	Functional categories from biological process IL8 and PMA		
Stimulating agent	Unique	Shared	Unique
	IL8	IL8 /PMA	PMA
No. of categories	17	13	17
Functional categories	Antigen receptor-mediated signaling pathway	Aromatic compound catabolic process	Cellular localization
	Cell activation	Cellular catabolic process	Cellular macromolecule localization
	Cellular response to mineralocorticoid stimulus	Cellular nitrogen compound catabolic process	Cellular protein localization
	DNA catabolic process	Granulocyte activation	Establishment of localization in cell
	DNA catabolic process, endonucleolytic	Heterocycle catabolic process	Exocytosis
	Fc receptor signaling pathway	Leukocyte degranulation	Interspecies interaction between organisms
	Fc-epsilon receptor signaling pathway	Myeloid cell activation involved in immune response	Intracellular protein transport
	Immune response	Neutrophil activation	Intracellular transport
	Immune response-activating cell surface receptor signaling pathway	Neutrophil activation involved in immune response	Macromolecule localization
	Immune response-regulating cell surface receptor signaling pathway	Neutrophil degranulation	Nitrogen compound transport
	Macromolecule catabolic process	Neutrophil mediated immunity	Organic substance transport
	MRNA metabolic process	Nucleobase-containing compound catabolic process	Regulated exocytosis
	Regulation of cellular catabolic process	Organic cyclic compound catabolic process	Regulation of biological quality
	Regulation of mRNA stability		RNA catabolic process
	Regulation of RNA stability		Symbiont process
	Response to organic substance		Vesicle-mediated transport
	Tumor necrosis factor-mediated signaling pathway		Viral process

discussed among experts, whereas other Fc receptor types, such as low-affinity Fc-gamma receptors, are commonly expressed on granulocytes playing an important role in immune complex mediated activation of neutrophils through their downstream pathways (58). Furthermore, Fc receptors are unlikely to mediate PMA-induced cell activation (59), which is consistent with our findings on PMA-stimulated granulocytes, where we found no allocation of uniquely expressed proteins to Fc receptor signaling pathways (**Figures 2**, **3**, **Tables 2**, **3**).

Our findings undermine the ongoing appreciation of granulocyte function toward finely tuned, heterogeneous, specific reactions of more than one subpopulation of neutrophils (4, 7, 8, 60–63). Furthermore, our data shows that a stimulation time of only 30 min is sufficient to initiate substantial and specific changes in granulocyte proteome as reaction to individual stimulating agents (**Figure 1**, **Table 1**, **Supplemental Table 2**). These rapid changes most likely occur due to gene induction of early responding genes but may also be the result of posttranslational modifications mediated by proteins that are activated early in neutrophil responses to stimuli, such as phosphatidylinositol 3-kinase (PI 3-kinase). Interestingly, PI 3-kinase activity appears as functional category from enrichment analysis of our IL8 data (**Figure 2A**, **Table 2**), supporting the involvement of PI 3-kinase in IL8-induced protein changes. With its ability to phosphorylate molecules acting as second messengers and thereby switch on downstream intracellular signaling (64), and its involvement in neutrophil chemokinesis and phagosome formation (49, 50, 65), PI 3-kinase merits further investigations in future functional studies. No matter the origin of the changed granulocyte protein repertoire described in our data, it gives insight into early onset of granulocyte activation on protein level, which may be useful to modulate granulocyte mediated pathological processes in future functional experiments. However, more experiments are needed, not only for determination of minimal stimulation times triggering regulation of protein expression levels in granulocytes, but also for analysis of expression kinetics in course of longer stimulation assays. From other comprehensive studies on equine neutrophils we know, that neutrophil extracellular traps (NETs) readily occur in response to adequate stimuli (66) as opposed to cells from other animal models (67). Our protein data however, lack association to this process (**Figure 2**, **Table 2**). We assume that the expression differences of proteins associated to NET formation occur after longer stimulation time, as recently described (66). Therefore, increasing the stimulation time in these assays could address protein repertoire changes associated to NET-relevant biological processes such as DNA decondensation, histone citrullination, and related signal transduction. Also, we would expect more prominent clustering of IL8 induced protein changes to cytoskeleton dynamics involved in chemotaxis and phagosome formation, as functional answers of cells to stimuli fluctuate over time (68). Keeping in mind the dynamic character of protein expression patterns in course of cell activation, our data put a spotlight merely on the first reaction to stimuli. This is a very interesting time point in our opinion, because it shows the initiating functional answers in activated cells, which are potentially accessible to experimental modulation. Adding proteomic data from more stimulation times would give a more precise insight into dynamic whole-cell proteome changes throughout the activation process of granulocytes, similar to previous analysis of pre-determined cytokines and degranulation markers by kinetic flow cytometry (69), which needs to be addressed in future studies.

CONCLUSION

With our data we provide a fundamental study on activation of primary granulocytes and regulation of downstream immune response by showing that different stimuli provoke divergent and rapid downstream responses through regulation of protein expression in these cells. These expression differences show involvement in various different pathways and biological processes which, among some similarities, differ between stimuli and support knowledge on heterogeneity of granulocytes and their highly selective response to stimuli. The presented data may therefore act as a guide for further, in-depth research on granulocyte response patterns and behavior in health and disease.

DATA AVAILABILITY STATEMENT

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (70) partner repository with the dataset identifier PXD013648.

ETHICS STATEMENT

Collection of blood was permitted by the local authority, Regierung von Oberbayern (Permit number: ROB-55.2Vet-2532.Vet_03-17-88).

AUTHOR CONTRIBUTIONS

CD conceived and designed the experiments. RD and SH performed the experiments. RD, SH, MW, and CD analyzed the data. RD wrote the manuscript. All authors critically read the manuscript and approved the final version to be published.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.03064/full#supplementary-material

REFERENCES

1. van der Linden M, Meyaard L. Fine-tuning neutrophil activation: strategies and consequences. *Immunol Lett.* (2016) 178:3–9. doi: 10.1016/j.imlet.2016.05.015
2. de Buhr N, Bonilla MC, Jimenez-Soto M, von Kockritz-Blickwede M, Dolz G. Extracellular trap formation in response to *Trypanosoma cruzi* infection in granulocytes isolated from dogs and common opossums, natural reservoir hosts. *Front Microbiol.* (2018) 9:966. doi: 10.3389/fmicb.2018.00966
3. Chakravarti A, Rusu D, Flamand N, Borgeat P, Poubelle PE. Reprogramming of a subpopulation of human blood neutrophils by prolonged exposure to cytokines. *Lab Invest.* (2009) 89:1084–99. doi: 10.1038/labinvest.2009.74
4. Eruslanov EB, Singhal S, Albelda SM. Mouse versus human neutrophils in cancer: a major knowledge gap. *Trends Cancer.* (2017) 3:149–60. doi: 10.1016/j.trecan.2016.12.006
5. Deniset JF, Kubes P. Neutrophil heterogeneity: bona fide subsets or polarization states? *J Leukoc Biol.* (2018) 103:829–38. doi: 10.1002/JLB.3RI0917-361R
6. Grieshaber-Bouyer R, Nigrovic PA. Neutrophil heterogeneity as therapeutic opportunity in immune-mediated disease. *Front Immunol.* (2019) 10:346. doi: 10.3389/fimmu.2019.00346
7. Ng LG, Ostuni R, Hidalgo A. Heterogeneity of neutrophils. *Nat Rev Immunol.* (2019) 19:255–65. doi: 10.1038/s41577-019-0141-8
8. Kubes P. The enigmatic neutrophil: what we do not know. *Cell Tissue Res.* (2018) 371:399–406. doi: 10.1007/s00441-018-2790-5
9. Degroote RL, Hauck SM, Amann B, Hirmer S, Ueffing M, Deeg CA. Unraveling the equine lymphocyte proteome: differential septin 7 expression associates with immune cells in equine recurrent uveitis. *PLoS ONE.* (2014) 9:e91684. doi: 10.1371/journal.pone.0091684
10. Degroote RL, Helm S, Klein U, Schmitt R, Ueffing M, Hauck SM, et al. The equine CD4+ lymphocyte proteome. *Dataset Pap Sci.* (2014) 2014:4. doi: 10.1155/2014/105312
11. Degroote RL, Uhl PB, Amann B, Krackhardt AM, Ueffing M, Hauck SM, et al. Formin like 1 expression is increased on CD4+ T lymphocytes in spontaneous autoimmune uveitis. *J Proteomics.* (2017) 154:102–8. doi: 10.1016/j.jprot.2016.12.015
12. Schauer M, Kleinwort KJH, Degroote RL, Wiedemann C, Kremmer E, Hauck SM, et al. Interaction of septin 7 and DOCK8 in equine lymphocytes reveals novel insights into signaling pathways associated with autoimmunity. *Sci Rep.* (2018) 8:12332. doi: 10.1038/s41598-018-30753-7
13. Degroote RL, Hauck SM, Kremmer E, Amann B, Ueffing M, Deeg CA. Altered expression of talin 1 in peripheral immune cells points to a significant role of the innate immune system in spontaneous autoimmune uveitis. *J Proteomics.* (2012) 75:4536–44. doi: 10.1016/j.jprot.2012.01.023
14. Degroote RL, Hauck SM, Treutlein G, Amann B, Frohlich KJ, Kremmer E, et al. Expression changes and novel interaction partners of Talin 1 in effector cells of autoimmune uveitis. *J Proteome Res.* (2013) 12:5812–9. doi: 10.1021/pr400837f
15. Kanamaru R, Ohzawa H, Miyato H, Matsumoto S, Haruta H, Kurashina K, et al. Low density neutrophils (LDN) in postoperative abdominal cavity assist the peritoneal recurrence through the production of neutrophil extracellular traps (NETs). *Sci Rep.* (2018) 8:632. doi: 10.1038/s41598-017-19091-2
16. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* (1976) 72:248–54. doi: 10.1016/0003-2697(76)90527-3
17. Grosche A, Hauser A, Lepper MF, Mayo R, von Toerne C, Merl-Pham J, et al. The proteome of native adult muller glial cells from murine retina. *Mol Cell Proteomics.* (2016) 15:462–80. doi: 10.1074/mcp.M115.052183
18. Hauck SM, Hofmaier F, Dietter J, Swadzba ME, Blindert M, Amann B, et al. Label-free LC-MSMS analysis of vitreous from autoimmune uveitis reveals a significant decrease in secreted Wnt signalling inhibitors DKK3 and SFRP2. *J Proteomics.* (2012) 75:4545–54. doi: 10.1016/j.jprot.2012.04.052
19. Hauck SM, Lepper MF, Hertl M, Sekundo W, Deeg CA. Proteome dynamics in biobanked horse peripheral blood derived lymphocytes (PBL) with induced autoimmune uveitis. *Proteomics.* (2017) 17:1700013. doi: 10.1002/pmic.201700013
20. Ge S, Jung D. ShinyGO: a graphical enrichment tool for animals and plants. *bioRxiv.* (2018) 315150. doi: 10.1101/315150
21. Li Y, Wang W, Yang F, Xu Y, Feng C, Zhao Y. The regulatory roles of neutrophils in adaptive immunity. *Cell Commun Signal.* (2019) 17:147. doi: 10.1186/s12964-019-0471-y
22. Saito T, Takahashi H, Doken H, Koyama H, Aratani Y. Phorbol myristate acetate induces neutrophil death through activation of p38 mitogen-activated protein kinase that requires endogenous reactive oxygen species other than HOCl. *Biosci Biotechnol Biochem.* (2005) 69:2207–12. doi: 10.1271/bbb.69.2207
23. Yipp BG, Kim JH, Lima R, Zbytnuik LD, Petri B, Swanlund N, et al. (2017). The lung is a host defense niche for immediate neutrophil-mediated vascular protection. *Sci Immunol.* 2:eaam8929. doi: 10.1126/sciimmunol.aam8929
24. French SW, Mendoza AS, Afifiyan N, Tillman B, Vitocruz E, French BA. The role of the IL-8 signaling pathway in the infiltration of granulocytes into the livers of patients with alcoholic hepatitis. *Exp Mol Pathol.* (2017) 103:137–40. doi: 10.1016/j.yexmp.2017.08.005
25. Bassel LL, Caswell JL. Bovine neutrophils in health and disease. *Cell Tissue Res.* (2018) 371:617–37. doi: 10.1007/s00441-018-2789-y
26. de Buhr N, Reuner F, Neumann A, Stump-Guthier C, Tenenbaum T, Schroten H, et al. Neutrophil extracellular trap formation in the Streptococcus suis-infected cerebrospinal fluid compartment. *Cell Microbiol.* (2017) 19:e12649. doi: 10.1111/cmi.12649
27. Lominadze G, Powell DW, Luerman GC, Link AJ, Ward RA, McLeish KR. Proteomic analysis of human neutrophil granules. *Mol Cell Proteomics.* (2005) 4:1503–21. doi: 10.1074/mcp.M500143-MCP200
28. Xu P, Crawford M, Way M, Godovac-Zimmermann J, Segal AW, Radulovic M. Subproteome analysis of the neutrophil cytoskeleton. *Proteomics.* (2009) 9:2037–49. doi: 10.1002/pmic.200800674
29. Rorvig S, Ostergaard O, Heegaard NH, Borregaard N. Proteome profiling of human neutrophil granule subsets, secretory vesicles, and cell membrane: correlation with transcriptome profiling of neutrophil precursors. *J Leukoc Biol.* (2013) 94:711–21. doi: 10.1189/jlb.1212619
30. Chapman EA, Lyon M, Simpson D, Mason D, Beynon RJ, Moots RJ, et al. Caught in a trap? proteomic analysis of neutrophil extracellular traps in rheumatoid arthritis and systemic lupus erythematosus. *Front Immunol.* (2019) 10:423. doi: 10.3389/fimmu.2019.00423
31. Petretto A, Bruschi M, Pratesi F, Croia C, Candiano G, Ghiggeri G, et al. Neutrophil extracellular traps (NET) induced by different stimuli: a comparative proteomic analysis. *PLoS ONE.* (2019) 14:e0218946. doi: 10.1371/journal.pone.0218946
32. Fessler MB, Malcolm KC, Duncan MW, Worthen GS. A genomic and proteomic analysis of activation of the human neutrophil by lipopolysaccharide and its mediation by p38 mitogen-activated protein kinase. *J Biol Chem.* (2002) 277:31291–302. doi: 10.1074/jbc.M200755200
33. Aquino EN, Neves AC, Santos KC, Uribe CE, Souza PE, Correa JR, et al. Proteomic analysis of neutrophil priming by PAF. *Protein Pept Lett.* (2016) 23:142–51. doi: 10.2174/0929866523666151202210604
34. Tomazella GG, da Silva I, Laure HJ, Rosa JC, Chammas R, Wiker HG, et al. Proteomic analysis of total cellular proteins of human neutrophils. *Proteome Sci.* (2009) 7:32. doi: 10.1186/1477-5956-7-32
35. Lippolis JD, Reinhardt TA. Proteomic survey of bovine neutrophils. *Vet Immunol Immunopathol.* (2005) 103:53–65. doi: 10.1016/j.vetimm.2004.08.019
36. Piubelli C, Galvani M, Hamdan M, Domenici E, Righetti PG. Proteome analysis of rat polymorphonuclear leukocytes: a two-dimensional electrophoresis/mass spectrometry approach. *Electrophoresis.* (2002) 23:298–310. doi: 10.1002/1522-2683(200202)23:2<298::AID-ELPS298>3.0.CO;2-I
37. Bright LA, Dittmar W, Nanduri B, McCarthy FM, Mujahid N, Costa LR, et al. Modeling the pasture-associated severe equine asthma bronchoalveolar lavage fluid proteome identifies molecular events mediating neutrophilic airway inflammation. *Vet Med.* (2019) 10:43–63. doi: 10.2147/VMRR.S194427
38. Khandpur R, Carmona-Rivera C, Vivekanandan-Giri A, Gizinski A, Yalavarthi S, Knight JS, et al. NETs are a source of citrullinated autoantigens and stimulate inflammatory responses in rheumatoid arthritis. *Sci Transl Med.* (2013) 5:178ra140. doi: 10.1126/scitranslmed.3005580
39. Zschaler J, Schlorke D, Arnhold J. Differences in innate immune response between man and mouse. *Crit Rev Immunol.* (2014) 34:433–54. doi: 10.1615/CritRevImmunol.2014011600

40. Horohov DW. The equine immune responses to infectious and allergic disease: a model for humans? *Mol Immunol.* (2015) 66:89–96. doi: 10.1016/j.molimm.2014.09.020
41. Rickards KJ, Page CP, Lees P, Cunningham FM. Phosphodiesterase activity in neutrophils from horses with chronic obstructive pulmonary disease. *Vet Immunol Immunopathol.* (2000) 76:319–30. doi: 10.1016/S0165-2427(00)00220-8
42. Deeg CA, Hauck SM, Amann B, Pompetzki D, Altmann F, Raith A, et al. Equine recurrent uveitis–a spontaneous horse model of uveitis. *Ophthalmic Res.* (2008) 40:151–3. doi: 10.1159/000119867
43. Herteman N, Vargas A, Lavoie JP. Characterization of circulating low-density neutrophils intrinsic properties in healthy and asthmatic horses. *Sci Rep.* (2017) 7:7743. doi: 10.1038/s41598-017-08089-5
44. Vargas A, Boivin R, Cano P, Murcia Y, Bazin I, Lavoie JP. Neutrophil extracellular traps are downregulated by glucocorticosteroids in lungs in an equine model of asthma. *Respir Res.* (2017) 18:207. doi: 10.1186/s12931-017-0689-4
45. Sheats MK. A comparative review of equine SIRS, sepsis, and neutrophils. *Front Vet Sci.* (2019) 6:69. doi: 10.3389/fvets.2019.00069
46. Busch M, Wefelmeyer KL, Walscheid K, Rothaus K, Bauer D, Deeg CA, et al. Identification of ocular autoantigens associated with juvenile idiopathic arthritis-associated uveitis. *Front Immunol.* (2019) 10:1793. doi: 10.3389/fimmu.2019.01793
47. Scudamore CL, Pemberton A, Watson ED, Miller HR. Neutrophil chemotaxis in the horse is not mediated by a complex of equine neutrophil elastase and equine alpha-1-proteinase inhibitor. *Br Vet J.* (1993) 149:331–8. doi: 10.1016/S0007-1935(05)80074-0
48. Brazil TJ, Dixon PM, Haslett C, Murray J, McGorum BC. Constitutive apoptosis in equine peripheral blood neutrophils *in vitro*. *Vet J.* (2014) 202:536–42. doi: 10.1016/j.tvjl.2014.08.029
49. Ferguson GJ, Milne L, Kulkarni S, Sasaki T, Walker S, Andrews S, et al. PI(3)Kgamma has an important context-dependent role in neutrophil chemokinesis. *Nat Cell Biol.* (2007) 9:86–91. doi: 10.1038/ncb1517
50. Feng S, Zhou L, Zhang Y, Lu S, Long M. Mechanochemical modeling of neutrophil migration based on four signaling layers, integrin dynamics, and substrate stiffness. *Biomech Model Mechanobiol.* (2018) 17:1611–30. doi: 10.1007/s10237-018-1047-2
51. Schmidt M, Finley D. Regulation of proteasome activity in health and disease. *Biochim Biophys Acta.* (2014) 1843:13–25. doi: 10.1016/j.bbamcr.2013.08.012
52. Wang X, Cimermancic P, Yu C, Schweitzer A, Chopra N, Engel JL, et al. Molecular details underlying dynamic structures and regulation of the human 26S proteasome. *Mol Cell Proteomics.* (2017) 16:840–54. doi: 10.1074/mcp.M116.065326
53. Pashevin DO, Nagibin VS, Tumanovska LV, Moibenko AA, Dosenko VE. Proteasome inhibition diminishes the formation of neutrophil extracellular traps and prevents the death of cardiomyocytes in coculture with activated neutrophils during anoxia-reoxygenation. *Pathobiology.* (2015) 82:290–8. doi: 10.1159/000440982
54. Wang J, Maldonado MA. The ubiquitin-proteasome system and its role in inflammatory and autoimmune diseases. *Cell Mol Immunol.* (2006) 3:255–61.
55. Sjakste T, Paramonova N, Osina K, Dokane K, Sokolovska J, Sjakste N. Genetic variations in the PSMA3, PSMA6 and PSMC6 genes are associated with type 1 diabetes in Latvians and with expression level of number of UPS-related and T1DM-susceptible genes in HapMap individuals. *Mol Genet Genomics.* (2016) 291:891–903. doi: 10.1007/s00438-015-1153-0
56. Shi CX, Kortum KM, Zhu YX, Bruins LA, Jedlowski P, Votruba PG, et al. CRISPR genome-wide screening identifies dependence on the proteasome subunit PSMC6 for bortezomib sensitivity in multiple myeloma. *Mol Cancer Ther.* (2017) 16:2862–70. doi: 10.1158/1535-7163.MCT-17-0130
57. Dempsey PW, Doyle SE, He JQ, Cheng G. The signaling adaptors and pathways activated by TNF superfamily. *Cytokine Growth Factor Rev.* (2003) 14:193–209. doi: 10.1016/S1359-6101(03)00021-2
58. Futosi K, Fodor S, Mocsai A. Neutrophil cell surface receptors and their intracellular signal transduction pathways. *Int Immunopharmacol.* (2013) 17:638–50. doi: 10.1016/j.intimp.2013.06.034
59. Spagnuolo PJ, Fain M, Bass SN. Dissociation of neutrophil aggregation, adhesiveness, and Fc receptor activity. *Am J Hematol.* (1987) 26:221–8. doi: 10.1002/ajh.2830260303
60. Jablonska J, Granot Z. Neutrophil, quo vadis? *J Leukoc Biol.* (2017) 102:685–8. doi: 10.1189/jlb.3MR0117-015R
61. Garley M, Jablonska E. Heterogeneity among neutrophils. *Arch Immunol Ther Exp.* (2018) 66:21–30. doi: 10.1007/s00005-017-0476-4
62. Battaglia M, Petrelli A, Vecchio F. Neutrophils and type 1 diabetes: current knowledge and suggested future directions. *Curr Opin Endocrinol Diabetes Obes.* (2019) 26:201–6. doi: 10.1097/MED.0000000000000485
63. Rungelrath V, Kobayashi SD, DeLeo FR. Neutrophils in innate immunity and systems biology-level approaches: an update. *Wiley Interdiscip Rev Syst Biol Med.* (2019) 12:e1458. doi: 10.1002/wsbm.1458
64. Karlsson A, Nixon JB, McPhail LC. Phorbol myristate acetate induces neutrophil NADPH-oxidase activity by two separate signal transduction pathways: dependent or independent of phosphatidylinositol 3-kinase. *J Leukoc Biol.* (2000) 67:396–404. doi: 10.1002/jlb.67.3.396
65. Koga H, Kasprick A, Lopez R, Auli M, Pont M, Godessart N, et al. Therapeutic effect of a novel phosphatidylinositol-3-kinase delta inhibitor in experimental epidermolysis bullosa acquisita. *Front Immunol.* (2018) 9:1558. doi: 10.3389/fimmu.2018.01558
66. Fingerhut L, Ohnesorge B, von Borstel M, Schumski A, Strutzberg-Minder K, Morgelin M, et al. Neutrophil extracellular traps in the pathogenesis of equine recurrent uveitis (ERU). *Cells.* (2019) 8:E1528. doi: 10.3390/cells8121528
67. Neubert E, Senger-Sander SN, Manzke VS, Busse J, Polo E, Scheidmann SEF, et al. Serum and serum albumin inhibit *in vitro* formation of neutrophil extracellular traps (NETs). *Front Immunol.* (2019) 10:12. doi: 10.3389/fimmu.2019.00012
68. Takami M, Terry V, Petruzzelli L. Signaling pathways involved in IL-8-dependent activation of adhesion through Mac-1. *J Immunol.* (2002) 168:4559–66. doi: 10.4049/jimmunol.168.9.4559
69. Naegelen I, Beaume N, Plancon S, Schenten V, Tschirhart EJ, Brechard S. Regulation of neutrophil degranulation and cytokine secretion: a novel model approach based on linear fitting. *J Immunol Res.* (2015) 2015:817038. doi: 10.1155/2015/817038
70. Perez-Riverol Y, Csordas A, Bai J, Bernal-Llinares M, Hewapathirana S, Kundu DJ, et al. The PRIDE database and related tools and resources in 2019: improving support for quantification data. *Nucleic Acids Res.* (2019) 47:D442–50. doi: 10.1093/nar/gky1106

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REVIEW
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Mouse Models and Tools for the *in vivo* Study of Neutrophils

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Neutrophils are the most abundant leukocytes in human blood and critical actors of the immune system. Many neutrophil functions and facets of their activity *in vivo* were revealed by studying genetically modified mice or by tracking fluorescent neutrophils in animals using imaging approaches. Assessing the roles of neutrophils can be challenging, especially when exact molecular pathways are questioned or disease states are interrogated that alter normal neutrophil homeostasis. This review discusses the main *in vivo* models for the study of neutrophils, their advantages and limitations. The side-by-side comparison underlines the necessity to carefully choose the right model(s) to answer a given scientific question, and exhibit caveats that need to be taken into account when designing experimental procedures. Collectively, this review suggests that at least two models should be employed to legitimately conclude on neutrophil functions.

Keywords: neutrophils, Cre-recombinase, diphtheria toxin, mouse models, depletion, Gfi-1, NETosis

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INTRODUCTION

Neutrophils are key players of the immune system. They are the first leukocytes to be recruited to inflammatory sites (1) and they can contribute to inflammation through several mechanisms. These include their capacity to engulf and eliminate pathogenic agents or particles through phagocytosis, an NADPH oxygenase-dependent mechanism with reactive oxygen species synthesis and antibacterial enzymes mobilization (2). Moreover, neutrophils can also release lipid mediators, such as PAF or LTB4, thereby facilitating the recruitment of circulating cells (3). Furthermore, neutrophils can release Neutrophil Extracellular Traps (NETs), which are composed of DNA decorated with proteins such as cathepsins, histones, neutrophil elastase and myeloperoxidase (MPO) (4). NETs are sticky weblike structures, which are thought to trap pathogens and toxins, thereby inactivating them and preventing them from spreading (4, 5).

Mouse models of human diseases have progressively been developed in parallel with techniques and strategies to deplete neutrophils, to knockout genes for key neutrophil enzymes, and to visualize these cells *in vivo*. Traditional models for the study of neutrophils, including models of depletion or mutant mice, allowed a global comprehension of neutrophil biology. Nevertheless, these models notably raised questions of specificity, and hence justified the generation of novel models in recent years (6, 7). Together, these advances rendered mouse models advantageous for the study neutrophils in health and disease. Here, we summarize and discuss currently available mouse models for neutrophil depletion and highlight the main models deficient in key neutrophil enzymes. Moreover, strategies to visualize neutrophils and NETs *in vivo* are examined.

INDUCIBLE DEPLETION OF NEUTROPHILS

One commonly used approach to study the role of a given cell type is to deplete the cell type of interest *in vivo* in order to characterize the resulting phenotype. Numerous studies have used inducible neutrophil depletion strategies, as they enable to control neutrophil deficiency at different stages of interest. Furthermore, drugs and depleting antibodies can be used in virtually all mouse strains, and are thus convenient and versatile tools for the study of neutrophil biology.

Cyclophosphamide

Cyclophosphamide is a pro-drug that is used in humans as an antitumor agent (8). The designation "prodrug" is due to the fact that cyclophosphamide needs to be metabolized by liver enzymes such as cytochrome P450 for the formation of alkylating cytotoxic agents (9). Metabolized cyclophosphamide triggers the formation of DNA crosslinks and lesions (9) that lead to cell cycle arrest and cell death, thereby limiting the proliferation of dividing cells (10). This explains its use as an antitumor drug. Treatment of mice with cyclophosphamide increases the susceptibility of mice to pathogenic agents and has been used for the development of mouse models of infection (11, 12). Indeed, intraperitoneal injection of cyclophosphamide triggers the death of hematopoietic stem cells and incapacitates remaining cells preventing their proliferation and differentiation (13). Neutrophils are rather short-lived cells (14, 15). Hence, pharmacological depletion of hematopoietic stem cells is associated with an almost complete disappearance of blood neutrophils as early as 3–4 days after intraperitoneal or subcutaneous cyclophosphamide injection (11, 16).

This background explains the choice of this drug to assess the role of neutrophils *in vivo* (17–19). Mice are usually treated with a high dose of cyclophosphamide (150 mg/kg) on day 0 and with a low dose (100 mg/kg) 3 days later (11, 16, 17, 20). Three to four days after the last treatment with cyclophosphamide, mice exhibit a strong neutrophilia with a 3-fold increase of blood neutrophils compared to untreated mice (11, 16). Indeed, repetitive injections are necessary if long-term effects of neutrophil depletion are to be evaluated (17). A clear advantage of the use of cyclophosphamide to induce neutropenia in mice is the relatively low price of this drug, and its capacity to render any mouse strain neutropenic. However, a major limitation of this approach is the fact that cyclophosphamide is all but neutrophil specific. Indeed, cyclophosphamide-treated mice also exhibit markedly reduced numbers of circulating monocytes, B and T cells (11, 16, 17, 20). These confounding factors render the interpretation of results obtained in cyclophosphamide-treated animals challenging. For example, after treatment with cyclophosphamide, *Streptococcus pneumoniae*-infected mice showed a higher number of lung colony-forming units (CFUs) (21). However, the role of neutrophils in controlling lung CFU numbers has since come into question. Marks et al. treated *S. pneumoniae*-infected mice with neutrophil depleting antibodies and revealed they had similar CFUs to mice treated with an isotype control antibody, a difference that was explained by the lack of specificity of cyclophosphamide (22). In conclusion, while cyclophosphamide represents a convenient first tool to predict the role of neutrophils *in vivo*, findings obtained using this drug require confirmation with other approaches to address neutrophil biology.

Vinblastine

Vinblastine is another cytostatic drug (23) that can bind to tubulin molecules, thereby disrupting the assembly of microtubules (24) and interfering with cytoskeletal dynamics. Thus, vinblastine targets dividing cells and leads to mitotic arrest (25). Treatment of mice with vinblastine induces strong neutropenia (26, 27). Nevertheless, this drug is rarely used to study neutrophils *in vivo*. Overall, vinblastine exhibits the same disadvantages as cyclophosphamide. It displays a poor selectivity in blood (as illustrated by a respective 35 and 39% reduction in blood monocytes and lymphocytes) (26), and induces cytotoxicity in other cell types such as pancreatic cells (28) and during spermatogenesis (29). Furthermore, vinblastine might affect any cellular process involving microtubule assembly, limiting its use for *in vivo* experiments.

Depleting Antibodies

Neutrophil depletion can also be induced by the systemic administration of specific antibodies. As pharmacological drugs, depleting antibodies are efficient in WT mice and most knockout mice, which circumvents the necessity to generate mutant mice.

Anti-Gr-1

The monoclonal rat IgG2b antibody RB6-8C5 was originally reported to specifically bind to neutrophils (30, 31), and recognize the surface molecule Gr-1. Treatment of mice with RB6-8C5 anti-Gr-1 antibodies leads to a profound neutropenia (32–34) that lasts for up to 3–5 days depending on the injected dose (32, 35). Early reports suggested that RB6-8C5-mediated depletion was neutrophil-specific and would not affect other cell types such as monocytes (31, 33). These findings were however challenged by the findings that mice infected with the helminth *Nippostrongylus brasiliensis* also exhibited a severe reduction of blood eosinophils upon RB6-8C5 injection (36), and that RB6-8C5 treatment could induce a decrease of blood and spleen monocytes and memory-type $CD8^+$ T cells (35, 37). A more precise analysis of Gr-1 revealed that Gr-1 represents a family of two GPI-anchored proteins, Ly6C, and Ly6G (30). Ly6G is specifically expressed on the surface of mouse neutrophils (30), and thus represents a good candidate to selectively target neutrophils and trigger their depletion *in vivo*. Ly6C, however, is mainly expressed on neutrophils and monocytes but also in macrophages, and subpopulations of $CD8^+$ T cells (38–41), which could account for the lack of selectivity of RB6-8C5 antibodies.

Neutrophil depletion by RB6-8C5 was reported to be unaffected by the treatment of mice with Fc-block (anti-FcγRII/III antibodies) or in FcRγ-knockout mice (34). Disruption of complement activation by pre-treatment with cobra venom factor delayed—but did not prevent—the RB6-8C5-induced neutrophil depletion (34), suggesting that neutrophil depletion occurs through complement-dependent and -independent pathways. The role of complement is not

likely to be dependent on the membrane attack complex since neutrophils are not sensitive to membrane attack complex-induced death (42). However, C3-deficient mice were shown to exert no depletion upon RB6-8C5 treatment (43), indicating that opsonization of neutrophils is a prerequisite for their depletion. Moreover, sterile inflammation in the peritoneum can be induced by injection of thioglycolate triggering a peritonitis with a characteristic sequential change in cell population and cell maturation. Following activation of receptor for Advanced Glycation Endproducts (44, 45), neutrophils are the first cells to be recruited to the injection sites followed by monocytes, which become the main cell population between day 3 and 6 of peritonitis. In this context, pre-treatment of mice with RB6-8C5 antibodies induced neutrophil apoptosis, suggesting that other pathways could participate in neutrophil depletion in thioglycolate-induced inflammatory conditions (46). Injection of RB6-8C5 antibody in TNF-α pre-treated mice induces death due to microvessel obstruction and coagulation resulting in respiratory defects (34, 47). This is thought to be a consequence of neutrophil activation following binding of RB6-8C5 antibodies to TNF-α-primed cells (34, 47). These observations further reveal that RB6-8C5-induced depletion cannot be used in every context due to potential adverse reactions triggered by the antibody itself (as observed in TNF-α-primed mice). Other limitations of RB6-8C5-induced depletion include a side-specific depletion pattern that needs to be considered when planning experiments. Indeed RB6-8C5 was reported to be inefficient in the liver (48). Finally, RB6-8C5 treatment may trigger other side effects, such as myeloid cell expansion and upregulation of macrophage markers, probably through STAT-1, 3, and 5 engagement (46).

Despite the now well-described lack of specificity, RB6-8C5 antibodies have been used extensively (alone or in combination with other approaches) to study the role of neutrophils in various disease models, including models of infection (35, 49, 50), arthritis (51, 52), wound repair (53) and anaphylaxis (54).

Anti-Ly6G

The rat IgG2a antibody 1A8 has been described as being specific for Ly6G (30), and therefore as interacting specifically with neutrophils (55). Intraperitoneal injection of 1A8 induces a nearly complete depletion of blood and spleen neutrophils (35, 55), and an 80% decrease of liver neutrophils (35), while monocyte counts remain unaffected (35, 55). However, 1A8 is less efficient than RB6-8C5 antibody for the induction of neutrophil depletion. Indeed, mice are usually treated with a 2-fold higher dose of 1A8 than RB6-8C5 (55, 56). Furthermore, 2 days post-treatment, neutrophil counts rise again in 1A8-treated mice while it is not the case in RB6-8C5-treated mice (55). Finally, 1A8 efficiency has recently been questioned, as 1A8-treated 24 weeks old C57BL/6J mice showed no neutrophil depletion (57). The same observation was conducted with bone marrow neutrophils from BALB/c mice (58). To circumvent this low efficacy, it was recently suggested that combining 1A8 treatment with a secondary antibody injection could lead to a 90% depletion of blood neutrophils (57). Nevertheless, this statement needs to be tempered since the 1A8-induced neutrophil depletion was still efficient in 9 and 24 weeks old BALB/c and FVB/N mice, and in 9 weeks old C57BL/6J mice (57). Furthermore, 1A8-induced neutrophil depletion was also questioned in PbNK65-infected C57BL/6 mice and CFA-challenged BALB/c mice (58) and further demonstrates the need to verify the depletion efficacy when using this antibody.

Studies investigating the *in vivo* mechanism underlying 1A8-induced depletion described that depletion of macrophages prior to 1A8-treatment decreases the efficiency of neutrophil depletion (59), suggesting that macrophages are key effector cells for neutrophil depletion (59). This was confirmed by intravital microscopy, revealing that neutrophils opsonized with fluorescently labeled 1A8 antibody were phagocytized by macrophages in the spleen, liver and bone marrow (60). Interestingly, the same group reported that the choice of fluorochrome had an influence on the depletion efficiency and suggested that this might be due to differences in the binding of the labeled antibodies to Ly6G. For instance, 1A8-FITC was more efficient at inducing neutrophil depletion than 1A8-APC (60).

1A8 antibody has been extensively used to study the contribution of neutrophils *in vivo*. It was for example used to show that neutrophils support metastasis formation (61), limit muscle alteration after intense exercise (62), and participate in protection against *S. pneumoniae* (63). Moreover, with the availability of this more neutrophil-specific antibody, several studies reassessed the role of neutrophils *in vivo*. For instance, in a context of Herpes Simplex Virus type-1 infection, treatment of mice with RB6-8C5 had resulted in an increased sensitivity of the mice toward the virus, suggesting a protective role of neutrophils (64, 65). However, the use of 1A8 antibody to deplete neutrophils did not alter the virus replication, questioning the previously described role of neutrophils in anti-viral reactions (65). Similar results were obtained for bacterial infections with *Listeria monocytogenes*. It was previously shown that RB6-8C5-treated mice failed to control *L. monocytogenes* infection and it was concluded that neutrophils played an important role in defense against this pathogen (49). More recently, it was verified that RB6-8C5-treated mice did not control *L. monocytogenes* infection. However, going further, it was also demonstrated that 1A8-treated mice showed the same *L. monocytogenes*-induced mortality as WT mice (66), questioning the requirement of neutrophils in this context. Using a model for monocyte specific depletion, it was demonstrated that monocyte-depleted mice died of infection (66), suggesting that monocytes are the key players in defense against *L. monocytogenes* whereas neutrophils are dispensable. This suggested that the phenotype of RB6-8C5-treated mice was due to the depletion of monocytes and not neutrophils.

NIMP-R14 is a second monoclonal antibody reported to recognize Ly6G (67). NIMP-R14 treatment leads to >95% depletion of spleen and blood neutrophils (20, 68–70). Neutrophil counts return to basal levels about 3 days post-NIMP-R14 injection (69), suggesting that repetitive injections are needed for long-term experiments (which is also the case for RB6-8C5 and 1A8). NIMP-R14 antibody has been used to study neutrophil functions in many disease models, including glomerulonephritis (71), anti-*Helicobacter pylori* immunity (72), autoimmune encephalomyelitis (73), and experimental

models of anaphylaxis (68, 70). In contrast to 1A8 antibody driven depletion for which no unwanted side-effects have been described, several reports described a 2-fold decrease in blood monocytes and Ly6C^{high} spleen monocytes after NIMP-R14 treatment (68, 70, 74), questioning the specificity of this antibody.

While high doses of anti-Gr-1 and anti-Ly6G antibodies induce neutrophil depletion, these antibodies have also been used at a low dose to bind neutrophils without inducing cell depletion. This "low dose" strategy was used as a mean to assess the *in vivo* role of Ly6G. Injection of 10 μg of RB6-8C5 anti-Gr-1 or 5 μg of 1A8 anti-Ly6G antibodies prevented neutrophils from migrating toward inflamed tissues in a model of arthritis, suggesting that Ly6G is a key regulator of neutrophil migration (75). These findings were, however, challenged by the observation that RB6-8C5 anti-Gr-1 antibody-covered neutrophils show normal recruitment to the tissues in a mouse model *of Staphylococcus aureus* cellulitis (76). Moreover, Ly6G knockout mice (called Catchup mice) also showed normal neutrophil migration and functions in multiple models of sterile or infections inflammation (77). The binding of anti-Gr-1 and anti-Ly6G antibodies to neutrophils in the absence of neutrophil depletion highlights the necessity to appropriately evaluate the efficacy of their depletion. This is particularly critical in an inflammatory context, in which large numbers of neutrophils are released from the bone marrow. For instance, the TLR4 agonist carrageenan has extensively been used to induce acute inflammatory reactions, leading to the recruitment of neutrophils to the injection site (78). It has been shown that pre-treating mice with RB6-5C8 was sufficient to reduce the number of peritoneal neutrophils to 99%, even if mice were later intraperitoneally injected with carrageenan, 36 h after RB6-5C8 treatment (79). Furthermore, it is to note that neutrophils might be covered by anti-Gr-1 or anti-Ly6G antibodies, and thus appear as Ly6G negative cells in flow cytometric analysis. For example, in mice treated with anti-Ly6G NIMP-R14, bone marrow or lung neutrophils were not fully depleted but appeared as Ly6G-negative cells in flow cytometry analysis, due to epitope masking by NIMP-R14 antibodies (58, 70). As neutrophils are the major side scatter-high (SSC^{high}) leukocyte population, we recommend to verify that treatment with a neutrophil-depleting antibody induces marked reduction in the number of SSC^{high} cells. Ideally, such analysis should be performed in combination with the use of complementary neutrophil markers to ascertain neutrophil depletion. These complementary markers include CD11b, Ly6C, or the 7/4-antigen, independently of the fact that these molecules are also expressed by other cell types such as monocytes or macrophages (80, 81). One possible strategy to identify "Ly-6G masked" neutrophils is to use either of the additional markers and subsequently exclude non-neutrophils from the gate by co-staining with other monocyte or lineage markers such as CD115 or B220 and including information from the forward vs. side scatter analysis (82). Alternatively, as anti-Ly6G and anti-Gr-1 antibodies are rat monoclonal antibodies, depleting antibody-covered neutrophils could be detected by revelation of the depleting antibodies themselves using fluorescently-labeled anti-rat IgG. Indeed, two studies used an anti-rat IgG2b to evaluate RB6-8C5-induced neutrophil depletion (48, 83).

The pharmacological approaches to deplete neutrophils are presented in **Table 1**.

PMN^{DTR} Mice

Diphtheria toxin (DT) is a protein composed of two subunits that is released by *Corynebacterium diphtheriae* during infection (84). The human membrane-anchored form of the heparin-binding EGF-like growth factor (HB-EGF precursor) acts as the DT receptor (DTR) (85). Mouse HB-EGF precursor does not bind efficiently to DT, due to several amino acid exchanges in the HB-EGF domain that is critical for DT-receptor interactions. As a consequence, mouse cells show significantly enhanced resistance to DT-induced death (86).

By introducing the simian DTR into specific mouse cells, it is possible to render such cell populations sensitive to DT, and induce depletion of these cells upon DT injection (87). Indeed, binding of subunit B to the diphtheria toxin receptor (DTR) on human or monkey target cells induces entry of DT into the cell through receptor-mediated endocytosis (88). Once in the prelysosomal vesicles, a drop of pH induces the penetration of a domain of the DT subunit A through the vesicle membrane, leading to the release of DT into the cytoplasm (89). There, the DT subunit A binds to elongation factor 2 and inhibits protein synthesis and consequently triggers apoptosis (90).

It has been postulated that one molecule of DT can kill a eukaryotic cell (91), rendering this model of depletion extremely efficient. This is generally achieved using the "Cre-Lox" system. First, a loxP-flanked STOP cassette was introduced in the DTR gene (92) and this construct was inserted into the mouse locus Gt(ROSA)26Sor. The Rosa 26 is constitutively active and ubiquitously expressed in mice, which accounts for its success to drive transgenes and reporter constructs (92, 93). In the presence of Cre-recombinase, the STOP cassette is removed, allowing expression of the DTR. In the following, these mice will be referred to as iDTR mice (92).

One caveat of this method is the requirement of a promoter that is specific for the cell type of interest, in order to restrict expression of DTR to that particular cell type. hMRP8-Cre-ires/GFP mice were used to assess the specificity of the human MRP8 (hMRP8) promoter (6, 94–97). The transgenic construct encompasses an internal ribosome entry sites (98) that allows the concomitant expression of both the recombinase Cre and GFP under control of the myeloid cell-specific human MRP8 (hMRP8) promoter (94, 99). Using these mice, it was shown that GFP expression in mainly found in neutrophils (6, 95–97). However, it is important to note that hMRP8 promoter activity was also reported in 10–20% into some monocyte/macrophages populations (6, 97). Residual promoter activity was also initially noticed in a portion of granulocyte-macrophage progenitors (GMPs) (95). However, these findings were not confirmed in a more recent study (6).

Based on these promising findings, iDTR mice were crossed with hMRP8-Cre mice to generate a new mouse model of inducible neutrophil depletion [hereafter called PMN^{DTR} mice (6)]. Indeed, these mice exhibit an almost complete neutrophil

TABLE 1 | Pharmacological approaches to deplete neutrophils.

	Name	Targets	Main advantages	Main limitations	References
Drugs	Cyclophos-phamide	DNA	. Rapid and efficient . Depletion can be performed in any mouse strain	. Poor specificity . Needs repetitive injections	(11, 16, 17)
	Vinblastine	Tubulin	. Rapid and efficient . Depletion can be performed in any mouse strain	. Poor specificity . Needs repetitive injections	(26, 27)
Depleting antibodies	RB6-8C5	Gr-1 (Ly6G; Ly6C)	. Rapid and efficient . Depletion can be performed in most mouse strains (exceptions include Ly6G$^{-/-}$ Catchup mice)	. Also affects other Gr-1 expressing cell types (monocytes, eosinophils, memory-type CD8^{+} T cells) . Repetitive injections needed for long-term depletion	(32, 34)
	1A8	Ly6G	. High specificity . Depletion can be performed in most mouse strains (exceptions include Ly6G$^{-/-}$ Catchup mice)	. Less efficient than anti-Gr-1 . Requires high dose of antibodies . Repetitive injections needed for long-term depletion	(35, 55, 56)
	NIMP-R14	Ly6G	. Rapid and efficient . Depletion can be performed in most mouse strains (exceptions include Ly6G$^{-/-}$ Catchup mice)	. Also affects Ly6C^{hi} monocytes . Repetitive injections needed for long-term depletion	(68, 69)

depletion in the blood, bone marrow and spleen 24 h after a single intraperitoneal injection of 500 ng DT (6, 20). These results were confirmed by another group, which showed that subcutaneous injection of DT (10 ng/g mouse body weight) for three consecutive days results in depletion of about 90% of neutrophils in the blood and lungs (100). DT-injection did not affect the counts of most of the leukocyte populations tested, such as blood and spleen eosinophils, basophils, B, and T cells (6). The same results are obtained with peritoneal lavage fluid macrophages, mast cells, dendritic cells, B and T cells, and bone marrow eosinophils, basophils, B cells and GMPs (6). Neutrophils started to re-appear in the blood 2 days after DT treatment, reaching normal levels at day 3 (6). This highlights the fact that repetitive DT injections are required for long-term neutrophil depletion using PMN^{DTR} mice. Importantly, neutrophil depletion in PMN^{DTR} mice was also accompanied by a reduction in blood Ly6C^{low} and Ly6C^{high} monocytes, and in spleen Ly6C^{high} monocytes (6), confirming that some hMRP8-driven expression occurs in the monocyte lineage. This will have to be taken into account when interpreting results obtained using PMN^{DTR} mice.

One major advantage of this model is the possibility to perform the adoptive transfer of purified WT or mutant neutrophils into DT-treated PMN^{DTR} mice. This is achievable due to the fact that non-DTR expressing neutrophils will not be depleted by the DT treatment. For instance, DT-treated PMN^{DTR} mice were shown to be more sensitive to LPS-induced endotoxemia. Whereas, engraftment of these mice with WT neutrophils restored their resistance (confirming the protective role of neutrophils in this setting), this was not the case when transferred neutrophils were MPO-deficient, suggesting that neutrophil-derived MPO is necessary for the resistance against LPS (6). Bowers et al. transplanted bone marrow cells from control (DTR^{-}) or PMN^{DTR} mice into recipient irradiated mice. One week later, mice were treated daily with DT for 7 days (i.p.), which resulted in a complete depletion of the granulocyte population (identified as Gr1^{+}CD115^{-} cells) in mice transplanted with bone marrow cells from PMN^{DTR} mice but not from DTR^{-} mice (101).

Besides using the hMRP8 promoter to control Cre-recombinase expression, the latter was also inserted in the neutrophil-specific ly6G locus (77). These mice were recently crossed with iDTR mice as a novel model for neutrophil depletion (57). Unfortunately, upon DT injection, no drop in blood neutrophil counts was reported (57).

MOUSE MODELS WITH CONSTITUTIVE NEUTROPENIA

The rapid turnover of neutrophils and the capacity of the bone marrow to quickly increase neutrophil output in

response to inflammatory signals in a process called emergency granulopoiesis (102) limits the use of inducible neutrophil depletion strategies for the study of long-term and inflammatory processes. Indeed, this would require repeated injections of DT or neutrophil-depleting antibodies. For instance, this could lead to the formation of anti-DT antibodies, lowering the efficiency of the depletion, as reported in CD11c-DTR mice (103). Several mouse models with constitutive neutropenia have been described and could theoretically circumvent these issues. However, constitutive neutropenia renders mice more susceptible to infections, and each of the constitutive neutropenic models presents with its own limitations that will be discussed below.

G-CSFR$^{-/-}$ Mice

A first mouse model with constitutive neutropenia was generated and described by Liu et al. (104). It is caused by the deletion of the Granulocyte Colony-Stimulating Factor Receptor (G-CSFR) gene. G-CSFR$^{-/-}$ mice exhibit 80 and 50% decreased counts of blood and bone marrow neutrophils, respectively, and residual bone marrow neutrophils are more prone to apoptosis (104). This model was used to show the pro-inflammatory role of neutrophils in collagen-induced and antibody-induced arthritis (105, 106), leading to the proposal of G-CSFR as a new target in human rheumatoid arthritis (106). Additionally, an immunosuppressive function of neutrophils was put forward using this model, characterized by the neutrophil-dependent regulation of antigen-presentation by dendritic cells *in vivo* (107). Recently G-CSFR$^{-/-}$ mice were shown to exhibit abnormally high responses in distal lymph nodes upon immunization, suggesting that neutrophils play a role in the restriction of the immune response to draining lymph nodes after immunization (108). Overall, however, G-CSFR$^{-/-}$ mice are rarely used, probably due to the already mentioned residual neutrophils (7, 104) and the anticipated side effects caused by G-CSFR deficiency. Indeed, G-CSFR plays a major role in endothelial cell regulation (109), in the induction of migration and proliferation (110), in bone regeneration through osteoblast regulation (111) and in sympathetic nerve neurons signaling (112). Although an effect of G-CSFR deletion on these processes has not been extensively described in the literature, G-CSFR$^{-/-}$mice likely exhibit several abnormalities that extend beyond their neutropenia.

Cxcr2$^{-/-}$ Mice

Neutrophil release from the bone marrow is tightly regulated by CXC chemokine receptors, such as CXCR2 and CXCR4. Whereas, neutrophils are retained in the bone marrow through constitutive expression of CXCL12 by bone marrow stromal cells acting on CXCR4, CXCR2 stimulation by Glu-Leu-Arg (ELR)$^{+}$ chemokines induces the mobilization of CXCR2^{+} neutrophils (113). Comprehensively, Cxcr2$^{-/-}$ mice (114) display a partial neutropenia with 60% decreased neutrophil counts in the blood compared to WT mice (113). Interestingly, spleen neutrophil counts are not affected by the deletion, whereas mature neutrophils in the bone marrow are twice more numerous in Cxcr2$^{-/-}$ mice than in WT mice (113). This accumulation is accompanied by a lack of mobilization of Cxcr2$^{-/-}$ neutrophils to the blood upon G-CSF treatment (113). Opposing the action of CXCR2, Cxcr4$^{-/-}$ mice and WT mice treated with the CXCR4 blocking agent AMD3100 exhibit elevated blood neutrophil counts (113, 115). These two models can thus be used in studies requiring mice presenting with neutrophilia in the blood, whereas Cxcr2$^{-/-}$ mice can be used as a model for mild neutropenia. A reduced neutrophil recruitment in Cxcr2$^{-/-}$ mice was for instance described in contexts of Dextran Sodium Sulfate-colitis-induced acute kidney injury (116), *Toxoplasma gondii* infection (117), autoantibody-mediated arthritis (118), or posttraumatic neutroinflammation (119). Nevertheless, this model is rarely used. This is likely due to the relatively high number of residual neutrophils found in Cxcr2$^{-/-}$ mice. Furthermore, CXCR2 is also expressed by monocytes/macrophages, mast cells, endothelial and epithelial cells (120–122), and the CXCR2 deficiency might thus also modify the biology of these cell populations. For instance, Cxcr2$^{-/-}$ mice were shown to exhibit exaggerated inflammatory response to cutaneous and peritoneal inflammatory stimuli (123). Using anti-Ly6G antibodies to deplete neutrophils, the authors suggested that the inflammatory phenotype observed in Cxcr2$^{-/-}$ mice might be neutrophil-independent, but depends on accumulation of a non-neutrophilic CXCR2 positive leukocyte population (123).

Gfi-1-Deficient Mice

In 2002, a new mouse model was generated, in which the Gfi-1 (Growth factor independence-1) gene was inactivated through the removal of the exons 2 to 4 and partial removal of exon 5 (124). Gfi-1 had been described 9 years earlier, as encoding a protein that, if activated by a promoter insertion of proviruses could abolish the dependency of thymoma cells toward IL-2 (125). Through the analyses of the Gfi-1 gene sequence, it was predicted that the encoded protein could bind DNA through the engagement of zinc finger domains, and suggested that this interaction could lead to a down-regulation of the transcription of specific genes (125), as verified 3 years later (126). Before the generation of Gfi-1$^{-/-}$ mice, Gfi-1 was therefore mainly investigated for its role in T cell differentiation and activation as well as in lymphomagenesis (125–128), thus restricting its spectrum of action to lymphoid cells.

However, following a more careful analysis of the Gfi-1 expression, it was first detected in the bone marrow (129) before it was demonstrated that its expression was not at all restricted to the lymphoid compartment. Indeed, granulocytes and LPS-activated macrophages also express the Gfi-1 gene (124).

One year after the first description of a Gfi-1$^{-/-}$ mouse (124), a second strain was generated, in which the exons 1, 2, and a part of 3 were deleted, resulting again in mice unable to express Gfi-1 (130). Not surprisingly, both Gfi-1$^{-/-}$ models develop a similar phenotype, with high mortality rates, reduced growth and decreased cell numbers in the thymus (124, 130). One of the most striking features of Gfi-1$^{-/-}$ mice is the quasi absence of mature neutrophil numbers in the blood, spleen and bone marrow (124, 130), highlighting the role of this gene in the differentiation and/or survival of neutrophils. Furthermore, neutropenia is accompanied by the appearance of an abnormal cell population, described as "blastoid monocytic cells" or "atypical myeloid

cells." This population exhibits specific characteristics of the granulocyte lineage as well as of the macrophage lineage (130), such as ring-shaped nuclei and low expression levels of Gr-1 and Mac-1 together with high expression of Mac-3 and M-CSFR (124, 130). Interestingly, these cells are still capable of phagocytosis and oxidative burst (124, 130). Altogether, these characteristics indicate that the atypical myeloid cells, which develop in Gfi-$1^{-/-}$ mice, share features of immature neutrophils and macrophage precursors (130).

Adding to the two Gfi-$1^{-/-}$ mouse strains, another model of Gfi-1 deficiency was developed using the Cre-Lox system (131). Exons 4 and 5 of the Gfi-1 gene were flanked by two *LoxP* sites and Gfi-$1^{fl/fl}$ mice were crossed with EIIa-Cre transgenic mice, to induce a constitutive deletion of Gfi-1 from the early embryo stage (132). Consistently with the two previous models, these Gfi-$1^{-/-}$ mice exhibit abnormal features such as small body size and a high percentage of atypical myeloid cells (131). Using the CD4-promoter, this model enabled the restriction of the mutation to CD4-T cells, and thus, the assessment of the specific function of Gfi-1 in T-cells (131, 133).

Finally, a knock-in mouse model was generated in which the Gfi-1 gene was replaced by the gene encoding GFP (which they called Gfi-$1^{GFP/GFP}$ mice) (134). This strain enabled the study of the expression and roles of Gfi-1 during T and B-cell differentiation and activation. Overall, Gfi-$1^{GFP/GFP}$ mice display the same phenotype as Gfi-$1^{-/-}$, including reduced growth and profound neutropenia (134, 135).

The constitutive neutropenia in Gfi-1-deficient mice can, to a certain extent and for a short period of time, be compensated by the adoptive transfer of neutrophils or their precursors. This approach has been used to study the role of neutrophils in the K/BxN model of antibody-mediated arthritis (136). Gfi-$1^{-/-}$ mice were resistant to the development of arthritis in this model. Arthritis could, however, be restored, following sub-lethal irradiation and engraftment with WT bone marrow cells (a process which resulted in the production of mature neutrophils within 2 weeks) before the transfer of K/BxN serum. By contrast, arthritis was not restored upon adoptive transfer of bone marrow cells from C5aR-, CD11a-, or FcRγ-deficient mice, suggesting that expression of these molecules on neutrophils is required for the development of arthritis in this model (136).

Although Gfi-1-deficient mice are useful tools to assess the role of neutrophils *in vivo*, many additional phenotypical abnormalities due to the lack of Gfi-1 expression have to be taken into account when interpreting findings obtained with these mice. First, disrupting neutrophil differentiation leads to the appearance of an abnormal myeloid cell population, which is still capable of inducing inflammation and can act as effector cells (124, 130). Moreover, Gfi-$1^{-/-}$ mice present with thymus aplasia (124, 130, 134), abnormal differentiation of dendritic cells (137), iNKT cells (138) and T-cells (124, 130, 134), aberrant production of IL-2, TNF, IL-10, and IL-1b (124, 133), development of eye inflammation and the presence of abscesses (124, 130). Gfi-1 was later shown to be also expressed in developing inner ear, retina, brain and dorsal root ganglia (139, 140). In accordance with these reports, Gfi-1 deficiency results in a complete loss of hair cells by apoptosis before birth as well as in a severe loss of spiral ganglion neurons that is visible 5 months after birth (140). This causes behavior defects as Gfi-1 mutant mice are ataxic, do not respond to noise, circle, and develop head tilt (140). Most importantly, Gfi-1 mutant mice exhibit delayed growth and higher mortality; these features are undoubtedly a limiting factor for long-term studies (124, 130). They are significantly restored when Gfi-$1^{-/-}$ mice are kept in SPF conditions (141). Along this line, the generation of Gfi-$1^{fl/fl}$ mice (133) provides a great tool for the restriction of the mutation to neutrophils and limitation of negative side effects. It was, however, reported that 10–20% of Gfi-$1^{fl/fl}$ mice exhibited features of Gfi-$1^{-/-}$ mice, such as reduced body size, even though no Cre was expressed (133).

Genista Mice

An additional mouse strain was generated and was named "*Genista*" mice. In these mice, Gfi-1 activity is impaired due to a point mutation in one of the zinc finger domains following N-ethyl-N-nitrosourea-induced random mutagenesis (142, 143). The C318Y point mutation in the third zinc finger domain in the Gfi-1 gene does not affect Gfi-1 expression but is likely to affect the interaction of Gfi-1 with DNA. Strikingly, it leads to the generation of highly neutropenic mice but did not result in delayed growth or increased mortality, even in conventional non-SPF conditions (142, 143). Furthermore, although the authors reported reduced cell numbers in the thymus of *Genista* mice, and reduced B cell precursors, these mice exhibited normal T and B cell differentiation and function (142). Thus, this model of neutropenia exhibits less phenotypic abnormalities than Gfi-$1^{-/-}$ mice (142, 143).

Nevertheless, NK cells from *Genista* mice have been shown to exhibit impaired responsiveness *in vivo* and *in vitro* (143). Indeed, neutrophils are thought to be central actors for the differentiation and activation of NK cells (143, 144) and their absence leads to impaired maturation and function of NK cells (143). Furthermore, it has also been shown that in *Genista* mice, interfering with neutrophil differentiation leads to the appearance of abnormal $CD11b^{+}$/Ly-$6G^{int}$ myeloid cells. Interestingly, this population appears to be arrested right after the metamyelocytic stage (142) and thus, in a more mature stage than in Gfi-$1^{-/-}$ mice (124, 130). Mature neutrophils are thought to be necessary for autoantibody-induced arthritis (136, 145), and immune complex-mediated alveolitis (146). Surprisingly, and different to results obtained in Gfi-$1^{-/-}$ mice, *Genista* mice still developed significant inflammation in these two models, albeit at reduced levels when compared to WT mice (142). This suggests that residual $CD11b^{+}$/Ly-$6G^{int}$ cells even though not fully mature are capable of sustaining inflammatory processes, and thus, question the use of *Genista* mice for *in vivo* studies of neutrophil functions. Intriguingly, however, *Genista* mice failed to provide resistance to acute bacterial infection (142), implying that neutrophil features acquired after the metamyelocytic stage are required for these effector functions. Consecutively, *Genista* mice have been used to study neutrophil functions in different models, for example in host defense against certain pathogens (67, 147, 148).

LysM-Cre Mcl-$1^{fl/fl}$

Myeloid cell leukemia-1 (Mcl-1) belongs to the anti-apoptotic Bcl-2 family. It is expressed by neutrophils and plays a role

in delaying apoptosis, especially when neutrophils are activated (149, 150). Neutrophils rely on Mcl-1 for survival (151), as it represents the only member of the Bcl-2 family expressed by neutrophils (149). Using the Mcl-$1^{fl/fl}$ mice expressing Cre under the LysM promoter (152), Dzhagalov et al. deleted Mcl-1 in neutrophils and macrophages (153). LysM-Cre Mcl-$1^{fl/fl}$ mice exhibited a 3-fold decrease in blood neutrophils and a high percentage of apoptotic neutrophils as compared to WT mice, whereas the phenotype of macrophages did not seem to be affected (153). Residual neutrophils still expressed Mcl-1 and thus, had escaped Cre-mediated gene deletion, which represents a potential limitation for the use of LysM-Cre Mcl-$1^{fl/fl}$ mice (153).

A few years later, $LysM^{Cre/Cre}$ Mcl-$1^{fl/fl}$ mice that are homozygous for the Cre gene were described (7), as opposed to the previously described LysM-Cre Mcl-$1^{fl/fl}$ mice (153). This bi-allelic expression of the Cre-recombinase resulted in severely neutropenic mice with a reduction of over 93% in the spleen and bone marrow, and a 98% reduction of circulating neutrophils in homeostatic conditions (7), and which remained neutropenic even upon thioglycolate-induced peritonitis (7). None of the other cell populations assessed—circulating monocytes, eosinophils, B and T cells, bone marrow dendritic cells and macrophages, splenic dendritic cells, T and B cells—seems to be affected by this mutation, with the exception of a decrease in bone marrow B cells and an increase in splenic macrophages (7). $LysM^{Cre/Cre}$ Mcl-$1^{fl/fl}$ mice can breed as homozygous but display higher mortality rates and reduced offspring numbers in SPF and non-SPF conditions (7). $LysM^{Cre/Cre}$ Mcl-$1^{fl/fl}$ mice were used to emphasize the fact that neutrophils are necessary for the sensitization and elicitation phases of 2,4,6-trinitrochlorobenzene-induced contact hypersensitivity (154), and were, as expected, protected against K/BxN serum-transfer arthritis and anti-CVII antibody–induced dermatitis, while displaying an increased susceptibility to infection with *S. aureus* and *C. albicans* (7). When interpreting results obtained with these mice, one should bear in mind that these mice are also knockout for Lysozyme M in all cells.

MRP8-Cre Mcl-$1^{fl/fl}$

The same authors also crossed Mcl-$1^{fl/fl}$ mice with mice expressing Cre under the control hMRP8 promoter (7). Indeed, MRP8-Cre Mcl-$1^{fl/fl}$ mice showed severe neutropenia (with more than 99% reduction in circulating neutrophils as compared to WT mice, whereas blood monocytes, eosinophils, T and B cells were not affected by the mutation (7). Unfortunately, this severe neutropenia was accompanied by high mortality (only 30% survival at 1 year of age) and poor breeding productivity (7), hampering the use of these mice for *in vivo* studies.

Foxo3a:Foxo3a-Deficient Mice

The forkhead class O (FOXO) subfamily of transcription factors are key in regulating the apoptosis, proliferation and control of oxidative stress in immune cells (155). Foxo3a is the main FOXO member expressed in lymphoid organs, and Foxo3a$^{-/-}$ mice (156) were shown to exhibit exaggerated lymphoproliferation and inflammatory reactions in various organs (156). This phenotype was accompanied with the presence of over-activated T helper cells releasing high amounts of Th1 and Th2 cytokines (156). It was further shown that Foxo3a was an inhibitor of the NF-κB pathway, and that its absence unleashed pro-inflammatory gene signature in Foxo3a$^{-/-}$ mice (156). With respect to its action in neutrophils, it was shown that Foxo3a$^{-/-}$ mice were resistant to neutrophil-dependent inflammatory reactions, such as immune complex-mediated arthritis, or thioglycollate-induced peritonitis (157). Indeed, Foxo3a-deficient neutrophils were more susceptible to Fas ligand-induced apoptosis (157) which engendered an incapacity of neutrophils to persist in inflamed tissues and subsequently a neutropenia (157). This suggests Foxo3a$^{-/-}$ mice could be used as a model to assess the function of neutrophils during inflammation, although the inflammatory state of these mice as well as the high numbers of apoptotic neutrophils could be limitations to the use of these mice.

The characteristics of genetic models of inducible and constitutive neutropenia are presented in **Table 2**.

KNOCKOUT OF KEY NEUTROPHIL MEDIATORS

Neutrophils exert a multitude of mechanisms of action that are based on different key enzymes. Knockout models of these enzymes have been developed in the last decades and allowed the study of their precise roles. The main models are summarized in **Table 3**, as well as their limitations that need to be kept in mind when using these mice.

First, one has to bear in mind the abnormalities that can be induced by the knockout itself. For instance, knockout of the iron-binding protein lactoferrin (an enzyme produced by neutrophils and which has anti-microbial as well as anti-inflammatory properties) leads to a 6% decrease in circulating lymphocyte counts, but to an increase in circulating neutrophil and eosinophil numbers (of respectively 46 and 89%) (177). This could lead to phenotypic particularities that are not directly linked to a role of lactoferrin.

Neutrophil enzymes are often closely related and can exhibit redundant roles, which can limit the use of mice deficient for only one of these enzymes. For instance, neutrophil elastase (NE), cathepsin G and proteinase 3 are 3 serine proteases (180). Double mutants have therefore recently been developed to demonstrate the roles of NE and cathepsin G in the endotoxic shock cascade (181) and in lung defense against *S. pneumoniae* (182), and of NE and proteinase 3 in immune complex-induced neutrophil activation (183).

In addition, neutrophil enzymes are generally not restricted to neutrophils but are also expressed by other cell populations. This suggests that the exclusive use of enzyme-knockout models might lead to an over-estimation of the roles of neutrophils. For instance, MPO is also expressed by monocytes, macrophages and peritoneal B lymphocytes (184); and Cathepsin G by splenic dendritic cells and microglia (185). Furthermore, in addition to its role in NETosis, protein arginine deiminase 4 (PAD4) is implicated in macrophage extracellular trap release (186). To circumvent these difficulties, a conditional model of PAD4

TABLE 2 | Characteristics of genetic models of inducible and constitutive neutropenia.

	Mice	Neutrophil numbers	Advantages	Potential limitations	References
Inducible	PMN^{DTR} (hMRP8-Cre $iDTR^{fl}$)	. Marked reduction (>90%) in blood, spleen, bone marrow and lung neutrophils upon DT injection	. Normal number of neutrophils until injection of DT . Neutrophil population can be restored in DT-treated PMN^{DTR} mice upon adoptive transfer of DTR^{-} neutrophils	. DT injection reduces levels of blood and spleen monocytes . Depletion is transient and needs repetitive injections for long-term experiments . Possible off-target or other side effects of repeated treatment with DT	(6)
Constitutive	$CXCR2^{-/-}$	. Reduction of 60% of blood neutrophils . No reduction of spleen neutrophils and higher counts of bone marrow mature neutrophils	. Neutrophil population can be restored upon adoptive transfer of WT neutrophils . Depletion effective from birth and does not require any treatment	. High numbers of residual neutrophils . Also expressed by other cell populations (such as monocytes/macrophages, mast cells, endothelial and epithelial cells) . Side effects caused by CXCR2 deficiency such as neutrophil-independent exaggeration of inflammatory reactions	(113)
	$G\text{-}CSFR^{-/-}$	. Reduction of 80 and 50% of blood and bone marrow neutrophils	. Neutrophil population can be restored upon adoptive transfer of WT neutrophils . Depletion effective from birth and does not require any treatment	. High numbers of residual neutrophils . Side effects caused by G-CSFR deficiency . Potential appearance of compensatory mechanisms due to constitutive neutrophil deficiency	(104)
	$Gfi\text{-}1^{-/-}$	. Quasi absence of mature neutrophil numbers in the blood, spleen and bone marrow	. Neutrophil population can be restored upon adoptive transfer of WT neutrophils . Depletion effective from birth and does not require any treatment	. Emergence of an abnormal myeloid cell population that can induce inflammation reactions . Thymus aplasia . Abnormal differentiation of immune cell populations (such as dendritic cells, iNKT cells and T-cells) . Potential appearance of compensatory mechanisms due to constitutive neutrophil deficiency . Aberrant production of cytokines . Manifestation of eye inflammation and abscesses . Behavior abnormalities (ataxia, no response to noise and head tilt) . Delayed growth and high mortality	(124, 130)
	Ella-Cre $Gfi\text{-}1^{fl/fl}$	. NA	. Neutrophil population can be restored upon adoptive transfer of WT neutrophils . Depletion effective from birth and does not require any treatment . $Gfi\text{-}1^{fl/fl}$ mice could be used to restrict the mutation to neutrophils	. Emergence of an abnormal myeloid cell population that can induce inflammation reactions . Delayed growth and high mortality of Ella-Cre^{+}; $Gfi\text{-}1^{fl/fl}$ mice . 10–20% of $Gfi\text{-}1^{fl/fl}$ mice exhibited features of $Gfi\text{-}1^{-/-}$ mice, such as reduced body size, even with no Cre expression . Thymus aplasia . Potential appearance of compensatory mechanisms due to constitutive neutrophil deficiency	(131, 133)
	$Gfi\text{-}1^{GFP/GFP}$	. Quasi absence of mature neutrophil numbers in the blood and bone marrow	. Neutrophil population can be restored upon adoptive transfer of WT neutrophils . Depletion effective from birth and does not require any treatment . Gfi-1 expressing cells are GFP^{+} and allow the study of Gfi-1 expressing cells	. Emergence of an abnormal myeloid cell population that is capable of inducing inflammation reactions . Thymus aplasia . Abnormal differentiation of immune cell populations (such as T-cells) . Potential appearance of compensatory mechanisms due to constitutive neutrophil deficiency . Delayed growth and high mortality	(134, 135)

(Continued)

TABLE 2 | Continued

Mice	Neutrophil numbers	Advantages	Potential limitations	References
Genista (Gfi-1^{C318Y})	▪ Marked reduction (around 90%) in blood neutrophils	▪ Normal growth and mortality, even in conventional non-SPF conditions ▪ Normal T and B cell differentiation and function ▪ Neutrophil population can be restored upon adoptive transfer of WT neutrophils ▪ Depletion effective from birth and does not require any treatment	▪ Impaired responsiveness of NK cells ▪ Emergence of abnormal CD11b^{+}/Ly-6Gint myeloid cells capable of sustaining inflammatory processes ▪ Thymus aplasia ▪ Potential appearance of compensatory mechanisms due to constitutive neutrophil deficiency	(142, 143)
LysM-Cre Mcl-1$^{fl/fl}$	▪ 3-fold decrease in circulating neutrophils ▪ High percentage of apoptotic neutrophils	▪ Neutrophil population can be restored upon adoptive transfer of WT neutrophils ▪ Depletion effective from birth and does not require any treatment	▪ High numbers of residual neutrophils escaping deletion ▪ Knock-out of Lysozyme M ▪ Appearance of compensatory mechanisms due to constitutive neutrophil deficiency	(153)
LysM$^{Cre/Cre-}$ Mcl-1$^{fl/fl}$	▪ Marked reduction of neutrophil counts in the blood (98%), spleen and bone marrow (>93%).	▪ Most of the other immune cell populations seem unaffected by the mutation ▪ Neutrophil population can be restored upon adoptive transfer of WT neutrophils ▪ Depletion effective from birth and does not require any treatment	▪ Decrease in bone marrow B cells and increase in splenic macrophages ▪ Higher mortality rate and reduced offspring numbers in SPF and non-SPF conditions ▪ Knock-out of Lysozyme M ▪ Potential appearance of compensatory mechanisms due to constitutive neutrophil deficiency	(7)
hMRP8-Cre Mcl-1$^{fl/fl}$	▪ Marked reduction (>99%) in blood neutrophil counts	▪ Other immune cell populations seem unaffected by the mutation ▪ Neutrophil population can be restored upon adoptive transfer of WT neutrophils ▪ Depletion effective from birth and does not require any treatment	▪ High mortality (only 30% survival at 1 year of age) ▪ Low breeding productivity ▪ Appearance of compensatory mechanisms due to constitutive neutrophil deficiency	(7)
Foxo3a $^{-/-}$	▪ 50% reduction of circulating neutrophils	▪ Neutrophil population can be restored upon adoptive transfer of WT neutrophils ▪ Depletion effective from birth and does not require any treatment	▪ High numbers of apoptotic neutrophils ▪ Exaggerated lymphoproliferation and more prone to inflammation reactions ▪ Overactivated T-helper cells	(157)

knockout has been developed by flanking the PAD4 gene with *loxP* sequences (167). Using the neutrophil-specific promoter MRP8 (95), MRP8-Cre PAD4$^{fl/fl}$ have been generated to restrict PAD4 deletion to neutrophils, and to demonstrate that NETs can facilitate the formation of premetastatic niches in a context of ovarian cancer (187). This approach could in principle be adapted to all neutrophil key enzymes for the generation of neutrophil-restricted knockouts, to facilitate the analysis of the real contribution of neutrophils *in vivo*.

Finally, it is noteworthy that a new mouse model has recently been generated in which the caspase recruitment domain-containing protein 9 (CARD9) is selectively knockout in neutrophils (Mrp8-Cre^{+} *Card9*$^{flox/flox}$) (188). Mice carrying this mutation do not develop autoimmune syndromes such as autoantibody-induced arthritis or skin blistering (188), due to a lack of chemokine/cytokine release from CARD9-defient neutrophils through Src-family kinases, Syk, PLCγ2, Bcl10/Malt1, and NFκB (188). This model provides therefore a possibility to investigate the functions of neutrophil-released chemokine/cytokine in diverse physiological and pathological settings.

IN VIVO TRACKING OF NEUTROPHILS

Tracking neutrophils temporally and spatially, or assessing their activity can be informative when studying these cells *in vivo*. Indeed, it gives clear evidence of their dynamics, and it may help to dissect the cellular and molecular pathways implicated in their release from the bone marrow as well as in their recruitment to sites of inflammation.

Assays Using *in vitro* Imaging System (IVIS)

Luminol is a chemical molecule that can directly be oxidized by HOCl produced by myeloperoxidase (MPO), or by MPO using other reactive oxygen species (ROS) (189). Oxidized luminol emits photons and can therefore be used as a mean to reveal the presence of active MPO (190). Using a *in vivo* imaging system (IVIS), it has been shown that systemic treatment of mice with luminol lead to the formation of a bioluminescent signal in tissues infiltrated by activated neutrophils in contexts of acute dermatitis, and arthritis (190, 191). No signal was generated in inflamed tissues of MPO$^{-/-}$ mice, even if these mice showed the infiltration of activated eosinophils (190). This

TABLE 3 | Characteristics of models of knockouts of neutrophil key enzymes.

Mutant	Gene name	Phenotype or limitations	References	Pharmacological inhibitor
MPO$^{-/-}$	Myeloperoxidase	. Increased ROS production by neutrophils . Impaired PMA-induced NETosis . Normal neutrophil counts in diverse tissues tested . Expressed by other cell types (such as monocytes, macrophages, peritoneal B lymphocytes)	(158–162)	4-Aminobenzoichydrazide
NE$^{-/-}$	Elane	. Impaired neutrophil functions (NETosis, pro-inflammatory cytokines release, phagocytosis and transmigration) . Normal circulating leukocyte counts	(159, 163–165)	Sivelestat
Cathepsin G$^{-/-}$	Cathepsin G	. Decreased production of mast cell protease-2. . Normal hematopoietic population counts in diverse tissues tested . Expressed by other cell types (such as splenic dendritic cells and microglia).	(166)	β-keto-phosphonic acid
PAD4$^{-/-}$	Protein arginine deiminase 4	. Increased bone marrow hematopoietic multipotent progenitor numbers . Normal neutrophil morphology and differentiation . Normal circulating neutrophil counts . Expressed by other cell types (such as circulating eosinophils and monocytes) . Impaired NETosis	(167–170)	Cl-Amidine
Ncf1$^{*/*}$	Neutrophil cytosolic factor 1	. Development of spontaneous chronic severe arthritis postpartum . Impaired ROS production by neutrophils and macrophages . Disrupted T cell functions . Impaired NETosis	(171–175)	
Proteinase 3$^{-/-}$	Proteinase 3	. Increased bone marrow hematopoietic stem progenitor numbers	(176)	
Lactoferrin$^{-/-}$	Lactoferrin	. Normal neutrophil morphology and differentiation . Normal neutrophil functions (phagocytosis, granule mobilization) although defects in PMA-induced oxidative burst . Decrease in circulating lymphocyte counts but increase in circulating neutrophil and eosinophil numbers . Also expressed in monocytes, macrophages and subpopulations of dendritic cells	(177–179)	

suggests that eosinophil peroxidase failed to induce a luminescent signal and further shows that luminol-induced signal is specific to MPO. Luminol has extensively been used as a mean to demonstrate the occurrence of inflammation reactions as well as the infiltration of neutrophils in tissues, for instance during anaphylaxis reaction (191), tumorigenesis (192) or acute and chronic intestinal inflammation (193). One caveat of luminol-based assays is the fact that neutrophils are not the exclusive producers of MPO, since this enzyme is also produced by monocytes/macrophages (184).

Other fluorescent probes have been developed following the same model, such as the Neutrophil Elastase 680 FAST imaging agent, allowing the imaging of NE activity in mice (194), as shown in a context of occurrence of colorectal cancer (195). Finally, probes binding specifically to neutrophil membrane have been generated, such as the cFIFIF-PEG-Cy7 that interacts with neutrophil formyl peptide receptor (196). This probe was for example validated in a context of ear inflammation (196).

Neutrophil Tracking Using Intravital Microscopy

The development of imaging technologies such as intravital two- and multi-photon microscopy gave rise to the development of new strategies to track neutrophils *in vivo*. Indeed, these technologies allow the live imaging of neutrophils deep in the tissues.

First, *in vivo* tracking of neutrophils can be performed using adoptive transfers of pre-stained neutrophils. This can be performed by isolating neutrophils from the bone marrow of donor mice, and staining these cells with a fluorescent dye such as CarboxyFluorescein Succinimidyl Ester [CFSE, that is cell permeable and binds to covalently to intracellular proteins (197)]

prior to transfer of the cells into recipient mice (198). Although this requires a heavy experimental procedure, it allows the use of mutant donor mice to target the molecular pathways implicated in the process of interest. This strategy was for instance employed to show that expression of the receptor IL-1R on the surface of neutrophils does not play a major role in the recruitment of these cells in inflamed tissues in a context of *Staphyloccocus aureus* infection (198).

Furthermore, labeled antibodies can be systemically injected to stain neutrophils *in vivo*. Indeed, It has been shown that low doses of anti-Ly6G antibodies do not interfere with neutrophil biology (76). No difference was thereby shown in the lung trafficking of red blood cells and neutrophils in a mouse model of Sickle cell disease when compared with WT mice (199).

To circumvent the need to treat mice with antibodies, it is possible to use or generate new strains of mice expressing reporter genes in neutrophils. For instance, LysM-EGFP mice were generated by inserting the *GFP* gene into the lysozyme M locus (152). In these mice, GFP^+ cells are mainly neutrophils and monocytes/macrophages (152). LysM-EGFP mice were used to track neutrophils *in vivo* using two-photon intravital microscopy to show for instance that neutrophils do not directly interact with mycobacteria in the liver of infected mice (200). LysM promoter is also active in monocytes and macrophages (152) neurons (201) and alveolar type II cells in the lungs (202). This lack of cell specificity could thus potentially limit the use of LysM-EGFP mice for the imaging of neutrophils. One strategy to bypass this has been to deplete monocytes and macrophages by treating LysM-EGFP mice with clodronate liposomes to visualize the recruitment of neutrophils in atherosclerotic lesions (203). Nevertheless, the side effects of the clodronate liposome treatment need to be taken into account when analyzing the data. Another strategy could be to target a more selective promoter, such as the neutrophil-related protein Ly6G (30).

Catchup mice were generated by replacing the first exon of *Ly6g* by the "*Cre-tom*" sequence corresponding to the sequences of the Cre recombinase and the fluorescent molecule tdTomato (77). Homozygous mice exhibit endogeneous staining of neutrophils in red, but are *Ly6g* deficient. To increase the levels of the tdTomato reporter gene expression and to restore the expression of Ly6G, Catchup mice were crossed with mice expressing the fluorescent molecule tdTomato under control of the Cre-dependent promoter in the ubiquitous *ROSA26* locus (204). These *Ly6g*(Cre-tom; wt) *ROSA26*(tom; wt) mice were called $Catchup^{IVM-red}$ mice. These mice express high levels of tdTomato in 80–90% of bone marrow, blood and spleen neutrophils, while red fluorescence appears to be restricted to neutrophils. Nevertheless, it is to note that $Catchup^{IVM-red}$ neutrophils express slightly decreased levels of Ly6G when compared to WT neutrophils (77). Neutrophils from $Catchup^{IVM-red}$ mice function normally *in vitro* and *in vivo*, they exhibit the same morphology as WT neutrophils and are easy to track, being endogenously stained in red. Using intravital two-photon microscopy, it was possible to show that neutrophils from $Catchup^{IVM-red}$ mice lacking FcγRIV exhibit impaired recruitment to inflamed tissues in a context of experimental epidermolysis bullosa.

MRP8-Cre mice express the Cre recombinase followed by an IRES-GFP reporter gene. This allows detection of GFP at low levels in neutrophils by flow cytometry (6, 95). However, GFP expression is likely too low to permit detection of neutrophils by microscopy using MRP8-Cre mice. However, MRP8-Cre mice were crossed with mice expressing a Cre-inducible YFP gene under the control of the ROSA26 locus. This lead to high levels of YFP expression in $\sim$ 80% bone marrow, blood and spleen neutrophils (6, 97). However, YFP expression was also reported in 10–20% of some monocyte and macrophages populations in MRP8-Cre YFP^{fl} mice (97), which limits the use of these mice for imaging approaches.

IN VIVO CHARACTERIZATION OF NETs

NETosis is an innate immune mechanism that involves the release of DNA decorated with histones and granule enzymes upon neutrophil activation (205). Since their first description in 2004 (4), NETs have extensively been studied and their contribution to human diseases has been suggested in many different settings. These include atherosclerosis (206), cancer metastasis (61), or thrombosis and allergic conditions (207–209).

Despite their reported multiple involvements, characterizing NETs *in vivo* remains challenging. Neutrophils (and other cells) can release DNA through multiple processes, including necrosis, and it is often difficult to stain DNA in-depth within the tissues and combine this staining with appropriate markers to distinguish NETs from other forms of tissular DNA. Methods to study NETosis have been reviewed elsewhere (210) and will only be briefly discussed here.

The occurrence of NETosis *in vivo* is often shown using fluorescent dyes revealing the colocalization of neutrophils (211), extracellular DNA [using non-permeable DNA intercalating agents such as Sytox Green (212), Sytox Orange (213–215) or propidium iodide (PI) (213–215)], and NET-associated molecules [i.e., NE, MPO or citrullinated histones (213–215)]. Images are then acquired by use of intravital microscopy [two-photon (206, 211, 215), multiphoton (214), or spinning disk (212, 213)]. 3D reconstructions of NETting neutrophils can even be performed to assess the morphological changes happening during NETosis (213).

CONCLUDING REMARKS

The use of mouse models for the study of neutrophils *in vivo* represents a milestone in the understanding of the biology of these cells, and has enabled the deep interrogation of a number of pathways leading to diverse pathologies. For instance, DNase I, that digests the DNA backbone of NETs, has been approved as a therapeutic drug to treat Cystic Fibrosis after the discovery that NETs play a pivotal role in the disease (216). Whereas, early neutrophil depletion approaches often resulted in a more generalized deficiency of hematopoietic cells, recent advances in our understanding of neutrophil biology lead to the generation

of more specific ways of depleting neutrophils. Nevertheless, the selectivity, specificity and the side effects of each of the different strategies need to be carefully evaluated, before concluding on neutrophil functions. The use of at least two different models seems indeed to be recommended to undoubtedly define the roles of neutrophils. Furthermore, the use of mutant mice helps to decipher the molecular pathways of interest. The expression of neutrophil key enzymes is often shared with other cell populations and the Cre-lox system will at term allow the generation of a library of mice exhibiting a deficiency of key enzymes specifically in neutrophils.

Although visualization of neutrophil activity using IVIS is an easy tool to assess the contribution of neutrophils *in vivo*, recent advances in intravital microscopy allow more precise tracking of neutrophils *in vivo*. These imaging approaches permit the observation of the behavior of the neutrophils, their interactions with other cell populations, and may finally help to answer some debated aspects of neutrophil biology. However, the analysis of deep organs still remains challenging.

Today, *in vivo* tracking of NETosis clearly remains one of the most challenging tasks. While it is relatively straightforward to visualize neutrophils and extracellular DNA, this co-staining is not sufficient to provide convincing proof of NETs. Indeed evidence for the presence of histones or signature enzymes associated to the DNA is a minimum necessity. Further difficulties result from the fact that there is an ongoing debate on the definition of NETosis, and as a consequence NETs. Indeed many different signaling pathways have been put forward and proposed to result in either suicidal, nuclear NETosis or mitochondrial vital NETosis (217). Moreover, the observation of NETs in a given pathology does not necessarily mean that NETs play a role in such disease, and proving so can be very challenging.

In conclusion, there is now a wide range of tools for assessing the functions of neutrophils *in vivo*. The main issue is therefore the choice of the methods used. Therefore, one needs to consider each advantage and caveat of these methods. Furthermore, confirming the results using at least two different approaches seems necessary to avoid overestimating or underestimating the true contribution of neutrophils *in vivo*.

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JS, FJ, and LR wrote the review.

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REFERENCES

1. Capoccia BJ, Gregory AD, Link DC. Recruitment of the inflammatory subset of monocytes to sites of ischemia induces angiogenesis in a monocyte chemoattractant protein-1-dependent fashion. *J Leukoc Biol.* (2008) 84:760–8. doi: 10.1189/jlb.1107756
2. Rosales C, Uribe-Querol E. Phagocytosis: a fundamental process in immunity. *Biomed Res Int.* (2017) 2017:9042851. doi: 10.1155/2017/9042851
3. Kolaczkowska E, Kubes P. Neutrophil recruitment and function in health and inflammation. *Nat Rev Immunol.* (2013) 13:159–75. doi: 10.1038/nri3399
4. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, et al. Neutrophil extracellular traps kill bacteria. *Science.* (2004) 303:1532–5. doi: 10.1126/science.1092385
5. Von Kockritz-Blickwede M, Blodkamp S, Nizet V. Interaction of bacterial exotoxins with neutrophil extracellular traps: impact for the infected host. *Front Microbiol.* (2016) 7:402. doi: 10.3389/fmicb.2016.00402
6. Reber LL, Gillis CM, Starkl P, Jonsson F, Sibilano R, Marichal T, et al. Neutrophil myeloperoxidase diminishes the toxic effects and mortality induced by lipopolysaccharide. *J Exp Med.* (2017) 214:1249–58. doi: 10.1084/jem.20161238
7. Csepregi JZ, Orosz A, Zajta E, Kasa O, Nemeth T, Simon E, et al. Myeloid-specific deletion of Mcl-1 yields severely neutropenic mice that survive and breed in homozygous form. *J Immunol.* (2018) 201:3793–803. doi: 10.4049/jimmunol.1701803
8. Colvin OM. An overview of cyclophosphamide development and clinical applications. *Curr Pharm Des.* (1999) 5:555–60.
9. Hengstler JG, Hengst A, Fuchs J, Tanner B, Pohl J, Oesch F. Induction of DNA crosslinks and DNA strand lesions by cyclophosphamide after activation by cytochrome P450 2B1. *Mutat Res.* (1997) 373:215–23. doi: 10.1016/S0027-5107(96)00200-X
10. Hung DT, Jamison TF, Schreiber SL. Understanding and controlling the cell cycle with natural products. *Chem Biol.* (1996) 3:623–39. doi: 10.1016/S1074-5521(96)90129-5
11. Zuluaga AF, Salazar BE, Rodriguez CA, Zapata AX, Agudelo M, Vesga O. Neutropenia induced in outbred mice by a simplified low-dose cyclophosphamide regimen: characterization and applicability to diverse experimental models of infectious diseases. *BMC Infect Dis.* (2006) 6:55. doi: 10.1186/1471-2334-6-55
12. Manepalli S, Gandhi JA, Ekhar VV, Asplund MB, Coelho C, Martinez LR. Characterization of a cyclophosphamide-induced murine model of immunosuppression to study *Acinetobacter baumannii* pathogenesis. *J Med Microbiol.* (2013) 62:1747–54. doi: 10.1099/jmm.0.060004-0
13. Hellman S, Grate HE. Effect of cyclophosphamide on the murine hematopoietic stem cell compartment as measured by different assay techniques. *Blood.* (1971) 38:706–14. doi: 10.1182/blood.V38.6.706.706
14. Summers C, Rankin SM, Condliffe AM, Singh N, Peters AM, Chilvers ER. Neutrophil kinetics in health and disease. *Trends Immunol.* (2010) 31:318–24. doi: 10.1016/j.it.2010.05.006
15. Timar CI, Lorincz AM, Ligeti E. Changing world of neutrophils. *Pflugers Arch.* (2013) 465:1521–33. doi: 10.1007/s00424-013-1285-1
16. Van't Wout JW, Linde I, Leijh PC, Van Furth R. Effect of irradiation, cyclophosphamide, and etoposide (VP-16) on number of peripheral blood and peritoneal leukocytes in mice under normal conditions and during acute inflammatory reaction. *Inflammation.* (1989) 13:1–14. doi: 10.1007/BF00918959
17. Katkar GD, Sundaram MS, Naveenkumar SK, Swethakumar B, Sharma RD, Paul M, et al. NETosis and lack of DNase activity are key factors in Echis carinatus venom-induced tissue destruction. *Nat Commun.* (2016) 7:11361. doi: 10.1038/ncomms11361

18. Abraham E, Carmody A, Shenkar R, Arcaroli J. Neutrophils as early immunologic effectors in hemorrhage- or endotoxemia-induced acute lung injury. *Am J Physiol Lung Cell Mol Physiol.* (2000) 279:L1137–45. doi: 10.1152/ajplung.2000.279.6.L1137
19. Parsey MV, Tuder RM, Abraham E. Neutrophils are major contributors to intraparenchymal lung IL-1 beta expression after hemorrhage and endotoxemia. *J Immunol.* (1998) 160:1007–13.
20. Stackowicz J, Balbino B, Todorova B, Godon O, Iannascoli B, Jonsson F, et al. Evidence that neutrophils do not promote Echis carinatus venom-induced tissue destruction. *Nat Commun.* (2018) 9:2304. doi: 10.1038/s41467-018-04688-6
21. Wang E, Simard M, Ouellet N, Bergeron Y, Beauchamp D, Bergeron MG. Pathogenesis of pneumococcal pneumonia in cyclophosphamide-induced leukopenia in mice. *Infect Immun.* (2002) 70:4226–38. doi: 10.1128/IAI.70.8.4226-4238.2002
22. Marks M, Burns T, Abadi M, Seyoum B, Thornton J, Tuomanen E, et al. Influence of neutropenia on the course of serotype 8 pneumococcal pneumonia in mice. *Infect Immun.* (2007) 75:1586–97. doi: 10.1128/IAI.01579-06
23. Radford JA, Knight RK, Rubens RD. Mitomycin C and vinblastine in the treatment of advanced breast cancer. *Eur J Cancer Clin Oncol.* (1985) 21:1475–7. doi: 10.1016/0277-5379(85)90241-X
24. Gigant B, Wang C, Ravelli RB, Roussi F, Steinmetz MO, Curmi PA, et al. Structural basis for the regulation of tubulin by vinblastine. *Nature.* (2005) 435:519–22. doi: 10.1038/nature03566
25. Jordan MA, Thrower D, Wilson L. Effects of vinblastine, podophyllotoxin and nocodazole on mitotic spindles. Implications for the role of microtubule dynamics in mitosis *J Cell Sci.* (1992) 102:401–16.
26. Getting SJ, Flower RJ, Perretti M. Inhibition of neutrophil and monocyte recruitment by endogenous and exogenous lipocortin 1. *Br J Pharmacol.* (1997) 120:1075–82. doi: 10.1038/sj.bjp.0701029
27. Looney MR, Su X, Van Ziffle JA, Lowell CA, Matthay MA. Neutrophils and their Fc gamma receptors are essential in a mouse model of transfusion-related acute lung injury. *J Clin Invest.* (2006) 116:1615–23. doi: 10.1172/JCI27238
28. Nevalainen TJ. Cytotoxicity of vinblastine and vincristine to pancreatic acinar cells. *Virchows Arch B Cell Pathol.* (1975) 18:119–27.
29. Jagetia GC, Krishnamurthy H, Jyothi P. Evaluation of cytotoxic effects of different doses of vinblastine on mouse spermatogenesis by flow cytometry. *Toxicology.* (1996) 112:227–36. doi: 10.1016/0300-483X(96)03402-6
30. Fleming TJ, Fleming ML, Malek TR. Selective expression of Ly-6G on myeloid lineage cells in mouse bone marrow. RB6-8C5 mAb to granulocyte-differentiation antigen (Gr-1) detects members of the Ly-6 family. *J Immunol.* (1993) 151:2399–408.
31. Lewinsohn DM, Bargatze RF, Butcher EC. Leukocyte-endothelial cell recognition: evidence of a common molecular mechanism shared by neutrophils, lymphocytes, and other leukocytes. *J Immunol.* (1987) 138:4313–21.
32. Jensen J, Warner T, Balish E. Resistance of SCID mice to *Candida albicans* administered intravenously or colonizing the gut: role of polymorphonuclear leukocytes and macrophages. *J Infect Dis.* (1993) 167:912–9. doi: 10.1093/infdis/167.4.912
33. Han Y, Cutler JE. Assessment of a mouse model of neutropenia and the effect of an anti-candidiasis monoclonal antibody in these animals. *J Infect Dis.* (1997) 175:1169–75. doi: 10.1086/516455
34. Abbitt KB, Cotter MJ, Ridger VC, Crossman DC, Hellewell PG, Norman KE. Antibody ligation of murine Ly-6G induces neutropenia, blood flow cessation, and death via complement-dependent and independent mechanisms. *J Leukoc Biol.* (2009) 85:55–63. doi: 10.1189/jlb.0507305
35. Carr KD, Sieve AN, Indramohan M, Break TJ, Lee S, Berg RE. Specific depletion reveals a novel role for neutrophil-mediated protection in the liver during *Listeria monocytogenes* infection. *Eur J Immunol.* (2011) 41:2666–76. doi: 10.1002/eji.201041363
36. Tepper RI, Coffman RL, Leder P. An eosinophil-dependent mechanism for the antitumor effect of interleukin-4. *Science.* (1992) 257:548–51. doi: 10.1126/science.1636093
37. Matsuzaki J, Tsuji T, Chamoto K, Takeshima T, Sendo F, Nishimura T. Successful elimination of memory-type CD8+ T cell subsets by the administration of anti-Gr-1 monoclonal antibody *in vivo*. *Cell Immunol.* (2003) 224:98–105. doi: 10.1016/j.cellimm.2003.08.009
38. Jutila MA, Kroese FG, Jutila KL, Stall AM, Fiering S, Herzenberg LA, et al. Ly-6C is a monocyte/macrophage and endothelial cell differentiation antigen regulated by interferon-gamma. *Eur J Immunol.* (1988) 18:1819–26. doi: 10.1002/eji.1830181125
39. Walunas TL, Bruce DS, Dustin L, Loh DY, Bluestone JA. Ly-6C is a marker of memory CD8+ T cells. *J Immunol.* (1995) 155:1873–83.
40. Sato, N., Yahata, T., Santa, K., Ohta, A., Ohmi, Y., Habu, S., et al. Functional characterization of NK1.1 + Ly-6C+ cells. *Immunol Lett.* (1996) 54:5–9. doi: 10.1016/S0165-2478(96)02632-6
41. Lee PY, Wang JX, Parisini E, Dascher CC, Nigrovic PA. Ly6 family proteins in neutrophil biology. *J Leukoc Biol.* (2013) 94:585–94. doi: 10.1189/jlb.0113014
42. Morgan BP, Dankert JR, Esser AF. Recovery of human neutrophils from complement attack: removal of the membrane attack complex by endocytosis and exocytosis. *J Immunol.* (1987) 138:246–53.
43. Morton J, Coles B, Wright K, Gallimore A, Morrow JD, Terry ES, et al. Circulating neutrophils maintain physiological blood pressure by suppressing bacteria and IFNgamma-dependent iNOS expression in the vasculature of healthy mice. *Blood.* (2008) 111:5187–94. doi: 10.1182/blood-2007-10-117283
44. Baron EJ, Proctor RA. Elicitation of peritoneal polymorphonuclear neutrophils from mice. *J Immunol Methods.* (1982) 49:305–13. doi: 10.1016/0022-1759(82)90130-2
45. Chavakis T, Bierhaus A, Al-Fakhri N, Schneider D, Witte S, Linn T, et al. The pattern recognition receptor (RAGE) is a counterreceptor for leukocyte integrins: a novel pathway for inflammatory cell recruitment. *J Exp Med.* (2003) 198:1507–15. doi: 10.1084/jem.20030800
46. Ribechini E, Leenen PJ, Lutz MB. Gr-1 antibody induces STAT signaling, macrophage marker expression and abrogation of myeloid-derived suppressor cell activity in BM cells. *Eur J Immunol.* (2009) 39:3538–51. doi: 10.1002/eji.200939530
47. Norman KE, Cotter MJ, Stewart JB, Abbitt KB, Ali M, Wagner BE, et al. Combined anticoagulant and antiselectin treatments prevent lethal intravascular coagulation. *Blood.* (2003) 101:921–8. doi: 10.1182/blood-2001-12-0190
48. Ma C, Kapanadze T, Gamrekelashvili J, Manns MP, Korangy F, Greten TF. Anti-Gr-1 antibody depletion fails to eliminate hepatic myeloid-derived suppressor cells in tumor-bearing mice. *J Leukoc Biol.* (2012) 92:1199–206. doi: 10.1189/jlb.0212059
49. Czuprynski CJ, Brown JF, Maroushek N, Wagner RD, Steinberg H. Administration of anti-granulocyte mAb RB6-8C5 impairs the resistance of mice to *Listeria monocytogenes* infection. *J Immunol.* (1994) 152:1836–46.
50. Easton A, Haque A, Chu K, Lukaszewski R, Bancroft GJ. A critical role for neutrophils in resistance to experimental infection with *Burkholderia pseudomallei*. *J Infect Dis.* (2007) 195:99–107. doi: 10.1086/509810
51. Tanaka D, Kagari T, Doi H, Shimozato T. Essential role of neutrophils in anti-type II collagen antibody and lipopolysaccharide-induced arthritis. *Immunology.* (2006) 119:195–202. doi: 10.1111/j.1365-2567.2006.02424.x
52. Reber LL, Gaudenzio N, Starkl P, Galli SJ. Neutrophils are not required for resolution of acute gouty arthritis in mice. *Nat Med.* (2016) 22:1382–4. doi: 10.1038/nm.4216
53. Nishio N, Okawa Y, Sakurai H, Isobe K. Neutrophil depletion delays wound repair in aged mice. *Age.* (2008) 30:11–9. doi: 10.1007/s11357-007-9043-y
54. Tsujimura Y, Obata K, Mukai K, Shindou H, Yoshida M, Nishikado H, et al. Basophils play a pivotal role in immunoglobulin-G-mediated but not immunoglobulin-E-mediated systemic anaphylaxis. *Immunity.* (2008) 28:581–9. doi: 10.1016/j.immuni.2008.02.008
55. Daley JM, Thomay AA, Connolly MD, Reichner JS, Albina JE. Use of Ly6G-specific monoclonal antibody to deplete neutrophils in mice. *J Leukoc Biol.* (2008) 83:64–70. doi: 10.1189/jlb.0407247
56. Ribeiro-Gomes FL, Peters NC, Debrabant A, Sacks DL. Efficient capture of infected neutrophils by dendritic cells in the skin inhibits the early anti-leishmania response. *PLoS Pathog.* (2012) 8:e1002536. doi: 10.1371/journal.ppat.1002536
57. Faget J, Boivin G, Ancey P-B, Gkasti A, Mussard J, Engblom C, et al. Efficient and specific Ly6G$^+$ cell depletion: a change in the current practices toward

more relevant functional analyses of neutrophils. *bioRxiv.* (2018) 498881. doi: 10.1101/498881
58. Pollenus E, Malengier-Devlies B, Vandermosten L, Pham TT, Mitera T, Possemiers H, et al. Limitations of neutrophil depletion by anti-Ly6G antibodies in two heterogenic immunological models. *Immunol Lett.* (2019) 212:30–6. doi: 10.1016/j.imlet.2019.06.006
59. Bruhn KW, Dekitani K, Nielsen TB, Pantapalangkoor P, Spellberg B. Ly6G-mediated depletion of neutrophils is dependent on macrophages. *Results Immunol.* (2016) 6:5–7. doi: 10.1016/j.rinim.2015.12.001
60. Bucher K, Schmitt F, Autenrieth SE, Dillmann I, Nurnberg B, Schenke-Layland K, et al. Fluorescent Ly6G antibodies determine macrophage phagocytosis of neutrophils and alter the retrieval of neutrophils in mice. *J Leukoc Biol.* (2015) 98:365–72. doi: 10.1189/jlb.1AB1014-488RR
61. Wculek SK, Malanchi I. Neutrophils support lung colonization of metastasis-initiating breast cancer cells. *Nature.* (2015) 528:413–7. doi: 10.1038/nature16140
62. Kawanishi N, Mizokami T, Niihara H, Yada K, Suzuki K. Neutrophil depletion attenuates muscle injury after exhaustive exercise. *Med Sci Sports Exerc.* (2016) 48:1917–24. doi: 10.1249/MSS.0000000000000980
63. Deniset JF, Surewaard BG, Lee WY, Kubes P. Splenic Ly6G(high) mature and Ly6G(int) immature neutrophils contribute to eradication of *S. pneumoniae J Exp Med.* (2017) 214:1333–50. doi: 10.1084/jem.20161621
64. Thomas J, Gangappa S, Kanangat S, Rouse BT. On the essential involvement of neutrophils in the immunopathologic disease: herpetic stromal keratitis. *J Immunol.* (1997) 158:1383–91.
65. Wojtasiak M, Pickett DL, Tate MD, Bedoui S, Job ER, Whitney PG, et al. Gr-1+ cells, but not neutrophils, limit virus replication and lesion development following flank infection of mice with herpes simplex virus type-1. *Virology.* (2010) 407:143–51. doi: 10.1016/j.virol.2010.08.001
66. Shi C, Hohl TM, Leiner I, Equinda MJ, Fan X, Pamer EG. Ly6G+ neutrophils are dispensable for defense against systemic *Listeria monocytogenes* infection. *J Immunol.* (2011) 187:5293–8. doi: 10.4049/jimmunol.1101721
67. Charmoy M, Milon G, Tacchini-Cottier F. Role of neutrophils in the early shaping of the *Leishmania major* specific immune response in experimental murine cutaneous Leishmaniasis. In: Tacchini-Cottier F, van Zandbergen G, editors. *Neutrophils in Infectious Diseases.* Sharjah: Bentham Science Publishers Ltd (2011). p. 49–58. doi: 10.2174/978160805023911101010049
68. Gillis CM, Jonsson F, Mancardi DA, Tu N, Beutier H, Van Rooijen N, et al. Mechanisms of anaphylaxis in human low-affinity IgG receptor locus knock-in mice. *J Allergy Clin Immunol.* (2017) 139:1253–65 e1214. doi: 10.1016/j.jaci.2016.06.058
69. Tacchini-Cottier F, Zweifel C, Belkaid Y, Mukankundiye C, Vasei M, Launois P, et al. An immunomodulatory function for neutrophils during the induction of a CD4+ Th2 response in BALB/c mice infected with *Leishmania major*. *J Immunol.* (2000) 165:2628–36. doi: 10.4049/jimmunol.165.5.2628
70. Beutier H, Gillis CM, Iannascoli B., Godon O, England P, Sibilano R, et al. IgG subclasses determine pathways of anaphylaxis in mice. *J Allergy Clin Immunol.* (2017) 139:269–80 e267. doi: 10.1016/j.jaci.2016.03.028
71. Xiao H, Heeringa P, Liu Z, Huugen D, Hu P, Maeda N, et al. The role of neutrophils in the induction of glomerulonephritis by anti-myeloperoxidase antibodies. *Am J Pathol.* (2005) 167:39–45. doi: 10.1016/S0002-9440(10)62951-3
72. Delyria ES, Redline RW, Blanchard TG. Vaccination of mice against H pylori induces a strong Th-17 response and immunity that is neutrophil dependent. *Gastroenterology.* (2009) 136:247–56. doi: 10.1053/j.gastro.2008.09.017
73. Wu F, Cao W, Yang Y, Liu A. Extensive infiltration of neutrophils in the acute phase of experimental autoimmune encephalomyelitis in C57BL/6 mice. *Histochem Cell Biol.* (2010) 133:313–22. doi: 10.1007/s00418-009-0673-2
74. Ribeiro-Gomes FL, Roma EH, Carneiro MB, Doria NA, Sacks DL, Peters NC. Site-dependent recruitment of inflammatory cells determines the effective dose of *Leishmania major*. *Infect Immun.* (2014) 82:2713–27. doi: 10.1128/IAI.01600-13
75. Wang JX, Bair AM, King SL, Shnayder R, Huang YF, Shieh CC, et al. Ly6G ligation blocks recruitment of neutrophils via a β2-integrin-dependent mechanism. *Blood.* (2012) 120:1489–98. doi: 10.1182/blood-2012-01-404046
76. Yipp BG, Kubes P. Antibodies against neutrophil LY6G do not inhibit leukocyte recruitment in mice *in vivo*. *Blood.* (2013) 121:241–2. doi: 10.1182/blood-2012-09-454348
77. Hasenberg A, Hasenberg M, Mann L, Neumann F, Borkenstein L, Stecher M, et al. Catchup: a mouse model for imaging-based tracking and modulation of neutrophil granulocytes. *Nat Methods.* (2015) 12:445–52. doi: 10.1038/nmeth.3322
78. Bhattacharyya S, Xue L, Devkota S, Chang E, Morris S, Tobacman JK. Carrageenan-induced colonic inflammation is reduced in Bcl10 null mice and increased in IL-10-deficient mice. *Mediators Inflamm.* (2013) 2013:397642. doi: 10.1155/2013/397642
79. Pagano RL, Dias MA, Dale CS, Giorgi R. Neutrophils and the calcium-binding protein MRP-14 mediate carrageenan-induced antinociception in mice. *Mediators Inflamm.* (2002) 11:203–10. doi: 10.1080/0962935029000050
80. Springer T, Galfre G, Secher DS, Milstein C. Mac-1: a macrophage differentiation antigen identified by monoclonal antibody. *Eur J Immunol.* (1979) 9:301–6. doi: 10.1002/eji.1830090410
81. Rosas M, Thomas B, Stacey M, Gordon S, Taylor PR. The myeloid 7/4-antigen defines recently generated inflammatory macrophages and is synonymous with Ly-6B. *J Leukoc Biol.* (2010) 88:169–80. doi: 10.1189/jlb.0809548
82. Breslin WL, Strohacker K, Carpenter KC, Haviland DL, Mcfarlin BK. Mouse blood monocytes: standardizing their identification and analysis using CD115. *J Immunol Methods.* (2013) 390:1–8. doi: 10.1016/j.jim.2011.03.005
83. Moses K, Klein JC, Mann L, Klingberg A, Gunzer M, Brandau S. Survival of residual neutrophils and accelerated myelopoiesis limit the efficacy of antibody-mediated depletion of Ly-6G+ cells in tumor-bearing mice. *J Leukoc Biol.* (2016) 99:811–23. doi: 10.1189/jlb.1HI0715-289R
84. Collier RJ. Diphtheria toxin: mode of action and structure. *Bacteriol Rev.* (1975) 39:54–85.
85. Naglich JG, Metherall JE, Russell DW, Eidels L. Expression cloning of a diphtheria toxin receptor: identity with a heparin-binding EGF-like growth factor precursor. *Cell.* (1992) 69:1051–61. doi: 10.1016/0092-8674(92)90623-K
86. Pappenheimer AM Jr, Harper AA, Moynihan M, Brockes JP. Diphtheria toxin and related proteins: effect of route of injection on toxicity and the determination of cytotoxicity for various cultured cells. *J Infect Dis.* (1982) 145:94–102. doi: 10.1093/infdis/145.1.94
87. Saito M, Iwawaki T, Taya C, Yonekawa H, Noda M, Inui Y, et al. Diphtheria toxin receptor-mediated conditional and targeted cell ablation in transgenic mice. *Nat Biotechnol.* (2001) 19:746–50. doi: 10.1038/90795
88. Drazin R, Kandel J, Collier RJ. Structure and activity of diphtheria toxin. II Attack by trypsin at a specific site within the intact toxin molecule. *J Biol Chem.* (1971) 246:1504–10.
89. Marnell MH, Shia SP, Stookey M, Draper RK. Evidence for penetration of diphtheria toxin to the cytosol through a prelysosomal membrane. *Infect Immun.* (1984) 44:145–50.
90. Honjo T, Nishizuka Y, Hayaishi O. Diphtheria toxin-dependent adenosine diphosphate ribosylation of aminoacyl transferase II and inhibition of protein synthesis. *J Biol Chem.* (1968) 243:3553–5.
91. Yamaizumi M, Mekada E, Uchida T, Okada Y. One molecule of diphtheria toxin fragment A introduced into a cell can kill the cell. *Cell.* (1978) 15:245–50. doi: 10.1016/0092-8674(78)90099-5
92. Buch T, Heppner FL, Tertilt C, Heinen TJ, Kremer M, Wunderlich FT, et al. A Cre-inducible diphtheria toxin receptor mediates cell lineage ablation after toxin administration. *Nat Methods.* (2005) 2:419–26. doi: 10.1038/nmeth762
93. Zambrowicz BP, Imamoto A, Fiering S, Herzenberg LA, Kerr WG, Soriano P. Disruption of overlapping transcripts in the ROSA beta geo 26 gene trap strain leads to widespread expression of beta-galactosidase in mouse embryos and hematopoietic cells. *Proc Natl Acad Sci USA.* (1997) 94:3789–94. doi: 10.1073/pnas.94.8.3789
94. Lagasse E, Weissman IL. Mouse MRP8 and MRP14, two intracellular calcium-binding proteins associated with the development of the myeloid lineage. *Blood.* (1992) 79:1907–15. doi: 10.1182/blood.V79.8.1907.1907
95. Passegue E, Wagner EF, Weissman IL. JunB deficiency leads to a myeloproliferative disorder arising from hematopoietic stem cells. *Cell.* (2004) 119:431–43. doi: 10.1016/j.cell.2004.10.010
96. Elliott ER, Van Ziffle JA, Scapini P, Sullivan BM, Locksley RM, Lowell CA. Deletion of Syk in neutrophils prevents immune complex arthritis. *J Immunol.* (2011) 187:4319–30. doi: 10.4049/jimmunol.1100341

97. Abram CL, Roberge GL, Pao LI, Neel BG, Lowell CA. Distinct roles for neutrophils and dendritic cells in inflammation and autoimmunity in motheaten mice. *Immunity*. (2013) 38:489–501. doi: 10.1016/j.immuni.2013.02.018
98. Brydges SD, Mueller JL, Mcgeough MD, Pena CA, Misaghi A, Gandhi C, et al. Inflammasome-mediated disease animal models reveal roles for innate but not adaptive immunity. *Immunity*. (2009) 30:875–87. doi: 10.1016/j.immuni.2009.05.005
99. Bouabe H, Fassler R, Heesemann J. Improvement of reporter activity by IRES-mediated polycistronic reporter system. *Nucleic Acids Res.* (2008) 36:e28. doi: 10.1093/nar/gkm1119
100. Casanova-Acebes M, Nicolas-Avila JA, Li JL, Garcia-Silva S, Balachander A, Rubio-Ponce A, et al. Neutrophils instruct homeostatic and pathological states in naive tissues. *J Exp Med.* (2018) 215:2778–95. doi: 10.1084/jem.20181468
101. Bowers E, Slaughter A, Frenette PS, Kuick R, Pello OM, Lucas D. Granulocyte-derived TNFalpha promotes vascular and hematopoietic regeneration in the bone marrow. *Nat Med.* (2018) 24:95–102. doi: 10.1038/nm.4448
102. Manz MG, Boettcher S. Emergency granulopoiesis. *Nat Rev Immunol.* (2014) 14:302–14. doi: 10.1038/nri3660
103. Hochweller K, Striegler J, Hammerling GJ, Garbi N. A novel CD11c.DTR transgenic mouse for depletion of dendritic cells reveals their requirement for homeostatic proliferation of natural killer cells. *Eur J Immunol.* (2008) 38:2776–83. doi: 10.1002/eji.200838659
104. Liu F, Wu HY, Wesselschmidt R, Kornaga T, Link DC. Impaired production and increased apoptosis of neutrophils in granulocyte colony-stimulating factor receptor-deficient mice. *Immunity*. (1996) 5:491–501. doi: 10.1016/S1074-7613(00)80504-X
105. Eyles JL, Hickey MJ, Norman MU, Croker BA, Roberts AW, Drake SF, et al. A key role for G-CSF-induced neutrophil production and trafficking during inflammatory arthritis. *Blood.* (2008) 112:5193–201. doi: 10.1182/blood-2008-02-139535
106. Campbell IK, Leong D, Edwards KM, Rayzman V, Ng M, Goldberg GL, et al. Therapeutic targeting of the G-CSF receptor reduces neutrophil trafficking and joint inflammation in antibody-mediated inflammatory arthritis. *J Immunol.* (2016) 197:4392–402. doi: 10.4049/jimmunol.1600121
107. Yang CW, Strong BS, Miller MJ, Unanue ER. Neutrophils influence the level of antigen presentation during the immune response to protein antigens in adjuvants. *J Immunol.* (2010) 185:2927–34. doi: 10.4049/jimmunol.1001289
108. Yang CW, Unanue ER. Neutrophils control the magnitude and spread of the immune response in a thromboxane A2-mediated process. *J Exp Med.* (2013) 210:375–87. doi: 10.1084/jem.20122183
109. Kojima H, Otani A, Oishi A, Makiyama Y, Nakagawa S, Yoshimura N. Granulocyte colony-stimulating factor attenuates oxidative stress-induced apoptosis in vascular endothelial cells and exhibits functional and morphologic protective effect in oxygen-induced retinopathy. *Blood.* (2011) 117:1091–100. doi: 10.1182/blood-2010-05-286963
110. Bussolino F, Wang JM, Defilippi P, Turrini F, Sanavio F, Edgell CJ, et al. Granulocyte- and granulocyte-macrophage-colony stimulating factors induce human endothelial cells to migrate and proliferate. *Nature.* (1989) 337:471–3. doi: 10.1038/337471a0
111. Christopher MJ, Link DC. Granulocyte colony-stimulating factor induces osteoblast apoptosis and inhibits osteoblast differentiation. *J Bone Miner Res.* (2008) 23:1765–74. doi: 10.1359/jbmr.080612
112. Lucas D, Bruns I, Battista M, Mendez-Ferrer S, Magnon C, Kunisaki Y, et al. Norepinephrine reuptake inhibition promotes mobilization in mice: potential impact to rescue low stem cell yields. *Blood.* (2012) 119:3962–5. doi: 10.1182/blood-2011-07-367102
113. Eash KJ, Greenbaum AM, Gopalan PK, Link DC. CXCR2 and CXCR4 antagonistically regulate neutrophil trafficking from murine bone marrow. *J Clin Invest.* (2010) 120:2423–31. doi: 10.1172/JCI41649
114. Cacalano G, Lee J, Kikly K, Ryan AM, Pitts-Meek S, Hultgren B, et al. Neutrophil and B cell expansion in mice that lack the murine IL-8 receptor homolog. *Science.* (1994) 265:682–4. doi: 10.1126/science.8036519
115. Silvestre-Roig C, Braster Q, Wichapong K, Lee EY, Teulon JM, Berrebeh N, et al. Externalized histone H4 orchestrates chronic inflammation by inducing lytic cell death. *Nature.* (2019) 569:236–40. doi: 10.1038/s41586-019-1167-6
116. Ranganathan P, Jayakumar C, Manicassamy S, Ramesh G. CXCR2 knockout mice are protected against DSS-colitis-induced acute kidney injury and inflammation. *Am J Physiol Renal Physiol.* (2013) 305:F1422–7. doi: 10.1152/ajprenal.00319.2013
117. Del Rio L, Bennouna S, Salinas J, Denkers EY. CXCR2 deficiency confers impaired neutrophil recruitment and increased susceptibility during *Toxoplasma gondii* infection. *J Immunol.* (2001) 167:6503–9. doi: 10.4049/jimmunol.167.11.6503
118. Jacobs JP, Ortiz-Lopez A, Campbell JJ, Gerard CJ, Mathis D, Benoist C. Deficiency of CXCR2, but not other chemokine receptors, attenuates autoantibody-mediated arthritis in a murine model. *Arthritis Rheum.* (2010) 62:1921–32. doi: 10.1002/art.27470
119. Semple BD, Bye N, Ziebell JM, Morganti-Kossmann MC. Deficiency of the chemokine receptor CXCR2 attenuates neutrophil infiltration and cortical damage following closed head injury. *Neurobiol Dis.* (2010) 40:394–403. doi: 10.1016/j.nbd.2010.06.015
120. Boisvert WA, Rose DM, Johnson KA, Fuentes ME, Lira SA, Curtiss LK, et al. Up-regulated expression of the CXCR2 ligand KC/GRO-alpha in atherosclerotic lesions plays a central role in macrophage accumulation and lesion progression. *Am J Pathol.* (2006) 168:1385–95. doi: 10.2353/ajpath.2006.040748
121. Hallgren J, Jones TG, Abonia JP, Xing W, Humbles A, Austen KF, et al. Pulmonary CXCR2 regulates VCAM-1 and antigen-induced recruitment of mast cell progenitors. *Proc Natl Acad Sci USA.* (2007) 104:20478–83. doi: 10.1073/pnas.0709651104
122. Reutershan J, Morris MA, Burcin TL, Smith DF, Chang D, Saprito MS, et al. Critical role of endothelial CXCR2 in LPS-induced neutrophil migration into the lung. *J Clin Invest.* (2006) 116:695–702. doi: 10.1172/JCI27009
123. Dyer DP, Pallas K, Medina-Ruiz L, Schuette F, Wilson GJ, Graham GJ. CXCR2 deficient mice display macrophage-dependent exaggerated acute inflammatory responses. *Sci Rep.* (2017) 7:42681. doi: 10.1038/srep42681
124. Karsunky H, Zeng H, Schmidt T, Zevnik B, Kluge R, Schmid KW, et al. Inflammatory reactions and severe neutropenia in mice lacking the transcriptional repressor Gfi1. *Nat Genet.* (2002) 30:295–300. doi: 10.1038/ng831
125. Gilks CB, Bear SE, Grimes HL, Tsichlis PN. Progression of interleukin-2 (IL-2)-dependent rat T cell lymphoma lines to IL-2-independent growth following activation of a gene (Gfi-1) encoding a novel zinc finger protein. *Mol Cell Biol.* (1993) 13:1759–68. doi: 10.1128/MCB.13.3.1759
126. Zweidler-Mckay PA, Grimes HL, Flubacher MM, Tsichlis PN. Gfi-1 encodes a nuclear zinc finger protein that binds DNA and functions as a transcriptional repressor. *Mol Cell Biol.* (1996) 16:4024–34. doi: 10.1128/MCB.16.8.4024
127. Karsunky H, Mende I, Schmidt T, Moroy T. High levels of the onco-protein Gfi-1 accelerate T-cell proliferation and inhibit activation induced T-cell death in Jurkat T-cells. *Oncogene.* (2002) 21:1571–9. doi: 10.1038/sj.onc.1205216
128. Zornig M, Schmidt T, Karsunky H, Grzeschiczek A, Moroy T. Zinc finger protein GFI-1 cooperates with myc and pim-1 in T-cell lymphomagenesis by reducing the requirements for IL-2. *Oncogene.* (1996) 12:1789–801.
129. Tong B, Grimes HL, Yang TY, Bear SE, Qin Z, Du K, et al. The Gfi-1B proto-oncoprotein represses p21WAF1 and inhibits myeloid cell differentiation. *Mol Cell Biol.* (1998) 18:2462–73. doi: 10.1128/MCB.18.5.2462
130. Hock H, Hamblen MJ, Rooke HM, Traver D, Bronson RT, Cameron S, et al. Intrinsic requirement for zinc finger transcription factor Gfi-1 in neutrophil differentiation. *Immunity.* (2003) 18:109–20. doi: 10.1016/S1074-7613(02)00501-0
131. Zhu J, Jankovic D, Grinberg A, Guo L, Paul WE. Gfi-1 plays an important role in IL-2-mediated Th2 cell expansion. *Proc Natl Acad Sci USA.* (2006) 103:18214–9. doi: 10.1073/pnas.0608981103
132. Lakso M, Pichel JG, Gorman JR, Sauer B, Okamoto Y, Lee E, et al. Efficient *in vivo* manipulation of mouse genomic sequences at the zygote stage. *Proc Natl Acad Sci USA.* (1996) 93:5860–5. doi: 10.1073/pnas.93.12.5860
133. Shi LZ, Kalupahana NS, Turnis ME, Neale G, Hock H, Vignali DA, et al. Inhibitory role of the transcription repressor Gfi1 in the generation of thymus-derived regulatory T cells. *Proc Natl Acad Sci USA.* (2013) 110:E3198–205. doi: 10.1073/pnas.1300950110
134. Yucel R, Kosan C, Heyd F, Moroy T. Gfi1:green fluorescent protein knock-in mutant reveals differential expression and autoregulation of the growth

factor independence 1 (Gfi1) gene during lymphocyte development. *J Biol Chem.* (2004) 279:40906–17. doi: 10.1074/jbc.M400808200
135. Vassen L, Duhrsen U, Kosan C, Zeng H, Moroy T. Growth factor independence 1 (Gfi1) regulates cell-fate decision of a bipotential granulocytic-monocytic precursor defined by expression of Gfi1 and CD48. *Am J Blood Res.* (2012) 2:228–42. doi: 10.1182/blood.V118.21.3217.3217
136. Monach PA, Nigrovic PA, Chen M, Hock H, Lee DM, Benoist C, et al. Neutrophils in a mouse model of autoantibody-mediated arthritis: critical producers of Fc receptor gamma, the receptor for C5a, and lymphocyte function-associated antigen 1. *Arthritis Rheum.* (2010) 62:753–64. doi: 10.1002/art.27238
137. Rathinam C, Geffers R, Yucel R, Buer J, Welte K, Moroy T, et al. The transcriptional repressor Gfi1 controls STAT3-dependent dendritic cell development and function. *Immunity.* (2005) 22:717–28. doi: 10.1016/j.immuni.2005.04.007
138. Yasuoka T, Kuwahara M, Yamada T, Maruyama S, Suzuki J, Taniguchi M, et al. The transcriptional repressor Gfi1 plays a critical role in the development of NKT1- and NKT2-type iNKT cells. *PLoS ONE.* (2016) 11:e0157395. doi: 10.1371/journal.pone.0157395
139. Yang H, Gan J, Xie X, Deng M, Feng L, Chen X, et al. Gfi1-Cre knock-in mouse line: a tool for inner ear hair cell-specific gene deletion. *Genesis.* (2010) 48:400–6. doi: 10.1002/dvg.20632
140. Wallis D, Hamblen M, Zhou Y, Venken KJ, Schumacher A, Grimes HL, et al. The zinc finger transcription factor Gfi1, implicated in lymphomagenesis, is required for inner ear hair cell differentiation and survival. *Development.* (2003) 130:221–32. doi: 10.1242/dev.00190
141. Geissler S, Textor M, Stumpp S, Seitz S, Lekaj A, Brunk S, et al. Loss of murine Gfi1 causes neutropenia and induces osteoporosis depending on the pathogen load and systemic inflammation. *PLoS ONE.* (2018) 13:e0198510. doi: 10.1371/journal.pone.0198510
142. Ordonez-Rueda D, Jonsson F, Mancardi DA, Zhao W, Malzac A, Liang Y, et al. A hypomorphic mutation in the Gfi1 transcriptional repressor results in a novel form of neutropenia. *Eur J Immunol.* (2012) 42:2395–408. doi: 10.1002/eji.201242589
143. Jaeger BN, Donadieu J, Cognet C, Bernat C, Ordonez-Rueda D, Barlogis V, et al. Neutrophil depletion impairs natural killer cell maturation, function, and homeostasis. *J Exp Med.* (2012) 209:565–80. doi: 10.1084/jem.20111908
144. Sporri R, Joller N, Hilbi H, Oxenius A. A novel role for neutrophils as critical activators of NK cells. *J Immunol.* (2008) 181:7121–30. doi: 10.4049/jimmunol.181.10.7121
145. Mancardi DA, Jonsson F, Iannascoli B, Khun H, Van Rooijen N, Huerre M, et al. Cutting Edge: the murine high-affinity IgG receptor FcgammaRIV is sufficient for autoantibody-induced arthritis. *J Immunol.* (2011) 186:1899–903. doi: 10.4049/jimmunol.1003642
146. Cochrane CG, Weigle WO, Dixon FJ. The role of polymorphonuclear leukocytes in the initiation and cessation of the *Arthus vasculitis*. *J Exp Med.* (1959) 110:481–94. doi: 10.1084/jem.110.3.481
147. Hurrell BP, Regli IB, Tacchini-Cottier F. Different Leishmania species drive distinct neutrophil functions. *Trends Parasitol.* (2016) 32:392–401. doi: 10.1016/j.pt.2016.02.003
148. Mora-Cartin R, Gutierrez-Jimenez C, Alfaro-Alarcon A, Chaves-Olarte E, Chacon-Diaz C, Barquero-Calvo E, et al. Neutrophils dampen adaptive immunity in brucellosis. *Infect Immun.* (2019) 87:e00118–19. doi: 10.1128/IAI.00118-19
149. Moulding DA, Quayle JA, Hart CA, Edwards SW. Mcl-1 expression in human neutrophils: regulation by cytokines and correlation with cell survival. *Blood.* (1998) 92:2495–502. doi: 10.1182/blood.V92.7.2495
150. El Kebir D, Damlaj A, Filep JG. Toll-like receptor 9 signaling delays neutrophil apoptosis by increasing transcription of Mcl-1. *PLoS ONE.* (2014) 9:e87006. doi: 10.1371/journal.pone.0087006
151. Leuenroth SJ, Grutkoski PS, Ayala A, Simms HH. The loss of Mcl-1 expression in human polymorphonuclear leukocytes promotes apoptosis. *J Leukoc Biol.* (2000) 68:158–66. doi: 10.1189/jlb.68.1.158
152. Clausen BE, Burkhardt C, Reith W, Renkawitz R, Forster I. Conditional gene targeting in macrophages and granulocytes using LysMcre mice. *Transgenic Res.* (1999) 8:265–77. doi: 10.1023/A:1008942828960
153. Dzhagalov I, St John A, He YW. The antiapoptotic protein Mcl-1 is essential for the survival of neutrophils but not macrophages. *Blood.* (2007) 109:1620–6. doi: 10.1182/blood-2006-03-013771
154. Weber FC, Nemeth T, Csepregi JZ, Dudeck A, Roers A, Ozsvari B, et al. Neutrophils are required for both the sensitization and elicitation phase of contact hypersensitivity. *J Exp Med.* (2015) 212:15–22. doi: 10.1084/jem.20130062
155. Birkenkamp KU, Coffer PJ. FOXO transcription factors as regulators of immune homeostasis: molecules to die for? *J Immunol.* (2003) 171:1623–9. doi: 10.4049/jimmunol.171.4.1623
156. Lin L, Hron JD, Peng SL. Regulation of NF-kappaB, Th activation, and autoinflammation by the forkhead transcription factor Foxo3a. *Immunity.* (2004) 21:203–13. doi: 10.1016/j.immuni.2004.06.016
157. Jonsson H, Allen P, Peng SL. Inflammatory arthritis requires Foxo3a to prevent Fas ligand-induced neutrophil apoptosis. *Nat Med.* (2005) 11:666–71. doi: 10.1038/nm1248
158. Aratani Y, Koyama H, Nyui S, Suzuki K, Kura F, Maeda N. Severe impairment in early host defense against *Candida albicans* in mice deficient in myeloperoxidase. *Infect Immun.* (1999) 67:1828–36.
159. Belaaouaj A, Mccarthy R, Baumann M, Gao Z, Ley TJ, Abraham SN, et al. Mice lacking neutrophil elastase reveal impaired host defense against gram negative bacterial sepsis. *Nat Med.* (1998) 4:615–8. doi: 10.1038/nm0598-615
160. Brennan M, Gaur A, Pahuja A, Lusis AJ, Reynolds WF. Mice lacking myeloperoxidase are more susceptible to experimental autoimmune encephalomyelitis. *J Neuroimmunol.* (2001) 112:97–105. doi: 10.1016/S0165-5728(00)00392-1
161. De Araujo TH, Okada SS, Ghosn EE, Taniwaki NN, Rodrigues MR, De Almeida SR, et al. Intracellular localization of myeloperoxidase in murine peritoneal B-lymphocytes and macrophages. *Cell Immunol.* (2013) 281:27–30. doi: 10.1016/j.cellimm.2013.01.002
162. Metzler KD, Fuchs TA, Nauseef WM, Reumaux D, Roesler J, Schulze I, et al. Myeloperoxidase is required for neutrophil extracellular trap formation: implications for innate immunity. *Blood.* (2011) 117:953–9. doi: 10.1182/blood-2010-06-290171
163. Kolaczkowska E, Jenne CN, Surewaard BG, Thanabalasuriar A, Lee WY, Sanz MJ, et al. Molecular mechanisms of NET formation and degradation revealed by intravital imaging in the liver vasculature. *Nat Commun.* (2015) 6:6673. doi: 10.1038/ncomms7673
164. Rossaint J, Herter JM, Van Aken H, Napirei M, Doring Y, Weber C, et al. Synchronized integrin engagement and chemokine activation is crucial in neutrophil extracellular trap-mediated sterile inflammation. *Blood.* (2014) 123:2573–84. doi: 10.1182/blood-2013-07-516484
165. Young RE, Thompson RD, Larbi KY, La M, Roberts CE, Shapiro SD, et al. Neutrophil elastase (NE)-deficient mice demonstrate a non-redundant role for NE in neutrophil migration, generation of proinflammatory mediators, and phagocytosis in response to zymosan particles *in vivo*. *J Immunol.* (2004) 172:4493–502. doi: 10.4049/jimmunol.172.7.4493
166. Macivor DM, Shapiro SD, Pham CT, Belaaouaj A, Abraham SN, Ley TJ. Normal neutrophil function in cathepsin G-deficient mice. *Blood.* (1999) 94:4282–93. doi: 10.1182/blood.V94.12.4282
167. Hemmers S, Teijaro JR, Arandjelovic S, Mowen KA. PAD4-mediated neutrophil extracellular trap formation is not required for immunity against influenza infection. *PLoS ONE.* (2011) 6:e22043. doi: 10.1371/journal.pone.0022043
168. Li P, Li M, Lindberg MR, Kennett MJ, Xiong N, Wang Y. PAD4 is essential for antibacterial innate immunity mediated by neutrophil extracellular traps. *J Exp Med.* (2010) 207:1853–62. doi: 10.1084/jem.20100239
169. Nakashima K, Arai S, Suzuki A, Nariai Y, Urano T, Nakayama M, et al. PAD4 regulates proliferation of multipotent haematopoietic cells by controlling c-myc expression. *Nat Commun.* (2013) 4:1836. doi: 10.1038/ncomms2862
170. Tanikawa C, Espinosa M, Suzuki A, Masuda K, Yamamoto K, Tsuchiya E, et al. Regulation of histone modification and chromatin structure by the p53-PADI4 pathway. *Nat Commun.* (2012) 3:676. doi: 10.1038/ncomms1676
171. Efimova O, Szankasi P, Kelley TW. Ncf1 (p47phox) is essential for direct regulatory T cell mediated suppression of CD4+ effector T cells. *PLoS ONE.* (2011) 6:e16013. doi: 10.1371/journal.pone.0016013

172. Gelderman KA, Hultqvist M, Olsson LM, Bauer K, Pizzolla A, Olofsson P, et al. Rheumatoid arthritis: the role of reactive oxygen species in disease development and therapeutic strategies. *Antioxid Redox Signal.* (2007) 9:1541–67. doi: 10.1089/ars.2007.1569
173. Huang CK, Zhan L, Hannigan MO, Ai Y, Leto TL. P47(phox)-deficient NADPH oxidase defect in neutrophils of diabetic mouse strains, C57BL/6J-m db/db and db/+. *J Leukoc Biol.* (2000) 67:210–5. doi: 10.1002/jlb.67.2.210
174. Hultqvist M, Olofsson P, Holmberg J, Backstrom BT, Tordsson J, Holmdahl R. Enhanced autoimmunity, arthritis, and encephalomyelitis in mice with a reduced oxidative burst due to a mutation in the Ncf1 gene. *Proc Natl Acad Sci USA.* (2004) 101:12646–51. doi: 10.1073/pnas.0403831101
175. Sareila O, Jaakkola N, Olofsson P, Kelkka T, Holmdahl R. Identification of a region in p47phox/NCF1 crucial for phagocytic NADPH oxidase (NOX2) activation. *J Leukoc Biol.* (2013) 93:427–35. doi: 10.1189/jlb.1211588
176. Karatepe K, Zhu H, Zhang X, Guo R, Kambara H, Loison F, et al. Proteinase 3 limits the number of hematopoietic stem and progenitor cells in murine bone marrow. *Stem Cell Reports.* (2018) 11:1092–105. doi: 10.1016/j.stemcr.2018.10.004
177. Ward PP, Mendoza-Meneses M, Park PW, Conneely OM. Stimulus-dependent impairment of the neutrophil oxidative burst response in lactoferrin-deficient mice. *Am J Pathol.* (2008) 172:1019–29. doi: 10.2353/ajpath.2008.061145
178. Ward PP, Mendoza-Meneses M, Cunningham GA, Conneely OM. Iron status in mice carrying a targeted disruption of lactoferrin. *Mol Cell Biol.* (2003) 23:178–85. doi: 10.1128/MCB.23.1.178-185.2003
179. Ye Q, Zheng Y, Fan S, Qin Z, Li N, Tang A, et al. Lactoferrin deficiency promotes colitis-associated colorectal dysplasia in mice. *PLoS ONE.* (2014) 9:e103298. doi: 10.1371/journal.pone.0103298
180. Korkmaz B, Moreau T, Gauthier F. Neutrophil elastase, proteinase 3 and cathepsin G: physicochemical properties, activity and physiopathological functions. *Biochimie.* (2008) 90:227–42. doi: 10.1016/j.biochi.2007.10.009
181. Tkalcevic J, Novelli M, Phylactides M, Iredale JP, Segal AW, Roes J. Impaired immunity and enhanced resistance to endotoxin in the absence of neutrophil elastase and cathepsin G. *Immunity.* (2000) 12:201–10. doi: 10.1016/S1074-7613(00)80173-9
182. Hahn I, Klaus A, Janze AK, Steinwede K, Ding N, Bohling J, et al. Cathepsin G and neutrophil elastase play critical and non-redundant roles in lung-protective immunity against *Streptococcus pneumoniae* in mice. *Infect Immun.* (2011) 79:4893–901. doi: 10.1128/IAI.05593-11
183. Kessenbrock K, Frohlich L, Sixt M, Lammermann T, Pfister H, Bateman A, et al. Proteinase 3 and neutrophil elastase enhance inflammation in mice by inactivating antiinflammatory progranulin. *J Clin Invest.* (2008) 118:2438–47. doi: 10.1172/JCI34694
184. Strzepa A, Pritchard KA, Dittel BN. Myeloperoxidase: a new player in autoimmunity. *Cell Immunol.* (2017) 317:1–8. doi: 10.1016/j.cellimm.2017.05.002
185. Gao S, Zhu H, Zuo X, Luo H. Cathepsin G and its role in inflammation and autoimmune diseases. *Arch Rheumatol.* (2018) 33:498–504. doi: 10.5606/ArchRheumatol.2018.6595
186. El Shikh MEM, El Sayed R, Nerviani A, Goldmann K, John CR, Hands R, et al. Extracellular traps and PAD4 released by macrophages induce citrullination and auto-antibody production in autoimmune arthritis. *J Autoimmun.* (2019) 105:102297. doi: 10.1016/j.jaut.2019.06.008
187. Lee W, Ko SY, Mohamed MS, Kenny HA, Lengyel E, Naora H. Neutrophils facilitate ovarian cancer premetastatic niche formation in the omentum. *J Exp Med.* (2019) 216:176–94. doi: 10.1084/jem.20181170
188. Nemeth T, Futosi K, Sitaru C, Ruland J, Mocsai A. Neutrophil-specific deletion of the CARD9 gene expression regulator suppresses autoantibody-induced inflammation *in vivo*. *Nat Commun.* (2016) 7:11004. doi: 10.1038/ncomms11004
189. Eiserich JP, Baldus S, Brennan ML, Ma W, Zhang C, Tousson A, et al. Myeloperoxidase, a leukocyte-derived vascular NO oxidase. *Science.* (2002) 296:2391–4. doi: 10.1126/science.1106830
190. Gross S, Gammon ST, Moss BL, Rauch D, Harding J, Heinecke JW, et al. Bioluminescence imaging of myeloperoxidase activity *in vivo*. *Nat Med.* (2009) 15:455–61. doi: 10.1038/nm.1886
191. Jönsson F, Mancardi DA, Kita Y, Karasuyama H, Iannascoli B, Van Rooijen N, et al. Mouse and human neutrophils induce anaphylaxis. *J Clin Invest.* (2011) 121:1484–96. doi: 10.1172/JCI45232
192. Alshetaiwi HS, Balivada S, Shrestha TB, Pyle M, Basel MT, Bossmann SH, et al. Luminol-based bioluminescence imaging of mouse mammary tumors. *J Photochem Photobiol B.* (2013) 127:223–8. doi: 10.1016/j.jphotobiol.2013.08.017
193. Wirtz S, Popp V, Kindermann M, Gerlach K, Weigmann B, Fichtner-Feigl S, et al. Chemically induced mouse models of acute and chronic intestinal inflammation. *Nat Protoc.* (2017) 12:1295–309. doi: 10.1038/nprot.2017.044
194. Kossodo S, Zhang J, Groves K, Cuneo GJ, Handy E, Morin J, et al. Noninvasive *in vivo* quantification of neutrophil elastase activity in acute experimental mouse lung injury. *Int J Mol Imaging.* (2011) 2011:581406. doi: 10.1155/2011/581406
195. Ho AS, Chen CH, Cheng CC, Wang CC, Lin HC, Luo TY, et al. Neutrophil elastase as a diagnostic marker and therapeutic target in colorectal cancers. *Oncotarget.* (2014) 5:473–80. doi: 10.18632/oncotarget.1631
196. Xiao L, Zhang Y, Berr SS, Chordia MD, Pramoonjago P, Pu L, et al. A novel near-infrared fluorescence imaging probe for *in vivo* neutrophil tracking. *Mol Imaging.* (2012) 11:372–82. doi: 10.2310/7290.2011.00054
197. Wang XQ, Duan XM, Liu LH, Fang YQ, Tan Y. Carboxyfluorescein diacetate succinimidyl ester fluorescent dye for cell labeling. *Acta Biochim Biophys Sin.* (2005) 37:379–85. doi: 10.1111/j.1745-7270.2005.00051.x
198. Liese J, Rooijakkers SH, Van Strijp JA, Novick RP, Dustin ML. Intravital two-photon microscopy of host-pathogen interactions in a mouse model of *Staphylococcus aureus* skin abscess formation. *Cell Microbiol.* (2013) 15:891–909. doi: 10.1111/cmi.12085
199. Bennewitz MF, Watkins SC, Sundd P. Quantitative intravital two-photon excitation microscopy reveals absence of pulmonary vaso-occlusion in unchallenged Sickle Cell Disease mice. *Intravital.* (2014) 3:e29748. doi: 10.4161/intv.29748
200. Egen JG, Rothfuchs AG, Feng CG, Winter N, Sher A, Germain RN. Macrophage and T cell dynamics during the development and disintegration of mycobacterial granulomas. *Immunity.* (2008) 28:271–84. doi: 10.1016/j.immuni.2007.12.010
201. Orthgiess J, Gericke M, Immig K, Schulz A, Hirrlinger J, Bechmann I, et al. Neurons exhibit Lyz2 promoter activity *in vivo*: implications for using LysM-Cre mice in myeloid cell research. *Eur J Immunol.* (2016) 46:1529–32. doi: 10.1002/eji.201546108
202. Hume DA. Applications of myeloid-specific promoters in transgenic mice support *in vivo* imaging and functional genomics but do not support the concept of distinct macrophage and dendritic cell lineages or roles in immunity. *J Leukoc Biol.* (2011) 89:525–38. doi: 10.1189/jlb.0810472
203. Drechsler M, Megens RT, Van Zandvoort M, Weber C, Soehnlein O. Hyperlipidemia-triggered neutrophilia promotes early atherosclerosis. *Circulation.* (2010) 122:1837–45. doi: 10.1161/CIRCULATIONAHA.110.961714
204. Madisen L, Zwingman TA, Sunkin SM, Oh SW, Zariwala HA, Gu H, et al. A robust and high-throughput Cre reporting and characterization system for the whole mouse brain. *Nat Neurosci.* (2010) 13:133–40. doi: 10.1038/nn.2467
205. Yousefi S, Stojkov D, Germic N, Simon D, Wang X, Benarafa C, et al. Untangling "NETosis" from NETs. *Eur J Immunol.* (2019) 49:221–7. doi: 10.1002/eji.201747053
206. Megens RT, Vijayan S, Lievens D, Doring Y, Van Zandvoort MA, Grommes J, et al. Presence of luminal neutrophil extracellular traps in atherosclerosis. *Thromb Haemost.* (2012) 107:597–8. doi: 10.1160/TH11-09-0650
207. Lachowicz-Scroggins ME, Dunican EM, Charbit AR, Raymond W, Looney MR, Peters MC, et al. Extracellular DNA, neutrophil extracellular traps, and inflammasome activation in severe asthma. *Am J Respir Crit Care Med.* (2019) 199:1076–85. doi: 10.1164/rccm.201810-1869OC
208. Jonsson F, De Chaisemartin L, Granger V, Gouel-Cheron A, Gillis CM, Zhu Q, et al. An IgG-induced neutrophil activation pathway contributes to human drug-induced anaphylaxis. *Sci Transl Med.* (2019) 11:eaat1479. doi: 10.1126/scitranslmed.aat1479
209. De Boer OJ, Li X, Teeling P, Mackaay C, Ploegmakers HJ, Van Der Loos CM, et al. Neutrophils, neutrophil extracellular traps and interleukin-17 associate

with the organisation of thrombi in acute myocardial infarction. *Thromb Haemost.* (2013) 109:290–7. doi: 10.1160/TH12-06-0425
210. De Buhr N, Von Kockritz-Blickwede M. How neutrophil extracellular traps become visible. *J Immunol Res.* (2016) 2016:4604713. doi: 10.1155/2016/4604713
211. Doring Y, Manthey HD, Drechsler M, Lievens D, Megens RT, Soehnlein O, et al. Auto-antigenic protein-DNA complexes stimulate plasmacytoid dendritic cells to promote atherosclerosis. *Circulation.* (2012) 125:1673–83. doi: 10.1161/CIRCULATIONAHA.111.046755
212. Mcdonald B, Urrutia R, Yipp BG, Jenne CN, Kubes P. Intravascular neutrophil extracellular traps capture bacteria from the bloodstream during sepsis. *Cell Host Microbe.* (2012) 12:324–33. doi: 10.1016/j.chom.2012.06.011
213. Yipp BG, Petri B, Salina D, Jenne CN, Scott BN, Zbytnuik LD, et al. Infection-induced NETosis is a dynamic process involving neutrophil multitasking *in vivo*. *Nat Med.* (2012) 18:1386–93. doi: 10.1038/nm.2847
214. Tanaka K, Koike Y, Shimura T, Okigami M, Ide S, Toiyama Y, et al. *In vivo* characterization of neutrophil extracellular traps in various organs of a murine sepsis model. *PLoS ONE.* (2014) 9:e111888. doi: 10.1371/journal.pone.0111888
215. Von Bruhl ML, Stark K, Steinhart A, Chandraratne S, Konrad I, Lorenz M, et al. Monocytes, neutrophils, and platelets cooperate to initiate and propagate venous thrombosis in mice *in vivo*. *J Exp Med.* (2012) 209:819–35. doi: 10.1084/jem.20112322
216. Porto BN, Stein RT. Neutrophil extracellular traps in pulmonary diseases: too much of a good thing? *Front Immunol.* (2016) 7:311. doi: 10.3389/fimmu.2016.00311
217. Delgado-Rizo V, Martinez-Guzman MA, Iniguez-Gutierrez L, Garcia-Orozco A, Alvarado-Navarro A, Fafutis-Morris M. Neutrophil extracellular traps and its implications in inflammation: an overview. *Front Immunol.* (2017) 8:81. doi: 10.3389/fimmu.2017.00081

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METHODS
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Detection and Quantification of Histone H4 Citrullination in Early NETosis With Image Flow Cytometry Version 4

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Neutrophil extracellular traps (NETs) formation has been implicated in an increasing number of infectious and non-infectious pathologies. NETosis is a tightly regulated process; the end-stage and read-out is the formation of DNA strands extruded from the nuclei, and traditionally assessed by fluorescence microscopy. Since NETosis has emerged as a possible biomarker of the inflammatory process, there is a need for less time-consuming, consistent, and quantitative approaches to improve its application in clinical assessment of pro-inflammatory conditions. Imaging Flow Cytometry (IFC) combines features of conventional flow cytometry with qualitative power of fluorescence microscopy and has an added advantage of the capability of assessing the early processes leading up to extrusion of the DNA-scaffolded strands. We explored the optimal imaging-based tools that can be used to measure citrullination of H4 in early NETosis. IFC identified and quantified histone 4 citrullination (H4cit3) induced with several known NETosis stimuli (Ionophore, PMA, LPS, Hemin, and IL-8) following treatment periods ranging from 2 to 60 min. Its relationship with other alterations at nuclear and cellular level, such as nuclear decondensation and super-condensation, multi-lobulated nuclei vs. 1-lobe nuclei and cell membrane damage, were also quantified. We show that the early progress of the H4cit3 response in NETosis depends on the stimulus. Our method identifies fast (Ionophore and Hemin), intermediate and slow (PMA) inducers and shows that H4cit3 appears to have a limited contribution to both early LPS- and IL-8-induced NETosis. While this method is rapid and of a higher throughput compared to fluorescence microscopy, detection and quantification is limited to H4cit3-mediated nuclear events and is likely to be stimulus- and signaling pathway dependent.

Keywords: PMN, NETs, H4cit3, imaging flow cytometry, stimulus-dependent

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INTRODUCTION

Neutrophils are key players in the innate immunity system. Resting neutrophils patrol the bloodstream and are rapidly recruited to sites of infection and injury upon activation, contributing to host defense, and inflammation. Activated neutrophils respond directly by engulfing the offending microorganism (phagocytosis), by releasing the content of their granules into the environment (degranulation), by producing and releasing reactive oxygen species, or by making

TABLE 1 | NETs stimuli.

Reagents	Purified neutrophils (2 × 10^6 in 500 μl)						
	RPMI only (control)	Hemin 20 μM	Hemin 5 μM	LPS 1 μg/ml	PMA 100 nM	PMA 20 nM	IL-8 50 pg/ml
(μl)	100	100	100	100	100	100	100
Reaction vol. (μl)	600	600	600	600	600	600	600
PFA 8% (μl)	600	600	600	600	600	600	600

extracellular traps (NETs). NETs formation involves chromatin de-condensation and nuclear swelling, transfer of cytoplasmic granules to the nucleus, and mixing of these granules with nuclear material. Eventually, the cell lyses and releases the nuclear/cytoplasmic mix into the environment. The extracellular DNA string-like structures associate with cytoplasmic azurophilic granule components such as elastase (NE) or myeloperoxidase (MPO), two enzymes with significant anti-pathogen properties. NE is a potent serine-protease that can break down the outer membrane of bacteria, and MPO catalyzes production of hypochlorous acid (HOCl), thus contributing to a more effective local antimicrobial activity. While NETs formation is thought to play a critical role in both infectious and sterile inflammation, their contribution to these pathologies, is yet to be determined (1).

NETs are most commonly studied by fluorescence microscopy through identification of specific markers: citrullination of histone 3 or histone 4 and/or extracellular DNA co-localized with NE/MPO. New methodologies are being developed for both research and clinical purposes including ELISA-based assays (2), automated high throughput live detection of nuclear changes (3), and microfluidics (4). Conventional flow cytometry methods are also available although some limitation have been recognized, such as the choice of the probes or how samples are prepared, that can affect gating (5–7). Imaging Flow Cytometry (IFC) allows high-throughput analysis and surpasses the qualitative limitations of conventional flow cytometry. IFC allows visualization of whole cells and combines the analytical power of fluorescent microscopy with the cell detection and statistical robustness of conventional flow cytometry. IFC-based techniques that use nuclear morphological changes to assess NETosis have been described (8–10).

Given the increasingly recognized role of NETosis in infectious and sterile inflammatory environments, it would be pertinent to capture the early processes that leads to the final stage of NETs formation, as this may be relevant with regard to the infectious and non-infectious triggers. We aimed at developing a detection and quantification method that can measure NETs-related markers in minimally processed neutrophils that come from an environment rich in physiological NETs inducers (e.g., activating cytokines, hemolysis, or bacterial-related products). Here, we present an IFC-based method that permits specific detection and quantification of a recognized NETosis marker, histone H4 citrullination (H4cit3) in addition to morphological nuclear changes. Analysis of other parameters, such as nuclear super-condensation, presence of unsegmented nuclei, and cell membrane damage, can be further used to pinpoint toward additional NETs-related characteristics. These analytical parameters were established not only by using a pharmacological inducer (PMA), but also LPS and IL-8 (inducers associated with infectious pathogens) and Hemin (inducer associated with hemolysis). This method might offer a viable alternative of evaluating NETs for both research and clinical studies with an objective of determining whether *in vivo* inhibition of NETs might work as a therapeutic strategy for diverse pathologies.

MATERIALS, EQUIPMENT, AND METHOD

Human Samples

Samples were obtained from healthy volunteers (study protocol NCT0004799) after obtaining written informed consent; the study was approved by the NHLBI Institutional Review Board.

Neutrophils Isolation, NETosis Induction, and Quantification

Whole blood was collected in EDTA vacutainers and neutrophils were separated with sterile Polymorphprep gradient medium (Cosmo Bio USA, cat # AXS-1114683) as recommended by the manufacturer. The remaining red blood cells (RBCs) were removed by treatment with ice-cold sterile water for 30 s followed by the addition of sterile KCl 0.6 M. The purified neutrophils were allowed to rest in the incubator, at 37°C, for 30 min.

NETosis was induced with several reagents, as described in **Table 1**. The experimental scheme outlined in **Figure 1** describes treatment times with the agonists (between 2 and 60 min), and details of the fixing, permeabilization, and staining times, in minutes at room temperature (RT) or overnight (ON) at 4°C. Donors' contribution to experiments and the number of independent repeats are detailed in **Table S1**. To assess early histone citrullination response in the absence of an extracellular source of calcium, purified healthy neutrophils were resuspended in DPBS without $CaCl_2$ and $MgCl_2$, that was supplemented back with 4.5 mM $MgCl_2$ and then stimulated with A23187 4 μM as described in **Figure 1**. (Source of reagents—Calcium Ionophore A23187, Hemin, cat#: 4039 and PMA, cat#: P1585 were from Sigma Aldrich. LPS was from Invitrogen, cat#: tlrl-3pelps and human IL-8 was from Peprotech, cat#: 200-08). Reactions were terminated with 4% PFA and fixed cells were then sequentially stained for CD66b, H4cit3, MPO, and DNA. Briefly, neutrophils were first stained with anti-Human CD66b-PE, clone G10F5 (Biolegend, cat#: 305106) then permeabilized with BD Cytofix/Cytoperm (BD Biosciences, cat#: 554722). A blocking step with 3% BSA in 1xDPBS, no calcium, no magnesium, + 0.2% porcine skin gelatin type A (Sigma, cat#: G1890) was conducted before addition of the primary antibody, rabbit polyclonal anti-histone H4, citrulline 3 (H4cit3, EMD Millipore, cat#: 07-596). Secondary antibody goat anti-Rabbit IgG, DyLight680 (Thermo Fisher, cat#: 35568) and the MPO staining (MPO Polyclonal

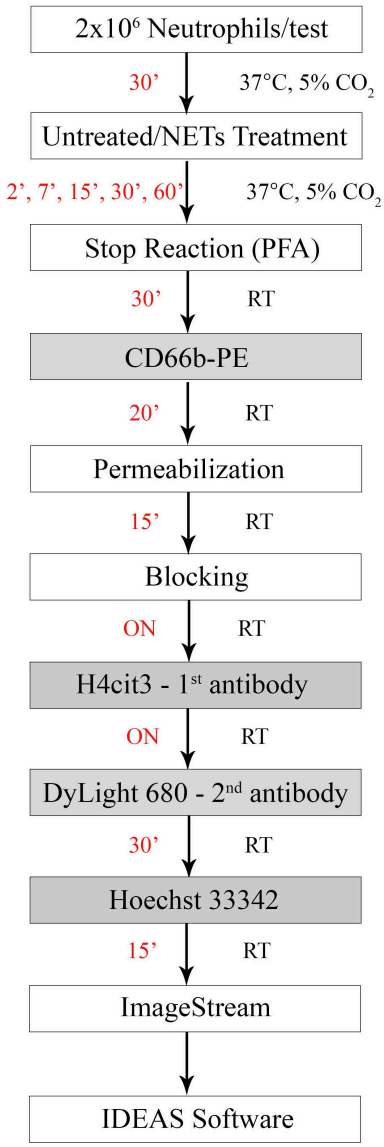

FIGURE 1 | Experimental staining scheme for specific early NETosis detection by imaging flow cytometry. Briefly, purified neutrophils were allowed to rest in the incubator for 30 min (30') prior to the treatment step. NETs agonists were added at the concentrations specified in **Table 1** for various times between 2 min (2') and 60 min (60'). Stimulation was stopped by adding paraformaldehyde (PFA) for 30 min (30') at room temperature. Staining steps are underlined in the gray boxes and staining times for each step are in red. Total protocol time from neutrophil isolation to stained samples ready for imaging flow cytometer acquisition is 3 days. (ON, over night; RT, room temperature).

AlexaFluor 594 Conjugated, Bioss Antibodies, cat#: bs-4943R-A594) were conducted together. The nuclear staining with Hoechst 33342 (BD Pharmingen, cat#: 561908) was last.

For each test 20,000 events were acquired with an ImageStream Mark II imaging flow cytometer (Millipore Sigma, Seattle, WA, USA). Single color controls for each marker were used as compensation controls and unstained cells were used to determine background. While initial tests were conducted including isotype controls, for experimental work FMOs (Fluorescence Minus One) were used to establish proper gating and identify possible unspecific binding or flaws in our panels. Efficiency of the blocking step was confirmed by quantifying the signal for the secondary antibody (DyLight680), in the absence of the primary antibody (H4cit3). Nuclear or cellular changes were identified and quantified by using the IDEAS software. These parameters included histone H4 citrullination signal, MPO colocalization with the nuclear compartment, nuclear decondensation, and super-condensation, and damage of the cellular membrane. Step-by-step acquisition and analysis guide are available in the **Supplemental Material**.

Quantification of Apoptotic Neutrophils

Purified neutrophils were treated with Hemin, LPS, PMA, and IL-8 as described in **Table 1** and then apoptotic and necrotic cells were detected with a FITC Annexin V Apoptosis Detection Kit with 7-AAD (Biolegend, cat#: 640922). Stained cells were acquired with a BD LSRFortessa (BD Bioscience) and data was analyzed with FlowJo.

Statistical Analysis

Data is presented as dot plots (RPMI and stimulus) with mean shown or, as percent change from the RPMI sample (i.e., untreated control) with the average value of all repeats per stimulus ± S.D. presented (100 = no change from the untreated control). Statistical analyses were performed with PRISM7 software (Graph Pad Software, CA). Students *t*-test was used for two group comparisons. ANOVA with Bonferroni or Dunnett's post tests were used to compare more than two groups. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

RESULTS

Progress of H4cit3-Dependent Early NETosis Is Stimulus-Dependent

Changes in nuclear decondensation and histone H4 citrullination (H4cit3)-expressing neutrophils were assessed with imaging flow cytometry (IFC) within 60 min (60′) of treatment with the stimuli. A change in the nuclear morphology from multi-lobular and well-organized to swelled and fuzzy-looking has been regarded as an early marker of neutrophils undergoing NETosis (10). This change can be monitored by using "Bright Detail Intensity_R3" (BDI_R3) feature in the IDEAS software that identifies normal, lobular nuclei as high intensity dye spots with a radius of three pixels or less, and fuzzy, decondensed nuclei as wide areas of low intensity staining (**Figure 2A**). Examples of H4cit3 positive staining in normal lobulated nuclei, and decondensed nuclei are shown in **Figure 2B**.

These features have been previously used to detect LPS-induced NETosis (10). We extended it to several stimuli (Hemin, PMA, and IL-8), some at two different concentrations (Hemin at 20 μM and 5 μM and PMA at 100 and 20 nM). We detected LPS- and IL-8-induced minimal changes in nuclear decondensation at any of the five tested time points. In contrast, PMA 100 nM already caused a significant increase in nuclear decondensation

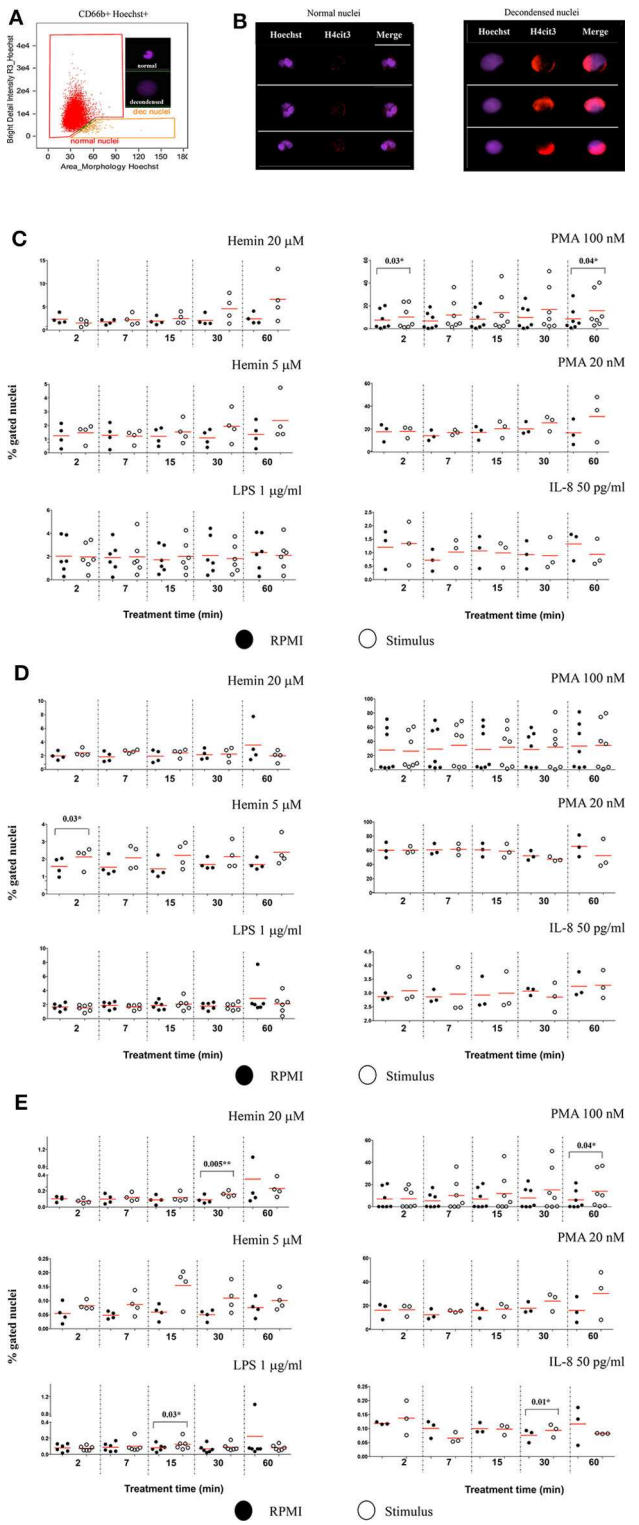

FIGURE 2 | Markers of NETosis can be identified after short and very short periods of treatment with the stimuli. **(A)** Identification of nuclear decondensation using dot-plot of area of the nuclear morphology mask vs. Bright Detail Intensity_R3 feature (normal, multi-lobed nuclei in the red gate, decondensed nuclei in the tangerine gate). **(B)** Examples of neutrophils

(Continued)

FIGURE 2 | specimens positive for H4cit3 (red) in the normal nuclei and decondensed nuclei (purple). Purified healthy neutrophils were left untreated in RPMI or treated with the specified NETs stimuli for the indicated lengths of time. **(C)** Percent neutrophils with decondended nuclei. **(D)** H4cit3 positive normal nuclei. **(E)** H4cit3 positive decondensed nuclei. Untreated cells (filled symbols); treated cells (empty symbols). A variable number of experiments were conducted for each stimulus ($N = 4$ for Hemin 20 µM; $N = 4$ for Hemin 5 µM; $N = 6$ for LPS 1 µg/ml; $N = 7$ for PMA 100 nM; $N = 3$ for PMA 20 nM; $N = 3$ for IL-8 50 pg/ml). Paired *t*-test was used to compare the response in untreated and treated neutrophils. $^{*}P < 0.05$, $^{**}P < 0.01$.

after only 2 min (2') of treatment ($P = 0.03^{*}$) and another strong increase at 60 min ($P = 0.04^{*}$). The two Hemin concentrations needed at least 30 min (30') to induce nuclear decondensation and while the response was clearly increased, the extent was below that elicited by the high PMA concentration and not yet statistically significant (**Figure 2C**).

We next quantified H4 citrullination (H4cit3), an accepted marker of NETosis, in both normal, segmented nuclei, and in the decondensed ones. With the exception of the two Hemin concentrations, none of the other stimuli induced significant changes in H4cit3 in the normal nuclei. The lower Hemin concentration, 5 µM, caused a rapid and statistically significant increase, only after 2 min of treatment ($P = 0.03^{*}$) (**Figure 2D**). In the decondensed nuclei, IL-8 did not appear to have an effect on H4cit3, while LPS had a limited one ($P = 0.03^{*}$ at 15 min), in agreement with the noted lack of nuclear decondensation. PMA 100 nM induced an increase in H4cit3 in a time-dependent manner, significant at the longest treatment time, 60 min ($P = 0.04^{*}$). The H4cit3 responses caused by the two Hemin concentrations were clearly increased and statistically significant at longer length of treatment ($P = 0.005^{**}$ at 30 min for Hemin 20 µM) (**Figure 2E**, **Figure S1C**) and matched an apparent increase in nuclear decondensation at these longer time points. A summary of by-stimulus changes in the nuclear decondensation (**Figure S1A**) and in the amount of the decondensed nuclei positive for H4cit3 (**Figure S1B**) confirmed that, following NETs stimulation, more neutrophils responded by increasing H4 citrullination rather than by increasing nuclear decondensation. Generally, the increase in the amount of cells with decondensed and H4cit3+ nuclei was significant at 30 min of treatment. Hemin 20 µM ($P = 0.02^{*}$) was more efficient than Hemin 5 µM, while both LPS and IL-8 caused a mild response ($P = 0.04^{*}$) (**Figure S1B**). The lack of nuclear decondensation and H4 citrullination with the LPS and IL-8 stimulations prompted additional testing to confirm strand-like NETs formation. Immunofluorescence microscopy confirmed that NETs agonists that did not elicit responses in the IFC testing (LPS and IL-8 in **Figure S2**), as well-agonists that caused responses (both Hemin concentrations in **Figure S3**) did form DNA-Elastase strands. Our data show that while NETs strands are produced following LPS and IL-8 stimulation, this process likely does involve extensive histone 4 citrullination. While our detection method does not provide mechanistic insights, the variability of the readouts suggests that early NETs H4cit3-mediated progression depends on the employed stimulus, and

likely on their associated signaling pathways. Follow up studies on the intracellular signaling mediated by TLR-4 (for LPS and Hemin), CXCR1/2 (for IL-8), and PKC (for PMA) should provide additional information on the involvement of these pathways to early NETosis events.

Calcium Ionophore Is a Fast Inducer of Histone H4 Citrullination in the Absence of Extracellular Calcium

Calcium ionophore is a noted NETs inducer, with fast kinetics and specific signaling pathways and a requirement for extracellular calcium to form NETs following H3 citrullination (11). As our detection method targets H4 citrullination as a NETs marker we asked whether the presence or absence of an extracellular source of calcium was relevant. Calcium ionophore A23187 was used to stimulate neutrophils without a source of extracellular calcium and nuclear decondensation and H4cit3 in normal and decondensed nuclei were evaluated. We found a significant increase in percent of neutrophils with decondensed nuclei following 30 min of treatment ($P = 0.03^*$) (**Figure 3A**). A significant decrease in neutrophils with H4cit3+ normal nuclei was notable after only 2 min of treatment ($P = 0.02^*$), and remained so for the immediately following tested time points ($P = 0.002^{**}$ at 7 min and $P = 0.01^*$ at 15 min). This decrease was complemented at these short treatment periods by a significant increase in neutrophils with H4cit3+ decondensed nuclei ($P = 0.01^*$ at 2 min and $P = 0.02^*$ at 7 min) (**Figure 3B**). An evaluation of the Mean Fluorescence Intensity (MFI) for H4cit3 in normal nuclei found a significant decrease at 7 min ($P = 0.04^*$) (**Figure S4A**). This matched the decrease in H4cit3-expressing cells at this time point. In decondensed nuclei H4cit3 signal intensity decreased significantly after 60 min of treatment ($P = 0.01^*$) (**Figure S4A**). By using IFC we were able to confirm the fast kinetics of calcium ionophore-induced NETosis and also show that nuclear changes associated with NETs in the absence of extracellular calcium are H4cit3-dependent.

The recruitment of the primary granules (MPO, elastase) to the nuclear compartment have been associated with NETs formation (12). We explored whether A23187 induced MPO translocation to the nucleus, and how it related with histone H4 citrullination and nuclear decondensation. For this, we employed an IDEAS analysis feature called Similarity, that calculates the degree to which two images (the nuclear stain and the MPO stain) correlated within a target area (i.e., the nuclear area), commonly defined as a mask. Briefly, when MPO is located in the cytoplasm the intensity of its staining is low at the nuclear location, (i.e., it has a dissimilar distribution compared to the Hoechst-stained nuclear mask). When the intensity of both dyes at the nuclear location is high, the image pair has a high degree of similarity and thus, high scores indicate co-localization. We did not confirm higher scores indicating DNA-MPO co-localization after A23187 stimulation, neither in the normal nuclei gate nor in the decondensed nuclei gate (**Figure 3C**). We did note, that in both normal and decondensed nuclei, the highest scores were found at the shortest stimulation times followed by consistent and time-dependent significant decrease. We asked whether this dissimilarity can be associated with a low amount of MPO available and determined the intensity of its fluorescence signal (**Figure S4B**). MPO signal was decreased in the cells with normal nuclei after only 2 min of treatment ($P = 0.03^*$) and remained significantly decreased at 60 min ($P = 0.01^*$), confirming lower MPO amounts in the cells. As A23187 induces neutrophil degranulation (13), release of the type I granules, containing MPO, might account for the decrease in the MPO availability.

Nuclear Citrullination but Not MPO Co-Localization Marks Early NETosis

We also checked the DNA-MPO Similarity scores in the normal and decondensed nuclei for neutrophils treated with Hemin, LPS, PMA, and IL-8. Confirming the viability of the method, decondensed nuclei had consistently higher DNA-MPO Similarity scores than the normal nuclei, both in untreated and treated samples (**Figures S5A,B**). We did not confirm higher scores indicating MPO translocation to the nucleus after NETs challenge, neither in the normal nuclei gate nor in the decondensed nuclei gate. Overall, our data indicated either no change, or a decrease in the DNA-MPO co-localization in the treated samples as compared to the untreated control (**Figure S5C**). We next sought to determine whether following stimulation with the NETs agonists the intracellular levels of H4cit3 or MPO changed (as exemplified in **Figure S6A**). None of the treatments except PMA 100 nM, increased H4cit3 signal intensity in the normal nuclei (**Figure 4A**, $P = 0.01^*$). In the decondensed nuclei 3 stimuli induced significant increase in H4cit3 signal at the longer stimulation. LPS-induced increase was significant at 30 min ($P = 0.001^*$) suggesting that H4 citrullination might only have a limited contribution to NETs formation by this inducer. Hemin 5 μM caused a notable increase at both 30 and 60 min (with a $P = 0.02^*$ for both time points) and Hemin 20 μM was efficient at even shorter stimulation ($P = 0.002^{**}$ at 15 min and $P = 0.01^*$ at 30 min). Overall, these data show that while IFC did not confirm DNA-MPO co-localization it did show significant stimulus-dependent changes in H4cit3 levels, consistent with NETs-associated morphological nuclear changes (decondensation).

Terminally differentiated neutrophils do not produce *de novo* MPO following stimulation thus no increase in signal was expected. Stimulus-induced degranulation might explain the mild decrease in the intensity of the MPO signal observed after the NETs treatments (**Figure S6B**).

In Early NETosis, Stimulus-Dependent Histone H4 Citrullination Occurs in 1-Lobe Nuclei

The IFC can provide an insight in the relationship between changes in histone H4 citrullination and nuclear segmentation.

The Lobe Count in IDEAS software uses "Symmetry 2, 3, 4" features on the DNA staining channel to count the nuclear lobes, by measuring the number of axis of elongation in a nucleus (for example a nucleus with a single axis will have two lobes and score high in the Symmetry 2 feature), and then

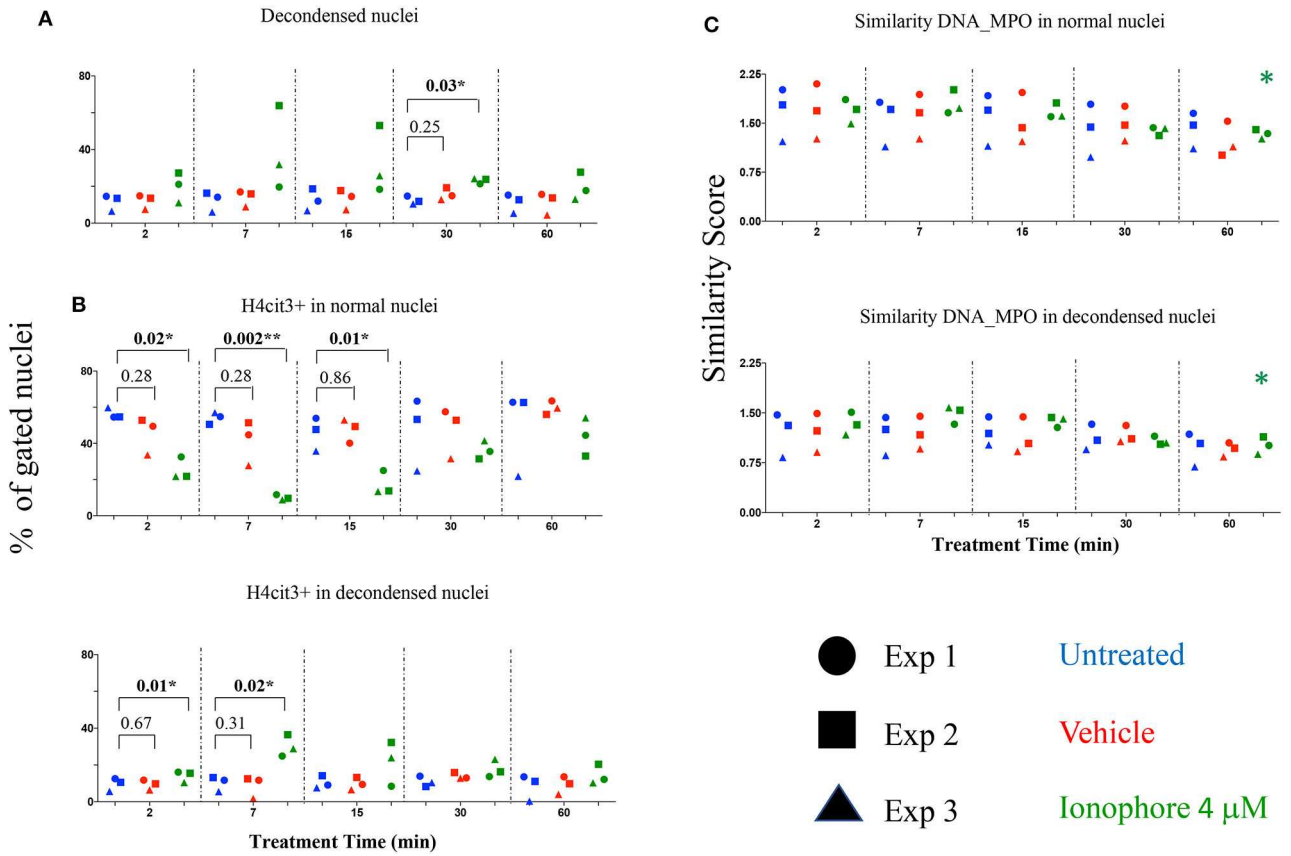

FIGURE 3 | Calcium ionophore treatment induces a rapid change in histone citrullination in both normal and decondensed neutrophil nuclei in the absence of extracellular calcium. Neutrophils purified from three healthy donors were left in DPBS (blue), or treated for the indicated times with vehicle only, DMSO (red), or A23187 4 μM (green) **(A)** Percent neutrophils with decondensed nuclei. **(B)** Percent neutrophils with normal and decondensed nuclei positive for H4cit3. **(C)** Similarity scores for DNA (Hoechst stain) and MPO, marking co-localization of two distinct cellular compartments, nucleus, and type I granules. Paired *t*-test was used to compare untreated with vehicle and ionophore 4 μM, respectively at each time point; Anova for panel ionophore-mediated response in **(C)** $^{*}P < 0.05$, $^{**}P < 0.01$.

bins together the cells that match the criteria. Neutrophils have two to five nuclear segments (i.e., lobes), 40–50% are 3-lobed, about 25%, 2-lobed or 4-lobed, and <10% are 5-lobed. In the untreated samples, we found that generally <1% neutrophils had unsegmented nuclei, and about 40% of the cells were 3-lobed (**Figure S7A**). After 60 min of treatment with PMA 100 nM, the number of cells with unsegmented nuclei increased by almost 10-fold while the number of those having 3-lobe nuclei decreased discernably (**Figure S7B**). LPS, IL-8, and PMA 20 nM had no effect on nuclear segmentation at any of the checked time points (**Figure 5A**). PMA 100 nM and Hemin 20 μM induced significant increases at few of the checked treatment time points, with the first showing the strongest increase after 15 min ($P = 0.001^{**}$) and the latter after 60 min ($P = 0.0003^{***}$). However, it was the low Hemin concentration that had the most substantial effect on nuclear segmentation. We found that Hemin 5 μM caused a significant increase in cells with 1-lobe nuclei already at 2 min of treatment ($P = 0.01^{*}$), up until 60 min ($P = 0.04^{*}$), with the biggest change at 30 min ($P = 0.001^{**}$) (**Figure 5A**, **Figure S7C**). We asked whether an increase in the amount of 1-lobe nuclei associated with an increase in H4cit3 signal in these nuclei. For this we looked at the 1-lobe nuclei amount (**Figure 5A**) vs. the H4cit3 signal intensity in these nuclei (**Figure 5B**). For the two Hemin concentrations the increase in H4cit3 intensity generally matched the increase in the 1-lobe nuclei. At the later time points, 30 and 60 min, Hemin 5 μM induced a significant increase in H4cit3 expression ($P = 0.03^{*}$ and $P = 0.007^{**}$, respectively), strongly suggesting that, for this stimulus, histone citrullination increased in the 1-lobe rather than in the segmented nuclei.

Imaging Flow Cytometry Offer Additional Analysis Features to Characterize Early NETosis Signature

Due to the subjective and semi-quantitative nature of the majority of methodology currently available for NETs studies, specific identification of NETs formation from other cellular processes is still a challenge (14). Our high throughput data suggested that H4 citrullination occurred in nuclei that would lose their organized structures and decondense. Depending on the stimulus, this process was rapid (Hemin) or slower

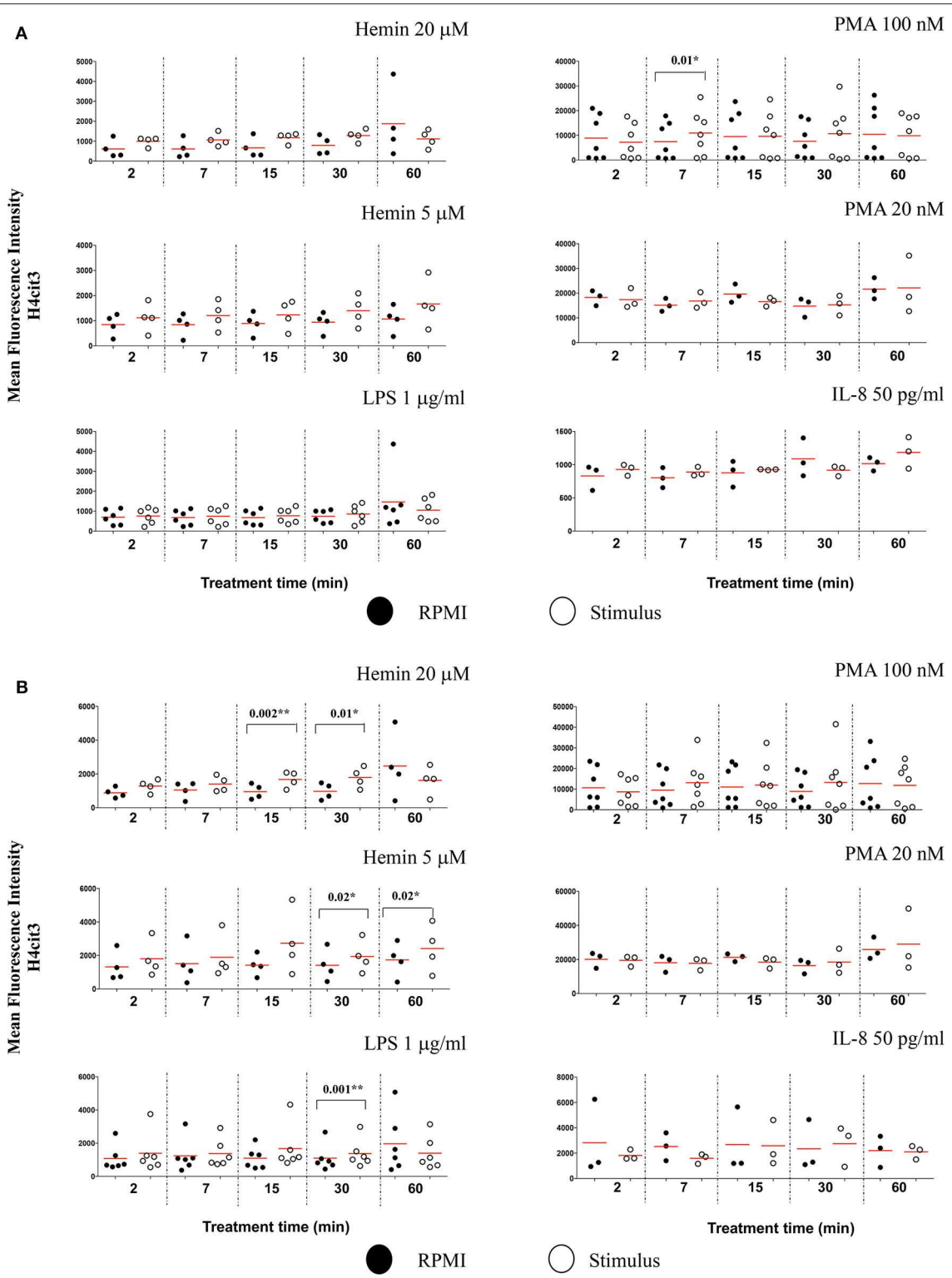

FIGURE 4 | Nuclear decondensation associates with intensity of the H4cit3 signal and is stimulus-dependent. Purified and rested healthy neutrophils were left in RPMI or treated for different lengths of time with NETs inducers as indicated. **(A)** Mean Fluorescence Intensity for H4cit3 in normal nuclei. **(B)** Mean Fluorescence Intensity for H4cit3 in decondensed nuclei. Paired *t*-test was used to compare the response in untreated and treated neutrophils. A variable number of experiments were conducted for each stimulus ($N = 4$ for Hemin 20 µM; $N = 4$ for Hemin 5 µM; $N = 6$ for LPS 1 µg/ml; $N = 7$ for PMA 100 nM; $N = 3$ for PMA 20 nM; $N = 3$ for IL-8 50 pg/ml). Filled symbols, RPMI control; empty symbols, stimulus. Paired *t*-test was used to compare the response in untreated and treated neutrophils. $^{*}P < 0.05$, $^{**}P < 0.01$.

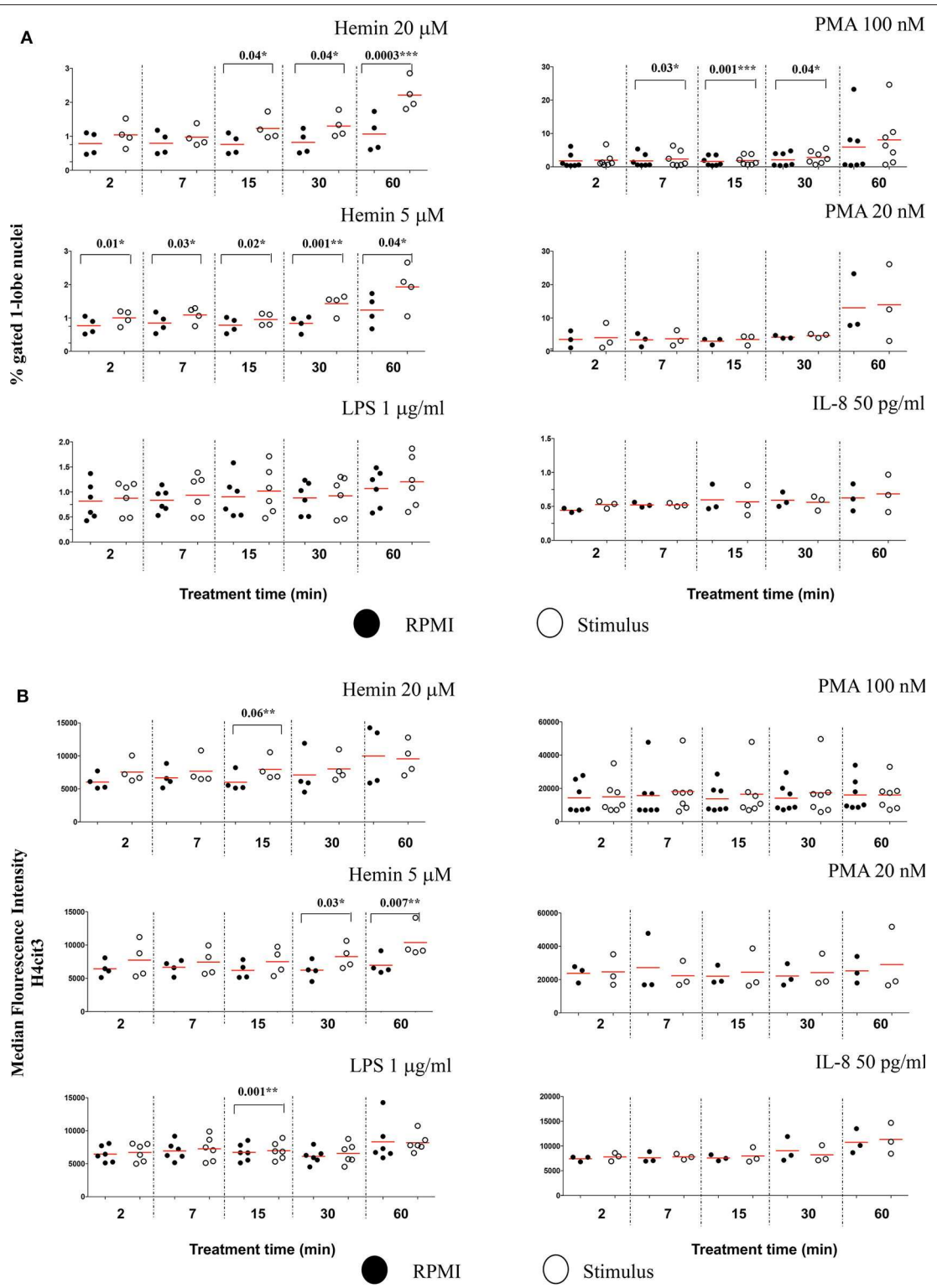

FIGURE 5 | Decrease in neutrophil nuclear segmentation is stimulus- and time-dependent and associates with increased H4 citrullination. **(A)** Purified healthy neutrophils were treated with NETs stimuli for the specified periods of time and percent of neutrophils with 1-lobe nuclei was quantified by using Lobe Count Feature in IDEAS software. **(B)** Median Fluorescence Intensity of the H4cit3 signal in 1-lobe normal nuclei. A variable number of experiments were conducted for each stimulus. Filled symbols, RPMI control; empty symbols, stimulus. Paired *t*-test was used to compare the response in untreated and treated neutrophils. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

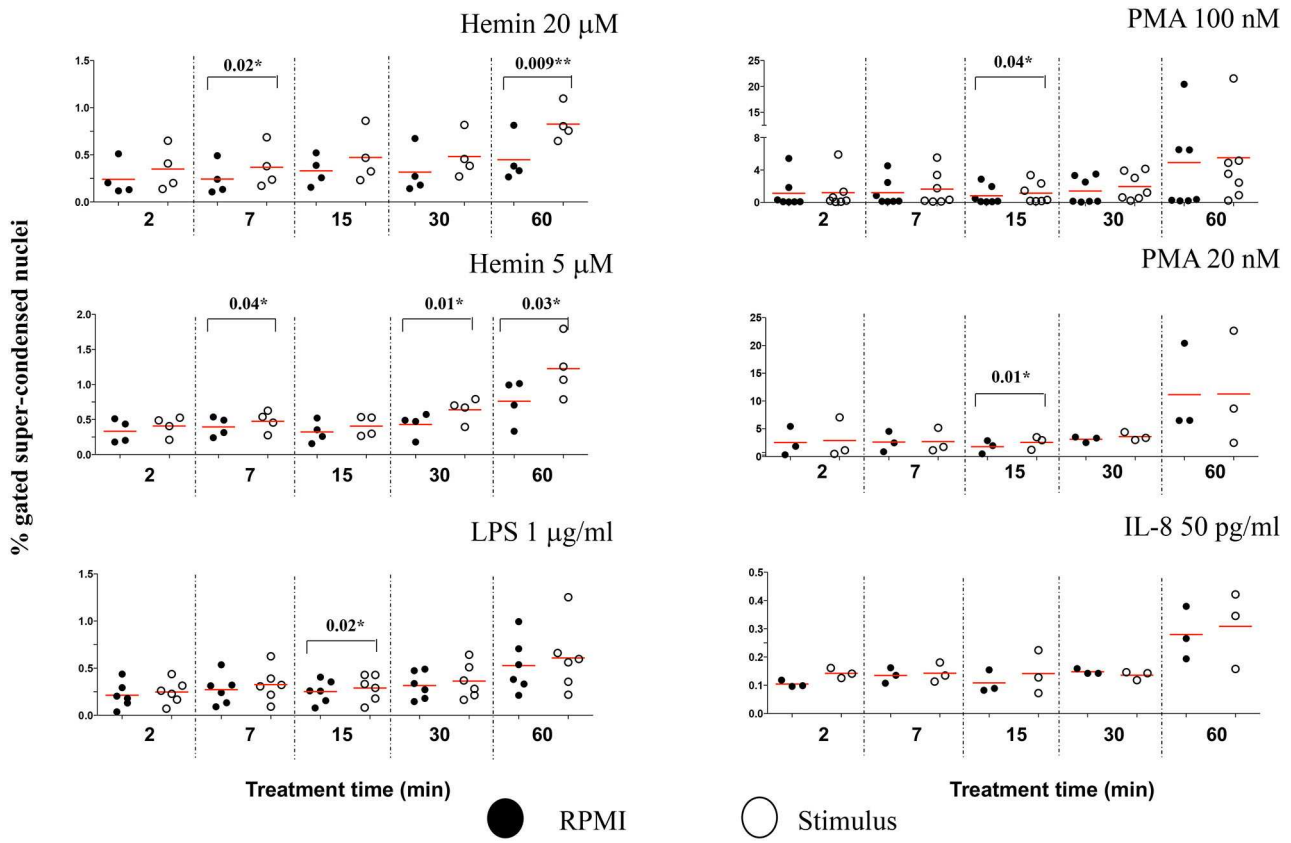

FIGURE 6 | The level of nuclear super-condensation caused by the NETs inducers varies with the stimulus. Purified healthy neutrophils were treated with the NETs stimuli for the shown lengths of time. Percent cells with super-condensed 1-lobe nuclei were identified and quantified by plotting nucleus/whole cell Area vs. Morphology Area (nuclear stain—Hoechst). An example of these analysis features is shown in **Figure S8A**. A variable number of experiments were conducted for each stimulus. Filled symbols, RPMI control; empty symbols, stimulus. Paired *t*-test was used to compare the response in untreated and treated neutrophils. $^{*}P < 0.05$, $^{**}P < 0.01$.

(PMA), and associated with loss in nuclear segmentation. We asked whether IFC could offer another tool for identification of morphological changes in these unsegmented nuclei. The chromatin undergoing strong condensation ahead of the dismantling of the nuclear envelope is recognized as a marker of cellular death (apoptosis). Used together, two analysis features in IDEAS software, dot plot of "Area of nucleus" vs. "Ratio of nuclear by the whole cell areas" can discriminate between levels of nuclear super-condensation in untreated and treated neutrophils (**Figure S8A**). With the exception of IL-8, all stimuli caused a statistically significant increase in the nuclear super-condensation. LPS, PMA 100 nM, and PMA 20 nM were particularly efficient at 15 min ($P = 0.02^{*}$, $P = 0.04^{*}$, and $P = 0.01^{*}$, respectively). Hemin was efficient as early as 7 min (Hemin 20 μM, $P = 0.02^{*}$ and for Hemin 5 μM, $P = 0.04^{*}$) and also after 60 min (Hemin 20 μM $P = 0.009^{**}$ and for Hemin 5 μM, $P = 0.03^{*}$) (**Figure 6**). Clear nuclear morphological differences were underlined in NETosis (decondensation) and apoptosis (super-condensation). While H4cit3 is a recognized marker of NETosis, Annexin V expression is often used as a marker of apoptosis. We employed conventional flow cytometry to determine whether we can complement our IFC findings for the Hemin-mediated stimulation with a positive detection of a known apoptosis marker, Annexin V. Both Hemin concentrations induced a time-dependent increase in Annexin V expression with a statistical significance at 60 min (Hemin 20 μM, $P = 0.02^{*}$ and Hemin 5 μM, $P = 0.03^{*}$) (**Figures S9B,C**). These finding are in agreement with the increase in super-condensation determined at 60 min with the IFC technique. While boosting confidence for accurate detection of NETosis, our findings also offer an option for preliminary detection of apoptosis-related events at the same time.

To determine whether other cellular changes can be associated with the observed increase in chromatin citrullination, we used "Standard deviation" and "Modulation" features in IDEAS analysis software to assess the cell membrane damage. The Standard Deviation Feature indicates the pixel intensity standard deviation values in the mask and provides an indication of the complexity of an object. Modulation Feature measures the intensity range of an image, and this can be used to quantify image quality and characterize contrast and texture in cells. High values for Standard deviation in darkfield (SSC channel) and

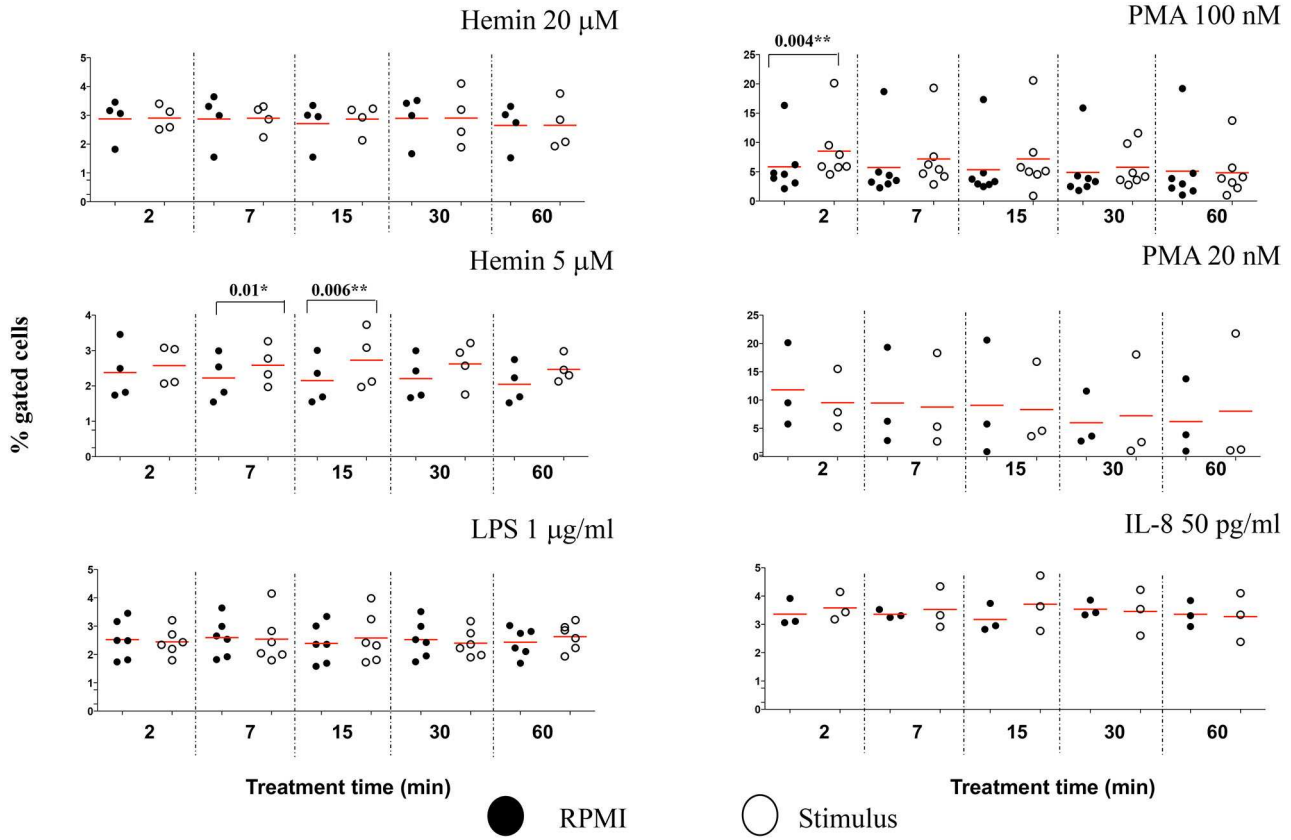

FIGURE 7 | NETs inducers cause minimal damage to neutrophil cell membranes. Following NETs stimulation neutrophils with damaged cell membranes were identified and quantified by plotting Standard Deviation feature on the SSC channel against Modulation in Brightfield, as exemplified in **Figure S10A**. A variable number of experiments were conducted for each stimulus. Filled symbols, RPMI control; empty symbols, stimulus. Paired *t*-test was used to compare the response in untreated and treated neutrophils. *$P < 0.05$, **$P < 0.01$.

for Modulation in brightfield indicate neutrophils with damaged cell membranes (**Figure S10A**). We showed that PMA 100 nM caused a rapid increased in the percentage of neutrophils with damaged cellular membranes, following just 2 min of treatment ($P = 0.004^{**}$), while the lower Hemin concentration efficiently induced cell membrane changes after 7 and 15 min of treatment ($P = 0.01^{*}$ and 0.006^{**}, respectively) (**Figure 7**). No significant changes for this analysis parameter were noted during the 60 min of the treatment with any of the used stimuli (**Figure S10B**). In comparison with healthy neutrophils, IFC competently detected changes cell membrane texture in neutrophils isolated from patients with sickle cell disease at steady state. This environment, known for its high inflammatory potential and high levels of free-circulating heme, was also directly linked with an increased potential for NETs production (15). Thus, while it does appear that NETs-associated cell membrane damage is stimulus-dependent, it might nevertheless be a useful parameter to follow up while characterizing NETosis.

DISCUSSION

The association of NETs with an increasing number of infectious and non-infectious pathologies calls for more effective and consistent approaches for NETosis assessments. Currently, a lot of the testing is conducted by using fluorescence microscopy to observe extracellular DNA co-localized with NE and/or MPO. Other approaches use quantification of extracellular DNA in cell-free supernatant or examine cellular viability with nuclear counterstains that are impermeant to live cells. Increased plasma levels of cell-free DNA, histones, or NETs associated molecules, such as NE or MPO, have been measured in some inflammatory pathologies (1). Presently, the NETotic capacity of pathological environments is generally assessed indirectly by treating healthy neutrophils with blood-derived fluids from patients and then measuring the NETs response. NETs might also function as biomarkers in certain diseases and provide information regarding the efficacy of a treatment regimen (16). Cell-free DNA or histones are certainly increased in many of the inflammatory diseases, but whether this reflects NETs production or simply the state of dying cells remains unclear. NETs-associated components such as NE or MPO are present in the plasma of individuals with many autoimmune diseases and also in infections, which complicates the specificity of these molecules as biomarkers. In this light there is a great need for alternative methodology for NETs characterization that circumvents the subjective nature of microscopy analysis and offers consistent

criteria between studies, reliable for both mechanistic and clinical applications.

We have developed this IFC protocol as a consistent approach for specific, objective and quantitative measurement of a well-established NETs marker (histone H4 citrullination) in combination with several other parameters that can further characterize nuclear and cellular changes associates with NETosis.

In our experience a few steps are critical for the experimental quality and reproducibility of this technique. Due to unavailability of a primarily conjugated antibody for this marker a primary antibody and fluorescent secondary antibody system are used for detection of citrullinated histone 4 in this protocol. For accurate detection and quantification of the H4cit3+ signal, the effectiveness of the blocking buffer is essential to prevent non-specific binding of the secondary antibody (DyLight680). It is thus strongly recommended that the efficiency of the blocking buffer should be confirmed, at the beginning of the experimental work by detecting the DyLight680 antibody signal in the absence of the H4cit3 primary antibody. For data analysis with the IDEAS software, custom masks should be employed because they represent more accurately the region of interest, rather than the default masks. Custom masks for total cell area and nucleus should be first validated for accuracy, by confirming that the masks are indeed masking the region(s) of interest (i.e., not missing parts of the target region or, on the opposite, masking "ghost" regions outside of what is relevant for the features) (17).

Marked variability in the specificity/reactivity of different antibodies targeting histones deimination on different histones has been reported (18). Multiple members of the PAD family mediate conversion of arginine to citrulline on different histones. PAD4 mediates deimination of histone 3 and histone 4, both routinely observed in NETs studies. Previous studies indicate that histone citrullination might be stimulus-depended. For example, PMA has been reported to induce histone H3 deimination in mouse (19). In humans, H3 citrullination might not be required for PMA-induced NETs formation after a 2-h long treatment (18). PAD4-mediated citrullination following shorter stimulation is less understood.

The protocol we have developed is sensitive enough to detect the H4cit3 marker in neutrophils treated for under 1 h with various NETs inducers. Our data show that H4cit3 site is relevant for PMA-induced NETosis, at both low and high concentrations, within the 1st hour of stimulation (**Figure 2** and **Table 2**). However, while pharmacological stimuli are convenient tools for research, they may not be so relevant and translatable for clinical purposes. Hence, in our study we included LPS, IL-8, and Hemin, products that are relevant clinically. We showed that the H4cit3 does not appear to be majorly involved in early NETosis induced with LPS and IL-8, but appears to strongly contribute to NETosis mediated by Hemin (**Figures 2D**, **4B**).

Because of its potential contribution to initiation and/or progression of pathological conditions, infection-related NETosis has been studied extensively in both mouse and human models (20, 21). Newer data shows that that PAD4-mediated citrullination of histone 3 is required for LPS-induced NETs formation (22, 23). Here, we showed that PAD-4-mediated H4cit3, did not appear to contribute notably to LPS-mediated NETosis. Nor was this site involved extensively in IL-8 mediated

TABLE 2 | NETs stimuli time-dependent response efficiency.

Parameters	Hemin 20 μM	Hemin 5 μM	LPS 1 μg/ml	PMA 100 nM	PMA 20 nM	IL-8 50 pg/ml
2 min						
Decondensed nuclei (%)					*	
H4cit3 in normal nuclei (%)					**	
H4cit3 in decondensed nuclei (%)					**	
H4cit3 in normal nuclei (MFI)					**	
H4cit3 in decondensed nuclei (MFI)					**	
1-lobe normal nuclei (%)					*	
Super-condensed normal nuclei (%)						
Damaged membrane and normal nuclei (%)						
7 min						
Decondensed nuclei (%)				*	**	
H4cit3 in normal nuclei (%)					**	
H4cit3 in decondensed nuclei (%)				*	*	
H4cit3 in normal nuclei (MFI)					*	
H4cit3 in decondensed nuclei (MFI)						
1-lobe normal nuclei (%)						
Super-condensed normal nuclei (%)						
Damaged membrane and normal nuclei (%)						
15 min						
Decondensed nuclei (%)				*	**	
H4cit3 in normal nuclei (%)					**	
H4cit3 in decondensed nuclei (%)					*	
H4cit3 in normal nuclei (MFI)					*	
H4cit3 in decondensed nuclei (MFI)					*	
1-lobe normal nuclei (%)					**	
Super-condensed normal nuclei (%)					**	
Damaged membrane and normal nuclei (%)						
30 min						
Decondensed nuclei (%)				*	**	
H4cit3 in normal nuclei (%)					*	
H4cit3 in decondensed nuclei (%)				*	**	
H4cit3 in normal nuclei (MFI)					*	
H4cit3 in decondensed nuclei (MFI)				*	*	
1-lobe normal nuclei (%)					**	
Super-condensed normal nuclei (%)					**	
Damaged membrane and normal nuclei (%)						
60 min						
Decondensed nuclei (%)				*	***	
H4cit3 in normal nuclei (%)						
H4cit3 in decondensed nuclei (%)				*	***	
H4cit3 in normal nuclei (MFI)					**	
H4cit3 in decondensed nuclei (MFI)					**	
1-lobe normal nuclei (%)					*	
Super-condensed normal nuclei (%)						
Damaged membrane and normal nuclei (%)						

*Anova with Dunnett's Multiple Comparison Test was used to compare stimuli response to the control group (RPMI) at each of the tested time points. (RPMI, N = 27; Hemin 20 μM, N = 4; Hemin 5 μM, N = 4; LPS 1 μg/ml, N = 6; PMA 100 nM, N = 7; PMA 20 nM, N = 3; IL-8 50 pg/ml, N = 3). *P < 0.05, **P < 0.01, ***P < 0.001.*

NETosis. IL-8 is an early marker of infection and its production is increased under LPS stimulation (24). Our observations suggest that NETs production under infectious and non-infectious conditions appear to follow distinct paths, likely from the initiation of the process.

Additionally, the Hemin-related results of our study might be of particular interest in chronic hemolytic conditions, where cell-free heme (Hemin), an oxidized product of the hemoglobin, is released from erythrocytes. Heme is a DAMP molecule and has been shown to be an effective trigger of NETosis (25) contributing to the sterile inflammatory condition. Our data show that IFC can confidently detect and quantify H4cit3 even after short exposure to Hemin.

As more evidence link NETs production with an increasing number of pathologies, it is becoming clearer that not all NETs are the same. Different stimuli have different potencies and initiation mechanisms, and the formed DNA-scaffolded strands appear to carry distinct cargo proteins. This variability, compounded by the high potential for non-specific *in vitro* activation of neutrophils, can hinder the development of reliable detection and quantification methods.

We aimed at developing a reliable detection method that would identify the earlier events, as neutrophils prepare for NETs production in response to physiological stimuli (such as cytokines—IL-8 or hemolysis product—Hemin). With our induction and detection methodology, the H4cit3 response for all tested stimuli was quantifiable, either in normal or decondensed nuclei. PMA and both Hemin concentrations were potent agonists that induced strong NETosis-related early nuclear changes; at the same time LPS and IL-8 had limited effects (**Figures 2**, **4**).

This methodology offers advantages for both research and clinical projects seeking reliable detection and quantification of markers involved in early NETosis. Our method does not require any additional information (e.g., the release of reactive oxygen species) to deliver quantifiable data on histone 4 citrullination and nuclear decondensation. Neutrophils are minimally handled (1-step isolation and no plating required) to reduce exposure to unspecific activation signals. This technique distinguishes and quantifies a known NETs marker (e.g., H4 histone citrullination) in addition to nuclear decondensation, in whole neutrophils with an intact cellular membrane, prior to the DNA extrusion. If required, this technique also allows quantitative evaluation and/or by imagery of DNA-free neutrophils as it allows specific gating of CD66b+Hoechst- cells. This can be particularly suitable for studies interested to look as NET-ing neutrophils as potential biomarkers for diseases.

Our method quantified NETosis-related events for potent and less potent stimuli. However, with the limited data from the LPS and IL-8 stimuli, it also emphasized that detection limitations might be associated with some stimuli.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The NHLBI Institutional Review Board approved the study that was conducted in accordance with the ethical principles stated in the Declaration of Helsinki. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

EB and VD wrote the manuscript. EB developed the staining technique. VD established the Imaging Flow Cytometry analysis parameters. LM performed experimental work and revised the manuscript. ST supervised the development of the project and revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.01335/full#supplementary-material

REFERENCES

1. Jorch SK, Kubes P. An emerging role for neutrophil extracellular traps in noninfectious disease. *Nat Med.* (2017) 23:279–87. doi: 10.1038/nm.4294
2. Thalin C, Daleskog M, Goransson SP, Schatzberg D, Lasselin J, Laska AC, et al. Validation of an enzyme-linked immunosorbent assay for the quantification of citrullinated histone H3 as a marker for neutrophil extracellular traps in human plasma. *Immunol Res.* (2017) 65:706–12. doi: 10.1007/s12026-017-8905-3
3. Gupta S, Chan DW, Zaal KJ, Kaplan MJ. A high-throughput real-time imaging technique to quantify netosis and distinguish mechanisms of cell death in human neutrophils. *J Immunol.* (2018) 200:869–79. doi: 10.4049/jimmunol.1700905
4. Muldur S, Marand AL, Ellett F, Irimia D. Measuring spontaneous neutrophil motility signatures from a drop of blood using microfluidics. *Methods Cell Biol.* (2018) 147:93–107. doi: 10.1016/bs.mcb.2018.07.005
5. Lee KH, Cavanaugh L, Leung H, Yan F, Ahmadi Z, Chong BH, et al. Quantification of NETs-associated markers by flow cytometry and serum

assays in patients with thrombosis and sepsis. *Int J Lab Hematol.* (2018) 40:392–9. doi: 10.1111/ijlh.12800
6. Zharkova O, Tay SH, Lee HY, Shubhita T, Ong WY, Lateef A, et al. A flow cytometry-based assay for high-throughput detection and quantification of neutrophil extracellular traps in mixed cell populations. *Cytometry A.* (2019) 95:268–78. doi: 10.1002/cyto.a.23672
7. Masuda S, Nakazawa D, Shida H, Miyoshi A, Kusunoki Y, Tomaru U, et al. NETosis markers: quest for specific, objective, and quantitative markers. *Clin Chim Acta.* (2016) 459:89–93. doi: 10.1016/j.cca.2016.05.029
8. Ginley BG, Emmons T, Lutnick B, Urban CF, Segal BH, Sarder P. Computational detection and quantification of human and mouse neutrophil extracellular traps in flow cytometry and confocal microscopy. *Sci Rep.* (2017) 7:17755. doi: 10.1038/s41598-017-18099-y
9. Pelletier MG, Szymczak K, Barbeau AM, Prata GN, O'Fallon KS, Gaines P. Characterization of neutrophils and macrophages from ex vivo-cultured murine bone marrow for morphologic maturation and functional responses by imaging flow cytometry. *Methods.* (2017) 112:124–46. doi: 10.1016/j.ymeth.2016.09.005
10. Zhao W, Fogg DK, Kaplan MJ. A novel image-based quantitative method for the characterization of NETosis. *J Immunol Methods.* (2015) 423:104–10. doi: 10.1016/j.jim.2015.04.027
11. Douda DN, Khan MA, Grasemann H, Palaniyar N. SK3 channel and mitochondrial ROS mediate NADPH oxidase-independent NETosis induced by calcium influx. *Proc Natl Acad Sci USA.* (2015) 112:2817–22. doi: 10.1073/pnas.1414055112
12. Papayannopoulos V, Metzler KD, Hakkim A, Zychlinsky A. Neutrophil elastase and myeloperoxidase regulate the formation of neutrophil extracellular traps. *J Cell Biol.* (2010) 191:677–91. doi: 10.1083/jcb.201006052
13. Lacy P. Mechanisms of degranulation in neutrophils. *Allergy Asthma Clin Immunol.* (2006) 2:98–108. doi: 10.1186/1710-1492-2-3-98
14. Naccache PH, Fernandes MJ. Challenges in the characterization of neutrophil extracellular traps: The truth is in the details. *Eur J Immunol.* (2016) 46:52–5. doi: 10.1002/eji.201546022
15. Barbu EA, Dominical VM, Mendelsohn L, Thein SL. Neutrophils remain detrimentally active in hydroxyurea-treated patients with sickle cell disease. *PLoS ONE.* (2019) 14:e0226583. doi: 10.1371/journal.pone.0226583
16. Barnado A, Crofford LJ, Oates JC. At the bedside: neutrophil extracellular traps (NETs) as targets for biomarkers and therapies in autoimmune diseases. *J Leukoc Biol.* (2016) 99:265–78. doi: 10.1189/jlb.5BT0615-234R
17. Dominical V, Samsel L, McCoy JP Jr. Masks in imaging flow cytometry. *Methods.* (2017) 112:9–17. doi: 10.1016/j.ymeth.2016.07.013
18. Neeli I, Radic M. Opposition between PKC isoforms regulates histone deimination and neutrophil extracellular chromatin release. *Front Immunol.* (2013) 4:38. doi: 10.3389/fimmu.2013.00038
19. Li P, Li M, Lindberg MR, Kennett MJ, Xiong N, Wang Y. PAD4 is essential for antibacterial innate immunity mediated by neutrophil extracellular traps. *J Exp Med.* (2010) 207:1853–62. doi: 10.1084/jem.20100239
20. Branzk N, Papayannopoulos V. Molecular mechanisms regulating NETosis in infection and disease. *Semin Immunopathol.* (2013) 35:513–30. doi: 10.1007/s00281-013-0384-6
21. Pieterse E, Rother N, Yanginlar C, Hilbrands LB, van der Vlag J. Neutrophils discriminate between lipopolysaccharides of different bacterial sources and selectively release neutrophil extracellular traps. *Front Immunol.* (2016) 7:484. doi: 10.3389/fimmu.2016.00484
22. Li RHL, Ng G, Tablin F. Lipopolysaccharide-induced neutrophil extracellular trap formation in canine neutrophils is dependent on histone H3 citrullination by peptidylarginine deiminase. *Vet Immunol Immunopathol.* (2017) 193–4:29–37. doi: 10.1016/j.vetimm.2017.10.002
23. Liang Y, Pan B, Alam HB, Deng Q, Wang Y, Chen E, et al. Inhibition of peptidylarginine deiminase alleviates LPS-induced pulmonary dysfunction and improves survival in a mouse model of lethal endotoxemia. *Eur J Pharmacol.* (2018) 833:432–40. doi: 10.1016/j.ejphar.2018.07.005
24. Weitkamp JH, Reinsberg J, Bartmann P. Interleukin-8 (IL-8) preferable to IL-6 as a marker for clinical infection. *Clin Diagn Lab Immunol.* (2002) 9:1401. doi: 10.1128/CDLI.9.6.1401.2002
25. Chen G, Zhang D, Fuchs TA, Manwani D, Wagner DD, Frenette PS. Heme-induced neutrophil extracellular traps contribute to the pathogenesis of sickle cell disease. *Blood.* (2014) 123:3818–27. doi: 10.1182/blood-2013-10-529982

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