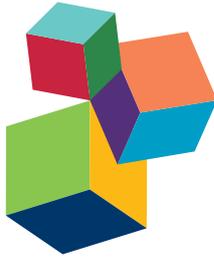




NETOSIS 2: THE EXCITEMENT CONTINUES

EDITED BY : Mariana J. Kaplan, Marko Radic and Martin Herrmann
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NETOSIS 2: THE EXCITEMENT CONTINUES

Topic Editors:

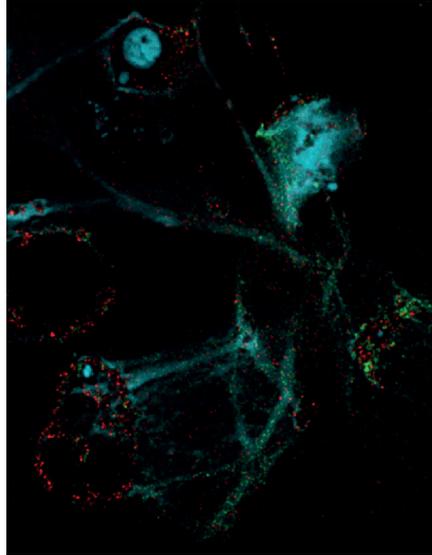
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NETosis, a form of cell death that manifests by the release of decondensed chromatin to the extracellular space, provides valuable insights into mechanisms and consequences of cellular demise. Because extracellular chromatin can immobilize microbes, the extended nucleohistone network was called a neutrophil extracellular trap (NET), and the process of chromatin release was proposed to serve an innate immune defense function. Extracellular chromatin NETs were initially observed in studies of neutrophils and are most prominent in these types of granulocytes. Subsequent studies showed that other granulocytes and, in a limited way, other cells of the innate immune response may also release nuclear chromatin following certain kinds of stimulation. Variations of NETosis were noted with cells that remain temporarily motile after the release of chromatin. Numerous stimuli for NETosis were discovered, including bacterial breakdown products, inflammatory stimuli, particulate matter, certain crystals, immune complexes and activated thrombocytes. Fundamental explorations into the mechanisms of NETosis observed that neutrophil enzyme activity (PAD4, neutrophil elastase, proteinase 3 and myeloperoxidase) and signal transduction pathways contribute to the regulation of NETosis. Histones in NET chromatin become modified by peptidylarginine deiminase 4 (PAD4) and cleaved at specific sites by proteases, leading to extensive chromatin externalization. In addition, NETs serve for attachment of bactericidal enzymes including myeloperoxidase, leukocyte proteases, and the cathelicidin LL-37. NETs are decorated with proteases and may thus contribute to tissue destruction. However, the attachment of these enzymes to NET-associated supramolecular structures restricts systemic spread of the proteolytic activity.

While the benefit of NETs in an infection appears obvious, NETs also participate as key protagonists in various pathologic states. Therefore, it is essential for NETs to be efficiently cleared; otherwise digestive enzymes may gain access to tissues where inflammation takes place. Persistent NET exposure at sites of inflammation may lead to a further complication: NET antigens may provoke acquired immune responses and, over time, could initiate autoimmune reactions, serve as antigen for nuclear autoantibodies and foster DNA immune complex-related inflammation. Neutrophil products and deiminated proteins comprise an important group of autoantigens in musculoskeletal disorders. Aberrant NET synthesis and/or clearance are often associated with inflammatory and autoimmune conditions. Recent evidence also implicates



Complement factor P deposits on PMA-induced NETs. Spinning-disk immunofluorescence microscopy images show that properdin and myeloperoxidase associate with cell bodies and PMA-induced NETs. For details see the article by Yuen et al.

Yuen J, Pluthero FG, Douda DN, Riedl M, Cherry A, Ulanova M, Kahr WHA, Palaniyar N and Licht C (2016) NETosing Neutrophils Activate Complement Both on Their Own NETs and Bacteria *via* Alternative and Non-alternative Pathways. *Front. Immunol.* 7:137. doi: 10.3389/fimmu.2016.00137

aberrant NET formation in the development of endothelial damage, atherosclerosis and thrombosis. Intravital microscopy provides evidence for conditions that induce NETosis *in vivo*. Furthermore, NETs can easily be detected in synovial fluid and tissue sections of patients with arthritis and gout. NETosis is thus of interest to researchers who investigate innate immune responses, host-pathogen interactions, chronic inflammatory disorders, cell and vascular biology, biochemistry, and autoimmunity. As we enter the second decade of research on NETosis, it is useful and timely to review the mechanisms and pathways of NET formation, their role in bacterial and fungal defense and their importance as inducers of autoimmune responses.

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Editorial: NETosis 2: The Excitement Continues

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Editorial on the Research Topic

NETosis 2: The Excitement Continues

INTRODUCTION

Chromatin externalization to form extracellular traps has first been described in neutrophils (1) but can also be observed in mast cells (Mollerherm et al.) as well as in other myeloid cells like eosinophils and basophils (2). DNA externalization-based defense emerged more than 1 billion years ago. Sentinel cells of the social amoeba *Dictyostelium discoideum* release redundant mitochondrial DNA and in an altruistic manner chromatin that sequester colony invading bacteria (Zhang and Soldati). Leukotoxic hypercitrullination and deficient mitophagy can initiate mitochondrial DNA expulsion (Konig and Andrade). These processes can be discriminated from canonical NET formation (1). However, since they cause DNA externalization they could be considered non-canonical forms of NET formation. Although the generation of reactive oxygen species is reportedly involved in several pathways of NET formation it is not strictly required for others (3). Furthermore, under hypoxic conditions, the PMA-induced NET formation is reported to be reduced, though not abrogated (Branitzki-Heinemann et al.). This editorial summarizes the collection of articles of the research topic “NETosis 2, the excitement continues.”

TO DIE OR NOT TO DIE

There is ongoing discussion whether the neutrophils are still viable when they release their chromatin and if they release chromatin at all. There are reports that claim the cells mainly externalize mitochondrial DNA (Yousefi and Simon). Some investigators question the notion that NETs form by cytolysis. They argue that it seems inconsistent with neutrophil “containment” processes (Malachowa et al.). Furthermore, the term “NETosis” has been criticized by some researchers who prefer the term “NET formation.”

Surveying the published data, including those published in this special issue, we want to summarize the current knowledge (I) Depending on the stimulus, NET formation can be a lytic death process eventually leading to disintegration of the neutrophil or a process where neutrophils remain migratory and impermeable to ionic dyes; (II) these processes have been observed *in vitro* as well as *in vivo*; (III) the inducer and its concentration determine, at least in part, these processes as well as the content of the NETs. For purposes of this review, we will use the term NET formation rather than NETosis to include the various forms of this cellular processes as described by various research groups.

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INDUCERS OF NET FORMATION

NET formation is favored by the higher pH at the borders of inflamed areas (Maueroder et al.). The neutrophil granule-derived enzyme peptidylarginine deiminase type 4 has been proposed to be involved in the formation of NETs and its pharmacological inhibition suppresses chromatin decondensation and NET formation following certain types of stimuli (Kusunoki et al.). In response to hyphae of *Candida albicans* or immobilized β -glucan neutrophils that are associated with the extracellular matrix rapidly form NETs independent of their ability to perform an oxidative burst (O'Brien and Reichner). The pathway of immune complexes to induce NET formation involves cross-linking of the receptor for IgG Fc fragments Fc γ RIIb, the TGF- β -activated kinase 1, and activation of MEK/ERK signaling (Aleman et al.). NET formation is also triggered by interaction of neutrophils with platelets (Carestia et al.) and by a complex interplay between neutrophils and bacterial lipopolysaccharides (LPS). Both "suicidal" and "vital" NET formation depend on the bacterial origin of the LPS and are modulated by the presence or absence of platelets (Pieterse et al.). NETs generated in the presence of propylthiouracil display a disordered phenotype which renders them resistant to treatment with DNaseI, and have been proposed to favor the generation of ANCA (Soderberg and Segelmark). During gestation, the capacity to form NETs increases and involves chorionic gonadotropin, G-CSF, and estrogen (Giaglis et al.).

INHIBITORS OF NET FORMATION

The increased capacity to form NETs during gestation is controlled by the inhibitory activity of progesterone. This hormone reduces the nuclear translocation of the neutrophil elastase leading to decreased NET formation (Giaglis et al.). Furthermore, Raloxifene, a selective modulator of the receptor for estrogen prevents NET formation induced by PMA (Flores et al.).

NET COMPONENTS

The NET backbones mainly consist of chromatin with modified, often citrullinated, histones and is overall similar in most NETs reported. However, there are considerable differences in their protein cargo (Mitsios et al.). These proteins may either be derived from the neutrophils' granules, from the vicinity of the NETs or from bystander cells. Neutrophils are endowed with a "tool kit" for alternative complement activation. They secrete properdin and deposit C3-derived complement fragments on NETs and on NET-bound bacteria (Yuen et al.). Since NETs are endowed with a potent toxic and proteolytic armament they need regulatory components to limit tissue injury. Hence, the secretory leukocyte protease inhibitor SLPI is bound to NETs. However, SLPI-NETs carry a certain risk; interacting with plasmacytoid dendritic cells, they induce type 1 interferon *in vitro*, cytokines that have been associated with the development of various autoimmune conditions (Majewski et al.). Extracellular histones are cytotoxic to endothelial cells

and thus may contribute to septic pathology and death. The NET component long pentraxin PTX3 protects against histone-mediated cytotoxicity (Daigo et al.).

THE ROLES OF HISTONES

Histones are endowed with bactericidal and fungicidal activities. This comes at the prize of bystander cytotoxicity toward endothelial cells. Thus, extracellular histones are partially responsible for tissue damage and septic death. To dampen overwhelming histone toxicity, NETs can be decorated with the long pentraxin PTX3 that limits histone-mediated tissue damage (Daigo et al.). Antibodies against posttranslational modified, citrullinated histones are pathognomonic for patients with rheumatoid arthritis (Neeli and Radic). In contrast to humans, some autoimmune mice are tolerant to citrullinated histones (Dwivedi et al.), a finding that highlights differences in PAD4 expression in human versus mouse antigen-presenting cells.

ANTIBODIES BINDING NETs AND THEIR COMPONENTS

Deimination is a physiological process that is amplified at sites of inflammation. However, only individuals with genetic predispositions for rheumatoid arthritis develop antibodies against deiminated proteins (ACPAs) (Corsiero et al.). Patients with malaria show circulating NET-binding P-ANCA (anti-MPO) and to a lesser extend C-ANCA (anti-PR3) which does not react with NETs. This supports the notion that NETs are involved in the etiopathogenesis of this, often fatal, disease (Boeltz et al.). Excessive NET formation in Balb/c wild type mice leads to generation of MPO-ANCA production *in vivo* (Kusunoki et al.). Importantly, the autoantibody binding to NETs may not only contribute to immune pathology but may aid in the clearance of NETs (Soderberg and Segelmark).

METHODS FOR THE ANALYSIS OF NETs

In general, NETs can be detected by staining of DNA and its co-localization with granular and modified nuclear proteins in web-like, spiky or cloudy structures, exceeding the size of a neutrophil. In neutrophil-rich areas like inflamed synovium or densely infiltrated tissues, these NETs tend to aggregate (4, 5) and form extended chromatin clumps decorated with an effective pathogenocidal armament (Brinkmann et al.). When exploring intravascular NET formation in disease, the triad neutrophils, platelets, and endothelium should be analyzed (Kazzaz et al.). For *in vitro* and *ex vivo* assays, the medium composition has to be precisely reported and strictly controlled during the entire assay and not just at its beginning (Maueroder et al.). Detection of deiminated histones is often used as an indication of NETosis but it should be stressed that the current methods for detection of citrullinated proteins require further optimization (Daigo et al.).

BARRIER FUNCTION OF AGGREGATED NETs

NETs generated at high neutrophil densities often aggregate and form extended matted structures (4, 5) decorated with a plethora of bactericidal and fungicidal molecules. Though these structures are based on a DNA meshwork, they only display a limited sensitivity against DNA degrading enzymes. Indeed, gouty tophi, an aggregate of NETs and MSU crystals, can persist in human tissues for several years where DNA is still detectable. In the periphery of necrotic areas, necrotic cells may recruit neutrophils and initiate and/or facilitate the formation of NET-based surrogate barriers [Bilyy et al.; Biermann et al.].

INTRAVASCULAR NET FORMATION AND VASCULAR DISEASES

Intravascular NETs have pro-coagulant activities associated to several pathologies (Kimball et al.). As in SLE, in ANCA associated vasculitis, the degradation pathways of NETs are perturbed. Due to low DNaseI activity, the degradation of intravascular NETs is slow leading to an increased amount of circulating NETs (Soderberg and Segelmark). This phenomenon may also contribute to the pathogenesis of severe malaria (Boeltz et al.). Similarly, NETs are discussed to contribute to the thrombotic pathologies observed in patients with cancer (Olsson and Cedervall).

After bone marrow transplantation, the capacity to form NETs is reportedly reduced for up to 200 days (Glenn et al.). In an animal model of paw edema by injection of nanodiamonds into wild-type mice and in those with deficient capacity for oxidative burst and NET formation (*Ncf1*** mice), the inflammatory response resolves in the former and becomes chronic in the latter as a result of the failure to dampen the neutrophil-driven inflammation (Biermann et al.). Importantly, ANCA in general are reported to drive immune complex-mediated pathologies but they may also aid in the clearance of circulating NETs or NET remnants (Soderberg and Segelmark).

NET FORMATION AND EXTRAVASCULAR DISEASE

Though NETs have initially been described as mechanism of bacterial defense, they must be considered as double-edged swords of innate immunity (Yang et al.). NETs are involved in a plethora of pathological conditions including autoimmunity, atherosclerosis, cancer, etc. (Mitsios et al.). Excess neutrophil recruitment to the alveolar space and NET formation in lungs reportedly cause inflammation and asthma (Akk et al.). NETs can easily expand in the pulmonary alveoli and cause lung injury. DNA disintegration by DNase, neutralization of NET proteins, anti-histone antibodies, and protease inhibition may alleviate NET-associated pathologies (Porto and Stein). Raloxifene, a selective modulator of the estrogen receptor inhibits the NET-based killing of the leading human bacterial pathogen MRSA (Flores et al.). Furthermore, it was recognized that viruses induce

NETs and target these for immune evasion (Schonrich and Rafferty). Harming of the blood-brain barrier and of neural cells by NETs was observed in humans with Alzheimer's disease and in a murine model of this disabling disease (Pietronigro et al.).

THERAPEUTIC INTERVENTIONS

NETs are discussed as a source of the citrullinated autoantigens pathognomonic for patients with rheumatoid arthritis and for the DNA observed in tissues and in the circulation of patients with SLE. Consequently, PAD4 is proposed to be a potential therapeutic target for these chronic inflammatory rheumatic diseases (Konig and Andrade). The synthetic peptide P140/Lupuzor™ selectively modulates chaperone-mediated autophagy but not NET formation in *sensu stricto* (Ramirez et al.). As high fat diet reportedly increases the formation of NETs dietary intervention and reduction of fat intake may be beneficial in NET-associated disorders (Moorthy et al.).

Targeting NET formation as well as NET-associated chromatin decondensation may delay the pathogenesis of Alzheimer's disease (Pietronigro et al.), the early inflammatory responses to Sendai virus infection (Akk et al.), and the disseminated intravascular coagulation observed in severe forms of malaria (Boeltz et al.). As already established for patients with cystic fibrosis, the clearance with recombinant human DNaseI of the NET-associated DNA, the neutralization of NET-borne proteins using anti-histone antibodies, as well as inhibitors for NET-bound proteases are discussed as therapeutic options for pulmonary diseases involving alveolar NETs in Porto and Stein.

From the papers included in this Research Topic, it is evident that the field of NET research is now more mature and sophisticated than even just a few years ago. Physiological and disease conditions that induce abundant NET formation are now firmly established and experimental methods for detection of NETs *in vivo* and *in vitro* have been carefully defined. It is the hope of the authors that the combined efforts presented here will contribute to further shape the consensus in the field, energize efforts to understand NET biology, and lead to novel therapies for major human disorders that present with abnormal NET release or impairments in NET degradation.

AUTHOR CONTRIBUTIONS

All authors wrote and revised the manuscript.

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New Insights into Neutrophil Extracellular Traps: Mechanisms of Formation and Role in Inflammation

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Recent data suggest that NETosis plays a crucial role in the innate immune response and disturbs the homeostasis of the immune system. NETosis is a form of neutrophil-specific cell death characterized by the release of large web-like structures referred to as neutrophil extracellular traps (NETs). NETs are composed of DNA strands associated with histones and decorated with about 20 different proteins, including neutrophil elastase, myeloperoxidase, cathepsin G, proteinase 3, high mobility group protein B1, and LL37. Reportedly, NETosis can be induced by several microbes, and particulate matter including sterile stimuli, *via* distinct cellular mechanisms. Meanwhile, suicidal NETosis and vital NETosis are controversial. As we enter the second decade of research on NETosis, we have partly understood NETs as double-edged swords of innate immunity. In this review, we will discuss the mechanisms of NETosis, its antimicrobial action, and role in autoimmune diseases, as well as the relatively new field of NET-associated mitochondrial DNA.

Keywords: NETosis, NETs, antimicrobial activity, autoimmune diseases, mitochondrial DNA

INTRODUCTION

Neutrophil granulocytes are the most abundant type of white blood cells in humans and play a vital role in innate immunity by defending the host against invading pathogens. The immune regulatory functions of neutrophils include phagocytosis, generation of reactive oxygen species (ROS), degranulation, and the formation of neutrophil extracellular traps (NETs), a process referred to as NETosis. NETosis is accepted as a specific form of cell death subroutine performed by granulocytes, differing from apoptosis and necrosis (1, 2). When neutrophils undergo NETosis, nuclear and granular membranes disintegrate, the chromatin decondenses, and it diffuses into the cytoplasm, mixing with cytoplasmic proteins. This is followed by plasma membrane rupture and the release of chromatin, decorated with granular proteins, into the extracellular space (2, 3). NETs consist of chromatin fibers with diameters of 15–17 nm that contain DNA and the histones H1, H2A, H2B, H3, and H4. Moreover, the DNA fibers are decorated with several proteins like neutrophil elastase (NE), myeloperoxidase (MPO), cathepsin G, proteinase 3 (PR3), high mobility group protein B1 (HMGB1), and LL37, thus displaying proinflammatory characteristics (1). In the past decade, new aspects of neutrophil functions have emerged unveiling their significance not only in defending the host against microbes but also in contributing to many autoimmune pathological conditions. Therefore, the purpose of this review is to present and discuss the current knowledge about the mechanisms of NETosis and its role in the pathogenesis of autoimmune diseases.

MECHANISMS OF NETosis

Neutrophil suicide, distinct from either necrosis or apoptosis, was first described following chemical stimulation with phorbol 12-myristate 13-acetate (PMA) in 1996 (4). This form of cell death was characterized by the disintegration of nuclear and granular membranes and by the release of decondensed chromatin into the cytoplasm. In 2004, Zychlinsky and colleagues reported that neutrophil suicide resulted in the release of large web-like structures composed of decondensed chromatin and neutrophil antimicrobial factors, and coined the name neutrophil extracellular traps (1). In their studies, they used PMA and interleukin-8 (IL-8) to elicit NETs *in vitro*. In 2007, it was reported that, going along with chromatin decondensation, neutrophils undergo an NADPH oxidase-dependent death process that includes nuclear envelope disintegration and the mixing of nucleic acids and granule proteins within a large intracellular vacuole (3). After the association of nucleic acids and granule proteins, NETs are released *via* plasma membrane perforation and cell lysis. This process is completed 1–4 h after the inciting stimulus. The released chromatin structures are prone to bind particular matter, e.g., bacteria. The authors concluded that PMA-induced NETosis is a form of a beneficial suicide (3). Apart from PMA and IL-8, bacteria, fungi, protozoa, antibody–antigen complexes (5), autoantibodies (6), tumor necrosis factor (TNF), interferon (IFN) (7), and further stimuli also trigger NETosis.

PATHWAYS

Conventional suicidal NETosis has long been recognized as a distinct form of active cell death. In addition, some researchers have described a different mechanism by which NETs are formed, termed vital NETosis. This non-suicidal pathway allows NET release from neutrophils staying viable (8–12).

CONVENTIONAL SUICIDAL NETosis

Conventional suicidal NETosis is frequently initiated by ligand binding to neutrophil toll-like receptors and receptors for IgG–Fc,

complement, or cytokines (1, 5, 13). Upon activation of these receptors, calcium storages of the endoplasmic reticulum release calcium ions into the cytoplasm. Elevated cytoplasmic calcium levels increase protein kinase C (PKC) activity and phosphorylation of gp91phox (14). This induces the assembly of the cytosolic and membrane-bound subunits of NADPH oxidase into functional complexes at cytoplasmic or phagosomal membranes (also called phagocytic oxidase, PHOX) and the subsequent generation of ROS (15). Under the influence of ROS, granules and the nuclear envelope rupture. Subsequently, the released nuclear, granular, and cytoplasmic contents blend. NE and MPO, usually stored in azurophilic granules, migrate to the nucleus. Here, NE degrades the linker histone H1 and processes the core histones, and MPO enhances chromatin decondensation (15). Histone deimination by peptidyl arginine deiminase 4 (PAD4) and proteolytic cleavage of histones initiated before nuclear breakdown additionally contribute to chromatin decondensation (16, 17). The rupture of the plasma membrane allows the release of NETs and leads to cell death and the loss of viable cell functions of like migration and phagocytosis (Figure 1) (15).

REACTIVE OXYGEN SPECIES

The generally accepted notion that ROS play a crucial role in the classical suicidal NETosis pathway is based on two important observations: (1) Neutrophils from patients with chronic granulomatous disease (CGD), not capable of performing the oxidative burst, show strongly reduced abilities to form NETs. This is independent of the type of mutation leading to a defective PHOX complex. CGD patients suffer from severe and often chronic infections (3, 18). Moreover, treatment with H₂O₂ rescued the production of NETs in neutrophils from CGD patients, downstream of the PHOX complex (3). (2) ROS scavengers, such as *N*-acetylcysteine, or trolox reportedly inhibit NETosis (3, 19). In fact, it remains unclear how ROS participate in the dismantling of the nuclear envelope or the mixing of the NET components. Some studies suggest that ROS directly promote the morphologic changes observed during NETosis (14). ROS may alternatively inactivate caspases, thereby inhibiting apoptosis and favoring

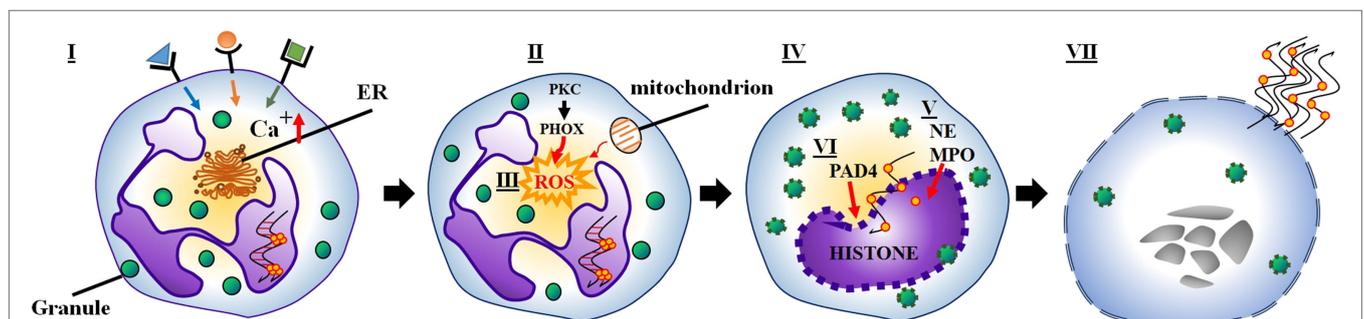


FIGURE 1 | (I) Several stimuli (e.g., bacteria, viruses, fungi) initiate NETosis by binding to neutrophil receptors (e.g., Fc receptors, TLRs), which activate the endoplasmic reticulum to release stored calcium ions. (II) Elevated cytoplasmic calcium levels increase PKC activity, which induces NADPH oxidase to assemble into a functional complex (PHOX). (III) Subsequently, PHOX (or alternatively the mitochondrial respiratory chain) generate ROS. (IV) ROS generation leads to the rupture of granules and the nuclear envelope. (V) Meanwhile, NE and MPO translocate to the nucleus. (VI) As a result, histone deimination and chromatin decondensation contribute to the formation of NETs. (VII) Finally, the rupture of the plasma membrane causes neutrophil lysis and allows the release of NETs.

autophagy. This leads to dissolution of cellular membranes (20). These two alternatives are not mutually exclusive: under certain experimental conditions, each of them can also act independently. There is now growing evidence that some stimuli induce NETosis independent of NADPH oxidase. Oxidant-independent release of NETs was studied in detail by Winterbourn and colleagues (21).

PEPTIDYL ARGININE DEIMINASE 4

Peptidyl arginine deiminase 4 catalyzes the conversion of arginine residues to citrulline in polypeptides, thereby eliminating a positive charge of the protein. Thus, citrullination of histones weakens the stability of nucleosomes (22, 23). Loss of positive charges causes the opening of the compact structure of chromatin and allows decondensation and dispersion of chromatin in the form of NETs. Consistently, neutrophils from mice with a PAD4 deficiency display impaired capacities to form NETs and are highly susceptible to severe skin infections *in vivo* (16, 17). However, PAD4 deficiency does not contribute to lung infections caused by influenza virus (16).

VITAL NETosis

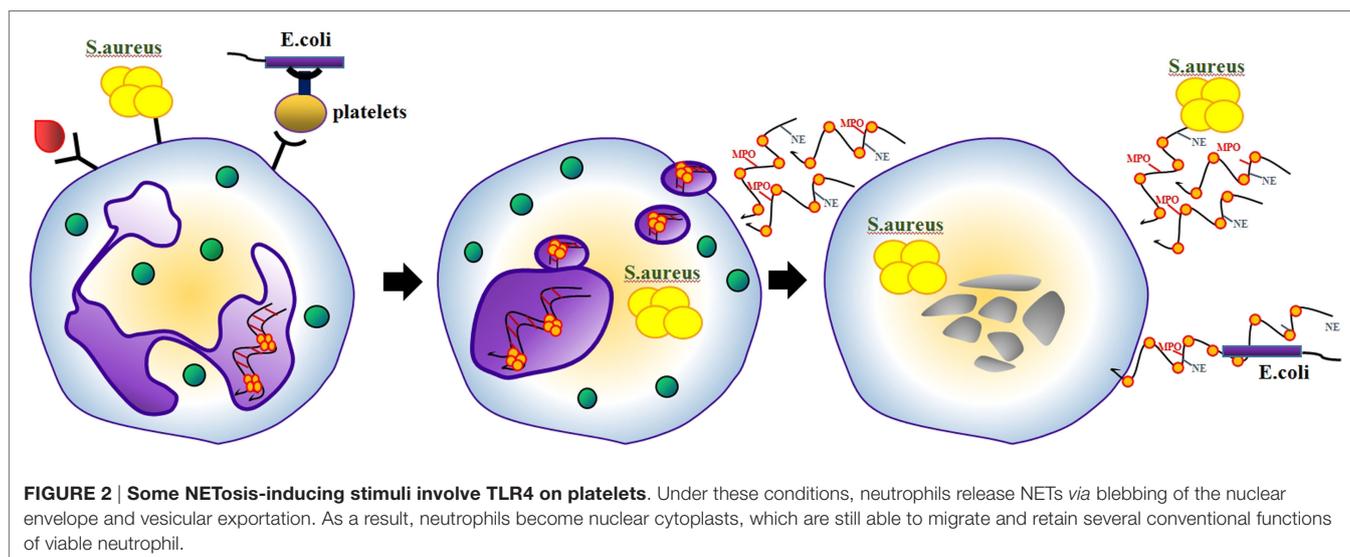
Contrary to previous studies describing the canonical pathways of NETosis as a process requiring several hours, Clark et al. reported in 2007 that lipopolysaccharide (LPS)-stimulated NETosis occurred within just 30 min involving TLR4 on platelets (8). It was demonstrated that neutrophils that released NETs remained impermeable for SYTOX Green, indicating that they remained structurally intact. Therefore, the authors later coined the term vital NETosis (12). Electron microscopy revealed that NET release induced by *Staphylococcus aureus* occurs via blebbing of the nuclear envelope and vesicular exportation *in vitro* and *in vivo* (9). As a result, this pathway preserved the integrity of the neutrophils' plasma membranes (Figure 2). NETting neutrophils became anuclear cytoplasts capable of chasing

and imprisoning live *Staphylococci* (10). *Candida albicans* was reported a further stimulus of vital NETosis (11). It still remains controversial whether and how suicidal and vital NETosis coexist. Furthermore, it is not clear if a neutrophil that has ejected (parts of) its DNA should be termed "viable."

MITOCHONDRIAL DNA

As mentioned above, ROS are indispensable for several kinds of NETosis (24). In mammals, both the mitochondrial respiration chain and the NADPH oxidase independently contribute to the production of ROS (25). Recently, it has been observed that *in vivo* inhibition of mitochondrial ROS production reduced intracellular ROS levels and NETosis (26). Ribonucleoprotein immune complexes (RNP ICs) were used to stimulate neutrophils and mitochondrial ROS generation. Mitochondria became hypopolarized, translocated to the cell surface, and were observed within the expelled NETs. Concomitantly, mitochondrial ROS oxidized mitochondrial DNA (mtDNA), which is proinflammatory *in vitro*. When injected into mice, oxidized mtDNA triggered inflammation and type I IFN production via a pathway dependent on the DNA sensor STING (7, 26). Mitochondria have evolved from bacteria and contain unmethylated CpG motifs (27) as well as *N*-formylated peptides (28). Similar to bacteria, extracellular mitochondria are stimulators of proinflammatory signaling. Several reports attribute this effect to the unmethylated CpG DNA repeats within the mtDNA (29), others highlight the effect of DNA oxidation (28).

In patients with systemic lupus erythematosus (SLE), abnormal NETosis and defects in the clearance of NETs were found to promote the production and release of type I IFN (30). In contrast, patients with CGD carry an increased risk to suffer from SLE, despite lacking functional NADPH oxidase activity (18), the major source of ROS in activated healthy neutrophils. Based on this observation, one might question whether increased NETosis is a factor contributing to the etiopathogenesis of SLE.



Instead, deficiency of the clearance of NETs is likely to foster the antinuclear autoimmunity in patients with SLE (30, 31). However, Kaplan and colleagues reexamined the importance of ROS in low-density granulocytes from patients with CGD and observed that this granulocyte subpopulation undergoes spontaneous NETosis and that their mitochondrial respiration produces sufficient amounts of ROS to execute NETosis. The levels of ROS derived from low-density granulocytes correlated with the levels of type I IFN in the corresponding patients. Accordingly, the authors not only confirmed that mitochondria drove NETosis but also concluded that NETosis is a pathological factor able to foster SLE (26, 32). Consistently, anti-mtDNA antibodies were elevated in the sera of patients with SLE, and antibody levels correlated with IFN scores and disease activity. Immune complexes containing mtDNA induced more IFN- α than those with nuclear dsDNA. Thus, anti-mtDNA antibodies can be considered as typical for driving both SLE and lupus nephritis (33). As a drug that selectivity inhibits mitochondrial respiratory chain complex I and decreases NADPH oxidase activity (34), metformin may be a new option to treat SLE (33).

ANTIMICROBIALS

Numerous microbes reportedly induce formation of NETs (Table 1). NET-inducing molecules include the bacterial cell surface components LPS, lipoteichoic acid, and their breakdown products. Several bacteria and fungi were reported to potently induce NET formation, such as *Staphylococcus aureus* (9, 35), *Streptococcus* sp. (36), *Haemophilus influenzae* (37), *Klebsiella pneumoniae* (15), *Listeria monocytogenes* (38), *Mycobacterium tuberculosis* (39), *Shigella flexneri* (1), *Aspergillus nidulans*, *Aspergillus fumigatus*, and *Candida albicans* (40–42). Further examples are pathogens, such as *Yersinia* (1) and members of the oral microbiome, including *Porphyromonas gingivalis* (43). NETs can immobilize and kill a broad range of microbes, including bacteria, fungi, and protozoa (1, 9, 15, 35–42), and thus prevent the dissemination of microbial pathogens (37). Some studies have questioned the killing capabilities of NETs since viable *Staphylococcus aureus* and *Candida albicans* blastospores were released from NETs by incubation with DNases (44). Branzk

et al. found that in response to large pathogens, like filamentous *Candida albicans*, neutrophils selectively released NETs (45). Intriguingly, NETosis did not occur in response to the yeast form of *Candida albicans* or single bacteria. Phagocytosis via dectin-1 acted as a sensor of microbial size and prevented NET release by downregulating translocation of NE to the nucleus. Apart from directly killing microbes, NETs inactivate microbial “virulence factors” that alter the function of host cells. NET-associated NE specifically cleaved virulence factors of *Shigella flexneri*, *Salmonella typhimurium*, and *Yersinia enterocolitica* (1). The serine proteases cathepsin G and PR3 may also destroy virulence factors of further classes of microbes (46). NETs contain several proteins that inhibit microbes, including enzymes, antimicrobial peptides, calgranulin, and histones. The microbicidal activity of NETs results from the combined action of several components being enhanced by the high local concentrations of mediators on the NETs’ surfaces (15).

Various components of NETs contribute to different aspects of microbicidal activity. It was shown that the activity of MPO on NETs is essential to eliminate *S. aureus* (47). The antifungal activity of NETs has been assigned to calgranulin (48), which chelates zinc, a cation required for fungal growth (15). Also, histones restrict microbial growth very efficiently, and antibodies against histones prevent NET-mediated microbicidal activity (1). Microbes are suggested to be entrapped due to electrostatic interactions between the positively charged bacterial surface and the negatively charged chromatin fibers based on electrostatic interactions (49). Encapsulated pathogens or those that can change their surface charge may escape entrapment (50). Importantly, several bacteria are able to degrade NETs by nucleases and thus escape NET-mediated entrapment (Table 2). These include the Gram-negative pathogen *Vibrium cholera* (51) and the Gram-positive bacteria *Streptococcus pneumoniae* (52), *Streptococcus pyogenes* (53), *Yersinia* ssp. (54), *Streptococcus agalactiae* (55), *Streptococcus suis* (56), *Staphylococcus aureus* (57), and *Aeromonas hydrophila* (58). This emphasizes the importance of nucleases as pathogenic factors.

THE ROLE OF NETosis IN AUTOIMMUNE DISEASES

Vasculitis

Vasculitis manifests in vessel wall inflammation and can affect any organ system of the body. ANCA-associated vasculitis (AAV), a subgroup of the vasculitides, is characterized by involvement of

TABLE 1 | Pathogens that induce NETs.

Species	Reference
<i>Staphylococcus aureus</i>	(9, 35)
<i>Streptococcus</i> sp.	(36)
<i>Haemophilus influenzae</i>	(37)
<i>Klebsiella pneumoniae</i>	(15)
<i>Listeria monocytogenes</i>	(38)
<i>Mycobacterium tuberculosis</i>	(39)
<i>Shigella flexneri</i>	(1)
<i>Aspergillus nidulans</i>	(40)
<i>Aspergillus fumigatus</i>	(41)
<i>Candida albicans</i>	(42)
<i>Yersinia</i>	(1)
<i>Porphyromonas gingivalis</i>	(43)
<i>V. cholera</i>	(51)
<i>Aeromonas hydrophila</i>	(58)

TABLE 2 | Pathogens which evade entrapments via degrading NETs.

Species	Reference
<i>V. cholera</i>	(51)
<i>Streptococcus pneumoniae</i>	(52)
<i>Streptococcus pyogenes</i>	(53)
<i>Yersinia</i>	(54)
<i>Streptococcus agalactiae</i>	(55)
<i>Streptococcus suis</i>	(56)
<i>Staphylococcus aureus</i>	(57)
<i>Aeromonas hydrophila</i>	(58)

the small vessels, a neutrophil-rich necrotizing inflammation, and the presence of anti-neutrophil cytoplasmic antibodies (ANCA) (59). AAV comprises granulomatosis with polyangiitis (formerly Wegener's granulomatosis), microscopic polyangiitis, and eosinophilic granulomatosis with polyangiitis (formerly Churg–Strauss syndrome). Many ANCA are directed against PR3 or MPO, enzymes typically found in the azurophilic granules of neutrophils and on the surfaces of NETs (60). NETs are reportedly released by ANCA-stimulated neutrophils and in turn contain the autoantigens PR3 and MPO (39). This suggests that NET formation triggers vasculitis and promotes the autoimmune response against neutrophil components in individuals with small-vessel vasculitis (61). Consistently, increased levels of NET remnants containing complexes of nucleosomes and MPO have been detected in the circulation of patients with active vasculitis (39) and in patients with active AAV (60). Neutrophils of patients with AAV exhibited an increased tendency for spontaneous cell death. The levels of NET remnants were positively correlated with disease activity and neutrophil count, but inversely with ANCA at least during remission.

Systemic Lupus Erythematosus

Systemic lupus erythematosus is a complex multifactorial autoimmune disease associated with severe organ damage. NETs are considered a potential source of autoantigens. Polymorphonuclear leukocytes (PMNs) of patients with SLE display an increased propensity to execute NETosis in conjunction with impaired degradation of NETs by circulating DNase1. The aberrant NETs induce type I IFN, which is associated with vascular complications and tissue damage (30, 62).

High numbers of low-density granulocytes have been identified as a particular subset of neutrophils in SLE patients. Low-density granulocytes persistently produced TNF and type I IFN, and spontaneously underwent NETosis (24). Furthermore, increased IFN- α in SLE patients is an important driving force that primes neutrophils for the execution of NETosis (63).

Not only production but also degradation of NETs is altered in SLE patients. Sera of a subgroup of SLE patients degrade NETs less efficiently than those of healthy controls (30). The deficient clearance of NETs in patients with SLE correlates with high titers of anti-NET antibodies and renal involvement (30). In healthy individuals, mononuclear phagocytes clear NETs in cooperation with DNase1 and C1q both synergizing in predigesting the chromatin part (64). The activities of serum DNase1 in patients with SLE are lower than that of healthy controls (65). Increased serum levels of DNase1 inhibitors, rare mutations in the gene of DNase1, and anti-DNase 1 antibodies may explain the decreased activity of DNase 1 (66, 67). Circulating chromatin in the form of immune complexes in individuals with SLE contains LL37, which triggers TLR9 in plasmacytoid dendritic cells, induces IFN- α synthesis, and protects nucleic acids from degradation by nucleases (68, 69). A study found that the individual NET degradation activity in the circulation of a given patient changed with disease activity. Sera of patients with SLE, which were not able to degrade NETs, showed increased complement consumption, since NETs activate the classical complement pathway due to their interaction with C1q (70). Thus, strategies that eliminate NETs and their components

from the circulation pose a promising therapeutic approach for the treatment of patients with SLE (70).

Thrombosis

Neutrophil extracellular traps promote thrombosis by providing a scaffold and stimulus for platelet and red blood cell adhesion and aggregation (71), thus enhancing coagulation (72). Neutrophils in thrombi are required for propagation of deep venous thromboses by binding factor XII and supporting its activation through NETosis (73). The major components of NETs (DNA, histones, and proteases) all display procoagulant properties. DNA induces thrombin generation in plasma and increases the protease activity of coagulation factors (74, 75). Histones may directly induce epithelial and endothelial cell death (76), and can mediate thrombosis *in vivo* (77). Histones were found to inhibit anticoagulation of plasma by promoting thrombin generation and hamper thrombomodulin function (78, 79). Elastase inactivated the tissue factor pathway inhibitor; thus, further increasing coagulation and fibrin deposition *in vivo* (72). Release of NETs in the vascular compartment triggered a procoagulant state and promoted binding and activation of platelets leading to thrombosis (80).

Rheumatoid Arthritis

In the autoimmune disease, rheumatoid arthritis (RA), the formation of autoantibodies to citrullinated proteins (ACPA) is thought to be a key pathogenic factor. Given that histone citrullination is implicated in NET formation, NETosis may play a critical role in RA (81). In 2013, Kaplan and colleagues found that neutrophils from patients with RA had a greater tendency to release NETs than neutrophils from healthy controls. RA serum and synovial fluid was a strong inducer of NETosis (82). Furthermore, NETosis resulted in the externalization of citrullinated protein antigens and immune-stimulatory molecules that may promote aberrant adaptive and innate immune responses in the joint.

Diabetes

Diabetes mellitus (or diabetes) is a chronic, lifelong condition, in which impaired insulin secretion and variable degrees of peripheral insulin resistance lead to hyperglycemia and affect the body's ability to use food energy. Under conditions of hyperglycemia, neutrophils reportedly produce more superoxide and cytokines, like TNF- α , which triggers NETosis (83, 84). Based on these studies, we speculated that hyperglycemia may facilitate NETosis. Recently, Wong et al. (85) isolated neutrophils from type 1 and type 2 diabetic humans and mice. Nearly twice as many neutrophils derived from patients released NETs in comparison to cells from healthy controls. The authors attributed this to PAD4 and revealed a fourfold upregulation of PAD4 protein expression in the neutrophils from individuals with diabetes as compared to healthy controls. It is well established that delayed wound healing is a hallmark of patients with diabetes. The authors reported that large quantities of NETs were found in excisional skin wounds of diabetic mice and that DNase1, which dismantled NETs, accelerated wound healing. Despite the triggers of NETosis in wounds remaining elusive, it has been confirmed that inhibiting NETosis or degrading NETs improved wound healing and reduced

NET-driven chronic inflammation in diabetes (85). However, the exact role of NETosis in wound healing remains to be revealed.

Cancer

NETosis may influence tumor development during many stages, including growth, angiogenesis, and metastasis. It has been observed that there is a large necrotic area of dead neutrophils and NET-like structures in Lewis lung carcinoma and Ewing sarcoma (86, 87). It remains to be clarified whether these NETs are responsible for the generation of the necrotic areas. Alternatively, NETs may serve to shield healthy tissues from necrotic areas. A study observed NET deposition on the microvasculature and subsequent local trapping of circulating cancer cells. The tumor cells, immobilized by NETs, survived and proliferated to form nodules. This suggests a role for NETs in enhancing tumor metastasis (88). However, whether NETs just protect or anchor cancer cells physically or whether they promote tumor growth is still elusive.

Sepsis

The pathology of sepsis results from infection, hyperinflammatory host response, and immune paralysis. During sepsis, NETs are released in the vascular system, where they trap bacteria (8, 12). Trapped bacteria can be killed, protecting patients from bacterial overflow (1, 44). In contrast, NET deposition in organs and their pro-thrombotic activities may also contribute to organ failure (89, 90). When researchers subjected mice to polymicrobial sepsis following cecal ligation and puncture, PAD4-deficient mice showed a similar survival rate when compared to wild-type controls (91). However, PAD4-deficient mice were partially protected from LPS-induced shock, indicating that NETs may contribute to the toxic inflammatory and procoagulant host response to bacteria in sepsis. The authors proposed that preventing NET formation by PAD4 inhibition in inflammatory or thrombotic diseases is not likely to increase host vulnerability to bacterial infections (91).

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CONCLUSION

Progress in the research on NETosis has greatly increased our understanding of its role in immunological processes and autoimmune disorders. Recent studies described how autoantigens, released during NETosis, activate immune cells and that cytokines in turn give rise to further NETosis. Aggregated NETs finally sequester and degrade proinflammatory mediators to avoid excessive inflammation (8, 14). The published data also revealed that blocking the process of NETosis or inhibiting the activity of components in NETs might be effective in the treatment of autoimmune diseases. Future work investigating the exact process of NETosis and the interplay of NET components and the immune system will contribute to a deeper understanding of the role of neutrophils in the induction and resolution of inflammation.

AUTHOR CONTRIBUTIONS

HY and YZ wrote the first draft of this article. HY and YL designed the figures. YL, MB, JB, and MH critically revised the manuscript for important intellectual content. All authors approved the final version.

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Of Amoebae and Men: Extracellular DNA Traps as an Ancient Cell-Intrinsic Defense Mechanism

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Since the discovery of the formation of DNA-based extracellular traps (ETs) by neutrophils as an innate immune defense mechanism (1), hundreds of articles describe the involvement of ETs in physiological and pathological human and animal conditions [reviewed in Ref. (2), and the previous Frontiers Research Topic on NETosis: http://www.frontiersin.org/books/NETosis_At_the_Intersection_of_Cell_Biology_Microbiology_and_Immunology/195]. Interestingly, a few reports reveal that ETs can be formed by immune cells of more ancient organisms, as far back as the common ancestor of vertebrates and invertebrates (3). Recently, we reported that the Sentinel cells of the multicellular slug of the social amoeba *Dictyostelium discoideum* also produce ETs to trap and kill slug-invading bacteria [see **Box 1**; and **Figure 1** Ref. (4)]. This is a strong evidence that DNA-based cell-intrinsic defense mechanisms emerged much earlier than thought, about 1.3 billion years ago. Amazingly, using extrusion of DNA as a weapon to capture and kill uningestable microbes has its rationale. During the emergence of multicellularity, a primitive innate immune system developed in the form of a dedicated set of specialized phagocytic cells. This professionalization of immunity allowed the evolution of sophisticated defense mechanisms including the sacrifice of a small set of cells by a mechanism related to NETosis. This altruistic behavior likely emerged in steps, starting from the release of “dispensable” mitochondrial DNA by *D. discoideum* Sentinel cells. Grounded in this realization, one can anticipate that in the near future, many more examples of the invention and fine-tuning of ETs by early metazoan ancestors will be identified. Consequently, it can be expected that this more complete picture of the evolution of ETs will impact our views of the involvement and pathologies linked to ETs in human and animals.

Keywords: amoebozoa, *Dictyostelium*, NOX, neutrophil extracellular traps, evolution, unicellular eukaryotes, multicellularity, innate immunity

During early evolution of multicellularity, when autonomous eukaryotic single-cell hosts were encountering prokaryotes, they either phagocytosed them as food or moved away to avoid being infected. However, when multicellular organisms evolved, they had to face more directly a serious problem, namely, infection of only parts or tissues of the organism. One solution is what happens in slugs of *D. discoideum*, in which invading bacteria are trapped by patrolling S cells that are subsequently shed behind during slug migration, keeping the multicellular structure free from infection (4, 8). The phagocytes in higher animals and men follow similar strategies to circumscribe the infection. For example, patrolling neutrophils catch the invaders and commit suicide, being finally

discarded by the intervention of macrophages (9). However, in plants, which are immobile and do not have circulating (innate immune) cells, and whose cells have a rigid wall, another strategy had to be co-opted to isolate infected parts from healthy tissues. One such solution is the formation of a callus or tumor induced by wounding and pathogen infections (10). Naturally, a logical question is whether extracellular DNA is also involved in plant callus and tumor formation, a topic unaddressed yet, but that might reveal interesting aspects of the evolution of innate immune defenses in the broadest way.

Dictyostelium discoideum is a remarkable model organism to study the functions of specific genes involved in the emergence

BOX 1 | *Dictyostelium discoideum* as a unique model to study evolution of innate immunity.

The social amoeba *Dictyostelium discoideum* belongs to the Amoebozoa, a sister group to the animals and fungi that branched after the divergence of plants (5, 6). The life cycle of *D. discoideum* comprises two major stages, a single-celled amoeboid stage and a “social” facultative multicellular stage. During the former, amoebae feed on bacteria and yeasts by phagocytosis, a biological process extremely well conserved in evolution and essentially shared between protozoan phagocytes and phagocytes of the animal innate immune system (7). These features make this genetically tractable organism a unique model to study the function of specific genes involved in the early evolution of innate immunity and the emergence of multicellularity.

of multicellularity and the early evolution of cell autonomous defenses (8). Our recent study revealed that extracellular trap (ET) generation evolved much earlier than the emergence of metazoan and that reactive oxygen species (ROS) generated by NADPH oxidases (NOX) are essential in this conserved process (4). Therefore, in this perspective article, we would like to present the provocative hypothesis that, within some limits, the evolutionary history of ROS-generating NOX enzymes may serve as a general signature, a guiding principle that will be useful for the future discovery and study of ETs in other ancient organisms. At this point and before we develop further our arguments in favor of this causal relationship, we would like the reader to note that NADPH- or ROS-independent pathways may also contribute to ET formation under specific stimulations and conditions (11–14), an emerging field that was comprehensively reviewed by Stoiber et al. (15). Although different groups of organisms may employ different molecular machineries to fine-tune the production of ETs, and the sources of DNA may also vary depending on the process involved (1, 2, 4, 16, 17), but from a conceptual point of view, the strategies are similar, using DNA as a weapon for host defense.

Nevertheless, based on our previous phylogenetic investigations, ROS-generating NOX enzymes evolved from metal reducing ferric reductases (FRE) through a functional shift (18). As shown in **Table 1**, one could speculate that the organisms

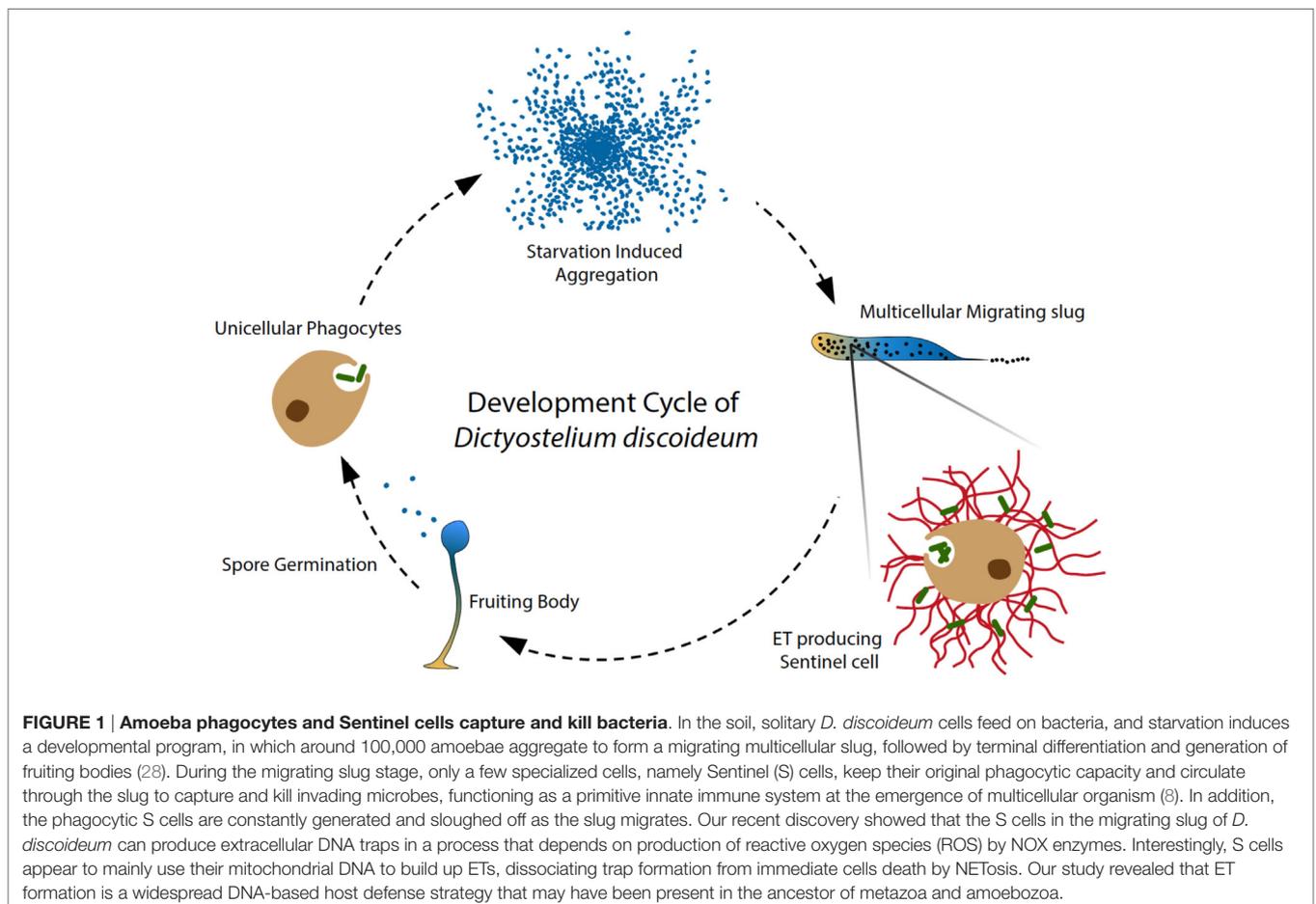


TABLE 1 | The co-emergence of NOX enzymes and multicellularity might also correlate with the origin of DNA-based defense strategies.

UniProt Mnemonic	Classification			Species	Number of NOX homologs	Multicellularity		
HUMAN	Eukaryotes	Opisthokonta	Metazoa	Vertebrates	<i>Homo sapiens</i>	7	YES	
MOUSE					<i>Mus musculus</i>	6	YES	
CHICK					<i>Gallus gallus</i>	6	YES	
ANOCA					<i>Anolis carolinensis</i>	5	YES	
XENTR					<i>Xenopus tropicalis</i>	6	YES	
TETNG					<i>Tetraodon nigroviridis</i>	4	YES	
ORYLA					<i>Oryzias latipes</i>	5	YES	
DANRE					<i>Danio rerio</i>	5	YES	
BRAFL					Invertebrates	<i>Branchiostoma floridae</i>	6	YES
CIOIN						<i>Ciona intestinalis</i>	6	YES
AEDAE						<i>Aedes aegypti</i>	2	YES
ANOGA						<i>Anopheles gambiae</i>	2	YES
DROME						<i>Drosophila melanogaster</i>	1	YES
PEDHC						<i>Pediculus humanus subsp. corporis</i>	1	YES
DAPPU			<i>Daphnia pulex</i>	5		YES		
IXOSC			<i>Ixodes scapularis</i>	1		YES		
CAEBR			<i>Caenorhabditis briggsae</i>	1		YES		
CAEEL			<i>Caenorhabditis elegans</i>	2		YES		
NEMVE			<i>Nematostella vectensis</i>	3		YES		
MONBE			<i>Monosiga brevicollis</i>	1		Transition		
NEUCR			Fungi	<i>Neurospora crassa</i>		2	YES	
PODAN				<i>Podospira anserina</i>		2	YES	
ASPTN				<i>Aspergillus terreus</i>	2	YES		
YEAST				<i>Saccharomyces cerevisiae</i>	0	NO		
SCHPO				<i>Schizosaccharomyces pombe</i>	0	NO		
DICDI			Amoebae	<i>Dictyostelium discoideum</i>	3	Transition		
POLPA				<i>Polysphondylium pallidum</i>	2	YES		
ENTHI				<i>Entamoeba histolytica</i>	0	NO		
ARATH	Plants	<i>Arabidopsis thaliana</i>	10	YES				
PHYPA		<i>Physcomitrella patens</i>	4	YES				
CHLRE		<i>Chlamydomonas reinhardtii</i>	0	NO				
MICPS		<i>Micromonas pusilla</i>	0	NO				
OSTLU		<i>Ostreococcus lucimarinus</i>	0	NO				
CYAME		<i>Cyanidioschyzon merolae</i>	0	NO				
PHATC	Other eukaryotes	<i>Phaeodactylum tricornutum</i>	0	NO				
PHYIT		<i>Phytophthora infestans</i>	2	YES				
TETHH		<i>Tetrahymena thermophila</i>	0	NO				
LEIMA		<i>Leishmania major</i>	0	NO				
TRYCC		<i>Trypanosoma cruzi</i>	0	NO				
NAEGR	<i>Naegleria gruberi</i>	2	NO					
AMYMU	Prokaryotes	<i>Amycolatopsis mediterranei</i>	0	NO				
ACTMD		<i>Actinosynnema mirum</i>	0	NO				
TRURR		<i>Truepera radiovictrix</i>	0	NO				
THASP		<i>Thauera</i> sp.	0	NO				
ECOLI		<i>Escherichia coli</i>	0	NO				
VIBF1		<i>Vibrio fischeri</i>	0	NO				
BACSU		<i>Bacillus subtilis</i>	0	NO				

Representative organisms from both eukaryotes and prokaryotes [see Ref. (18) for detailed presentation] were collected and organized by major branches in taxonomy. The number of NOX enzymes in each organism is indicated and color coded. Unicellular and multicellular organisms are indicated by a "NO" and "YES," respectively. Two organisms that are at the transition between the two life forms or have both life forms are indicated as "Transition." One exception is *Naegleria gruberi*, a single-celled organism well known for its capacity to transition from an amoeboid to a flagellated form. It is a free-living organism, but closely related to pathogenic, parasitic species. Therefore, it is plausible that the NOX gene of *Naegleria* might have been acquired from its host via horizontal gene transfer or that it derives from an organism that was at the transition to multicellularity, but lost this characteristic of multicellular organisms as it specialized to its environment. The discovery of NOX-dependent ET generation in the multicellular form of the amoeba *D. discoideum*, an organism that is at the transition to multicellularity, combined to the recognition of the apparent coemergence of multicellularity and NOX enzymes indicate that the origin of ET formation might be traced back to the emergence of multicellular organisms. It also suggests that variants and diverse evolutions of DNA-based defense strategies might be identified in other organisms with functional NOX enzymes, both in primitive metazoans and organisms close to the transition to multicellularity.

that express NOX homologs can generate ROS as signaling molecules to trigger ET formation for host defense. Interestingly, the evolutionary time of emergence of experimentally confirmed ET formation, NOX function, and multicellularity coincide well, possibly indicating that ROS-dependent DNA-based host defenses played a critical role in the early evolution of multicellular organisms guarded by an innate immune system.

In the near future, DNA-based host defense strategies will certainly be identified in a growing number of organisms. We propose that their study will reveal the fundamental significance in the relationship between host organisms and their coexisting commensals and pathogens and bring conceptual changes in the way we approach many relevant human diseases. For example, in higher plants, the roots have direct contact with various microbes in the soil, and among the various host defense mechanisms, the root border cells are able to secrete extracellular DNA to trap and kill bacteria and fungi (19, 20). In analogy, the human gut is colonized by large numbers of microbes, collectively referred to as the microbiota (21). While maintaining intimate contact with the normal microbiota, the intestinal epithelial cells are at the front of host–microbe interactions (22). Enteric pathogenic bacterial infection and antibiotic treatment are able to dramatically change the metabolic profile of the human microbiota and the gut ecosystem, sometimes leading to systemic inflammation and autoimmune responses. Importantly, unregulated ET formation is a major inducing factor of systemic inflammation and autoimmune

diseases in human (23–27). Therefore, understanding whether intestinal macrophages or neutrophils, or some other intestinal cell types, are able to excrete DNA during enteric pathogenic bacterial infections or antibiotic treatment is an interesting but underexplored area. In the future, understanding the chemical dialogs between gut microbiota and ET formation could potentially lead to new therapies to control and cure these diseases.

In conclusion, formation of ETs is an ancient cell-intrinsic defense mechanism that might have played a critical role in the evolution of multicellular organisms, and we need more systematic approaches and a broader perspective to recognize the importance of ETs in host–commensal and host–pathogen interactions. We expect that more related studies in the future will keep up the excitement in this field of research.

AUTHOR CONTRIBUTIONS

XZ and TS designed the experiments and interpreted the results. XZ performed the experiments and XZ and TS wrote the manuscript.

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NETopathies? Unraveling the Dark Side of Old Diseases through Neutrophils

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Neutrophil extracellular traps (NETs) were initially described as an antimicrobial mechanism of neutrophils. Over the last decade, several lines of evidence support the involvement of NETs in a plethora of pathological conditions. Clinical and experimental data indicate that NET release constitutes a shared mechanism, which is involved in a different degree in various manifestations of non-infectious diseases. Even though the backbone of NETs is similar, there are differences in their protein load in different diseases, which represent alterations in neutrophil protein expression in distinct disorder-specific microenvironments. The characterization of NET protein load in different NET-driven disorders could be of significant diagnostic and/or therapeutic value. Additionally, it will provide further evidence for the role of NETs in disease pathogenesis, and it will enable the characterization of disorders in which neutrophils and NET-dependent inflammation are of critical importance.

Keywords: neutrophil extracellular traps, neutrophil, thromboinflammation, autoimmunity, autoinflammation

INTRODUCTION

Neutrophils constitute an essential part of the innate immune system in host defense against pathogens, as shown more than 100 years ago (1, 2). Circulating neutrophils are recruited in vast numbers at the sites of infection or sterile inflammation, in response to a variety of pathogen and host-derived inflammatory mediators (3). There, *via* adhesive interactions with endothelial cells, neutrophils rapidly infiltrate the site of inflammation (4). Uncontrolled inflammation in turn results in the release of newly produced neutrophils from the bone marrow, in a process termed as emergency granulopoiesis (5).

In addition to phagocytosis and degranulation, it has been recently proposed that neutrophils employ an additional strategy, in order to restrain infection: the release of NETs (1, 2, 6, 7).

Neutrophil extracellular traps are extracellular chromatin structures, formed upon certain inflammatory stimuli and composed of cytoplasmic, granular, and nuclear components of neutrophils (1, 2, 6, 7). To date, it is known that they can entrap and possibly kill pathogens. It has been shown that NETs bind bacteria (6, 8, 9) as well as fungi (10). The antimicrobial activity of NETs relies on both cytoplasmic and granular proteins as well as histones. This suggests that the intact NET structure is

crucial for their antimicrobial function, enabling the increased local concentration of antimicrobial factors (1, 2, 6, 7, 11).

Besides their role in infectious disorders, studies carried out after 2008 support the role of NETs in the pathophysiology of non-infectious diseases, such as thrombosis (12–16), autoimmune diseases (14, 17–22), genetically driven autoinflammatory (23), and other inflammation-related diseases (24–26), metabolic disorders (27, 28), lung diseases (29–32), fibrosis (33), and cancer (34–36).

Herein, we seek to review current data regarding the proposed role of NETs in non-infectious human diseases. We also discuss the existing evidence supporting that these structures constitute a common mechanism of the pathophysiology of distinct diseases.

MECHANISM OF NET FORMATION

Despite the morphological similarities of NETs released by neutrophils in response to different stimuli and under diverse conditions, it is nowadays widely accepted that there is more than one mechanism involved in NET release (37). Additionally, mitochondrial DNA also contributes in NET formation (38, 39), whereas, even though *in vitro* NET formation leads to cell death

(40), it is reported that neutrophils that undergo NET release *in vivo* may remain active and functional, suggesting that NET formation may not necessarily be a terminal event (41, 42).

Activated neutrophils undergo dramatic morphological changes in order to release NETs (43–47). The nuclear and granular membranes disintegrate and elastase enters into the nucleus, followed by hypercitrullination of histones, chromatin decondensation into the cytoplasm, rupture of the plasma membrane, and extrusion of nuclear material from the cell into the extracellular space (43–47). The enzymes peptidyl arginine deiminase type IV (PAD4), neutrophil elastase (NE), and myeloperoxidase (MPO) have been implicated in the initial chromatin decondensation and in the degradation of the nuclear envelope (43–47). As a final step, extracellular DNA, histones, and granular enzymes form a network of NETs that entrap endogenous (e.g., platelets) and extrinsic (e.g., bacteria) particles and molecules (Figure 1A) (43–47). The negatively charged DNA acts as the backbone of the NET, interacting with other NET components through positive electrostatic charge (43–47). As it has been recently described, this scaffold is crucial for NET proteins to maintain their function (6, 43, 47), since dismantling of NET structures by DNase abolishes their antimicrobial activity (6). However, in the majority of

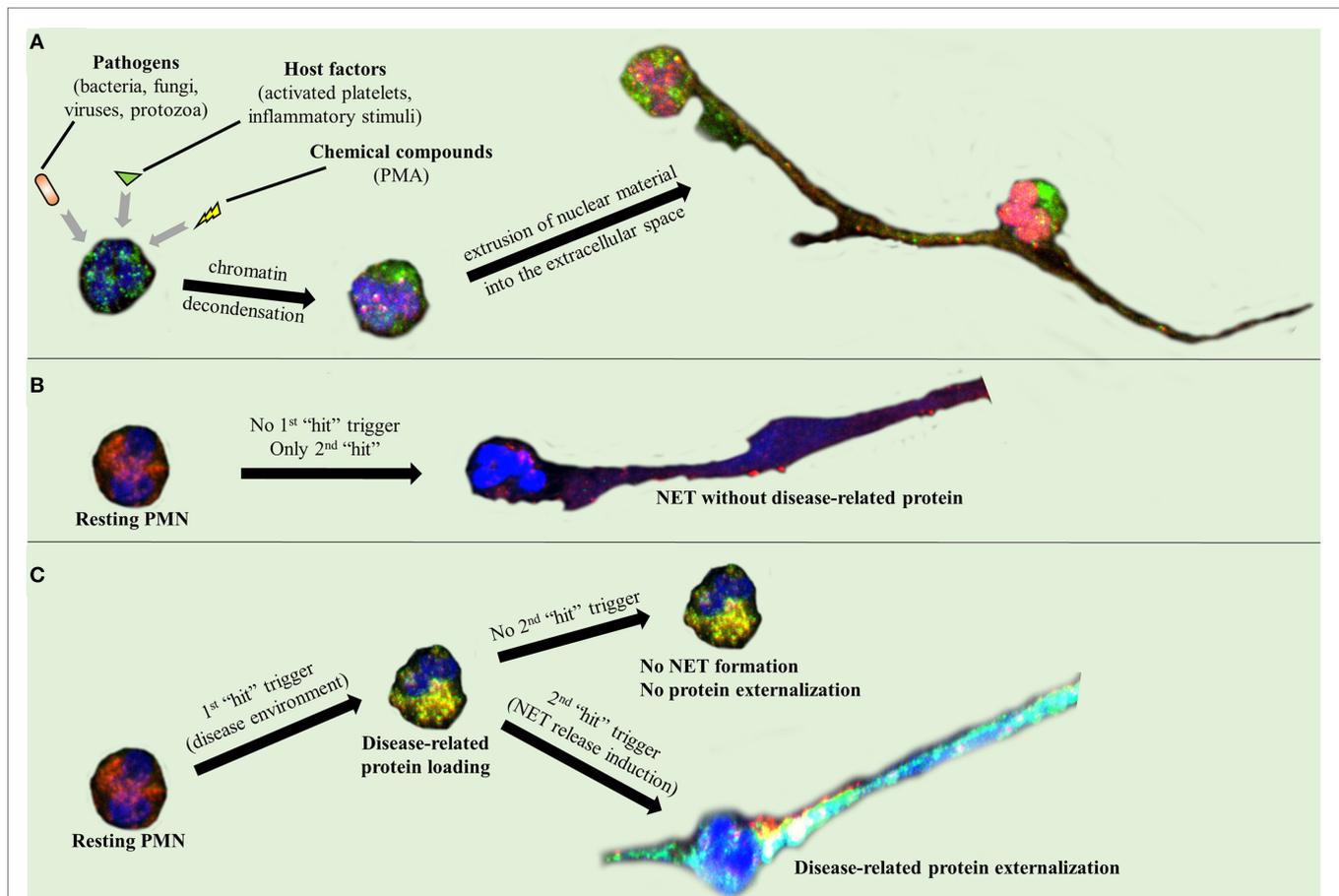


FIGURE 1 | Neutrophil extracellular trap (NET) formation and protein decoration. Representative images taken using confocal microscopy, demonstrating (A) NET formation mechanism and (B,C) the two-step process through which the disease-related protein is externalized.

these studies, PMA was used as a NET inducer (48, 49). Based on the criticism directed against the use of PMA as a NET inducer, the exact intracellular pathway that leads to NET release is still unclear (50).

At the molecular level, NET formation is still poorly understood and it is not defined whether neutrophils employ a similar mechanism to release NETs under different circumstances. However, there is strong evidence that the production of reactive oxygen species (ROS), the relocation of NE and MPO into the nucleus, histone citrullination, and eventually the rupture of the plasma membrane are, sequentially, involved in NETosis (43–47).

Cell metabolism has major contribution in immune cell function (51), including neutrophil activation. Neutrophils rely mainly on glycolysis for their metabolic needs, enabling their adaptation to the highly hypoxic inflammatory sites (52, 53).

Moreover, there is evidence that the metabolic shift to the pentose phosphate pathway is important for NET release, due to the involvement of glucose-6-phosphate dehydrogenase in fueling NADPH oxidase-2 with NADPH, to produce an effective amount of ROS and thus induce NETs. In contrast, mitochondrial ROS release, which is NADPH-independent, is not effective in signaling for NET production (54, 55).

Additionally, NET formation has been shown to require, at least in certain circumstances, the activation of autophagy (56). Autophagy is an anti-apoptotic mechanism activated in response to cell stress, in order to regulate protein and organelle turnover, ensuring cell survival (57). The protein kinase mammalian target of rapamycin (mTOR) negatively regulates autophagy, involved also in NET formation (58, 59). We and others (23, 24, 33, 56, 60–62) have shown that blocking autophagy through PI3K signaling, either at the initial levels by using 3-methyladenine (24, 60–62) or at the level of autophagosomal acidification by using wortmannin or bafilomycin (23, 33, 56, 62), inhibits the induction of NET release. However, more mechanistic studies are needed to identify how autophagy is involved in NET release, even though mTOR signaling and ROS production have been linked to both processes (7, 56, 59).

It is suggested that autophagy is crucial for NET release in both infectious and non-infectious diseases, including sepsis, familiar Mediterranean fever (FMF), gout, and inflammatory-driven fibrosis (12, 23, 56).

CAN NET CARGO DEFINE NEUTROPHIL ROLE IN DISEASE?

Independently of the stimulus, NETs are composed of DNA, citrullinated histone 3 (cit-H3), NE, and MPO, the three main proteins commonly used for their detection (43–45, 47). Even though a proteomic analysis of infiltrating neutrophils in diverse tissues and in different disorders could be the proof of concept, there is evidence proposing that neutrophils express and release in the form of NETs a variety of proteins, depending on the specific inflammatory environment (63). For example, tissue factor (TF) was detected on NETs in vein and arterial thrombosis (16, 64, 65), interleukin 1 beta (IL-1 β) in gouty arthritis (24) and FMF (23), interleukin 17 (IL-17) in psoriasis (66) and pulmonary fibrosis

(33), antimicrobial peptide LL-37 in systemic lupus erythematosus (SLE) (19), and PAD4 in rheumatoid arthritis (RA) (67).

Even though NETs constitute a common event in distinct pathophysiologic conditions, the expression of distinct bioactive proteins on NETs in different disorders might be the one that determines their specific function in disease pathogenesis.

A two-“hit” process has been proposed to explain the differential protein cargo of NETs in distinct disorders. The first “hit” in this process is the disease-specific environment that primes neutrophils to express disease-associated protein. A second “hit” is then required for the induction of NET formation (**Figures 1B,C**). However, this is a simplified model, and we cannot exclude the possibility that the same stimulus can drive both events. A typical paradigm of this two-“hit” model has been described in ST-segment elevation acute myocardial infarction (16). It has been shown in acute coronary syndromes that a variety of inflammatory stimuli trigger the cytoplasmic expression of TF in circulating neutrophils. At sites of atherosclerotic plaque rupture, locally activated platelets interact with TF-loaded neutrophils leading to the release of TF-bearing NETs inside the affected artery. The release of functional TF on NETs is able to further induce thrombin generation and platelet activation, creating a possible vicious cycle, that leads to thrombus propagation and stability (16).

The expression of these “disease-related” proteins on NETs could increase their local bioactivity (12, 14, 16, 23, 66, 68). On the other hand, it has been shown that, at high densities, NETs limit inflammation by degrading cytokines and chemokines (69). This balance between the pro-inflammatory and prothrombotic role of NETs, though the expression of cytokines like IL-1 β and IL-17, and their anti-inflammatory role, could be exploited for the development of new therapeutic approaches.

In the following section, we review the clinical and experimental data that link NETs with pathogenesis of several disorders. Even though the list of diseases in which NETs have been identified is extensive, we believe that the further characterization of the degree of NET involvement in such disorders could enable the classification of diseases in which NETs have a definite and strong involvement under the term of “NET-driven disorders” or “NETopathies.” The term NETopathy(ies) is derived from the abbreviation NET and the Greek word *πάθος* = *pathos*, which means disorder.

NETs in Thromboinflammation

The widely accepted cross talk between inflammation and thrombosis has led to the introduction of the term thromboinflammation (68). Cells of the hematopoietic system, including neutrophils, platelets, and monocytes, have a major role in this process (64). There is increasing evidence implicating NET release with the development of both vein and arterial thrombosis (12, 14, 16, 26, 65, 70–77). Extracellular deposition of DNA co-localized with neutrophil granule proteins has been shown in thrombi from patients with deep vein thrombosis (DVT) (78), especially at the phase of organization of the thrombus (70). Additionally, circulating extracellular DNA in the form of nucleosomes and DNA associated with neutrophil granule proteins, supporting the induction of NET release, has been identified in blood samples from

patients with DVT (79, 80). Similarly, NETs have been identified in thrombus specimens from patients undergoing thrombectomy in the context of myocardial infarction (15, 16, 62, 71). In a recent multicenter study in patients presenting with stent thrombosis, neutrophils were the more abundant leukocyte population in thrombus specimens, whereas NETs were identified in 23% of thrombi (71). Regarding specific disorders associated with thrombotic manifestations, NETs in thrombus specimens and/or increased levels of nucleosomes have been identified in disseminated intravascular coagulation in sepsis (73), in paroxysmal nocturnal hemoglobinuria (81), thrombotic microangiopathies (82), antiphospholipid syndrome (APS) (74), antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) (14), or hemodialysis-related thrombogenicity (83). These clinical data support a role for NETs in the development of both arterial and venous thrombosis.

The prothrombotic role of NETs was further confirmed in several experimental animal models. NETs were observed in thrombi, in a baboon model (75) and in several mouse models of DVT (64, 76, 84). In a mouse model of DVT, infusion of DNase I resulted in protection from thrombosis (76), whereas PAD4^{-/-} mice were protected from thrombosis (85), supporting the pathogenetic role of NETs in venous thrombosis, at least in this animal model. The *in vivo* role of NETs in the development of thrombosis was further shown in a mouse model of APS (86). Additionally, NETs contribute in cancer-induced venous thrombosis, as shown in a mouse model of chronic myelogenous leukemia (34) and in the RIP1-Tag2 model of insulinoma and MMTV-PyMT model of breast cancer (77). Brill et al. linked histones with the prothrombotic effect of NETs, since histone infusion also resulted in thrombosis. However, there is evidence that NETs participate in DVT *via* interaction with von Willebrand factor, a factor that potentially activates platelets (76). Furthermore, it has been reported that in a mouse model of DVT TF triggers intraluminal fibrin formation, while the release of NETs activates factor XII, consolidating DVT (64). The involvement of NET-bound TF, which is the main *in vivo* initiator of coagulation (87), in NET-dependent thromboinflammation has been shown in several studies, since TF has been identified in NETs released in neutrophils from patients with sepsis, APS, AAV, or myocardial infarction (12, 14, 16, 74) or in a mouse model of DVT (64).

The interplay between neutrophils and platelets has been shown to have a major contribution in NET release (16, 62, 72, 84, 88). Clark et al. have shown that upon toll-like receptor 4 (TLR4) activation platelets induce the formation of NETs in a mouse model of sepsis (72). This leads not only to bacterial but also to platelet entrapment in NETs, resulting in tissue damage (72). Several studies have further identified platelet derived high mobility group box 1 (HMGB1) as the factor that mediates platelet–neutrophil interaction and NET release (62, 84). HMGB1 released by platelets has been shown to promote thrombosis in a mouse model of DVT (84), whereas it mediates neutrophil activation in the context of myocardial infarction (62). The importance of platelet–neutrophil interaction is prominent in coronary artery thrombosis, since it was proved that coronary thrombi are mainly composed of interacting neutrophils and platelets (16, 62). The

rupture of the atherosclerotic plaque primes a cascade of events, which results in platelet activation and NET release, leading to thrombus formation and blood vessel occlusion. The expression of TF on NETs may propagate the further activation of the coagulation system, leading to thrombus expansion (16).

Taken together, there is strong evidence, derived by clinical and experimental observation, that neutrophils and NETs are major players in both venous and arterial thrombosis. The development and clinical use of factors that target NETs could provide, however, the definite proof for the role of NETs in thrombotic disorders.

NETs in Autoimmune Diseases

A growing number of studies demonstrate that NETs play a driving role in the pathogenesis of a variety of autoimmune disorders, such as SLE, AAV, RA, and psoriasis. In the aforementioned disorders, NETs are a main source of autoantigens, are present in excess amount, or are decorated with disease-specific proteins.

Systemic Lupus Erythematosus

Systemic lupus erythematosus is a systemic autoimmune disease and a well-studied model. SLE is characterized by systemic production of autoantibodies against a plethora of intracellular and extracellular targets. These autoantibodies are able to cause extensive tissue damage (89, 90).

There is evidence supporting the involvement of NETs in the pathophysiology of SLE. It has been shown that NETs are directly associated with the severity and the progression of the disease (91–95). Neutrophils from SLE patients are primed to undergo NET release (17, 96). Autoantibodies and more specifically antibodies against LL-37 have been shown to activate neutrophils for NET release (18, 19). On the other hand, NETs are composed of DNA, histones, and proteins-like LL-37, providing a possible source of autoantigens for the development of lupus-specific autoantibodies (**Figure 2B**) (17–19, 97–99). Interestingly, Villanueva et al. reported a neutrophil subpopulation in SLE, termed as low-density granulocytes (LDG), prone to release NETs, which promote vascular damage (18, 91, 100). It was further demonstrated that LL-37-bearing NETs fuel the immune response in SLE by activating plasmacytoid dendritic cells (pDCs) in an Immunoglobulin-Fc region receptor II-a (FcRIIa) and TLR9-dependent manner. This leads to interferon alpha (IFN α) production, which is a critical player in the pathogenesis of SLE. Furthermore, IFN α triggers NET generation and activates T and B cells leading to the production of antibodies against NETs, creating a vicious cycle (19, 97, 101, 102).

Interestingly, there is a disease-associated defect in the clearance of NETs, due to the reduced activity of DNase I and the increased amounts of DNase I inhibitors (17, 20, 94, 103–106), supporting the hypothesis that dysregulation of NET clearance may be one of the initial steps that lead to lupus-specific autoantibody production.

ANCA-Associated Vasculitis

Antineutrophil cytoplasmic antibody-associated vasculitis is described as a group of autoimmune diseases, characterized by the presence of autoantibodies against the neutrophil granule

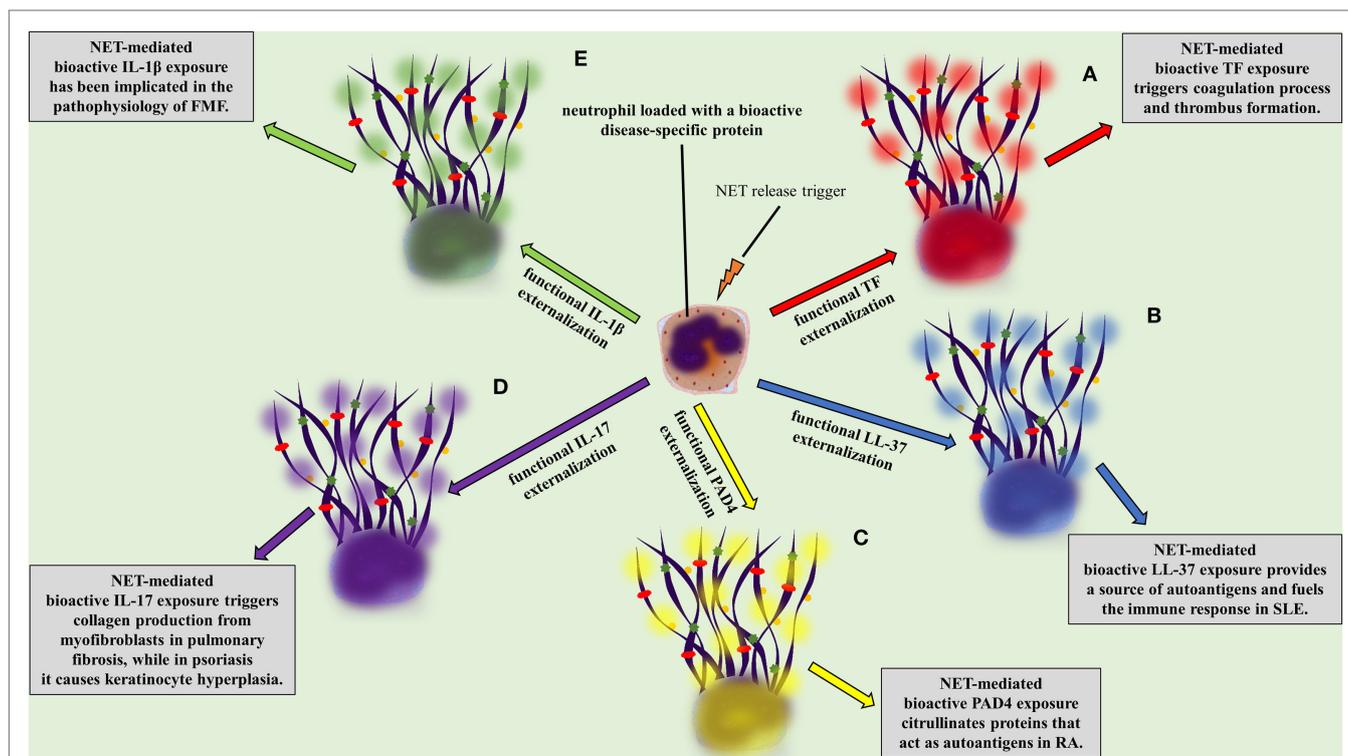


FIGURE 2 | NETopathies are ruled by the bioactive disease-related neutrophil extracellular trap (NET) proteins. The clinical manifestations of (A) thrombosis, (B) systemic lupus erythematosus, (C) rheumatoid arthritis (RA), (D) pulmonary fibrosis and psoriasis, (E) familial Mediterranean fever are determined by the NET-mediated exposure of bioactive disease-related proteins.

proteins, such as proteinase 3 (PR3) and MPO. The study by Kessenbrock et al. provided the initial evidence for the link between NETs and AAV. In this study, the intraglomerular deposition of NETs in biopsies from patients with small-vessel vasculitis was shown. Additionally, it was shown that neutrophils release NETs when activated with ANCA (107). Further studies confirmed the deposition of NETs in affected tissues from patients with AAV (14, 61, 108–110), whereas increased levels of circulating NET remnants were observed in patients with AAV (14, 22). Additionally, a recent study correlated AAV disease activity with the presence of NET-prone LDGs in peripheral blood (110). NETs were further associated with the AAV hypercoagulability, since NETs released during active disease are loaded with TF [Figure 2A (14, 111)].

Since PR3 and MPO are abundantly present in NETs, it has been proposed that NETs mediate the extracellular exposure of these potential autoantigens, having an important role in the initiation of the disease (17, 20, 21, 107). Sangaletti et al. have shown that myeloid DCs can acquire neutrophil proteins released in the form of NETs. Furthermore, immunization of mice with DCs co-cultured with NET remnants resulted in the development of MPO-ANCA and renal vasculitis (112). A common characteristic between SLE and AAV is the decreased degradation of NETs, attributed to the reduced activity and inhibition of DNase I, as well as to the protection over NETs by autoantibodies and components of the complement (17, 20, 107).

RA and Psoriasis

Rheumatoid arthritis is a chronic autoimmune disease that affects synovial joints. It is known that neutrophils are the most abundant cell type of synovial fluid in RA patients (113).

Recent studies identified the presence of NETs in the circulation and the release of NETs by synovial neutrophils (114, 115). Khandpur et al. have shown that TNF, IL-17, and anti-citrullinated protein antibodies (ACPA) promote NET release by neutrophils from patients with RA, whereas therapeutic blockade of TNF function has been shown to decrease the extensive NET generation that characterizes RA patients. Of interest, IL-17 was able to promote NET release only in neutrophils from patients with RA, which implies that the disease-specific inflammatory microenvironment primes neutrophils for NET formation (115).

Recent studies highlight that citrullinated histones in NETs consist autoantigens that stimulate and participate in the outset of the excessive inflammation, and more specifically in ACPA immune response, in RA (18, 115). It has been further demonstrated that RA-driven NETs are decorated with enzymatically active PAD4, which possibly further citrullinates targets, rendering them autoantigens (Figure 2C) (49, 67, 116). Finally, NETs in RA indirectly participate in the stimulation of distinct cell types, such as fibroblast-like synoviocytes, which invade and damage cartilage in RA (115, 117).

The possible involvement of NETs in the pathogenesis of psoriasis has been also proposed. Psoriasis is an autoimmune

skin disorder characterized by epidermal hyperplasia and neutrophil infiltration in the epidermis. Neutrophils are involved in the pathophysiology of psoriasis, linking innate and adaptive immune system, and acting as a main source of IL-17 (66, 118, 119).

Interleukin 17 has a significant role in the pathophysiology of psoriasis causing keratinocyte hyperplasia (119, 120), whereas therapeutic administration of antibodies against IL-17 is efficacious in the treatment for psoriasis (121–123). The externalization of IL-17 in a bioactive form is feasible through NET formation (Figure 2D) (66, 124), which has been also observed in models of RA (115) and pancreatitis (125). The fact that the active form of IL-17 lies on NETs renders it an easily accessible target.

Taken together, a significant amount of evidence suggests that NETs contribute in the pathogenesis of several autoimmune disorders, acting either at the initiation of disease, providing a source of autoantigens, or promoting tissue injury (66, 90, 93, 107, 109, 115). There are reports suggesting that NETs can activate other inflammatory cell populations and promote the activation of the adaptive immune system (97, 102, 115). However, whether the specific structure of NETs and the possible modification in proteins loaded on NETs have a major impact in the break of tolerance and induction of autoimmunity still remains elusive.

NETs in Autoinflammatory Diseases

Recent studies revealed a possible role for NETs in the inflammatory response that governs autoinflammatory syndromes, including gout and FMF.

Gout is an autoinflammatory type of arthritis caused by the intra-articular deposition of monosodium urate crystals (MSU crystals). The deposition causes inflammatory attacks due to innate immunity activation (126–129). Additionally, the chronic form of the disease is characterized by tophus formation, causing mechanical destruction of the joint (130). It has been shown that MSU crystals cause a strong induction of NETs (24, 131) which, in high neutrophil concentrations, ameliorates MSU crystal-induced inflammation by promoting the degradation of inflammatory cytokines and chemokines in a mouse model of MSU-induced inflammation (69, 132). Despite their protective role, NETs indirectly engender the destruction of the joint by easing the packing of MSU crystals and the formation of tophi (69, 132). However, whether NETs support the initiation of gouty inflammation in humans remains unanswered.

Familiar Mediterranean fever is a hereditary autoinflammatory disorder, characterized by inflammatory attacks and neutrophil infiltration into the affected sites (23). Moreover, it is an IL-1 β -mediated disease, and this is clear due to the fact that IL-1 β blockade constitutes an emerging treatment in FMF (23, 133, 134). During FMF attacks, neutrophils undergo excessive NET formation, which decreases after the inflammation dissolution (23).

During FMF attacks increased levels of circulating MPO-DNA complexes are detected, suggesting the release of NETs in the systemic circulation, whereas their levels normalize during the resolution phase of the disease (23). The detection of bioactive IL-1 β in NETs released *ex vivo* by patient neutrophils or

control neutrophils treated with FMF attack serum implies that neutrophils serve as critical effector cells in the amplification of inflammation in FMF (Figure 2E) (23).

NETs in Metabolic Disorders

In type II diabetes (T2D), immunological changes lead to altered levels of cytokines and changes in both number and activation status of various leukocytes, including neutrophils (135). Until recently, it was thought that inflammatory responses may have a dual role in T2D, as they seem to have a causal relationship leading to resistance to insulin, while on the other hand they seem to be intensified by the hyperglycemic state, resulting in T2D complications (135).

Bearing in mind that diabetes affects neutrophil count and activity, that hyperglycemia-driven oxidative stress facilitates diabetic complications, and that neutrophils generate oxidative stress in diabetes, it was assumed that a dysregulation in NETosis may represent the link among hyperglycemia, oxidative stress, inflammation, and diabetic complications (27). In this direction, a recent study demonstrated that high glucose *in vitro* and hyperglycemia *in vivo* induce release of NETs and their products (27). Another study provided evidence that hyperglycemic conditions lead to the formation of short-lived and unstable NETs, while also prime neutrophils and constitutively activate NET formation, leading to reduced response to subsequent external stimuli (136). Thus, it was hypothesized that neutrophils primed due to hyperglycemia may not respond to further external stimulus in T2D patients, making them susceptible to infections (136). Finally, a third study demonstrates that, in T2D patients, dysregulated NET release caused by hyperglycemia is responsible for impairment of wound healing as well as for diabetic complications (137). Even though these studies support a role for NETs in T2D, it is not clear to what extent manipulation of neutrophils could ameliorate or prevent diabetic complications.

Moreover, there is evidence that neutrophils and NETs have a potential role in the pathogenesis of type I diabetes (28, 138, 139); however, their implication in the onset and/or the development of this disease has not been investigated so far.

NETs in Lung Diseases and Fibrosis

Neutrophil extracellular traps have been implicated in inflammatory lung diseases and inflammatory-derived fibrosis (33). Several inflammatory lung diseases are characterized by the migration and detection of neutrophils and monocytes in the airway lumen and the bronchoalveolar lavage fluid (140). NETs have been associated with inflammatory diseases, such as chronic obstructive pulmonary disease (COPD), cystic fibrosis (CF), acute lung injury, acute respiratory distress syndrome, and asthma (29, 30).

Cystic fibrosis is characterized by abundant free DNA structures in airway fluids that increase the viscosity of the sputum and lead to airflow obstruction and tissue damage. Free DNA originates mainly from NETs released from neutrophils that are recruited to the area in an effort to kill the bacterial burden, but they finally contribute to the damage of lung tissue (31, 32). Additionally, it has been proved that NE plays an equally important role in CF, leading to tissue damage, especially in patients

under treatment that are characterized by increased DNA cleavage (141). Recombinant human deoxyribonuclease (rhDNase) is an adjunctive to antibiotics treatment for patients with CF over the last two decades, showing a beneficial effect at least in a subpopulation of patients with CF (142, 143). Moreover, it has been reported that DNase I and histone-blocking antibodies have been used in mice against transfusion-related acute lung injury, in which NETs play a crucial role (144). Inhibition of either NE or NET release in general could be a novel future therapeutic strategy in patients with CF (141, 145).

There is evidence that the inflammatory microenvironment developed in chronic lung diseases including COPD and interstitial lung disease contributes either to localized or to generalized fibrosis, respectively. Specific fibrosis-related agents, such as cigarette smoke, magnesium silicate, and bleomycin, stimulate neutrophils to undergo NETosis. NETs indirectly regulate fibrosis by activating lung fibroblasts and differentiating them into myofibroblasts, through autophagy and histone hypercytullination. Subsequently, NET remnants, such as IL-17, regulate connective tissue growth factor (CCN2) expression and collagen production by the differentiated fibroblasts and not their differentiation (**Figure 2D**). However, NET degradation significantly restricts these effects, indicating that it could be possibly used as a restraining mechanism against fibrosis (33).

NETs in Cancer

In the last few years, NETs have redefined the role of neutrophils in tumor biology (34–36, 146–150). It is suggested that NETs may act within the primary tumor promoting tumor progression (146–148), while at remote sites they might sequester circulating cancer cells favoring metastasis (35, 36, 149). Additionally, NETs have been implicated in cancer-associated thrombosis (34, 147).

There is increasing evidence supporting that, in both experimental models and cancer patients, NET deposition in the tumor mass is associated with tumor progression (35, 146, 150–153). A finding that supports the implication of NETs in tumor biology is that tumor cells predispose neutrophils to undergo NETosis (34, 146). Moreover, in the tumor microenvironment, NETs interact with tumor cells and expose them to bioactive proteins, possibly favoring their survival through induction of proliferation and inhibition of apoptosis, as well as supporting their escape from the primary tumor (148).

Excessive NET deposition leads to a persistent inflammatory state (154–156), which in cancer probably promotes the expression of adhesion molecules (157–159). Under inflammatory conditions, when NET formation is induced, circulating tumor cells are more prone to adhere to end organ vasculature (158–160). Thus, given that the entrapment of bacteria is one of the primary roles attributed to NETs, they probably act accordingly to capture circulating tumor cells. By entrapping tumor cells and exposing them to various neutrophil-derived factors, NETs may generate a microenvironment rich in proteins and enzymes that promote tumor cell survival and progression (35, 36, 149, 153). Taken together, these data support a potential pro-metastatic role for NETs, involved in early adhesion, proliferation, invasion, and angiogenesis.

Neutrophil extracellular traps have also been implicated in cancer-associated thrombosis, the second most common cause of death in cancer patients (34). Recently, it was demonstrated that, through the generation of NETs, neutrophils provide a scaffold and a stimulus for platelet adhesion and thrombus formation (75). NETs were shown to promote coagulation as well (68, 75). Moreover, a recent study based on murine models reported that both leukemia and solid tumors produce a factor, G-CSF, that primes neutrophils to undergo NETosis and predisposes the host to thrombosis (34). In conclusion, NETs have been identified as a key player in cancer-associated thrombosis.

The biological significance of NETs in cancer remains unclear. It is hypothesized that initially they represent a reaction of the tumor environment against the growing cancer. However, NETs seem to play an adverse role in tumor growth, offering a scaffold with an array of biologically active molecules attached on it, which may promote malignant cell survival, growth, and local tumor expansion.

THERAPEUTIC AND DIAGNOSTIC/ PROGNOSTIC POTENTIAL OF NETS

To date, clinical and experimental evidence highlight the significant role of NETs in the pathophysiology of the aforementioned diseases. Even though studies in animal models have shown the beneficial role of NET inhibition, especially in thrombosis, it is yet unknown whether NET-targeting therapies could be effective in clinic (161). NET induction or inhibition could be beneficial for patients with diseases that have been associated with restricted or excessive NET formation, respectively (**Table 1**). To this end, drug repositioning offers the opportunity for the immediate use of therapeutic agents that induce or inhibit NETs, which are already used in clinic (11).

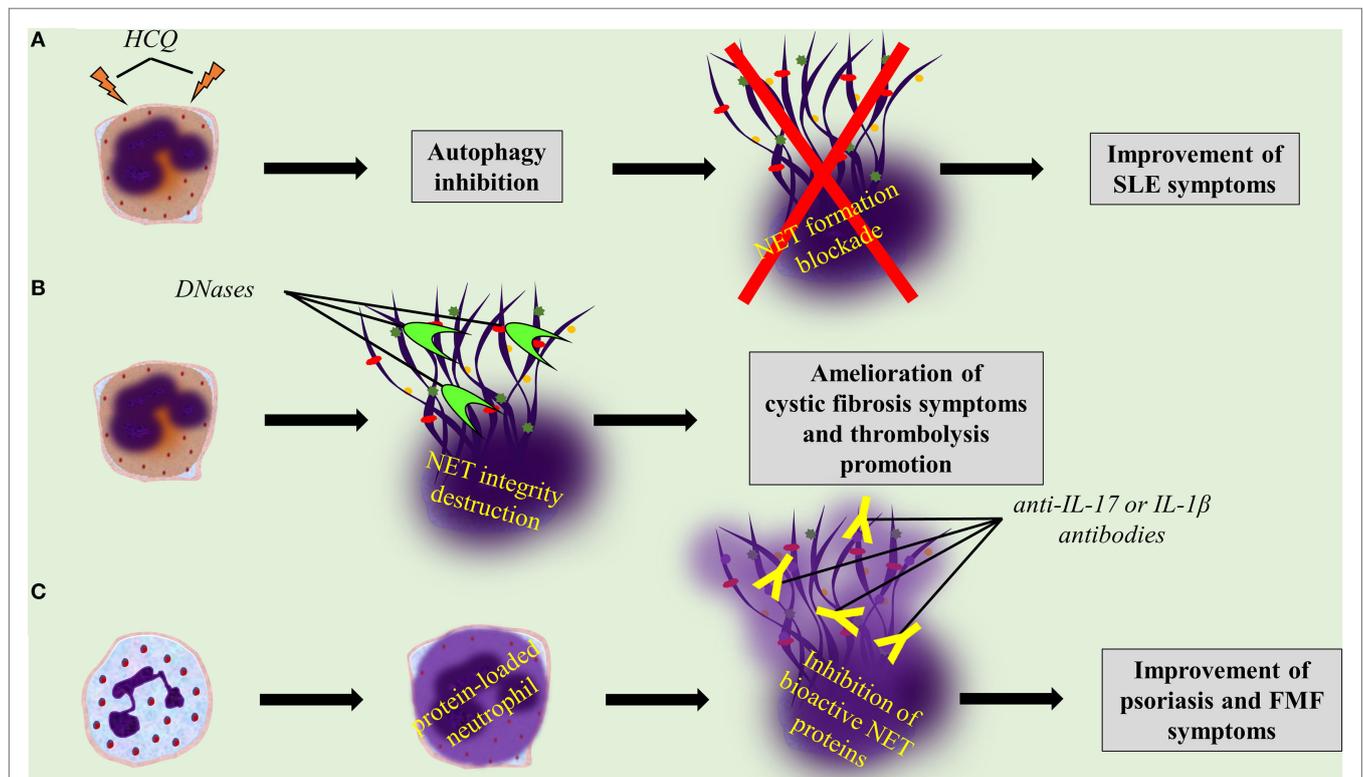
Several drugs already used in clinical practice might affect either NET formation or integrity, or the expression of NET proteins. For instance, it is known that hydroxychloroquine (HCQ), a drug that has been used for decades in the treatment of SLE, has anti-autophagic effect (162). Since the autophagic machinery is an essential step for NETosis, the effectiveness of HCQ may be mediated through the indirect inhibition of NET formation (**Table 1; Figure 3A**). In addition, rhDNase administration, a therapy used in patients with CF aiming to the liquefaction of mucus (142), may possibly target NET structures. DNase promotes thrombolysis *via* degradation of NETs in murine models (**Table 1; Figure 3B**) (64, 76). Moreover, monoclonal antibodies are widely used against bioactive NET proteins, externalized through NET formation. In psoriasis, treatment with anti-IL-17 antibodies (121), probably targets the IL-17-decorated NETs, the main origin of bioactive IL-17 in psoriasis (66). Finally, NET-bound IL-1 β may be one of the targets of anti-IL-1 β therapies, such as canakinumab which targets bioactive IL-1 β in FMF or gout patients (**Table 1; Figure 3C**) (134).

There are a few recent studies demonstrating that NETs could also have prognostic and/or diagnostic potential, as they could represent a disease activity marker for some of the aforementioned

TABLE 1 | Potential and applied therapeutic strategies targeting neutrophil extracellular traps (NETs).

NET formation blockade		NET integrity dismantling		NET components antagonism	
Drug (activity)	Disorder (species)	Drug (activity)	Disorder (species)	Drug (activity)	Disorder (species)
Hydroxychloroquine (<i>autophagy inhibition</i>)	SLE (h) (162)	DNases (<i>DNA dismantling</i>)	Thrombosis, cystic fibrosis (h) (64, 76, 142)	Secukinumab (<i>IL-17 inhibition</i>)	Psoriasis (h) (121)
N-acetylcysteine (<i>ROS reduction</i>)	SLE (h) (163)	Heparin (<i>chromatin dismantling</i>)	Thrombosis (h) (75)	Anakinra & Canakinumab (<i>IL-1β inhibition</i>)	FMF, gout (h) (23, 24, 134)
Sifalimumab (<i>IFN-α inhibition</i>)	SLE (h) (164)				
Cl-Amidine (<i>PAD family inhibition</i>)	RA, SLE (m) (165, 166)				
GSK199 (<i>PAD4 inhibition</i>)	(m) (167)				
Adalimumab (<i>TNF inhibition</i>)	RA, psoriasis (h) (115, 168)				
Roflumilast (<i>neutrophil-platelet interaction inhibition</i>)	Thrombosis (h) (169)				
Eculizumab (<i>C5a inhibition</i>)	PNH (h) (170)				

FMF, familial Mediterranean fever; h, human model; IFN- α , interferon alpha; IL-17, interleukin 17; IL-1 β , interleukin 1 beta; m, murine model; PAD4, peptidyl arginine deiminase type IV; PNH, paroxysmal nocturnal hemoglobinuria; RA, rheumatoid arthritis; ROS, reactive oxygen species; SLE, systemic lupus erythematosus; TNF, tumor necrosis factor.

**FIGURE 3 | Targeting neutrophil extracellular trap (NET) formation or integrity, or specific NET proteins, promises novel therapeutic strategies.**

(A) Hydroxychloroquine inhibits NET formation through its anti-autophagic activity. (B) rDNase and DNase I dismantle NET structures. (C) Anti-interleukin 17 (IL-17) and anti-interleukin 1 beta (IL-1 β) antibodies blockade bioactive IL-17 and IL-1 β on NETs, respectively.

diseases (161). Furthermore, the measurement of NET release or specific NET protein expression in blood samples and biopsies could be a useful diagnostic tool (150, 171). Nevertheless, further experimental data are needed to evaluate the therapeutic, prognostic, and/or diagnostic potential of NETs.

CONCLUSION

The identification of NETs and the characterization of their role in disease pathogenesis. Phagocytosis of pathogens and limitation

of infection was considered the exclusive role of neutrophils. However, mechanistic studies in animal models and clinical observation dramatically altered our perception of the involvement of neutrophils in disease during the last decade. From a patrolling police force, neutrophils are considered nowadays an important player in autoimmune diseases or thrombotic disorders, which were previously thought to be exclusively mediated by adaptive immune system and platelet or endothelial cells, respectively. The characterization of the differential protein load and function of neutrophils, and subsequently of NETs, in distinct disorders can provide novel diagnostic targets and targets for therapeutic intervention. Additionally, the study on the role of NETs in modulation of tissue homeostasis, including the initiation and resolution of inflammation and the elucidation of the effect of NETs on different cell population involved in inflammatory, autoimmune, or thrombotic disorders, will increase our

knowledge in the mechanisms that govern the pathogenesis of complex disorders. The clarification of the role of NETs in the pathogenesis of such disorders and the clinical use of therapeutic agents that target NETs will enable the identification of a group of disorders that could be characterized by the term NET-associated diseases or NETopathies.

AUTHOR CONTRIBUTIONS

AM, AA, SA, IM, and KR wrote the manuscript and created the figures. IM and KR also revised the manuscript.

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Bet on NETs! Or on How to Translate Basic Science into Clinical Practice

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Neutrophil extracellular traps (NETs) comprise neutrophil DNA, citrullinated (deiminated) histones, and proteases (1, 2). NET formation *in vivo* mostly occurs at the sites of inflammation. Neutrophils, adhering to the endothelium or after extravasation, generate NETs upon interaction with microbes, activated platelets, cytokines, alarmins, such as high-mobility group box 1 (HMGB1), or uric acid. Environmental cues, such as hyperosmolarity or hyperglycemia, neurotransmitters, and some autoantibodies also trigger NET generation (3–7). *In vitro*, two main pathways are activated: suicidal and vital NETosis (8). In the former, membrane integrity is disrupted and neutrophils die. In contrast, after “vital” NETosis neutrophils still migrate, chase bacteria, and extrude residual nuclear material through exocytosis (9). Generation of reactive oxygen species (ROS) and fusion of neutrophil primary granules with the nuclear membrane that promote interactions between elastase, myeloperoxidase (MPO), and DNA are features of suicidal NETosis. Their role in vital NETosis is debated (8, 10–12). Activation of the autophagic pathway is intermingled with NET generation. Autophagy sustains the metabolic requirements of the extensive intracellular vesicular formation, transport, and fusion associated with NET generation. It also sustains neutrophil survival (5).

Neutrophils undergo extensive *ex vivo* manipulations and methodological approaches vary among research groups, possibly explaining some discrepancies and nurturing some healthy skepticism (13). Some reports indicate a role of the mitochondrial DNA, which is devoid of histones, in NET generation. The relative contribution of nuclear vs. mitochondrial DNA to extracellular traps generation remains a controversial issue (14).

Under physiological conditions, NETs enhance the host response to microbes by (i) providing a template concentrating humoral innate immune molecules, such as pentraxin 3 (PTX3) and complement, together with microbicidal molecules, such as histones, MPO, proteinase 3 (PR3), or cathelicidin, and (ii) contrasting the hematogenic spread of pathogens through immunothrombosis (i.e., activation of platelets and of the coagulation cascade through NET components) (15). The actions of NET-embedded von Willebrand factor, of citrullinated histones, and of negative charges on platelet recruitment/activation and on the progression of the coagulation cascade contribute to thrombosis. Excellent reviews on this issue have been published (16, 17). The bioactive molecules integrated within the chromatin threads may vary according to the inciting stimuli or the environment. The characterization of the NETs proteome is a fascinating challenge for the near future.

Neutrophils are abundant in the blood, and their concentration and activation state increase following surges of systemic cytokines during acute phase responses. Thus, NETs are readily available and easily renewable tools for the first-line response to infectious agents. However, there is a price to pay. During acute infections (e.g., during sepsis), NETs favor progression toward septic shock, disseminated intravascular coagulation, and acute respiratory distress syndrome (ARDS), as well as tissue damage and organ failure, possibly because of the deleterious effects of immunothrombosis. On the other hand, the imbalance between the rate formation and degradation of NETs might favor the persistence and the presentation of autoantigens and facilitate autoimmunity (1).

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Systemic lupus erythematosus (SLE) is a paradigm of the pathogenic *continuum* that links nuclear antigens exposure to autoimmunity. Neutrophil from patients with SLE show prolonged survival (possibly through enhanced autophagy) and are thus endowed with additional chances to generate NETs. In addition, the clearance of extracellular DNA in SLE is impaired either as a genetically determined trait influencing waste disposal or due to antibodies toward regulatory molecules, such as DNases (18, 19).

Antineutrophil cytoplasmic antibodies (ANCA) represent a variant on the theme of autoantibodies elicited following enhanced generation and/or persistence of NETs. The formation of NETs at sites of vascular inflammation in ANCA-associated vasculitides (AAV) and the ability of ANCA to perpetuate the generation of NETs were described in 2009 (3). Later on, dendritic cells have been found to capture ANCA targets (i.e., MPO and PR3) from NETs and to present them to T lymphocytes, while the susceptibility to ANCA generation was found to be deeply rooted in genetics. Neutrophil priming with HMGB1, a well-established inducer of NETs and of autophagy, contributes to the action of ANCA (20).

In rheumatoid arthritis, NETting neutrophils infiltrate the synovium and rheumatoid nodules, while anti-citrullinated peptides antibodies apparently boost the generation of NETs (21). B cells that expand and differentiate within ectopic synovial germinal centers frequently target deiminated antigens generated during NETosis (22, 23) and/or as a consequence of the release of bioactive peptidylarginine deiminases in the synovial fluid.

Anti-phospholipid antibodies in combination with ROS and platelet-assisted TLR4 stimulation induce neutrophils to form NETs, which in turn precipitate intravascular thrombosis (15). Conversely, NET degradation appears to be impaired in a fraction of patients with anti-phospholipid syndrome (19). Neutrophils infiltrate the pancreas of patients with type 1 diabetes mellitus (T1DM) (24) and, according to experimental models and indirect clinical evidence (systemic levels of NETs by-products in serum), cause an IFN α response, autoantibody generation, and β -cells destruction through *in situ* NETosis (25, 26).

INDICATORS AND MARKERS OF NETosis

The notion that NETs contribute to a wide range of autoimmune diseases so far has had little impact on the clinical practice. Reasons include

- (i) the low threshold of activation of neutrophils, which limits the development of robust, easy-to-perform, and cheap diagnostic assays;
- (ii) high costs of clinical trials; and
- (iii) the lack of insight on appropriate targets to safely target NETosis.

Three main approaches are currently used in research laboratories:

- analytical assays based on fluorimetry for cell-free DNA or on ELISAs for soluble NET by-products (DNA–MPO or DNA–neutrophil elastase complexes, citrullinated histones) (3, 5);
 - confocal microscopy for neutrophil enzymes along extracellular DNA lattices (3, 5); and
 - flow cytometry, based on nuclear morphology and variations in MPO distribution (11) or staining of citrullinated histones or DNA (27).
- Fluorimetry is not time-consuming and generate semi-quantitative information that can be associated with clinical variables. However, it does not unambiguously identify the source of DNA (neutrophils or other cells) or the process by which DNA was released (NETosis, necrosis, necroptosis, etc.). The association of DNA fragments to neutrophil enzymes and the citrullination of histones are relatively specific for NETosis. Thus, determination of MPO–DNA complexes and/or of citrullinated histones selectively reveals the amount of NET by-products in biological fluids. Human studies monitoring *in vivo* NETs formation (3, 5) also revealed a concordant rise in NETs by-products in plasma, suggesting that ELISA is sufficient *per se* to assess the degree of NET formation in human inflammatory diseases (Figures 1A–F). Furthermore, parallel quantitation of *in vitro*-generated NETs by confocal microscopy and concomitant measurement of DNA, DNA–MPO complexes, and citrullinated histones in cell-free supernatants drives to concordant results (Figures 1G–I). Thus, we believe that, at present, only ELISA assays on plasma samples meet the requirements of robustness, reproducibility, and easiness for widespread clinical use.
- However, it does not discriminate between vital or suicidal NETosis nor assess the relative contribution of NET generation and catabolism. Standardized protocols will be necessary for transfer these assays to the clinics (28). For example, DNases for sample enrichment influence the sensitivity of MPO/DNA-based analytical assays (Figures 1G,H).
- Microscopy allows a direct visualization of NETs. In expert hands, it remains a powerful and informative tool, even more when combined with the determination of NET by-products (Figures 1A–F) (3, 5) and with high-throughput proteomic assays. However, due to the high inter-observer variability, it is not routinely used in clinical practice. Novel semi-automated image analysis techniques might circumvent this limit. Flow cytometry yields accurate and reproducible data. Large trials are needed to pave the way to their widespread use (11, 28). Besides improvements in NET detection, caution in the pre-analytical sample handling is mandatory, since neutrophils and platelets respond to physical and chemical stimuli during blood sampling and transportation. Activated platelets, in particular, release various signals – both soluble and associated with microparticles – that impact on neutrophil functions, including NET generation (12, 29).

NETs, DRUG DEVELOPMENT, AND REPOSITIONING

Citrullination of histone residues is a key step in NETosis. Pharmacological or genetic tools to inhibit deiminating enzymes reduce the formation of NETs and their detrimental consequences in preclinical models (30–32). The actual selectivity and potential

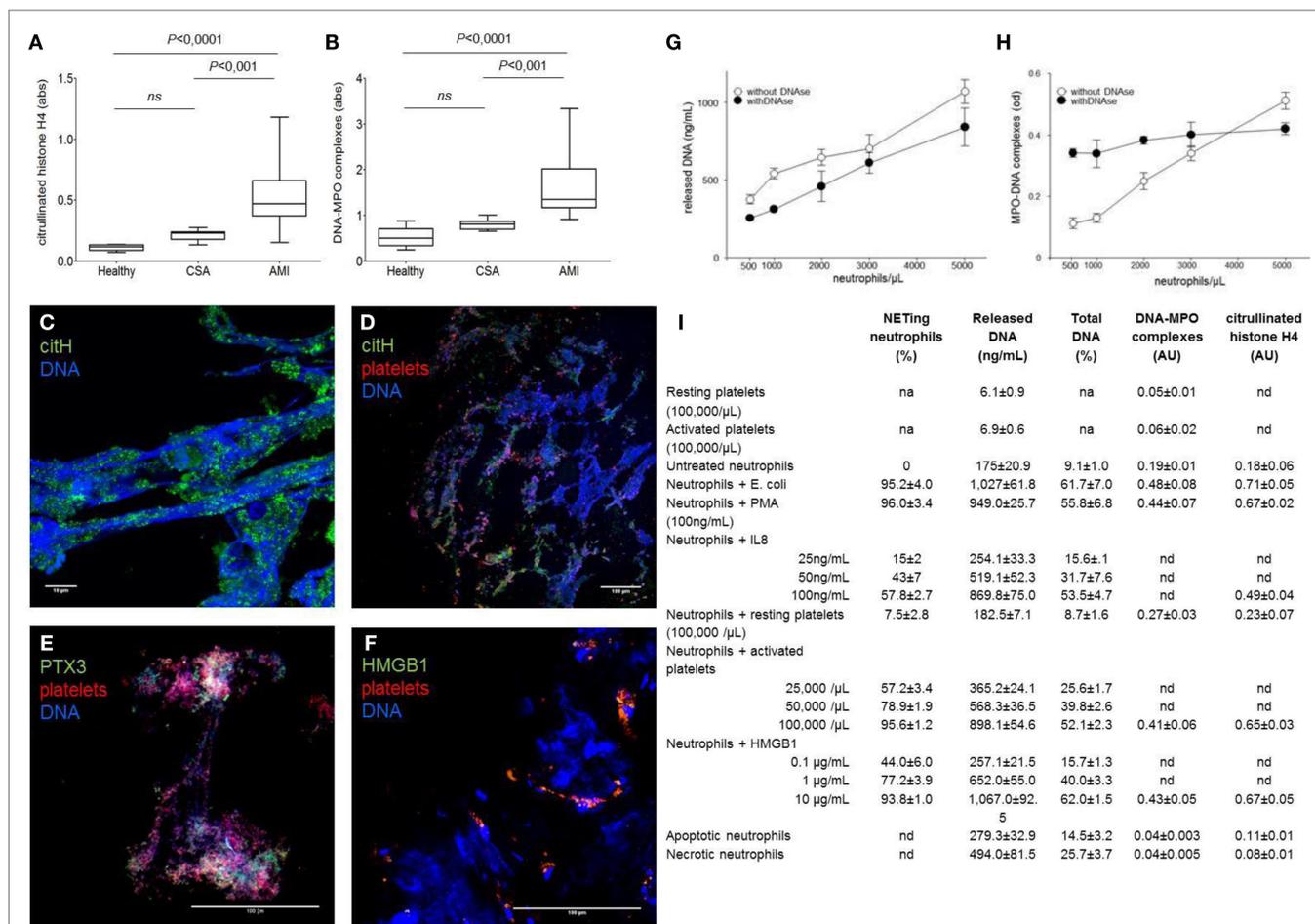


FIGURE 1 | NETs by-products are easily detected in the plasma of patients ongoing acute myocardial infarction and reflect the NETs that

characterized coronary thrombi. (A–F) Coronary thrombi and plasma samples were obtained from 26 consecutive patients with STEMI underwent percutaneous intervention within 1–6 h from the onset of chest pain. NETs by-products were determined in plasma samples (anticoagulated with EDTA, venipuncture through a 19-gauge butterfly needle) and processed within 15 min of sampling. Whole blood was centrifuged 15 min (320 g; 4°C), and retrieved plasma was further centrifuged (5 min at 100,000 g, 4°C) to eliminate any cellular debris. Obtained plasma was then aliquoted and frozen until de-determination of NETs by-products. All steps of samples preparation were performed in sterile conditions. **(A,B)** The amount of DNA–MPO complexes and the quantification of histone H4 citrullinated were assessed in the plasma samples of the same AMI patients in which were analyzed the composition of coronary thrombi, patients with chronic stable angina (CSA) candidates for coronary revascularization, and sex- and age-matched healthy donors. **(C–F)** Representative confocal analysis of coronary thrombi of AMI patients obtained during revascularization procedure. NETs were identified as DNA lattices with evident citrullination of histones **(C,D)** and by the presence of specific neutrophil proteins such as PTX3 **(E)**. We consistently detected the presence of NETs in close proximity of platelets **(D–F)** that frequently express HMGB1 **(F)** [adapted from Maugeri et al. (5)]. **(G,H)** The use of DNases impaired the sensitivity on NET determination: addition of DNase seems useful when low numbers of neutrophils are used. Freshly purified human neutrophils ($0.5\text{--}5 \times 10^6$) of healthy donors were placed on poly-L-lysine-coated slides for 20 min at 37°C, then were challenged with PMA (100 ng/mL) for 20 min at 37°C. Then, treated or not with DNase I, after incubation plates were centrifuged 5 min (320 g; 4°C), supernatants were retrieved and further cleared (5 min at 100,000 g) and frozen until determination of NETs by-products and cell-free DNA as we previously described [adapted from Maugeri et al. (5)]. **(I)** Quantitative assays for NET formation. The results of a series of independent experiments carried out with cells of different donors are presented in this back-to-back comparison of different quantitative assays to estimate the degree of NET generation. Neutrophils were stimulated with a large panel of agonists to reproduce the environmental variability that occurs under physiological conditions. Quantitation of cell-free DNA is consistent with the degree of NET generation as estimated by direct microscopy visualization and MPO/DNA- or citrullinated histone-based techniques in this set of experiments, where purified neutrophils were employed [adapted from Maugeri et al. (5)].

safety risks of the available pharmacological inhibitors are not yet established.

Mitochondrial generation of ROS triggers NET formation. In addition, oxidized mitochondrial DNA within NETs could contribute to its immunogenic potential. Interference with the respiratory chain and/or ROS scavenging exerts anti-inflammatory effects and clinical benefit in mice models of sepsis and SLE (33).

Targeting platelet/neutrophil reciprocal activation and platelet microparticle-associated moieties, HMGB1 in particular (5, 12, 34), and finding strategies aimed at restoring the phagocytosis of apoptotic substrates by neutrophils can exert a calming influence over NET generation (35) and thus appear promising. Other anti-NET treatments could aim at restoring and/or potentiating the NET clearance. NET-mediated lung injury in cystic

fibrosis abates in response to nebulized DNase supplementation. Studies improving the drug delivery could pave the way for the application of this anti-NETs treatment to inflamed joints, kidney, lung, or skin in the setting of autoimmune diseases.

The repositioning of known drugs and agents has advantages over the development of new drugs, since toxicity/safety profiles are usually known and cost and time to bring agents to market abate. A proof-of-concept trial links reduced SLE flares to the metformin inhibition of mtDNA-enriched NETs. An action of metformin on HMGB1 release (5, 33) could also be involved. Heparins are being used in a wide range of diseases for effects independent of their anticoagulant properties (36), including conditions, in which NETs generation possibly plays a role. Heparins might interfere with the metabolic needs for NET generation since they restrict the activation of the autophagic flux (29). Prophylactic doses of low-molecular weight heparins, which are routinely used for thromboprophylaxis and for the prevention of pregnancy complications, indeed interfere with autophagy induction of neutrophils of healthy subjects and virtually abrogate the ability to generate NETs in response to various stimuli (29). Thus, interference with NETosis might be involved in the benefit

of treatments selected mainly on empirical basis. Unfractionated heparin has also been shown to dismantle NETs after they have been generated, by liberating histones from the DNA backbone and destabilizing the chromatin threads (37).

In conclusion, accumulating data on the role of NETs in autoimmune diseases and in highly prevalent inflammatory conditions such as sepsis demonstrate that novel bioindicators and treatments might readily become available and improve the quality of patients care from tomorrow. Its time to take the next step.

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Intercellular Interactions as Regulators of NETosis

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Neutrophil extracellular traps (NETs) are chromatin-derived webs extruded from neutrophils in response to either infection or sterile stimulation with chemicals, cytokines, or microbial products. The vast majority of studies have characterized NET release (also called NETosis) in pure neutrophil cultures *in vitro*. The situation is surely more complex *in vivo* as neutrophils constantly sample not only pathogens and soluble mediators but also signals from cellular partners, including platelets and endothelial cells. This complexity is beginning to be explored by studies utilizing *in vitro* co-culture, as well as animal models of sepsis, infective endocarditis, lung injury, and thrombosis. Indeed, various selectins, integrins, and surface glycoproteins have been implicated in platelet–neutrophil interactions that promote NETosis, albeit with disparate results across studies. NETosis can also clearly be regulated by soluble mediators derived from platelets, such as eicosanoids, chemokines, and alarmins. Beyond platelets, the role of the endothelium in modulating NETosis is being increasingly revealed, with adhesive interactions likely priming neutrophils toward NETosis. The fact that the same selectins and surface glycoproteins may be expressed by both platelets and endothelial cells complicates the interpretation of *in vivo* data. In summary, we suggest in this review that the engagement of neutrophils with activated cellular partners provides an important *in vivo* signal or “hit” toward NETosis. Studies should, therefore, increasingly consider the triumvirate of neutrophils, platelets, and the endothelium when exploring NETosis, especially in disease states.

Keywords: neutrophil extracellular traps, platelets, endothelium, selectins, integrins

INTRODUCTION

Neutrophil extracellular traps (NETs), first described in 2004, are released by neutrophils via an active process coined NETosis (1, 2). While first characterized for their role in combatting infectious organisms (1), these tangles of chromatin and antimicrobial proteins are now known to play a role in pathogenic autoimmunity and other sterile inflammatory states (3, 4). NETs may place organ systems at risk, including the vasculature (5–7), central nervous system (8), lungs (5), and kidneys (9, 10). Organ failure and thrombotic vessel occlusions are even possible (11–13). Neutrophils, as one of the first responders to inflammatory insults have long been known to interact with other cell types (especially platelets and endothelial cells) with implications for neutrophil recruitment, generation of reactive oxygen species (ROS), and phagocytosis. This cell-to-cell crosstalk may be mediated by either direct cell contact or soluble mediators. In this review, we will focus on the implications of crosstalk for NETosis. Relevant studies have characterized not only *in vitro* systems (typically with human cells) but also more complex murine models of disease. There is significant heterogeneity

between studies, especially in terms of how NETosis is scored and the neutrophil pathways that are considered (which is probably not surprising as a canonical model of NETosis is still not established). Our goal is to highlight the similarities between studies and to point out the discrepancies that necessitate further research. Also, whenever possible, we will try to focus on the implications of these interactions for controlling infection and for regulating inflammation and end-organ damage.

PLATELET FUNCTION

Platelets are megakaryocyte-derived cell bodies that lack nuclei. They circulate in the bloodstream as well-established regulators of the hemostatic system (14). Platelets may be activated by the exposure of subendothelial matrix proteins, such as von Willebrand factor (vWF) and collagen, as might happen with mechanical vessel injury (15). Platelets recognize vWF via a glycoprotein receptor complex, glycoprotein Ib (GPIb)/IX/V (16), with the GPIb subunit playing a particularly key role (17). In parallel, collagen engages a different glycoprotein receptor, GPVI (18). Soluble plasma factors also activate platelets, including fibrinogen (via GPIIb/IIIa) (19) and thrombin (through protease-activated receptors or PARs) (20). When considering research studies, it is important to note that some studies may activate platelets with synthesized activators. An example is thrombin receptor activator peptide (TRAP), which acts as an agonist for all PARs (21), and the more specific TRAP-6, which binds specifically to PAR-1 (22).

These various activating signals lead to platelet aggregation and the release of copious amounts of preformed mediators from platelet granules, such as adenosine diphosphate (ADP) and thromboxane A₂ (TXA₂) – with the potential for potent local effects and feedforward into further platelet activation (14, 17). Platelet factor 4 (PF4, also known as C–X–C motif ligand 4) is another mediator released by platelets. In addition to functioning as a chemokine for cells, such as neutrophils, PF4 binds and neutralizes negatively charged cell surface glycosaminoglycans, such as heparan sulfate, dermatan sulfate, and chondroitin sulfate, thereby mediating several downstream effects, including platelet aggregation (23). Another soluble mediator that will be discussed in this article is high-mobility group box 1 (HMGB1), a protein “alarmin”/cytokine released by activated platelets (24). Finally, proteins such as P-selectin may be either released locally, or expressed on the platelet surface, thereby regulating the local environment (25, 26). For example, P-selectin has been implicated in platelet aggregation under pulsatile shear stress conditions (27).

While platelets clearly play a key role in stemming blood loss in the event of vessel injury, they also have well-established immunomodulatory properties, potentially acting as sentinels of infectious and inflammatory events (28, 29). In particular, the innate immune receptors toll-like receptor 2 (TLR2) and TLR4 (for Gram-positive and Gram-negative organisms, respectively) are expressed on the platelet surface (30, 31). Activation of these receptors may lead to release of platelet granules (32), PF4 upregulation (33), GPIIb/IIIa conformational changes (34), and ultimately feed forward to thrombin generation (30). Having said that, some studies have found less potent responses. For example,

exposure of platelets to triacylated lipoproteins (like Pam3CSK4, a TLR2 agonist) and lipopolysaccharide (LPS, a TLR4 agonist) does not always lead to significant P-selectin release (35).

PLATELET–NEUTROPHIL INTERPLAY

Platelets interact directly with neutrophils and thereby alter neutrophil function (17). Examples of ligand/receptor pairs that mediate direct platelet/neutrophil interactions include P-selectin/P-selectin glycoprotein ligand 1 (PSGL-1) (36, 37), intercellular adhesion molecule 2 (ICAM-2)/lymphocyte function-associated antigen (LFA-1) (38), and GPIb/macrophage-1 antigen (Mac-1) (17, 39). These interactions clearly support platelet adhesion to leukocytes (40, 41) and, in some cases, have been shown to be of fundamental importance for recruitment of neutrophils to sites of inflammatory insult (40). Furthermore, beyond traditional direct interaction, some molecules (such as GPIIb/IIIa) may be transferred from platelets to neutrophils via microparticles (MP), thereby regulating neutrophil function (an example being nuclear factor kappa B activation) (42).

There is also a key role for platelet-released soluble mediators (ADP, TXA₂, etc.) in both perpetuating platelet–neutrophil interplay and activating neutrophils. As an example, ADP (which would presumably be platelet-derived *in vivo*) induces platelet–neutrophil complexes through a mechanism that may be dependent upon P-selectin, but not PSGL-1 (41). TXA₂ augments multiple neutrophil functions, including neutrophil adhesiveness (43), oxidative burst (44), and diapedesis (45). Platelet-derived HMGB1 can engage/activate neutrophil TLRs (46). Beyond TLRs, another well-recognized receptor for HMGB1 is the receptor for advanced glycation end products (RAGE), with engagement by HMGB1 leading to neutrophil recruitment and neutrophil-mediated tissue injury (47). PF4 interacts with neutrophil chondroitin sulfate (48) and (in the presence of co-stimulatory tumor necrosis factor alpha) mediates neutrophil granule release and surface adherence (49). PF4 has also been implicated in neutrophil chemotaxis (50). Neutrophil-activating peptide 2 (NAP-2) released from platelets can regulate neutrophil polarization and motility through CXCR1/2 (51). CCL5 (another chemokine released by platelets) may also play a role in neutrophil infiltration (52).

PLATELETS AND NETosis

Platelets are far-and-away the most studied cellular regulators of NETosis. Most model systems have pointed to platelet activation as the first step. This is followed by platelet–neutrophil crosstalk, and ultimately regulation of neutrophil effector function. Studies have employed numerous platelet activators, including LPS, Pam3CSK4, thrombin, collagen, ADP, and TRAP-6 (53–55). These different strategies for activation, beyond anything else, make it challenging to compare studies side-by-side (**Table 1**).

Regarding *in vitro* studies, platelet–neutrophil interactions have been assessed under static conditions (53, 57), and also with the introduction of shear stress (53–56). It is worth noting that the methodology for quantifying NETosis has varied markedly across

TABLE 1 | Selected *in vitro* studies of platelet-stimulated NETosis.

Species	Platelet activator	Required mediator(s)	Not required	Reference
Human	LPS		P-selectin, Mac-1, GpIIb/IIIa	(54)
Mouse	LPS			(54)
Human	LPS	LFA-1		(56)
Human	<i>S. aureus</i> alpha toxin	hBD1		(57)
Human	TRAP	TXA ₂		(5)
Mouse		HMGB1 (via TLR4)	HMGB1 (via RAGE)	(58)
Human	Collagen, ADP, thrombin, TRAP-6	HMGB1	P-selectin, Mac-1, GpIIb/IIIa	(55)
Mouse	Collagen, ADP, thrombin, TRAP-6	HMGB1 (via RAGE)		(55)
Mouse	LPS		HMGB1	(55)
Human	TRAP, Pam3CSK4	TXA ₂ , leukotriene B4, GPIb, vWF, LFA-1	P-selectin, GpIIb/IIIa	(53)
Mouse	Thrombin	P-selectin		(59)

ADP, adenosine diphosphate; GPIb, glycoprotein Ib; GpIIb/IIIa, glycoprotein IIb/IIIa; hBD1, human beta-defensin-1; HMGB1, high-mobility group box 1; LFA-1 lymphocyte function-associated antigen 1; LPS, lipopolysaccharide; Mac-1, macrophage 1 antigen receptor; RAGE, receptor for advanced glycation end products; *S. aureus*, *Staphylococcus aureus*; TLR4, toll-like receptor 4; TRAP, thrombin receptor-activating peptide; TXA₂, thromboxane A2; vWF, Von Willebrand factor.

studies. Examples include cell-free DNA quantification (53, 55), myeloperoxidase-deoxyribonucleic acid (MPO-DNA) ELISA (5, 55, 60, 61), neutrophil elastase-DNA ELISA (53), neutrophil elastase concentration (57), or direct visualization of NETs by fluorescence microscopy (54). Microscopy samples have been scored by quantifying percent surface area of Sytox green staining (detects extracellular DNA) (54, 58), histone H2Ax percentage surface area (56), or citrullinated histone H3-positive cells per field (62).

We will first describe some notable *in vivo* studies in the field, which have focused on disease models (Table 2). We will then step through the various stages of platelet–neutrophil interplay, beginning with platelet activation and ending with NETosis (Figure 1).

Notable *In Vivo* Models

One of the first studies to consider the impact of activated platelets on NETosis *in vivo* utilized a mouse model of endotoxemia (sepsis) induced by intravenous LPS (54, 56). The authors found that LPS triggers the recruitment of neutrophils to liver sinusoids, which then facilitate recruitment of platelets (54) – with platelet recruitment dependent upon neutrophil LFA-1 (56). Importantly, NETosis is only triggered after engagement by the activated platelets (which seem to have been primed by LPS acting through platelet TLR4). This functionality presumably plays a key role in bacterial sequester, but also places the host at risk for significant endothelial damage (54). The authors further mimic these data *in vitro*, demonstrating that stimulation of platelets through TLR4 enhances both platelet–neutrophil adhesion and NETosis, but without upregulating P-selectin expression or platelet aggregation (54).

Another notable study investigated platelet–neutrophil interplay in the context of transfusion-related acute lung injury (TRALI). TRALI was modeled by treating BALB/c wild-type mice with the combination of LPS and an anti-MHC I monoclonal antibody (5, 64). NETosis was quantified in the lungs by either intravital microscopy or postmortem histological examination (5). Lung NETosis was dependent upon platelet–neutrophil interplay as NETosis was significantly mitigated by inhibiting platelet activation with aspirin (an irreversible inhibitor of platelet TXA₂

TABLE 2 | Selected *in vivo* models of platelet-stimulated NETosis.

Species	Model	Required mediator(s)	Reference
Mouse	Endotoxemia	LFA-1	(56)
Mouse	TRALI	GPIIb/IIIa	(5)
Mouse	ALI	HMGB1	(58)
Mouse	ALI	Mac-1, CXCL4/CCL5	(61)
Mouse	P-selectin overexpression	P-selectin	(59)
Rat	Endocarditis	P-selectin/PSGL-1	(63)
Mouse	IVC ligation	TXA ₂	(62)

ALI, acute lung injury; CCL5, chemokine (C–C motif) ligand 5; CXCL4, (C–X–C motif) ligand 4; GpIIb/IIIa, glycoprotein IIb/IIIa; HMGB1, high-mobility group box 1; IVC, inferior vena cava; LFA-1, lymphocyte function-associated antigen 1; Mac-1, macrophage 1 antigen; PMA, phorbol 12-myristate 13-acetate; PSGL-1, P-selectin glycoprotein ligand 1; TRALI, transfusion-related acute lung injury; TXA₂, thromboxane A2.

generation) or a GPIIb/IIIa inhibitor, tirofiban (5). *In vitro*, TRAP-activated platelets enhanced NETosis (5).

In a murine model of acute lung injury achieved with positive-pressure ventilation, platelet depletion led to depressed NETosis as measured in blood by MPO-DNA ELISA and in the lungs by microscopy (61). A critical role for Mac-1 was demonstrated with blocking antibodies and genetic knockout. By contrast, blocking LFA-1 did not suppress NETosis (61). Beyond integrin signaling, the authors argued that a second hit was also necessary for full neutrophil activation. Indeed, blocking platelet-derived CXCL4/CCL5 chemokine heterodimers reduced lung injury, while also explicitly mitigating NETosis in response to TRAP-activated platelets *in vitro* (61).

In a model of endocarditis, cultured bacteria from endocarditis patients were infused through carotid catheters into rats (63). By confocal microscopy, a platelet/bacteria layer was demonstrated inside the vegetation film, which was also intermixed with NETs (63). Furthermore, deoxyribonuclease (DNase, an enzyme that degrades DNA) proved to be an effective treatment (63). Platelets were deemed necessary for NETosis in this model, shown by inhibition with aspirin (63). Furthermore, NETosis was inhibited by P-selectin and PSGL-1 blocking antibodies (63).

In a final noteworthy study, the authors were interested in probing mechanisms by which aspirin might mitigate venous

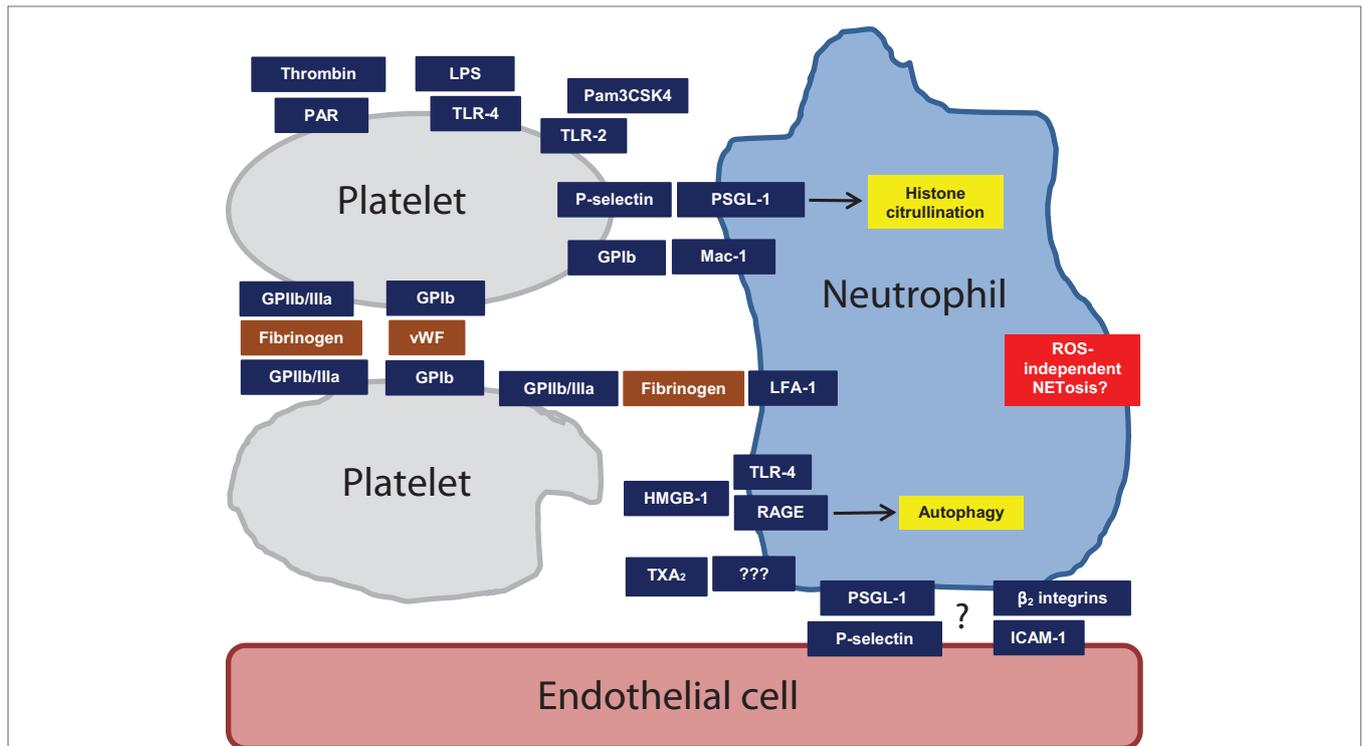


FIGURE 1 | Mechanisms of platelet activation and heterotypic intercellular interactions that may regulate NETosis. Some are more speculative than others, as described in the text. Abbreviations: GPIb, glycoprotein 1b; GPIIb/IIIa, glycoprotein IIb/IIIa; HMGB1, high-mobility group box 1; LFA-1, lymphocyte function-associated antigen 1; LPS, lipopolysaccharide; Mac-1, macrophage-1 antigen; PAR, protease-activated receptor; PSGL-1, P-selectin glycoprotein ligand 1; RAGE, receptor for advanced glycation end products; TLR2, toll-like receptor 2; TLR4, toll-like receptor 4; TXA₂, thromboxane A₂; vWF, Von Willebrand factor.

thrombosis (62). In a murine model of deep vein thrombosis (achieved by complete inferior vena cava ligation), both aspirin (which reduces the synthesis of TXA₂ by platelets) and a selective thromboxane receptor antagonist reduced thrombus size. This was accompanied by a reduction in neutrophil infiltration, as well as deposition of both fibrin and NETs.

Mediators of Direct Platelet–Neutrophil Interaction

P-Selectin/PSGL-1

If one considers *in vitro* studies with human neutrophils, then P-selectin has largely been judged dispensable for the ability of stimulated platelets to promote NETosis (53–55). In other species, the story may be different. For example, P-selectin has been implicated as required for thrombin-activated platelets to induce NETosis, as well as histone citrullination (a prerequisite for NETosis); this was demonstrated with cells isolated from knockout mice, and also by antibody-based inhibition (59). In the same study, mice overexpressing soluble P-selectin demonstrated higher neutrophil histone citrullination *in vivo*. Interestingly, P-selectin overexpression did not seem to regulate baseline NETosis, although accelerated NETosis could be unmasked in these mice with *ex vivo* stimulation (suggesting the neutrophils had been somehow primed by the overexpression) (59). Additionally, in the aforementioned rat model of infective endocarditis, platelet-induced NETosis was found to be

dependent upon P-selectin/PSGL-1 as demonstrated by blocking antibodies (63).

What explains these discrepancies? One simple possibility is species difference (human versus mouse/rat). Another consideration is that P-selectin/PSGL-1 interactions may already be established when neutrophils are purified for *in vitro* studies, and so blocking antibodies may be less effective in this context (5, 65). As hinted above, the method of platelet stimulation must also be kept in mind, as there was no apparent role for platelet P-selectin in studies in which platelets were stimulated with LPS (54) or TRAP-6 (56), as compared to a positive role in a study using thrombin as the stimulus (59). As P-selectin may serve a priming role *in vivo* more so than as the primary stimulus (59), and as P-selectin is also well-known to be expressed on endothelial cells (66, 67), intravital studies that can probe these interactions in real time will be important in sorting this out going forward.

Neutrophil Mac-1

There is a suggestion that the β_2 integrin Mac-1 is dispensable for platelet-induced NETosis based on *in vitro* studies with human neutrophils [with either TLR4 agonist (54) or TRAP-6 (5, 55) as the platelet stimulator]. By contrast, a study of acute lung injury demonstrated the requirement of Mac-1 for neutrophil-platelet aggregation as well as NETosis (61). Another interesting study recently revealed that neutrophil Mac-1 is required for crawling on the inflamed endothelium, a process that also requires

PSGL-1, albeit without direct PSGL-1/endothelium contact (39). The authors discovered that PSGL-1 instead concentrates in a uropod, which projects into the bloodstream where it receives activating signals from platelets. These PSGL-1-mediated signals then regulate Mac-1 distribution and ultimately crawling (39). This study nicely highlights the potential complexity of platelet–neutrophil interplay *in vivo*, and how a comprehensive model of neutrophil effector functions (such as NETosis) may not be possible without considering both platelets and the endothelium.

Neutrophil LFA-1

The β_2 integrin lymphocyte function-associated antigen 1 (LFA-1) is known to be the key receptor by which neutrophils interact with fibrinogen, an interaction that has been linked to an effective neutrophil oxidative burst (68). Beyond fibrinogen, platelet ICAM-2 may also interact with LFA-1 (38). *In vitro* studies with human platelets (activated with LPS, TRAP, or Pam3CSK4) have demonstrated that platelet–neutrophil interaction and resulting NETosis can be reversed with blockade of LFA-1 (53, 56), including under conditions of shear stress (56). Similarly, a mouse model of sepsis has supported a key role for LFA-1 in platelet-mediated NETosis, with either genetic deletion or blockade reducing NETosis in liver sinusoids (54, 56). However, in a different study focusing on murine neutrophils, TRAP-activated platelets signaled through neutrophil Mac-1, but not LFA-1, to induce NETosis (61). Differences in species, model, or culture conditions may have contributed to the discrepancies across studies.

Platelet GPIb

An *in vitro* study has suggested that GPIb (the classic receptor for vWF) is required for platelet-induced NETosis (53), although without a clear understanding of its counterpart on neutrophils. Interestingly, the authors also found that LPS-stimulated platelets increase expression and release of vWF, with blockade of vWF preventing platelet-induced NETosis (53). As GPIb can interact directly with neutrophils through Mac-1 (69, 70), and since vWF is also presented on the surface of endothelial cells, this pathway will need to be further dissected (including *in vivo*) before definitive conclusions can be drawn (71).

Platelet GPIIb/IIIa

In a mouse model of TRALI, blockade of GPIIb/IIIa (with tirofiban) reduced NETosis in lung tissue (5). This stands in contrast to *in vitro* human studies, which have not found a role for GPIIb/IIIa in platelet-induced NETosis (53–55). Interestingly, GPIIb/IIIa can be transferred from platelets to neutrophils through platelet-derived MP (42), an observation that could have implications for *in vitro* and *in vivo* discrepancies. It may also be that the key role of GPIIb/IIIa is to facilitate platelet–platelet or platelet–endothelial interactions (72–74), which would stand out in *in vivo* models, more so than the *in vitro* work.

Soluble Mediators Released by Platelets

Eicosanoids

Platelets stimulated with Pam3CSK4 and TRAP (5, 53) may utilize TXA₂ as a means of signaling to promote release of

NETs (53). Given that there is no well-characterized receptor for TXA₂ on neutrophils, mechanistic details remain to be determined.

Chemokines

PF4 (CXCL4) can play a role in regulating *in vitro* human NETosis, based on blocking experiments (53), and also direct stimulation of neutrophils with recombinant PF4 (53). *In vivo*, MKEY (a peptide inhibitor of CXCL4/CCL5 heterodimer formation) reduces NETosis in a model of acute lung injury (61).

Alarmins

Recombinant HMGB1 activates neutrophils to release NETs, dependent upon either neutrophil TLR4 (58) or neutrophil RAGE (55). Human beta defensin-1 (a microbicidal protein found in both neutrophils and platelets) is released by platelets exposed to *Staphylococcus aureus* alpha toxin, in a manner that then triggers NETosis (57).

Neutrophil Signaling in Response to Platelets

It should be noted that neutrophil signaling has not been characterized in most models of platelet-induced NETosis. When Pam3CSK4, LPS, or TRAP were used to stimulate platelets, the resulting NETosis was found to be ROS independent (5, 53, 55). This is in contrast to *S. aureus* alpha toxin-activated platelets, which promote NETosis in a ROS-dependent manner (57). Platelet HMGB1 seems to leverage neutrophil autophagy to induce NETosis (55). Another study has demonstrated that ERK and PI3K are required for platelet-induced NETosis, when platelets were activated with Pam3CSK4, LPS, or arachidonic acid (53). At this point, the data are too limited to predict whether a consensus signaling pathway will emerge, although there are hints that ROS may not be a critically important factor in a critically important factor in platelet-induced NETosis.

ENDOTHELIUM–NEUTROPHIL INTERPLAY

Neutrophils develop in the bone marrow from myeloid precursors, reaching sites of infection or inflammation via the vasculature. This migration of neutrophils from the bloodstream to inflamed tissues is mediated by the interaction of adhesion molecules on the neutrophil surface with their respective ligands on the vascular endothelium. Details regarding this well-coordinated series of events arise from intravital microscopy studies in animals, as well as observations of patients with leukocyte adhesion deficiency (75). As an initial step, neutrophils leverage specific surface ligands in order to tether to P- and E-selectin molecules expressed on activated endothelial cells (selectin ligands potentially expressed on neutrophils include PSGL-1, E-selectin ligand 1, and CD44). Tethering of neutrophils is followed by their rolling along the endothelium (76–80). Rolling neutrophils develop membrane extensions at their rear end (tethers) and front (slings), which stabilize neutrophil rolling and allow the process to proceed despite the high shear stress of flowing blood (81). Subsequently, neutrophils firmly adhere to endothelial cells, mediated by

the binding of neutrophil β_2 integrins (LFA-1 and Mac-1) to endothelial ligands such as intracellular adhesion molecule 1 (ICAM-1) and ICAM-2 (76, 78, 79, 82). β_2 integrins have two main states of activation: the first is an extended (but not open) form with low to intermediate affinity, and the second an extended and open form with high affinity (the form required for firm adhesion). Mechanisms and signaling pathways involved in these transitions have been delineated in great detail, and are reviewed elsewhere (82–84).

Rolling and adhesion may be followed by transmigration, when neutrophils pass between endothelial cells (paracellular) or through endothelial cells (transcellular). While many details remain to be determined, the paracellular process is more prevalent, occurring perhaps 90% of the time (76, 83, 85) and favored by neutrophils expressing Mac-1 (86, 87). By contrast, the transcellular route may be favored by increased endothelial expression of ICAM-1 (88) or by activation of endothelial cells by neutrophils through annexin A1 secretion (89). Beyond the above, adhesion molecules involved in the transmigration process include platelet endothelial cell adhesion molecule 1 (PECAM-1), CD99, ICAM-2, junctional adhesion molecules (JAMs), and cadherins (90). The roles of these adhesion molecules have primarily been demonstrated in mouse models wherein their deletion results in inhibition of transmigration and reduced accumulation of neutrophils in tissues (83, 85, 91).

Within inflamed tissues, neutrophils home via chemokine gradients. Interestingly, recent studies have demonstrated that neutrophils are able to undergo a “reverse transmigration” process such that tissue neutrophils may migrate back to the vascular lumen. Studies in mice have demonstrated that downregulation of JAM-C by neutrophil elastase plays a key role in the process (92). At present, the functional significance of reverse transmigration is not entirely clear. One idea is that the reverse transmigration has a significant downside, as it may contribute to dissemination of a local immune response into a systemic inflammatory phenomenon (93). Alternatively, it may play a role in dampening immune response as observed in zebrafish (94) and, we speculate patients with systemic inflammation (95).

Circulating neutrophils tend to be quiescent in nature, with their activation tightly linked to migration from circulation to tissue. Neutrophil activation can be thought of as a two-step process whereby exposure to one stimulus (priming) ensures a maximum response to a second. So, rolling and adhesion of neutrophils on the endothelium may initiate their activation, but full effector functions only become available to neutrophils once they encounter certain pro-inflammatory chemokines/cytokines or pathogen-derived ligands that can activate other receptors (G protein-coupled receptors and innate pattern-recognition receptors as classic examples). Neutrophils can then rapidly undergo degranulation, activation of their NADPH oxidase pathway for free radical generation, phagocytosis, and even NETosis (96–98). An example comes from studies of P-selectin overexpressing mice in which neutrophils seem to be sensitized to NETosis by excess P-selectin exposure, but do not actually release NETs unless confronted with a second stimulus (59).

THE ENDOTHELIUM AND NETosis

Netting neutrophils externalize not just chromatin but also a variety of antimicrobial peptides and proteases that target pathogens. Recent work has demonstrated that these mediators of host defense may also promote tissue damage (12). NETs induce endothelial cell death in a dose-dependent and partially DNA-independent manner (99). Rather than DNA, associated histones and to some extent myeloperoxidase may be most responsible for NET-mediated endothelial cytotoxicity (99). Another study demonstrated the externalization of matrix metalloproteinase-9 (MMP-9) and MMP-25 along with NETs. This externalized MMP-9 activates pro-MMP-2 produced by the endothelium, resulting in cytotoxicity and vessel dysfunction (100).

An interesting *in vitro* study investigated the implications of co-culture of activated endothelial cells with neutrophils (101). The result was not just increased NETosis by neutrophils, but also increased endothelial cell death (101). The death was attributable to increased IL-8 production by the endothelial cells themselves (101). One can imagine a scenario *in vivo* in which activated endothelial cells induce NETosis, followed by endothelial cytotoxicity and potentially the release of mediators that feed forward into more NETosis.

It should also be noted that although endothelial cells have not been the explicit focus of most NETs studies, they almost surely play a prominent role *in vivo*, either through direct regulation of neutrophil activity, or through modulation of other cellular elements, such as platelets (**Figure 1**). As an example, in the aforementioned sepsis model, liver sinusoids support neutrophil adhesion even in the absence of platelets, perhaps providing certain activating signals to the neutrophils that prime them for subsequent platelet capture (56). One might also point to the TRALI model (5). There, GPIIb/IIIa plays a key role in NETosis beyond anything that has been seen *in vitro* (53–55) – raising the question of whether additional synergistic signals may emanate from the endothelium *in vivo* (5). Finally, although studies focusing on platelet–neutrophil interactions *in vitro* have suggested contradictory roles for P-selectin (53–55), it is worth noting that P-selectin is also present on endothelial cells, which may help explain its more clear-cut role *in vivo* (59). We expect to see much more on this front in the coming years.

DENDRITIC CELLS

Dendritic cells (DCs) are best known for their role as professional antigen-presenting cells, bridging the gap between innate and adaptive immunity. In recent years, the intersection of neutrophils/NETosis and DCs has been increasingly considered. First, neutrophils are well established to play a role in the recruitment of DCs to sites of inflammation, and promote maturation of DCs via secretion of a variety of soluble mediators, such as CCL3, CCL4, CCL5 (RANTES), CCL20, tumor necrosis factor α , α -defensins, and cathelicidins (102–106). At the same time, *in vivo* immunization studies have demonstrated that neutrophils can dampen immune responses by competing for antigen with DCs and limiting contact between T cells and DCs (107). So, at least in some contexts, vaccination responses may improve with

temporary depletion of neutrophils. In other contexts, NETs seem to do the opposite, quite specifically transferring antigens to DCs, and thereby initiating autoimmune disorders, such as small vessel vasculitis (108).

With further implications for autoimmunity and sterile inflammation, NETs activate plasmacytoid DCs in lupus and atherosclerosis via TLR9. Activated plasmacytoid DCs produce interferons, which in turn prime neutrophils for more NETosis (thereby setting up a positive feedback loop) (109, 110).

Again pointing to different roles in different contexts, DCs may sometimes downregulate NETosis. This has been described in the specific context of human immunodeficiency virus (HIV), which acts through CD209 on DCs to produce interleukin 10 (IL-10). IL-10 then inhibits HIV/TLR7-mediated NETosis (111). Demonstrating at least some specificity, PMA-induced NETosis is not suppressed by IL-10 (111).

MICROPARTICLES

MP are small, cell membrane-derived vesicles (112). MP from endothelial cells (113, 114), platelets (115), and red blood cells (116) have all been implicated in activating neutrophils. Furthermore, both platelet-derived (115) and red blood cell-derived (116) MP induce Mac-1 expression on neutrophils and stimulate neutrophil phagocytic activity (115, 116). The role of MP in promoting NETosis was also demonstrated in a paper focusing on preeclampsia, in which placenta syncytiotrophoblast-derived MP seem to promote NETosis (117). In inflammatory bowel disease, MP also appear to activate NETosis (118).

CLEARANCE OF NETS

While NETs play a critical role in host defense, excessive formation or persistence of NETs may lead to adverse effects. Thus, clearance of NETs is an important physiological process that helps minimize excessive presentation of both toxic products and potential self-antigens. Degradation of NETs by serum DNase is one mechanism by which NETs are cleared, with impairment of this process leading to a lupus-like syndrome in mice (119). Interestingly, inadequate DNase activity has also been detected in the blood of patients with both lupus (119–121) and autoimmune vasculitis (122). Beyond the enzymatic activity of DNase, macrophages also play a role in the clearance of NETs. DNase processing of NETs prepares them for engulfment by macrophages, with the process further facilitated by the opsonization of NETs by complement C1q (123). Though this process was initially

thought to be immunologically silent, recent *in vitro* studies have demonstrated a potentially complicated response that depends upon macrophage polarization (124). The authors show that M2 macrophages induce a pro-inflammatory response when exposed to NETs (including the release of a variety of pro-inflammatory cytokines/chemokines). By contrast, M1 macrophages initially undergo cell death that leads to their own nuclear decondensation and DNA release. Interestingly, over time, M1 macrophages then degrade this macrophage-derived DNA in a caspase-activated DNase-dependent manner (124). The full implications of this interplay remain unclear *in vivo* (and in disease states) and will hopefully be elucidated by future studies.

FUTURE DIRECTIONS

This is a field in which much remains to be defined, as is especially highlighted by the various studies of platelet-induced NETosis. Studies in different systems and by different investigators have revealed surprisingly little mechanistic consensus, which probably points to an involvement of multiple pathways, thereby allowing certain aspects to be revealed by different groups. An obvious barrier is that platelet activation is achieved through different methodology in each study. It would be very interesting to see one group (or preferable a number of groups) take a systematic approach to this question, asking how the method of stimulation influences the specifics of platelet–neutrophil crosstalk. Given the highly regulated crosstalk that exists between the endothelium and neutrophils, endothelial cells surely play an important role in regulating NETosis *in vivo* – although relatively few studies have specifically probed that role. Studies should, therefore, increasingly consider the triumvirate of neutrophils, platelets, and the endothelium when exploring NETosis, especially in disease states.

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Platelets: New Bricks in the Building of Neutrophil Extracellular Traps

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In addition to being key elements in hemostasis and thrombosis, platelets have an important role in the inflammatory and innate immune response. This activity is associated with their capability to recognize pathogens through the expression of toll-like receptors, the secretion of various cytokines, chemokines, and growth factors stored within their granules, and the expression of cell adhesion molecules that allows interaction with other immune cells, mainly neutrophils and monocytes. As part of the first line of defense, neutrophils control invading pathogens by phagocytosis, the release of antimicrobial proteins during degranulation, or through the formation of web-like structures named neutrophil extracellular traps (NETs). NETs are formed by chromatin, proteases, and antimicrobial proteins, and their main function is to trap and kill bacteria, virus, and fungi, avoiding their dissemination. Besides microorganisms, NET formation is also triggered by proinflammatory molecules and platelets. The uncontrolled formation of NETs might exert tissue damage and has been involved in a pathogenic mechanism of autoimmune and prothrombotic clinical conditions. In this review, we discuss the role of platelets in NET generation highlighting the mediators, stimuli, and molecular mechanisms involved in this phenomenon, both in human and murine models.

Keywords: platelets, neutrophils, neutrophil extracellular traps, inflammation, platelet-neutrophil interaction

INTRODUCTION

Platelets are known to be central mediators of hemostasis and thrombosis; however, this role and the spatiotemporal relationship of the events that occur after tissue injury also link them directly to the host inflammatory and immune responses. The contribution of platelets to processes beyond hemostasis and thrombosis is associated with several properties, including the ability to store within intracellular granules – a broad array of biologically active molecules, which can be released into the circulation or translocated to the platelet surface during activation; and the recognition of pathogens through toll-like receptors (TLRs), and by the interaction of platelets directly with pathogens and other vascular cells, mainly endothelial cells and leukocytes (1).

Abbreviations: ADP, adenosine diphosphate; ALL, acute lung injury; ASA, acetylsalicylic acid; DNase, deoxyribonuclease; DVT, deep vein thrombosis; GP, glycoprotein; GPCR, G-protein-coupled receptor; HMGB1, high mobility group box 1; LPS, lipopolysaccharide; MI, myocardial infarction; MPO, myeloperoxidase; NADPH, nicotinamide adenine dinucleotide phosphate; NETs, neutrophil extracellular traps; PAD4, peptidylarginine deiminase 4; PF4, platelet factor 4; PMA, phorbol-12-myristate 13-acetate; PSGL-1, P-selectin glycoprotein ligand-1; ROS, reactive oxygen species; TLR, toll-like receptor; TRALI, transfusion-related acute lung injury; TXA₂, thromboxane A₂; VTE, venous thromboembolism; vWF, von Willebrand factor.

The release of DNA extracellular traps is a newly discovered defense mechanism of neutrophils in their fight against pathogen invasion. Neutrophil extracellular traps (NETs) occur as a result of a special cell death program named NETosis that involves the release of DNA webs decorated with all five types of histones and with neutrophil-derived granular proteins with antimicrobial activity, such as elastase, myeloperoxidase (MPO), and the bactericidal/permeability-increasing protein, among others (2). Even though these traps are important as anti-infection agents in the innate immune response, they can be a double-edged sword since, in excess, they are cytotoxic and induce tissue damage (3).

Increasing *in vitro* and *in vivo* evidence indicates that the ability of platelets to operate as innate immune cells is critical to the process of NET formation. In this review, we aim to discuss the role of platelets in the formation of NETs, identifying the stimuli, mediators, and molecular mechanisms involved as well as their pathiopathologic relevance, both in mice and humans.

PLATELETS

Platelets are anucleated cells derived from megakaryocytes, which play a crucial role in hemostasis and thrombosis. Under normal conditions, circulating platelets do not adhere to the wall of blood vessels, leukocytes, or between them due to the anti-thrombotic properties of vascular endothelium. However, during vascular injury or after endothelium activation under inflammatory conditions, platelets adhere to subendothelial molecules such as collagen or von Willebrand factor (vWF), triggering initial platelet activation characterized by the release of soluble mediators stored in their granules, such as adenosine diphosphate (ADP) and thromboxane A₂ (TXA₂). These molecules act as platelet activators amplifying cell activation in an autocrine manner and allowing the activation of other circulating platelets, favoring the formation of platelet-platelet aggregates. The interaction between platelets is called aggregation and occurs due to the principal integrin of these cells, $\alpha_{IIb}\beta_3$. Upon platelet activation, this integrin changes from a closed to an open conformation, enabling fibrinogen binding, which acts as a bridge between platelets. The amplification of platelet stimulation leads to procoagulant activity, thrombin generation, and formation of a thrombus that will repair the damaged vessel (4).

NEUTROPHIL EXTRACELLULAR TRAPS

The main function of NETs is to eliminate pathogens. Once attached to these webs, histones and microbicide proteins degrade virulence factors and kill pathogens. The NET is not only a trap, which prevents the propagation of pathogens from the initial site of infection, but also, by being attached to injurious stimuli, concentrates microbicide proteins to the site of infection (2). While NET formation was initially associated with pathogens such as bacteria, fungi, viruses, and parasites (5–9), it is now known that cytokines, chemokines, platelet agonists, and antibodies (10–14) are also capable of triggering this phenomenon. Depending on the location of the neutrophils when stimulated (extravasated versus vascular), these NETs can be either spread throughout

the interstitium of specific organs or released into the lumen of blood vessels, where they may attach to the vessel walls of narrow capillaries.

NETosis is initiated by the activation of peptidylarginine deiminase 4 (PAD4), which induces citrullination of histones 3 and 4 dismantling the nucleosome (15, 16). In addition, the activation of protein kinase C and nuclear factor kappa B (NF κ B) pathways (12) and RAF/MEK/ERK signaling (17) leads to phosphorylation of several kinases resulting in assembly of the functional nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex for reactive oxygen species (ROS) generation (18). ROS trigger the dissociation of neutrophil elastase from a membrane-associated complex (azurosome) into the cytosol and activate its proteolytic activity in an MPO-dependent manner. Once in the cytoplasm, elastase binds actin and is translocated to the nucleus where it promotes chromatin decondensation by cleavage of histones. After disintegration of the nuclear envelope and rupture of the cytoplasmic membrane, NETs are released (19, 20).

In addition to ROS generation, autophagy is also required for NET generation (21). Furthermore, recent evidence has also shown that activation of SK3 potassium channels, mediated by calcium influx, may lead to an alternative NADPH oxidase-independent mechanism of NETosis (22).

The above description is of what is called “suicidal” NETosis (6). However, it has been reported that a fraction of neutrophils can release NETs without dying, leaving behind cytoplasts that continue to ingest microbes. This highly efficient process is called “vital” NETosis (23, 24).

NETs form an important arm of antimicrobial innate immunity, which is exemplified by the fact that defects in NET generation, or experimental NET depletion, increase susceptibility to various kinds of infections in mice and humans (25–28).

ROLE OF PLATELETS IN NET FORMATION

Platelet involvement in DNA extracellular trap formation was first described in 2007, when Clark et al. showed in a mouse model of sepsis that lipopolysaccharide (LPS) binds to TLR4 present in the platelet membrane, allowing the binding of platelets to neutrophils and leading to rapid neutrophil activation and NET formation. These structures maintain their integrity under flow conditions and ensnare bacteria in the circulation (29). The finding that activation of TLR4 on platelets activates the release of NETs was a significant shift in understanding the inflammatory response to sepsis. The ability of platelets to promote NET formation was also observed in human cells, not only with Gram-negative bacterial components but also with Gram-positive bacteria (14).

Platelet-mediated NET formation is not restricted to activation by bacteria; it has been shown that platelets are also required for NETosis-mediated virus clearance. After systemic poxvirus challenge, mice exhibit thrombocytopenia and the recruitment of both neutrophils and platelets to the liver vasculature. Circulating platelets interact with, roll along, and adhere to the surface of adherent neutrophils, forming large aggregates that facilitate the release of NETs within the liver vasculature. Inhibition of this aggregation does not form NETs (30). The platelets’ participation

in the formation of NETs and virus clearance could represent another mechanism for the thrombocytopenia that is frequently observed in virus-infected patients (31). Although the central role of platelets in NETosis was initially identified in infectious processes, it also happens during sterile inflammation conditions. In fact, activation of platelets, not only by pathogens but also by classical platelet agonists such as thrombin, arachidonic acid, collagen, or ADP, results in NET induction (11, 13, 14, 32). These findings, together with the fact that besides exerting antimicrobial activity, NETs can also trigger procoagulant, prothrombotic, and proinflammatory responses and explain why NETs are increasingly being recognized as novel mediators of several non-infectious clinical conditions, such as thrombosis, pre-eclampsia, and autoimmune diseases (3).

CELL ADHESION MOLECULES INVOLVED IN PLATELET-MEDIATED NETosis

Platelet–neutrophil interaction takes place mainly due to the binding of P-selectin to its receptor P-selectin glycoprotein ligand-1 (PSGL-1) on the leukocyte surface (33). Consistent with this observation, recent studies in mice have shown that NET formation during sepsis or acute lung injury (ALI) experimental models is also dependent on cell to cell interaction *via* PSGL-1 and P-selectin (34, 35). However, other studies, including those from our group, have indicated that, in humans, platelet-mediated NET formation is a P-selectin-independent mechanism since its blockade does not modify DNA release and the incubation of neutrophils with recombinant P-selectin was not able to induce NETosis (13, 14). Platelet TLR4 appears to be a major target involved in platelet-mediated NETosis. As mentioned before, platelet TLR4 is involved in inducing NETs in mice and humans (14, 29). However, it is not yet known whether the LPS bound to platelet TLRs interacts with the neutrophils' TLR4 counter receptor, or if it is just necessary to trigger platelet activation and the release of mediators that, in turn, induce NETosis. Of note, the blockade of platelet TLR4 markedly impairs NETosis and this has been suggested as a new therapeutic approach for sepsis (29).

Cooperation between neutrophil $\beta 2$ integrin (CD18) and platelet glycoprotein (GP) Ib has been considered as another major event involved in platelet–neutrophil adhesion (36, 37). Studies using murine cells of wild type and mice deficient in CD18, as well as a murine model of sepsis, demonstrated that $\beta 2$ integrin is also involved in platelet-mediated NETosis. Furthermore, blocking Mac-1 (CD18/CD11b), but not LFA-1 (CD18/CD11a), in wild-type mice significantly reduced NET formation (38, 39). These data were corroborated in studies of mice and human cells where blocking CD18 markedly decreased the release of NETs mediated by activated platelets (14, 39). With regard to platelet GP Ib, its specific inhibition by gene deletion (40), or using blocking antibodies in human platelets (14), also affected the formation of NETs, demonstrating its participation in this process.

In a mouse model of ALI and using mice platelets and neutrophils, it has been shown that platelet-stimulated NETs require simultaneous activation of integrin-mediated outside-in and

G-protein-coupled receptor (GPCR) signaling. Blocking one of these pathways reduced NET formation *in vivo*, diminishing the severity of ALI (39).

Platelet integrin $\alpha_{IIb}\beta_3$ is the most abundant molecule in the platelet's surface that mediates interaction between platelets, and platelets with other cells. In a mouse model of lung injury, blocking the platelet integrin inhibited the formation of NETs and tissue damage (11), whereas a study of human platelets showed that $\alpha_{IIb}\beta_3$ seems not to participate in this process (13, 14).

Together, these observations suggest that the bridging between platelets and neutrophils in NET formation differs either between species or due to *in vivo* cofactors not involved in the *in vitro* models. In fact, while platelet-mediated NETosis in mice appears to be dependent on P-selectin, GPIb, TLR4, and integrin $\alpha_{IIb}\beta_3$ expression on the platelet surface; human NET formation by platelets seems to only require GPIb and TLR4. In contrast, integrin $\beta 2$ on the neutrophil's surface appears to be a major protein involved in platelet-mediated NETosis, either in mice or humans.

SOLUBLE MEDIATORS OF PLATELET-MEDIATED NETosis

Neutrophils and platelets activate each other. The crosstalk between these cells depends on cell-to-cell contact and secreted substances. Upon activation, platelets express or secrete various molecules capable of modulating the activation of neutrophils (41).

High mobility group box 1 (HMGB1) is a damage-associated molecular pattern molecule, which is released as a result of cell death and represents a crucial sign in the inflammatory response to tissue injury (42). Platelets contain HMGB1, which is released after activation (43). Recently, it has been shown that platelet-derived HMGB1 is a mediator of NET formation. Of interest, HMGB1, either soluble or presented from activated platelets, induces autophagy in neutrophils promoting NETosis and inhibiting apoptosis (13), supporting the notion that autophagy is a critical signal for the NETosis process. Of note, NETs induction by HMGB1 is independent of TLR4; thus, the axis HMGB1–receptor for advanced glycation endproducts (RAGE) represents a potential target to control NET formation in sterile inflammatory conditions.

Platelet-derived *chemokines* such as RANTES (CCL5) and platelet factor 4 (CXCL4, PF4) are well-known *molecules* responsible for neutrophil recruitment to sites of inflammation/infection (44). In a mouse model of lung injury, it has been shown that blocking these cytokines or their heterodimerization inhibited NETosis (39). We have confirmed the participation of PF4 in NETosis using human cells, while inhibition of PF4 abrogated the release of DNA, recombinant PF4 promoted the formation of these DNA traps (14). These observations suggest that, besides chemotaxis, RANTES and PF4 are involved in the inflammatory response, not only recruiting leukocytes to the site of injury but also being strong NETs inducers.

Venous thromboembolism is a GP stored in α granules of megakaryocytes and platelet and Weibel Palade bodies of endothelial cells. After vessel injury, vWF mediates platelet adhesion to the exposed subendothelium (45). Several studies

have suggested that vWF is a critical component of NETs. In a murine model of deep vein thrombosis (DVT), it was observed that citrullinated histone H3 (citH3) colocalized with this factor, suggesting that this molecule and NETs form a network that contributes to thrombus growth and stabilization (46). Furthermore, the ultralarge vWF fibers adhered to the endothelium that are observed during inflammation, bind and immobilize the DNA released by neutrophils acting as a linker between leukocyte adhesion to the endothelium and supporting leukocyte extravasation and inflammation (47). In addition, we found that incubation of human platelets with an antibody against vWF suppressed NET formation induced by activated platelets, indicating that not only vWF is a NET inducer but also revealing a functional role for this molecule is released by platelets (14). Together, these observations indicate that, besides its function as an adhesive molecule, vWF secreted either by platelets or endothelial cells has a central role in the inflammatory and thrombotic features of NETs.

Thromboxane A₂ is a potent platelet agonist, and its role in the formation of platelet-mediated NETs has been recently studied. In this context, it was reported that pharmacological

inhibition of TXA₂-mediated platelet activation by acetylsalicylic acid (ASA) suppressed the formation of platelet-mediated NET generation (11, 14). Furthermore, pretreatment of neutrophils with a selective thromboxane receptor antagonist (SQ29548) prior to the addition of thrombin receptor activating peptide (TRAP)-activated platelets reduced the production of NETs (11). The *in vivo* relevance of TXA₂ in platelet-mediated NET formation was first pointed out by Cadrillier et al. who showed in experimental transfusion-related acute lung injury (TRALI) that NETs are detectable in the lung microcirculation and they were prevented by inhibition of platelet aggregation using ASA (11). In addition, dipetalodipin and triplatin are triatomine salivary proteins that exhibit high affinity binding to prostanoids, such as TXA₂, inhibited platelet-mediated NET formation (32). Given that neutrophils do not express the TXA₂ receptor, the effect of TXA₂ is intriguing. However, as TXA₂ is a strong autocrine and paracrine activator, we have shown that this molecule, once released, acts on platelets triggering the release of platelet granules content, which in turn promote the formation of NETs (14) (Figure 1).

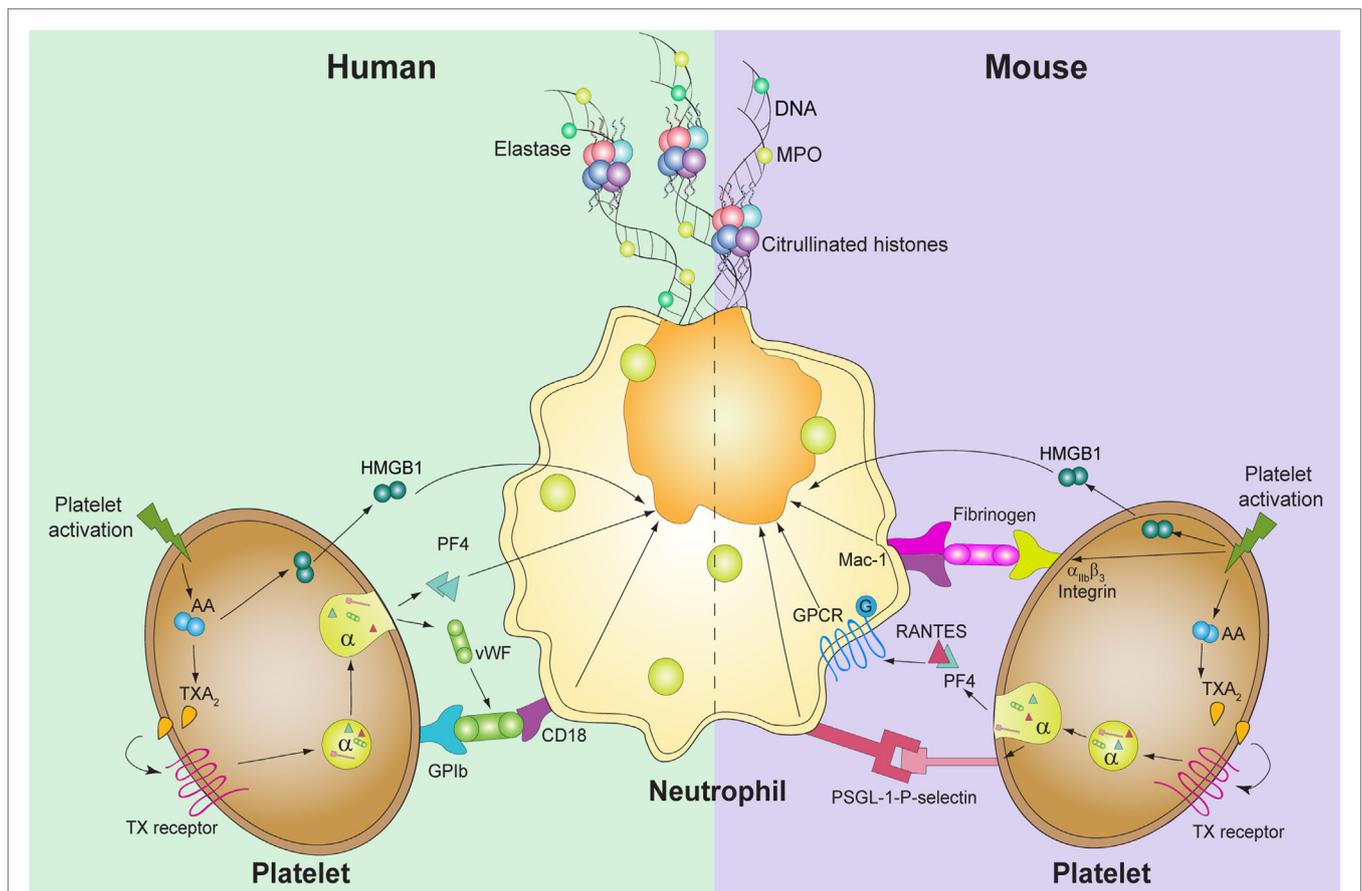


FIGURE 1 | Known molecules involved in platelet-mediated NET formation. In humans (left), platelet activation induces thromboxane A₂ (TXA₂) formation, which triggers the release of high mobility group box 1 (HMGB1), von Willebrand factor (vWF), and platelet factor 4 (PF4). vWF binds to its platelet receptor, glycoprotein (GP)Ib, which binds to CD18 in neutrophils. PF4 and HMGB1 act directly upon neutrophils and induce DNA release. In mice, not only TXA₂ generation and HMGB1 are involved but also PF4 and RANTES, through GPCR, induce platelet-mediated NET formation. Also, the interaction between P-selectin-PSGL-1 and $\alpha_{IIb}\beta_3$ -Mac-1 is required for the release of NETs.

SIGNALING INVOLVED IN PLATELET-MEDIATED NET FORMATION

Although the molecular basis governing the formation of NETs are not yet fully elucidated, the activation of the RAF/MEK/ERK, the generation of ROS by the enzyme NADPH oxidase, and activation of PAD4 seems to be of great importance in the NETosis induced by phorbol-12-myristate 13-acetate (PMA) (15–18). Phosphorylation of ERK both in platelets and neutrophils is also necessary for the formation of NETs mediated by activated platelets (11, 14, 48). Blocking ROS production, in both cell types, did not influence the release of DNA traps in human cells (14); however, in a murine model, NETosis was significantly decreased using diphenyleneiodonium (DPI) both *in vitro* and *in vivo* (39). Nevertheless, while platelet activation of Src is required for induction of platelet-mediated NETosis, in neutrophils both Src and PI3K activation are triggered during NET formation mediated by platelets (14, 48) (Figure 2). Further studies are required to elucidate the signaling pathways involved in platelet-mediated NETosis.

PLATELETS AND NETs IN DISEASE

Infectious Inflammation Sepsis

It is increasingly recognized that platelets play an important role in the host defense to pathogens. Several mechanisms appear to be responsible for this response, including the ability of platelets to directly recognize, sequester, and kill pathogens, to recruit leukocytes to the site of infection and to activate neutrophils, enhancing their ability to phagocytose and kill pathogens (49). In addition, a seminal study by Clark et al. (29) demonstrated that platelets induce neutrophil unique effector responses, such as the generation of NETs. In response to endotoxemia, neutrophils adhere to endothelium mainly in the sinusoids of the lungs and liver and then LPS-activated platelets anchor to the surface of neutrophils, promoting NETosis. Because these interactions were observed during severe sepsis, it was suggested that platelets, through the expression of TLR4, act as a barometer for systemic infection and, under high levels of LPS, the interplay between platelets and neutrophils constitutes an efficient mechanism in the fight against pathogens. Remarkably, the formation of NETs, if uncontrolled, is also responsible for tissue damage and organ dysfunction. The biological relevance of platelets in NET formation during sepsis was underscored by the observation that plasma from severely septic patients also triggered the formation of neutrophil DNA traps in the presence of platelets (29). In addition, in a *Streptococcus mutans* endocarditis rat model, it has been demonstrated that infective endocarditis-inducing pathogens activate platelets through specific IgG, which contributes to bacteria–platelet aggregate formation on the damaged valve; the bacteria also activate the infiltrated neutrophils and produce ROS, which triggers chromatin decondensation. The resulting NETs entrap the bacteria–platelets aggregates to promote vegetation formation (48). These observations lead the authors to suggest that a similar process could take place in human endocarditis, because NETs have been identified in human specimens.

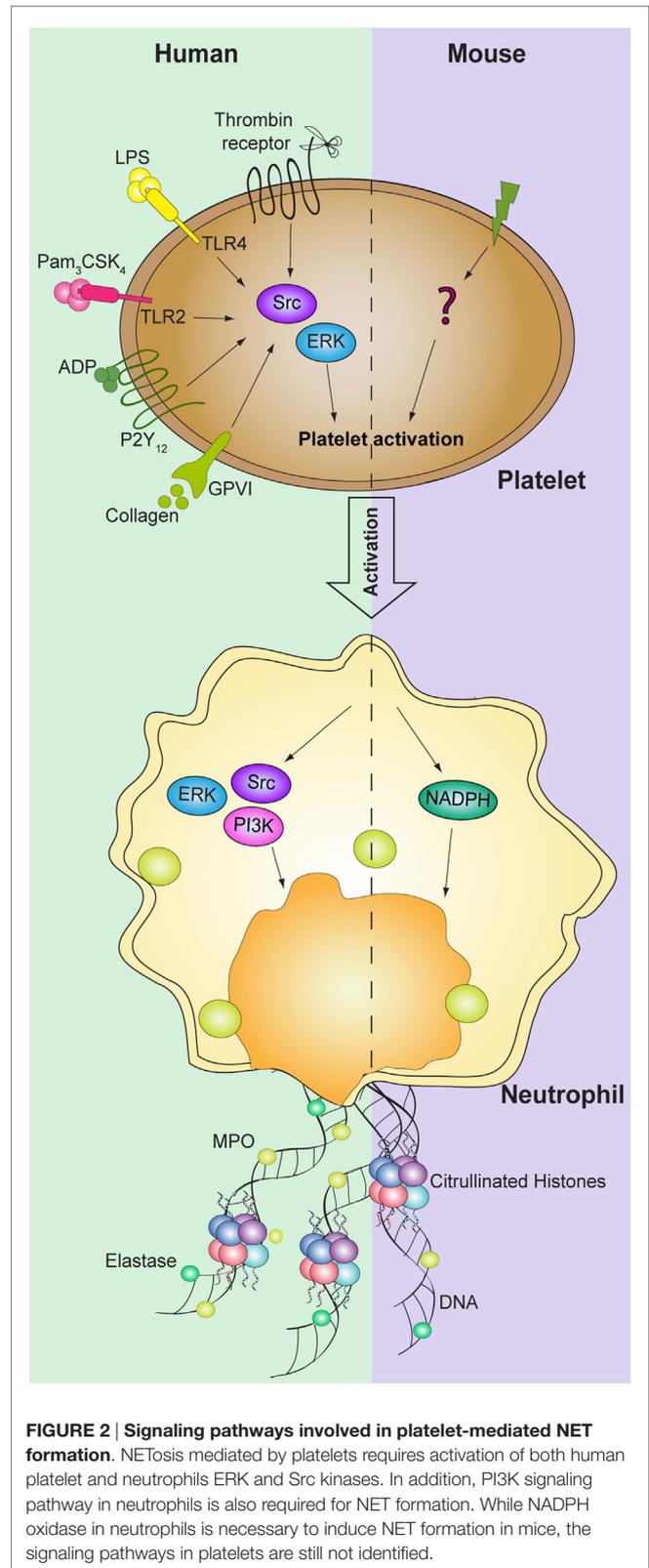


FIGURE 2 | Signaling pathways involved in platelet-mediated NET formation. NETosis mediated by platelets requires activation of both human platelet and neutrophils ERK and Src kinases. In addition, PI3K signaling pathway in neutrophils is also required for NET formation. While NADPH oxidase in neutrophils is necessary to induce NET formation in mice, the signaling pathways in platelets are still not identified.

Interestingly, it was recently proposed that the link between coagulation and the innate immune response might constitute an innate effector mechanism, named immunothrombosis, which

mediates the recognition of pathogens and damaged cells and inhibits pathogen dissemination and survival. Immunothrombosis is therefore considered to be a physiological process crucial for intravascular immunity, whereas its dysregulation may be one of the underlying events that trigger thrombotic disorders, such as VTE and disseminated intravascular coagulation. Neutrophils are major players in the development of immunothrombosis through the release of NETs. Moreover, NETs may also provide a scaffold to activate platelets and stimulate thrombus formation (50). Several studies have indicated the relationship between infection and thrombosis; however, the pathogenic mechanism was not clear. The discovery of NETs and the bidirectional interaction with platelets represents a link between these phenomena.

Sterile Inflammation

Acute Lung Injury

In a murine model of TRALI, NETs were found in the microcirculation of the lungs and components of these networks in plasma (11). When platelet function was inhibited, DNA content significantly decreased indicating that platelets are necessary for the generation of NETs. Similar results were demonstrated in a mouse model of ventilation-induced lung injury (39). In this study, platelet depletion resulted in a decreased neutrophil accumulation in alveolar compartments and release of these DNA traps. Furthermore, a similar result was obtained by specifically inhibiting the heterodimerization of RANTES and PF4, reaffirming the role of platelets in this mechanism, and increasing the severity of the disease. However, Thomas et al. reported that platelet depletion with neuraminidase in a murine model of TRALI did not prevent NET formation in the lungs (51). This observation was only qualitative, thus, it is conceivable that platelets potentiate the release of NETs induced by other agonists. Taking into account these discrepancies, more studies are needed to understand the relevance of platelets in the formation of NETs in TRALI and whether therapeutic inhibition of platelet–neutrophil aggregates and/or NET formation provide a useful therapeutic strategy.

Thrombosis

Venous Thrombosis

Traditionally, the pathogenic mechanism of venous thromboembolism (VTE) that includes DVT or pulmonary embolism was considered the result of reduction in blood flow, injury to the vascular endothelium and a hypercoagulable state (Virchow's Triad) (52). The discovery of NETs offered a very exciting explanation for the cellular events that trigger VTE. Initial studies showed that DNA traps promote platelet adhesion, activation, and aggregation and exert procoagulant activity, thus providing a scaffold for thrombus formation and growth (53). These effects are mainly caused by histones present in the NETs (54–56). Moreover, histones induce the release of platelet mediators that, in turn, induce NETosis, thus creating a positive feedback mechanism that favors thrombus formation. NETs also recruit red blood cells and promote vWF, fibrinogen, and fibrin deposition inducing a red thrombus, such as that found in veins. Remarkably, clot formation in the presence of activated neutrophils can be prevented only by simultaneous treatment with tissue plasminogen activator and

deoxyribonuclease (DNase), indicating that NETs may provide a scaffold independent from fibrin (53).

Using a mouse model of flow restriction-induced DVT, von Bruhl et al. and Brill et al. demonstrated that flow restriction triggers the rapid accumulation of leukocytes, mainly neutrophils (85%) and monocytes (15%), that initiate local fibrin formation through the expression of tissue factor. In addition, neutrophils form NETs that bind factor XII and initiate coagulation. Platelets were critical to foster neutrophil recruitment and NET formation through the GPIIb- β_2 integrin axis and supported fibrin formation by enhancing neutrophil-dependent coagulation. While DNase I and enoxaparin protected mice from DVT, infusion of an unfractionated mixture of calf thymus histones increased plasma vWF and promoted DVT early after stenosis application, indicating the relevant role of NETs in DVT (40, 46). In fact, these experimental models provided the first *in vivo* evidence that NETs can act as a platform for thrombogenesis in large veins in response to perturbations of flow and also that these DNA traps are a unique link between inflammation and DVT. Furthermore, these studies highlighted that platelets, commonly recognized as central mediators of arterial thrombosis, also contribute to inflammation and the propagation of DVT in the absence of endothelial disruption, mainly fostering NET formation.

The critical role of NETs in developing DVT was also demonstrated by Martinod et al., who showed that PAD4-deficient mice (PAD4^{-/-}) were protected from producing venous thrombi (57). However, in contrast to these observations, El-Sayed et al. found no differences in the stasis thrombus size either in WT animals that were treated with DNase I or in PAD4^{-/-} mice compared with controls (58). The differences between these studies are still not clear and require further investigation. Furthermore, unexpectedly, it was recently reported that neutrophils from elastase-deficient mice only showed a mild reduction in non-infectious stimuli-mediated NET formation *in vitro* and accordingly, elastase deficiency had no significant effect on thrombosis in the inferior vena cava stenosis model (59). These data revealed not only that neutrophil elastase is not essential for NETosis but also that even partial NET release seems sufficient to attract platelets, red blood cells, promote coagulation, and drive venous thrombus formation. Since all these findings were obtained in mice, the question arises whether similar molecular mechanisms are effective in humans.

The first clinical study of 150 patients showed that increased levels of circulating nucleosomes and neutrophil activation, as evidenced by the presence of elastase–antitrypsin complexes, were associated with a threefold risk of DVT (60). In another study, immunohistochemical analysis of thrombi obtained from patients at different stages of VTE demonstrated the presence of NETs during the organizing stage of thrombus maturation. In contrast, NETosis was absent in the mature regions of organized thrombi. CitH3-positive cells surrounded vWF-positive platelet islands in human DVT samples, and diffuse vWF-positive staining was associated with diffuse citH3 patterns in the organizing regions of thrombi (61). These findings support the notion that NETs, together with vWF, could enhance thrombus formation/stability in DVT.

Arterial Thrombosis

Increased levels of nucleosomes and cell-free DNA have frequently been shown to be associated with infarcted tissue in patients with ischemic stroke and myocardial infarction (MI) (62). However, because these biomarkers are not specific to tissue injury, it was not clear from these studies whether the DNA material was released from necrotic tissues or from NETs. Furthermore, the relationship was not established between the nucleosome of cell-free DNA and thrombi. The presence of citH3 neutrophils in heart sections and increased plasma nucleosomes levels in mice subjected to myocardial reperfusion injury was recently demonstrated. Also of note, monotherapy with DNase I significantly decreased the number of infiltrating neutrophils and plasma nucleosomes levels as well as reducing the infarcted area and improving cardiac function, indicating not only that NETs are components of the inflammatory milieu of the infarcted myocardium but also their direct involvement in cardiac injury (63). In a prospective, observational, cross-sectional cohort of 282 individuals with suspected coronary artery disease, Borissoff et al. found that markers of NETs, such as MPO–DNA complexes, predicted the number of atherosclerotic coronary vessels, the presence of a hypercoagulable state, and the occurrence of major adverse cardiac events (64). Moreover, they also identified high nucleosome levels as an independent risk factor of severe coronary stenosis. Of note, this study only found a weak correlation of platelet activation markers (measured by PF4 plasmatic levels) and citH4 levels. However, in another study, Maugeri et al. demonstrated that in patients with acute MI, coronary artery thrombi obtained during percutaneous coronary intervention were made up mainly of platelets and neutrophils and contained large amounts of DNA strands that were decorated with citrullinated histones and granule proteins such as MPO and pentaxin; platelets were identified close to DNA webs (13). In support of these observations, citH3 was detected in the cerebral microthromboses of ischemic stroke patients. Interestingly, the patients also had an unexpectedly higher prevalence of cancer; half of the cancers were diagnosed post-mortem (65). Furthermore, cancer patients had increased plasmatic markers of NETosis that correlated positively with thrombin–antithrombin levels and soluble P-selectin. These data not only further support the link between NETosis and thrombosis but also highlight the relevance of NET formation as biomarkers detecting cancer in arterial microthrombosis, or vice versa.

The role of NETs and platelets was also confirmed in patients with MI in selective samplings of thrombotic material and surrounding blood from the infarct-related coronary artery during primary percutaneous revascularization. The study showed that interaction of thrombin-activated platelets with neutrophils at

the site of plaque rupture during an acute MI results in local NET formation and delivery of functional tissue factor (66).

CONCLUSION

The presented evidence indicates that platelets, when stimulated by bacteria, viruses, or traditional agonists, are capable of binding to neutrophils, activating them to release NETs. This interaction can be beneficial to protect the host against pathogens but, if uncontrolled, can cause tissue and organ damage. Of note, while experiments performed *in vitro* are compelling in their collective confirmation that platelet–neutrophil interactions can be induced to generate NETs, the relevance of this interaction *in vivo* still contains elements of ambiguity and leads to numerous unresolved questions often involving pathway differences, both between species and cell types.

The interaction of platelets and neutrophils in NETosis induction appears to be a relevant pathogenic mechanism for both arterial and venous thrombosis; prevention of platelet- and neutrophil-mediated thrombogenesis is now being considered as a potential promising new target for anti-thrombotic therapy.

Although we have witnessed great advances during the last decade in the comprehension of NETosis and the involvement of platelets in this process, several questions still remain to be answered: are circulating nucleosomes and activated neutrophils causative of thrombus formation or are they rather a consequence of its development? Is there any degree of selectivity regarding the decondensation and release of DNA during NETosis mediated by platelets in sterile or infectious conditions? Is there any relationship between platelets and NETs formation in atherosclerotic plaque progression? Is NETosis mediated by platelets required for hemostasis?

It is clear that more experimental and clinical studies are required to further understand the physiopathologic relevance of this newly discovered interaction between platelets and neutrophils.

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

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Neutrophil Integrins and Matrix Ligands and NET Release

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Neutrophils are motile and responsive to tissue injury and infection. As neutrophils emigrate from the bloodstream and migrate toward a site of affliction, they encounter the tissue extracellular matrix (ECM) and thereby engage integrins. Our laboratory studies the neutrophilic response to the fungal pathogen *Candida albicans* either in the filamentous state of the microbe or to the purified pathogen-associated molecular pattern, β -glucan. We have gained an appreciation for the role of integrins in regulating the neutrophil anti-*Candida* response and how the presence or absence of ECM can drive experimental outcome. The $\beta 2$ integrin CR3 (complement receptor 3; $\alpha M\beta 2$; Mac-1; CD11b/CD18) plays an important role in fungal recognition by its ability to bind β -glucan at a unique lectin-like domain. The presence of ECM differentially regulates essential neutrophil anti-fungal functions, including chemotaxis, respiratory burst, homotypic aggregation, and the release of neutrophil extracellular traps (NETs). We have shown that NET release to *C. albicans* hyphae or immobilized β -glucan occurs rapidly and without the requirement for respiratory burst on ECM. This is in contrast to the more frequently reported mechanisms of NETosis to other pathogens without the context of ECM, which occur after a prolonged lag period and require respiratory burst. As expected for an ECM-dependent phenotype, NETosis and other neutrophil functions are dependent on specific integrins. The focus of this review is the role of ECM ligation by neutrophil integrins as it pertains to host defense functions with an emphasis on lessons we have learned studying the anti-*Candida* response of human neutrophils.

Keywords: NETs, extracellular matrix, neutrophils, integrins, *Candida*

PREVALENCE AND RISK FACTORS FOR CANDIDIASIS

Candida albicans exists as normal flora of the skin and GI tract but can become a serious and life-threatening infection. Candidiasis can present either locally as mucocutaneous infection or as the more severe invasive form of the disease. Predisposing factors lending to loss of host control of the colonized organism are likely to be a combination of host as well as microbial factors (1). Invasive candidiasis continues to be a significant medical problem and *Candida* ranks as the fourth leading pathogen in causing nosocomial infection with mortality up to 40% in spite of available anti-fungal

Abbreviations: CARD9, caspase recruitment domain-containing protein 9; CR3, complement receptor 3; ECM, extracellular matrix; LAD, leukocyte adhesion deficiency; NETs, neutrophil extracellular traps; PAD4, peptidyl arginine deiminase type IV; PAMP, pathogen-associated molecular pattern; PRR, pattern recognition receptor; ROS, reactive oxygen species.

therapy (2). Infection can take place in any bodily organ and systemic infection can involve coincident infection of multiple organs, as well as the blood.

Clinical risk factors for acquisition of *Candida* infection include neutropenia or a neutrophil defect whether heritable or epigenetic, systemic antibiotic usage, central venous catheter, mucosal damage, and prolonged stay in the ICU even in the presence of surfeit neutrophils (3, 4). *Candida* infection is remarkably high in non-trauma emergency surgical patients with a prolonged ICU stay, reaching a rate of 21.7/100 discharges, higher than other established high-risk populations (5, 6).

FUNGAL RECOGNITION

Candida albicans is a polymorphic fungal pathogen that can grow as yeast, pseudohyphae, and true hyphae and the ability to switch between phenotypic states is an essential virulence factor complicating immune detection (7–9). Neutrophils respond to infectious fungi in a variety of ways, including phagocytosis, production of reactive oxygen species (ROS), degranulation, recruitment of other leukocytes, and the more recently recognized release of neutrophil extracellular traps (NETs). In its budding yeast form, *C. albicans* is small enough for neutrophils to phagocytose. This response involves uptake of microbes into the phagosome, where fusion of cytotoxic granules and oxidative products facilitate microbial killing (10). The invasive filamentous forms of *C. albicans* are too large to be engulfed, necessitating other cellular strategies for anti-fungal response and clearance (11–16). The recently described process of NETosis, where NETs consisting primarily of DNA studded with histones and components of cytotoxic granules are extruded into the extracellular space, accomplishes the dual functions of both immobilizing and killing harmful microbes where phagocytosis is not feasible (17).

Innate immune cells recognize *C. albicans* by binding to molecules present in the fungal cell wall. β -glucans are a class of long-chain polymers of glucose in β -(1,3) (1,6)-linkages that are conserved in microbial structures but not found in mammalian cells and, thus, are considered a pathogen-associated molecular pattern (PAMP) (11, 18, 19). Pattern recognition receptors (PRRs) on cells of the innate immune system discern PAMPs as being non-self and initiate antimicrobial host defense mechanisms through activation of intracellular signaling pathways. With regard to recognition of β -glucan, two receptors have received the most attention; the integrin CR3 and the C-type lectin Dectin-1 that may exert non-overlapping roles in clinical and experimental host defense. To parse the relative roles of these receptors, one must take into account the species of the host and immune cell type being studied as the anti-fungal role of these receptors can differ between monocyte/macrophages and neutrophils. Differences may also lie in the specific immune function being assayed and the morphological form of the *Candida*.

Dectin-1 plays a key role in *C. albicans* control in mice such that mice defective in Dectin-1 are susceptible to fungal infections while CR3 knockout mice are more resistant to challenge with disseminated *C. albicans*, suggesting that CR3 has a non-protective, or suppressive effect on murine host defense (20). In humans, Dectin-1 has been shown to be important in control

of mucocutaneous but not systemic infection (21, 22). This was supported by a study of a family with a mutation of caspase recruitment domain-containing protein 9 (CARD9), a signaling molecule downstream of Dectin-1 (23). In this family, the CARD9 defect presented as a predisposition to mucocutaneous candidiasis similar to the absence of Dectin-1, mediated by a cytokine production defect of monocytes and macrophages (21, 23). Neutrophils from leukocyte adhesion deficiency (LAD) type 1 patients that are devoid of CD11b/CD18 but which express Dectin-1 failed to internalize *Saccharomyces cerevisiae* or unopsonized zymosan demonstrating the primacy of CR3 in phagocytosis of unicellular yeast and β -glucan-containing particles (24). In short, phagocytosis of unopsonized yeast or β -glucan-containing particles is primarily mediated by CR3 in human phagocytes and by Dectin-1 in murine cells (25). It is not clear why the genetic absence of CR3 has such different implications for anti-fungal immunity in mice and humans. This is often correlated with the notion that CR3 ligation by β -glucan particles fails to induce respiratory burst thereby limiting this host defense mechanism (26). However, we and others have shown that human neutrophils induce a CR3-dependent respiratory burst to fungal hyphae or immobilized purified β -glucan as a model of the response to non-phagocytosable filaments (11, 12, 15). Given the multifaceted role of CR3 in immune response to a pathogen, it is difficult to ascribe a mechanism to the increased resistance in CR3 knockout mice.

With regard to recognition of non-phagocytosable fungal hyphae, our laboratory showed that antibody blockade of either fungal cell wall β -glucan or neutrophil CR3 was sufficient to obviate the respiratory burst of human neutrophils; antibody blockade of Dectin-1 had no effect (11, 13). In addition to the host defense mechanisms affected by CR3 and Dectin-1 individually, there is solid evidence for a crosstalk pathway connecting these PRRs. Li et al. showed a mechanism dependent on the RhoGTPase exchange factor, Vav, through which binding of β -glucan to Dectin-1 resulted in CR3 activation in both murine and human cells (27). This highlights the potential complexity of working toward a more complete understanding of the differential nature of immune recognition of *C. albicans* hyphae and yeast forms. A significant step forward in this regard is found in a report by Lowman et al. (22) in which a novel cyclical, or “closed chain” structure of β -glucan was found in *C. albicans* hyphae but not in yeast. These authors purified β -glucan from *C. albicans* yeast and hyphae into water-insoluble microparticulate form and showed that the β -glucan extracted from hyphae, but not yeast, produced a potent IL-1 response by human monocytes and macrophages, which was Dectin-1-dependent. Whether monocyte Dectin-1 can recognize cyclical hyphal β -glucan within the cell wall of the organism remains to be seen. Findings to date suggest that Dectin-1 recognition of *Candida* hyphae is limited to bud scars where β -glucan is particularly exposed, it does not appear to recognize β -glucan along hyphal filaments (28). Whether neutrophils exhibit differential responsiveness to these β -glucan isoforms has not yet been determined. Therefore, the differential responsiveness of innate immune cells to the yeast and hyphal forms of *C. albicans* may well be due to variance in the structure of the prominent fungal PAMP β -glucan. Work from our laboratory and others show that CR3 is most likely the prominent immune

receptor on human neutrophils and is able to detect β -glucan within fungal filaments (11, 13, 15, 29). As CR3 serves as both a PRR and an extracellular matrix (ECM)-binding integrin, it plays a critical role in integrating tissue environment and microbial recognition, driving neutrophil anti-fungal immunity.

ROLE OF INTEGRINS IN ANTI-FUNGAL IMMUNITY

All cell–cell and cell–ECM adhesive events occur extracellularly but are translated into cellular responses by communication across the plasma membrane through the action of integrins (30). Integrins are essential for proper regulation of a number of fundamental physiological processes, including tissue morphogenesis, inflammation, immune responsiveness, wound healing, and regulation of cell growth and differentiation. All cells express a contingent of integrins and respond to integrin activation by cytoskeletal-dependent processes, such as shape change, adhesion, spreading, migration, and/or phagocytosis (31). Among 24 $\alpha\beta$ heterodimers that have been reported in vertebrates, the $\beta 2$ family ($\alpha L\beta 2$, $\alpha M\beta 2$, $\alpha X\beta 2$, and $\alpha D\beta 2$) are specifically expressed on leukocytes (31). Leukocyte $\beta 2$ integrins regulate many aspects of immune or inflammatory responses because, unlike cells that reside within solid tissues, circulating leukocytes by necessity relocate during the course of immune reactions. In so doing, they dynamically adhere and de-adhere to cells of the vasculature, to other immune cells, and to components of the ECM, in order to ultimately contact the foreign body or pathogen at the site of infection or injury. Evidence for the physiological significance of leukocyte integrins is highlighted by the recurrent, life-threatening infectious episodes observed in LAD patients that are genetically deficient for expression of $\beta 2$ integrins (32). In stark contrast to impaired host defense found in the absence of $\beta 2$ integrins, sustained and improper activation of these integrins contributes to the pathogenesis of autoimmune diseases, chronic inflammatory disorders, and ischemic stroke (33).

Complement receptor 3 (CR3; $\alpha M\beta 2$; CD11b/CD18; Mac-1) is a member of the $\beta 2$ integrin family, yet it functions like no other integrin and, in some ways, like no other receptor yet described in nature. In general, receptors can be defined as having a canonical ligand that binds with characteristic affinity to a single binding site which, in turn, leads to a characteristic intracellular response. In stark contrast, CR3 has two spatially distinct binding sites, the so-called I-domain and the lectin-like domain, that bind completely different ligands and results in differing cellular responses. The I-domain itself is a highly promiscuous binding site with over 30 structurally unrelated ligands shown to be capable of binding at that domain alone, including iC3b, fibrinogen, ICAM-1, fibronectin, heparan sulfate, and factor X (34, 35). I-domain ligands are both host- and microbial-derived such that a multitude of immune effector functions executed by inflammatory neutrophils are entirely mediated, or regulated, by CR3. As with other integrins, ligand binding is regulated by the structural state of activation such that when in a bent conformation the integrin is in a low-affinity state that is modulated upon activating signals that can originate internally (inside-out) or

externally (outside-in). In either case, the receptor assumes an upright conformation consistent with high-affinity ligand binding that can be further regulated by receptor clustering resulting in avidity modulation (36). The lectin-like domain is spatially distinct from the I-domain, and is noted for its ability to bind the glucose polymer β -glucan (11, 18, 37–39). Ligation of purified fungal β -glucan to CR3 is sufficient to induce a signaling response (39). The ability of CR3 to mediate neutrophil recognition of fungi and initiate signaling identifies it as the only integrin that also serves as a PRR.

A novel aspect of CR3 bioactivity is that the manner in which it is ligated at its two binding domains has a profound effect on cellular responsiveness. This concept was first posited where Vetvicka et al. reported that murine and human natural killer cells could acquire cytotoxic capability for resistant tumor cells if targets were opsonized with iC3b, a well-described CR3 I-domain ligand, and effector cells were exposed to β -glucan, but not by either ligand alone (40–42). This increased cytotoxic activity could be inhibited with CR3-specific antibodies. Administration of β -glucan enhanced the activity of complement-fixing, anti-tumor antibodies *in vivo*, causing tumor regression and increased survival as compared to mice receiving either antibody or β -glucan alone (43–45). Surprisingly, this adjuvant activity of β -glucan in reducing tumor burden was shown to be mediated by neutrophils and did not occur in mice lacking either CR3 or complement or in mice depleted of neutrophils.

ROLE OF EXTRACELLULAR MATRIX IN ANTI-FUNGAL IMMUNITY

In a seminal paper by Carl Nathan in 1989, the respiratory burst of human neutrophils to soluble proinflammatory mediators was shown to be adhesion dependent and require attachment to either ECM components or human umbilical vein endothelial cells (46, 47). This adhesion dependence of neutrophil effector function provided an early the basis for suggesting the coordination of integrin and non-integrin stimuli to drive host defense in tissues. As CR3 mediates cellular interactions with ECM, and since all neutrophilic responses to tissue infections necessitate ECM contact, we focused our attention on investigating the role of CR3 as a fungal PRR via the lectin-like domain in the presence of fibronectin, a ubiquitous ECM molecule and I-domain ligand. We showed that the effect of CR3 on the anti-fungal response of human neutrophils to *Candida* is not a straightforward consequence of receptor ligation, but is directed by how it is ligated (11–13, 39, 48–52). We have found that upon dual ligation of CR3 by fibronectin and β -glucan, neutrophils demonstrated enhanced chemotaxis, swarming and aggregation, NETosis, and an actively suppressed respiratory burst (**Figure 1**). Antibody-blocking studies were used to show coincident ligation of CR3 at both the I-domain with the ECM component fibronectin and the lectin-like domain control neutrophil effector functions differently than ligation of either site alone (11–13, 39, 49–52). These studies additionally identified a CR3-mediated regulation of $\beta 1$ integrins, driving a shift in fibronectin binding from $\alpha 5\beta 1$ to $\alpha 3\beta 1$ (12, 50, 52).

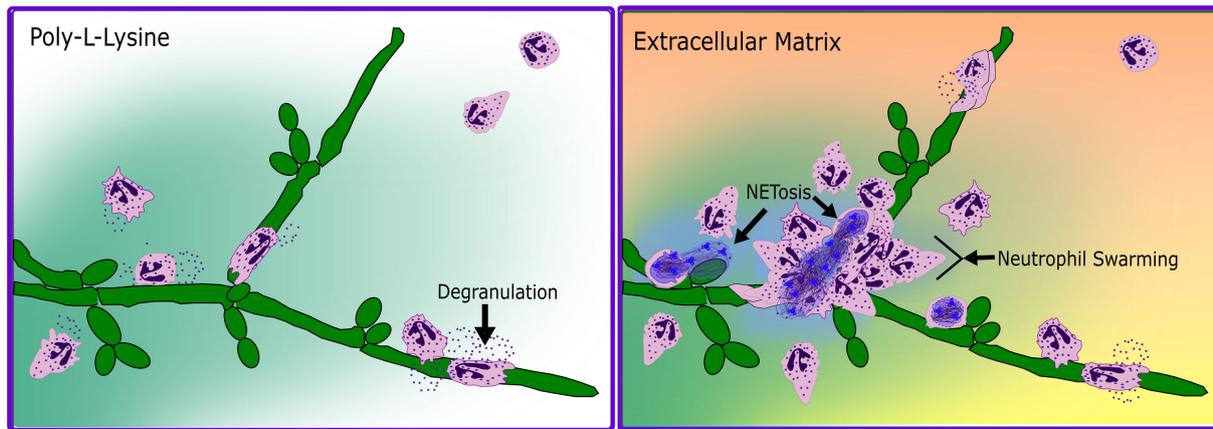


FIGURE 1 | Schematized neutrophil response to *C. albicans* hyphae in the absence and presence of extracellular matrix. In the absence of ECM (poly-L-lysine, left panel), neutrophils respond to hyphae by chemotaxis, by degranulation and respiratory burst, and by wrapping around fungal filaments in a form of frustrated phagocytosis. In the presence of ECM (Extracellular Matrix, right panel), neutrophils chemotaxis to fungal filaments is faster and more directed, with degranulation and respiratory burst being actively suppressed until the multifocal contact of frustrated phagocytosis. Additionally, in a subset of cells contacting the fungal hyphae, a rapid, respiratory burst independent NETotic response is induced and followed by neutrophil swarming.

In order to be virulent, *C. albicans* must be capable of transitioning between yeast and hyphal forms (53). The yeast form is readily cleared by neutrophil phagocytosis, the mechanisms of which have been studied extensively. Less work has focused on the neutrophilic response to this filamentous form of the microbe. As *Candida* destruction necessitates a full-blown response to both forms of the organism, work in our lab has focused on this gap in our understanding of anti-fungal host defense. We found that *C. albicans* hyphae growing in the kidney of an infected rat induced massive clustering of inflammatory neutrophils that entirely surrounded the hyphae (11). This clustering of human neutrophils could be replicated *in vitro* with *C. albicans* hyphae plated on fibronectin but not on hyphae plated in the absence of fibronectin (13). Pretreating hyphae with an anti- β -glucan antibody prevented clustering of neutrophils suggesting that the β -glucan component of the fungal cell wall is important for neutrophil responsiveness (13). Immobilization of purified β -glucan in the presence of fibronectin was a biomimic for *Candida* hyphae within tissue ECM suggesting that fungal β -glucan is necessary and sufficient for homotypic aggregation (13). Furthermore, this swarming and aggregation took place rapidly, being evident in less than 30 min *in vitro* (13).

ADDING NETosis TO THE REPERTOIRE OF NEUTROPHIL-MEDIATED IMMUNITY

NETosis was initially described as a pathway of chromatin decondensation and release with requisite NADPH oxidase, elastase, and myeloperoxidase activity in response to activating stimuli (54–56). The initial reports showed relatively slow kinetics, occurring hours following exposure to stimuli, including bacteria, fungi, or PMA (14, 17, 54, 55, 57, 58), though evidence suggests that no *de novo* gene synthesis is required (59). As additional investigators explored conditions necessary and sufficient to

NET release within their experimental systems, some variance in the original paradigm emerged. The “classical” pathway involves entry of the neutrophil into a cell death program that requires ROS and manifests in plasma membrane disruption and NET release 1–4 h after stimulation. This pathway utilized peptidyl arginine deiminase type IV (PAD4) for histone citrullination that leads to chromatin decondensation due to neutralization of histone electrostatic charge normally imparted by arginine but lost upon conversion to citrulline (60). Elastase and myeloperoxidase serve to digest nuclear histones after translocation such that absence of these enzymes impairs NET release (61). A more recently identified early/rapid, or “vital,” NET release was identified that can result in extrusion in minutes, independently of ROS and without compromising cell viability, in response to *Staphylococcus aureus*, *C. albicans*, and *Leishmania* promastigotes (13, 62, 63). The “classical” and “vital” NETosis pathways need not be mutually exclusive, as the context of NETotic stimuli presentation, such as timing, viability, size, or morphotype, can drive differential response patterns and kinetics (13, 14, 54, 57, 62–64). ECM ligands in the context of tissue infection can also drive differential neutrophil responses.

ROLE OF INTEGRINS AND EXTRACELLULAR MATRIX IN THE REGULATION OF NETosis

Our laboratory has demonstrated an integrin-dependent ECM response that both actively suppresses the respiratory burst to *Candida* hyphae, or immobilized fungal β -glucan, while driving a robust, rapid NETotic response (12, 13). Additionally, work with neonatal neutrophils show that this NETotic anti-fungal pathway is active even though neonatal neutrophils have been shown to be deficient in NETotic responses to other initiating agents,

underscoring the importance of stimuli context in evaluating effector function (65, 66).

In addition to our work, evidence to date describes the role of $\beta 2$ integrins in NET release as it occurs along liver sinusoids or vascular endothelium. Platelet–neutrophil interactions have been shown to occur under conditions of severe sepsis (67) or endotoxemia in which activation of platelet TLR4 promotes platelet binding to neutrophils with ensuing NET release (68). Two recent studies differ with regard to which $\beta 2$ integrin mediates platelet–neutrophil binding. McDonald et al. (69) show a role for LFA1, although the ligand on the platelet remains to be defined. Rossaint et al. (70) showed that incubation of stimulated platelets with neutrophils *ex vivo* induced NETosis that could be blocked with anti-CR3 antibodies but not with antibodies against LFA1. Mohanty et al. (71) recently identified that neutrophils form NETs from saliva exposure in a $\beta 2$ integrin-independent fashion, as LAD1 patients form NETs to saliva and PMA but not to unopsonized *S. aureus*.

Complement receptor-3 has been shown to regulate apoptosis of neutrophils such that the genetic absence of CR3 delayed the onset of apoptosis of neutrophils after thioglycollate injection (72). Given that CR3 determines a NETotic pathway for *Candida*, it is of interest to consider whether or not NETotic and apoptotic pathways have common points of regulation. Evidence to date suggests that NETosis and apoptosis both require calcium for initiation but then show divergence in the sense that PAD4 activation does not depend on downstream components of the apoptotic pathway, such as activated caspase, and apoptosis does not depend on PAD4 (73). Indeed, histone citrullination in neutrophils is induced by inflammatory stimuli and not by treatments that induce apoptosis (73). Moreover, treatment of neutrophil-differentiated HL60 cells with calcium ionophore showed that histone citrullination preceded PARP cleavage, such that the decision-making events may be temporal.

The occupancy of one integrin by ligand has been shown to be capable of suppressing the function of other integrins in a phenomenon referred to as trans-dominant inhibition, or integrin crosstalk. For example, activating antibodies specific for the $\alpha v \beta 3$ integrin suppress $\alpha 5 \beta 1$ -dependent phagocytosis and ligation of $\alpha 4 \beta 1$ inhibits $\alpha 5 \beta 1$ -dependent expression of metalloproteinases (74, 75). Ligation of $\alpha IIb \beta 3$ induces trans-dominant suppression

of target integrins $\alpha 5 \beta 1$ and $\alpha 2 \beta 1$ (76). Additionally, antibody activation of $\beta 1$ integrins was shown to increase CR3 adhesion to fibronectin (77) and outside-in activation of $\beta 2$ integrins via crosslinking was demonstrated to upregulate the expression of $\beta 1$ integrins (78). These studies suggest that certain integrin-specific ligands provoke integrin crosstalk that could result in alterations in cell migration and invasion. With regard to the anti-*Candida* response of human neutrophils, we discovered a temporal, interregulatory relationship between the $\beta 2$ integrin CR3 and regulation of $\beta 1$ family members and this modulates the response to β -glucan or *C. albicans* hyphae in the context of ECM (12, 13, 49–52). The extent to which integrin crosstalk operates as a regulatory pathway for other innate immune functions is not well understood.

NEUTROPHILS, INTEGRINS, EXTRACELLULAR MATRIX, NETosis, AND BEYOND

The host response of neutrophils to *C. albicans*-infected tissues necessitates ECM contact. Our work and others have clearly demonstrated a regulatory role of ECM in determining neutrophil function, including NETosis. The focus of this review, the role of ECM ligation by neutrophil integrins as it pertains to both host defense functions and the kinetics of these functions, has implications that reach far beyond the anti-fungal response. The totality of ECM involvement in neutrophil host defense in tissues makes accounting for both its presence and the role of integrin engagement an important and under-examined mechanistic aspect of inflammation.

AUTHOR CONTRIBUTIONS

XO and JR conceived, designed, and wrote the manuscript.

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Inhibitors of Serine Proteases in Regulating the Production and Function of Neutrophil Extracellular Traps

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Neutrophil extracellular traps (NETs), DNA webs released into the extracellular environment by activated neutrophils, are thought to play a key role in the entrapment and eradication of microbes. However, NETs are highly cytotoxic and a likely source of autoantigens, suggesting that NET release is tightly regulated. NET formation involves the activity of neutrophil elastase (NE), which cleaves histones, leading to chromatin decondensation. We and others have recently demonstrated that inhibitors of NE, such as secretory leukocyte protease inhibitor (SLPI) and SerpinB1, restrict NET production *in vitro* and *in vivo*. SLPI was also identified as a NET component in the lesional skin of patients suffering from the autoinflammatory skin disease psoriasis. SLPI-competent NET-like structures (a mixture of SLPI with neutrophil DNA and NE) stimulated the synthesis of interferon type I (IFN1) in plasmacytoid dendritic cells (pDCs) *in vitro*. pDCs uniquely respond to viral or microbial DNA/RNA but also to nucleic acids of “self” origin with the production of IFN1. Although IFN1s are critical in activating the antiviral/antimicrobial functions of many cells, IFN1s also play a role in inducing autoimmunity. Thus, NETs decorated by SLPI may regulate skin immunity through enhancing IFN1 production in pDCs. Here, we review key aspects of how SLPI and SerpinB1 can control NET production and immunogenic function.

Keywords: neutrophil extracellular traps, neutrophil elastase, SLPI, serpin B1, plasmacytoid dendritic cells, psoriasis

NEUTROPHIL SERINE PROTEASES IN NEUTROPHIL BIOLOGY

Neutrophils, key immune cells for protection against microbial infection, are also associated with a range of pathologies, including autoinflammatory diseases, such as systemic lupus erythematosus (SLE) and psoriasis (1–3). Neutrophils are a rich source of proteolytic enzymes, including serine proteases. The enzymatic activity of serine proteases depends on a catalytic triad that contains a serine residue. Four active serine proteases, neutrophil elastase (NE), cathepsin G (CatG), proteinase 3 (PR3), and neutrophil serine protease 4 (NSP4), as well as azurocidin, an enzymatically inactive

Abbreviations: CatG, cathepsin G; DPP1, dipeptidyl peptidase I; IFN1, interferon type I; NE, neutrophil elastase; MPO, myeloperoxidase; MSU, monosodium urate; PAD4, peptidylarginine deiminase 4; pDCs, plasmacytoid dendritic cells; PMA, phorbol ester; PR3, proteinase 3; SLPI, secretory leukocyte protease inhibitor; TFPI, tissue factor pathway inhibitor.

serine protease homolog, were characterized in neutrophils (4, 5). Serine proteases are synthesized early in granulocyte development, during the promyelocytic stage of granulopoiesis in the bone marrow, and require N-terminal trimming by dipeptidyl peptidase I (DPPI) for activation (6–8). Under homeostatic conditions, the proteases are stored in a catalytically active form in the azurophilic granules of circulating granulocytes (4). Neutrophils are equipped with heterogeneous granules, which are classified into four subsets: primary or azurophilic granules, formed first during granulopoiesis and containing myeloperoxidase (MPO) and the serine proteases as their hallmark proteins; secondary or specific granules, containing lactoferrin and cathelicidin; tertiary or gelatinase granules, with gelatinase and lysozyme; and finally, secretory granules, with complement and chemotaxis receptors (4, 9). As pre-stored agents, neutrophil serine proteases can be quickly engaged to provide protection against microbial challenge, either by degrading internalized microbes or upon release from activated neutrophils. The serine proteases are important contributors to the physiological response to infection, both as antimicrobial agents and as immunomodulators. Although serine proteases, such as NE and CatG, can kill microbes by virtue of their antimicrobial activity unrelated to their digestive potential (10), these enzymes can also restrain microbial growth through the processing of microbial and host proteins. For example, they cleave virulence factors of enterobacteria (11) or liberate host antimicrobial peptides from their inert precursor proteins. The latter mechanism was reported for human cathelicidin hCAP-18, which is cleaved into the potent antimicrobial peptide LL37 by PR3 (12). Serine proteases also participate in a defense against microbes through limiting microbial spreading. The underlying mechanism involves degradation of an inhibitor of coagulation, tissue factor pathway inhibitor (TFPI) by NE, thereby fostering production of intravascular fibrin barriers that sequester bacteria (13).

The immunomodulatory function of neutrophil serine proteases depends to a large extent on the regulation of the bio-availability of adhesion molecules, cell surface receptors, growth factors, cytokines, and chemoattractants (4). For example, several cytokines belonging to the IL1 superfamily, such as IL1 β , IL18, and IL33, have been reported to be processed into biologically active forms by NE, CatG, and/or PR3 (14–16). Given the crucial role of these cytokines in inflammatory responses to infection or sterile tissue damage, processing of these cytokines alone by neutrophil serine proteases may have far-reaching consequences for a number of the host defensive strategies. Likewise, by triggering the chemotactic activity of the inert chemoattractant proteins, such as chemerin (17, 18), or increasing the chemotactic potential of chemokines, such as CXCL8 (19), neutrophil serine proteases may mobilize specific immune cells to sites of inflammation. On the other hand, the NE-mediated proteolytic degradation of the chemokine CXCL12 and its receptor CXCR4, which disrupts the CXCL12/CXCR4 chemotactic pathway in the bone marrow, facilitates the mobilization of hematopoietic stem cell precursors from the bone marrow into the circulation in response to mobilizing agents, such as G-CSF (20). Together, these findings indicate that by activating or deactivating cell-guiding molecules, the serine proteases provide an important layer of control over cell recruitment.

The enzymes also influence neutrophil development and the functional state of the cell, including apoptosis and the formation of neutrophil extracellular traps (NETs). Mutations in the gene encoding NE-*ELANE* are a leading cause of severe congenital neutropenia (SCN), a disorder leading to a lack of mature neutrophils (21, 22). However, pathogenic *ELANE* mutations are distributed throughout NE, and at least some *ELANE* mutants retain NE activity (23), indicating that neutropenia is not a result of impaired NE proteolytic function. Indeed, recent advances suggest that the pathogenesis of *ELANE* mutations is associated with NE mislocalization, the accumulation of NE in the ER and other cytosolic regions outside of the azurophilic granules, and the activation of the unfolded protein response/ER stress. These alterations in turn lead to the death and differentiation arrest of granulocytic precursors (promyelocytes). Notably, the sequestration of mutated NE in azurophilic granules of myeloid precursor cells, as well as neutrophil maturation, can be rescued by a small, cell-permeable NE inhibitor, sivelestat, given in combination with low-dose G-CSF (23). Although sivelestat may also affect cellular responses in a manner independent of NE inhibition (24), these findings suggest that a NE inhibitor protects differentiating granulocytes against the activity of the mislocalized NE and that the impaired intracellular trafficking of NE can be corrected in the presence of a NE inhibitor.

Neutrophils have a short life span relative to other cells and are subjected to caspase-3-mediated spontaneous death, which phenotypically fits the profile of apoptotic cell death (25). Apoptosis is triggered and executed *via* intracellular cysteine proteases-caspases. The main effector protease that drives the terminal stages of cell death is caspase-3. This protein requires proteolytic cleavage for apoptotic activity. Among the key activatory enzymes are caspase-9 and caspase-8. It was recently reported that although cleavage of caspase-3 was integral to the death of aging neutrophils, it was independent of the proteolytic activity of caspase-8 or caspase-9. Instead, PR3 leaking from azurophilic granules into the cytosol was found to regulate caspase-3 activation and cell death in aging neutrophils (25).

Similar to apoptosis, neutrophil serine proteases have been shown to contribute to the formation of NETs. This process, called NETosis, is associated with irreversible cell state changes, but in a manner distinct from apoptotic death (26).

ROLE OF SERINE PROTEASES IN NET FORMATION

Neutrophil extracellular traps are web-like DNA structures extruded into the extracellular environment by activated neutrophils. A wide range of stimuli triggers NETosis, including Gram-positive and Gram-negative bacteria, such as *Staphylococcus aureus* (26, 27) and *Shigella flexneri* (28); the fungus *Candida albicans* (29); parasites, such as *Leishmania amazonensis* (30); and viruses, such as HIV-1 (31). NET formation is also induced by host-derived inflammatory mediators, such as hydrogen peroxide (H₂O₂) (26), the cytokines, such as IL17 and TNF α (32), chemokines, such as CXCL8 (28), monosodium urate (MSU) (33), cholesterol (34) or calcium carbonate crystals (35), antibodies (36), or antibody-antigen complexes (2). Synthetic

chemicals, such as phorbol ester (PMA) or ionophores, are the most potent inducers of NETosis commonly used in experimental systems.

Neutrophil extracellular traps are thought to represent a unique defense strategy against microbial infection. NET microbicidal function is aided by antibacterial proteins and peptides that are complexed with decondensed chromatin and mitochondria-derived DNA. These proteins include histones, which account for ~70% of all detected proteins in PMA-stimulated neutrophils, as well as serine proteases. Notably, NE is the most abundant non-histone NET-protein, accounting for ~5% of the total protein (29). In agreement with the high levels of NE in NETs revealed by proteomic analysis, a dominant role was also found for the NE-mediated proteolytic signature in NETs based on a functional activity assay (37). Whereas the major proteolytic activity associated with NETs derived from PMA-stimulated neutrophils was attributed to NE (~70%), all remaining neutrophil serine proteases (CatG, PR3, and NSP4), most notably CatG, contributed to the cleavage sites that were profiled in NET samples (37). These data suggest the proteolytic involvement of all neutrophil serine proteases in NET formation and/or function.

The mechanisms that underlie NET release are not yet fully characterized but are proposed to involve at least two strategies. One pathway can be triggered by specific microbes or PMA, takes 2–4 h to culminate in NET release, and is associated with plasma membrane perforation and neutrophil cell death (26). After activation, neutrophils undergo step-wise morphological changes, including chromatin decondensation, nuclear expansion, and nuclear envelope disintegration, which is followed by the intracellular assembly of nuclear and cytoplasmic components. Finally, DNA coated with nuclear and cytoplasmic proteins is deposited into the environment following plasma cell rupture and cell lysis (26). In the other pathway, neutrophils undergoing NET formation can extrude a fraction or their entire nuclear DNA mainly through nuclear budding and vesicular release. During this process, neutrophils can maintain their integrity and live cell function, such as migration and phagocytosis, at least for a couple of minutes when tested *in vivo* (38). This form of non-lytic NET extrusion, also known as vital NETosis, is triggered by complement-opsonized targets and occurs much more rapidly (<30 min). Since NET release that involves cell lysis is a slow process, potentially allowing microbes to exploit the open time window for infection, the rapid NETosis might prove particularly beneficial against infection (38).

However, rapid NET release by neutrophils was also reported in response to collagen-activated platelets as well as danger signals, such as MSU crystals (13, 33, 39), suggesting that rapid NETosis is not limited to microbes. Platelet and neutrophil dialog through NETs supports blood clotting (13, 40). NETs that are formed within the vasculature capture anticoagulants, such as TFPI, enabling proteolytic inactivation of TFPI on NETs by NE. As a consequence of TFPI degradation, fibrin formation is rapidly enhanced (13). NETs are not only induced by activated platelets but can also serve as a scaffold to platelets aggregation and red blood cells adhesion, thereby accelerating coagulation (40). On the other hand, MSU crystal deposits in joints can

induce gouty arthritis. These MSU crystals required 10 min to trigger NETosis and NET aggregation in human neutrophils (33). Aggregated NET structures were formed when neutrophils at high densities were stimulated with the crystals, mimicking dense neutrophil infiltrates in the synovial fluid of individuals with gouty arthritis. The aggregation of NETs was found to be beneficial in the setting of inflammatory arthritis, since it promoted degradation of chemokines and cytokines, such as IL1 β , that were entrapped in NETs, providing a potential mechanism for resolution of inflammation (33). Thus, rapid NETosis may potentially serve multiple functions.

One of the best characterized models of NET formation is based on the activation of neutrophils purified from human blood by PMA. The model depends on the production of reactive oxygen species (ROS) by the NADPH oxidase complex and involves NE as one of the major contributors to chromatin decondensation (41, 42).

Several lines of evidence support the critical involvement of NE in NET generation. First, inhibitors of NE proteolytic activity, such as small β -lactam-based, cell-permeable NE inhibitors, blocked chromatin decondensation and NET release in neutrophils derived from healthy volunteers (41). Moreover, neutrophils isolated from patients suffering from Papillon-Lefevre syndrome (PLS) failed to release NETs or were severely impaired in NET formation (43, 44). PLS is a disorder caused by loss-of-function mutations in the gene encoding DPPI, resulting in marked defects in the activities of serine proteases, including NE (43). Finally, NETs were not detected in the lungs of mice deficient in NE in a pulmonary model of *Klebsiella pneumoniae* infection (41) or in mice double-deficient in NE and PR3 in an experimental model of atherosclerosis (34). Although mouse neutrophils are much less prone to NET formation than human granulocytes, together, these data indicate that the genetic or functional deficiency of NE severely inhibits NETosis. However, the lack of NE did not prevent NET generation in an experimental model of deep vein thrombosis (24). These data suggest that NE, although linked to NETosis, is not a causative agent in this process, or that neutrophils do not exclusively rely on NE for NET formation. Indeed, chromatin decondensation, a critical step in NETosis, was reported to also be mediated by peptidylarginine deiminase 4 (PAD4). Whereas NE destabilizes chromatin structure *via* the processing of specific histones (41), PAD4 mediates chromatin decondensation through converting histone tail arginine residues to citrullines (45). PAD4 is the main PAD isozyme expressed in neutrophils out of five PADs present in human cells. Moreover, histone citrullination is catalyzed primarily by PAD4, whereas other PADs citrullinate multiple substrates out of the nucleus (46). These data suggest that chromatin decondensation and subsequent NETosis relies mainly on PAD4.

Although the relative contribution of NE and PAD4 to histone modification and the alteration of chromatin structure in NETosis remain to be determined, it is likely that both enzymes act as co-regulators or separate regulators of chromatin decondensation, depending on the type of NET stimulus. For example, PAD4 requires calcium for its enzymatic activity (47), and NETosis triggered by calcium influx is associated with the

presence of citrullinated histones, such as citrullinated histone H3, in the activated neutrophils (48, 49). However, in contrast to the calcium ionophore-stimulated granulocytes, citrullinated histone H3 was hardly observed in neutrophils triggered to form NETs by PMA, suggesting that PAD4 is less required for the PMA-regulated NETosis pathway (24, 42).

As mentioned earlier, NE is confined to azurophilic granules in resting neutrophils. However, upon neutrophil activation, NE can translocate to the nucleus and aid in chromatin decondensation *via* core histone cleavage (41). NE translocation from the primary granules to the nucleus is dependent on ROS generated by NADPH oxidase and MPO, which assists in releasing active NE from the granules into the cytosol (50). According to the recently proposed model of NE translocation, NE in azurophilic granules is associated with several other granule proteins, including MPO, PR3, CatG, azurocidin, eosinophil cationic protein, defensin-1, lysozyme, and lactoferrin. This association is supported by the immunoprecipitation of these proteins with anti-NE antibodies from isolated and detergent-solubilized azurophilic granules. H₂O₂, the secondary product of NADPH oxidase, triggers the dissociation of the NE protein complex from intact azurophilic granules, releasing NE when the enzyme is still assembled with CatG and azurocidin. In the cytoplasm, NE binds to the actin cytoskeleton and possibly degrades F-actin to reach the nucleus (50). Notably, the accumulation of NE in the insoluble cytoskeleton fraction isolated from the activated neutrophils is facilitated in the presence of a small molecule inhibitor of NE. Because blocking of NE activity markedly reduces NE entry into the nucleus (41), together, these findings are consistent with the model in which active NE interacts with the cytoskeleton en route to the nucleus and the inhibition of NE activity arrests NE on the cytoskeleton, preventing NE from translocating to the nucleus (50). Defining F-actin as a potential cytoplasmic substrate for NE also indicates that this protease may regulate neutrophil migration *via* the disassembly of the actin cytoskeleton during NETosis. NE interference with actin dynamics is likely to disable cell movement and confine NETting neutrophils to the NET trigger site. This strategy, embraced by neutrophils undergoing PMA- or *C. albicans*-induced NETosis, differs considerably from the rapid, vital NETosis that coexists with the ability of neutrophils to crawl (38, 50). Although these differences imply that NE might not be involved in rapid NETosis, early NET release that occurred 10 min after neutrophil stimulation with *L. amazonensis* was reduced by a NE inhibitor but was not significantly affected by diphenyleneiodonium (DPI), which mainly inhibits NADPH oxidase-mediated ROS production (30). These data suggest that NE might also be involved in rapid, ROS-independent NETosis.

INHIBITORS OF SERINE PROTEASES IN NET GENERATION

Small molecule, exogenous NE inhibitors suppressed NET formation, suggesting that endogenous inhibitors of serine proteases might regulate NETosis in similar fashion. Notably, neutrophils contain multiple serine protease inhibitors (51, 52), but the roles

of SerpinB1 and secretory leukocyte protease inhibitor (SLPI) are the best known in the context of neutrophil function (52, 53).

SERPINB1

SerpinB1, also known as leukocyte elastase inhibitor (LEI) or monocyte/NE inhibitor (MNEI), is a member of the serpin family of serine protease inhibitors. Serpins are proteins characterized by a unique tertiary structure that employs a suicide-substrate-like mechanism to deactivate their target proteases (54). The inhibitors expose their reactive site loop as a substrate for a cognate protease. The protease cleaves the loop, which leads to extensive conformational changes of the serpin, resulting in protease entrapment in a tight covalent complex (55). Human serpins are divided into nine clades, named from A to I (56). SerpinB1 is a 42-kDa protein and is a member of the clade B serpins. Among the neutrophil serine proteases, SerpinB1 inhibits NE, PR3, and CatG. Notably, SerpinB1 is one of the most efficient inhibitors of NE (57, 58). SerpinB1 is mainly expressed in macrophages and neutrophils and accumulates at high levels in the cytoplasm and granules of neutrophils (59). It lacks a signal peptide and is not secreted to the extracellular environment *via* the classic secretory pathway. However, the detection of SerpinB1 in extracellular localizations, possibly as a result of cell necrosis, was also reported (60, 61).

This multifunctional cytoplasmic protein acts as a protease inhibitor in its native form, but the cleavage of SerpinB1 by its cognate proteases can lead to the loss of antiprotease properties and gaining other functions, such as DNase activity. A SerpinB1 derivative equipped with nuclease activity, called L-DNase II, was isolated from porcine spleen (62). NE-mediated SerpinB1 conversion from an antiprotease to an endonuclease resulted from conformational alteration that exposed the endonuclease active site and a nuclear localization signal. The SerpinB1 derivative L-DNase II was reported to have pro-apoptotic effects (63). The main features of SerpinB1 are summarized in **Table 1**.

By contrast, data have been accumulating that SerpinB1 plays a pro-survival role in neutrophils. This role is exemplified by the recently reported regulation of the spontaneous death of aging neutrophils by SerpinB1, *via* counterbalancing the activity of PR3 during leakage of the protease from azurophilic granules (25). Whereas cytosolic SerpinB1 was found to form a complex with PR3 in neutrophils undergoing spontaneous death but not in freshly isolated neutrophils, the rate of spontaneous death was increased in neutrophils isolated from SerpinB1-deficient mice

TABLE 1 | The main characteristics of SerpinB1 and SLPI.

	Main expression	Main localization	Main functions	Proteolytic targets in neutrophils
SerpinB1	Macrophages, neutrophils	Intracellular	Inhibitor of serine proteases, DNase	NE, CatG, PR3
SLPI	Epithelial cells, neutrophils	Secreted	Inhibitor of serine proteases, Antimicrobial protein, Inhibitor of NFκB	NE, CatG

(25). These findings are consistent with the protective role of SerpinB1 against apoptosis.

An intrinsic defect in survival observed in neutrophils derived from SerpinB1 knock-out mice may also be caused by a higher propensity of these cells to die by NETosis. In a model of *Pseudomonas aeruginosa* lung infection, neutrophils infiltrating the lungs of SerpinB1-deficient mice exhibited excessive death. The cell death was accompanied by the presence of free NE, MPO, and DNA in the bronchoalveolar lavage fluid (BALF) (52). In *ex vivo* experiments, neutrophils devoid of SerpinB1 that were isolated from the BALF of the infected mice also generated more NETs than those from control mice. These findings demonstrated that NETosis was increased in SerpinB1-deficient mice in the setting of infection. Higher susceptibility of SerpinB1-deficient neutrophils to form NETs was also found when neutrophils isolated from the bone marrow of uninfected mice were subjected to treatment with native proinflammatory mediators, such as the chemokine CXCL2, or PMA, suggesting that SerpinB1 controls NETosis (52). The addition of recombinant SerpinB1, but not related serpins, to these *in vitro* activated neutrophils abrogated NET production (52). These findings indicated that SerpinB1 is a negative regulator of NETosis.

In response to PMA, SerpinB1-deficient mouse neutrophils demonstrated a higher tendency to expand their nuclei, indicative of chromatin decondensation. Moreover, in PMA-treated human neutrophils, SerpinB1, similar to NE, migrated to the nucleus and co-stained with NE and DNA on NETs formed by the stimulated cells. These results raised the possibility that SerpinB1 blocks NET formation *via* interfering with NE-mediated chromatin decondensation. However, SerpinB1 localization to the nucleus did not seem to involve forming a complex with NE. When NE was confined to the cytoplasmic region by neutrophil pretreatment with a chemical protease inhibitor, SerpinB1 could still translocate to the nucleus (52). Moreover, the enhanced NET formation observed in SerpinB1-deficient mice was not reversed by NE deletion (24). Therefore, NE might not be an exclusive SerpinB1 target in restricting NETosis. Given the multiplicity of its cognate proteases, it is also possible that, in the absence of NE, SerpinB1 might select other proteolytic targets to limit NET generation. As an alternative mechanism, nuclear SerpinB1 was proposed to interfere with PAD4 by blocking PAD4 access to histone tails (52). This role for SerpinB1 in regulating chromatin decondensation is supported by the ability of the inhibitor to tightly associate with condensed chromatin (64).

Secretory Leukocyte Protease Inhibitor

Secretory leukocyte protease inhibitor, a single polypeptide cationic protein of 107 amino acids, is also known as antileukoprotease (ALP), bronchial secretory inhibitor (BI), human seminal inhibitor I (HUSI-I), cervix uterine secretion inhibitor (CUSI), mucous proteinase inhibitor (MPI), or secretory leukoprotease inhibitor (SLI) (65). The most well-documented role of SLPI is the inhibition of serine proteases, including human NE and CatG but not PR3 (66), **Table 1**. Beyond a role in neutralizing proteases, SLPI is also microbicidal and suppresses the activity

of the transcription factor NF κ B (67). SLPI is a canonical type of serine protease inhibitor, binding to proteases through the exposed binding loop, which in conformation is complementary to the enzyme's active site (66). The inhibitor is composed of two four-disulfide core domains, also called whey acidic protein (WAP) domains. The N-terminal WAP I and C-terminal WAP II domains share 35% homology (68), but their biological function is distinct. The WAP II domain is primarily responsible for the antiprotease activity of the SLPI. The biological function of the N-terminal WAP I domain is less well understood, although the antimicrobial potential of SLPI is thought to mainly reside in this domain (67).

In contrast to SerpinB1, SLPI is predominantly secreted and found primarily at mucosal surfaces as a product of epithelial cells. The inhibitor is also expressed in leukocytes, including neutrophils, macrophages, and dendritic cells (67). Despite the presence of a signal peptide, indicative of cell secretion, SLPI has intracellular targets, suggesting that it might be retained in cells. However, the inhibitor can penetrate cellular membranes and potentially be acquired from adjacent cells. Such loading with SLPI, mimicked in experimental systems by cell treatment with the exogenous inhibitor, appears to be functionally relevant. For example, SLPI produced by epithelial cells lining tonsillar crypts restrains the production of antibodies in adjacent B cells (69).

Although SLPI was shown to inhibit a wide spectrum of proteases, one of its main actions is likely to be the inhibition of NE because the complex of SLPI with NE is the strongest among complexes of SLPI with any other proteases (66, 70). Notably, SLPI is thought to be the major inhibitor of NE present in the neutrophil cytosol (51). According to another report, SLPI is primarily stored in secondary granules in neutrophils (71). As SLPI, similar to NE, is likely to migrate between different cell compartments in response to neutrophil stimulation, the presence of SLPI in the cytosol or in specific granules might reflect different activation statuses of neutrophils. The main features of SLPI are summarized in **Table 1**.

Secretory leukocyte protease inhibitor plays a regulatory role in granulopoiesis (72) and, similar to SerpinB1, inhibits apoptosis in circulating neutrophils (73). The mechanism underlying its antiapoptotic activity remains to be determined. However, the protective role of SLPI in apoptosis might be reminiscent of SerpinB1 counteracting PR3, although its activity must be directed against other proteases because SLPI does not inhibit PR3.

Secretory leukocyte protease inhibitor may also serve to protect cells from the harmful effects of NETosis. This conclusion stems from the observation that stimulation of human neutrophils with PMA, TNF α , or *S. aureus* in the presence of exogenous SLPI, but not another native NE inhibitor, α 1-proteinase inhibitor, substantially decreased NET release. Exogenous SLPI mainly localized to the cytoplasm of resting neutrophils, but upon stimulation it relocated to the nucleus and inhibited histone cleavage. Endogenous SLPI was also found to co-localize with NE in the nuclei of *in vitro* activated human neutrophils, or in neutrophils infiltrating the skin of patients with the autoinflammatory skin disease psoriasis (53). Together, these data suggested that the protective

effect of SLPI in NET formation might result from constraining NE-mediated histone processing. Notably, although histone cleavage was efficiently blocked by added SLPI independently of the NET-triggering stimulus, in response to PMA, neutrophils appeared to degrade more histones than neutrophils stimulated with *S. aureus* (53). This observation further points to divergent mechanisms underlying NETosis, with *S. aureus* possibly relying more on other pathways. Moreover, the inhibition of NE activity was unlikely to be solely responsible for the SLPI tailoring effect on NETosis because SLPI mutants devoid of inhibitory activity against NE were still capable of restraining NET formation in stimulated neutrophils, albeit to a lower degree than the fully active SLPI (53). Given the inhibitory effects of SLPI against multiple proteolytic enzymes, one possible mechanism whereby SLPI may interfere with NETosis is to counteract other proteases. In another scenario, the independent antiprotease activity of SLPI might be involved in blocking NET release. In agreement with the anti-NET effect of SLPI, the inhibitor deficiency led to excessive NETosis in *in vitro* activated bone marrow neutrophils. The negative regulation of NET formation by SLPI was further supported by an *in vivo* psoriasis model. This model demonstrated that SLPI^{-/-} neutrophils infiltrating psoriatic skin formed twice as many NETs as WT neutrophils (53).

Because neutrophil treatment with exogenous SLPI resulted in a partial decrease in NET generation, other inhibitory parallel pathways must exist. The overlapping but distinct activities of SLPI and SerpinB1 against neutrophil proteases (Table 1), as well as the structural and functional heterogeneity between the inhibitors, suggest that SLPI and SerpinB1 may act in synergy to control NETosis.

INHIBITORS OF SERINE PROTEASES IN NET FUNCTION

Uncontrolled NET production in chronic inflammatory states has serious consequences. For example, NET formation may contribute to the pathogenesis of autoimmune diseases, as NETs are cytolytic and are a potential source of autoantigens (32, 74, 75). Multiple NET components, including DNA, histones, MPO, PR3, LL37, CatG, and NE, are recognized by autoantibodies (76–78). The clinical measures of disease severity are often positively correlated with the titers of antibodies directed against NET components (79). As discussed earlier, SerpinB1 and SLPI may be a part of the defense system to cope with challenges imposed on the host by NET deposition. However, as regulators of NETosis, they may be externalized together with other NET components and impact immunity after extrusion from neutrophils. Although SLPI, as a secretory protein, is unlikely to be intrinsically immunogenic, intracellular SerpinB1, normally hidden in the cell, might be revealed as a consequence of NETosis and potentially provoke or enhance immune responses. Immunohistochemistry data indicated that *in vitro* activated human neutrophils release NETs with SerpinB1 and SLPI attached to DNA (52, 80). Likewise, SLPI was also found to decorate NETs in the affected skin of patients suffering from psoriasis (80, 81), suggesting that the presence of SLPI on NETs might be functionally relevant.

Psoriasis is a skin condition affecting on average 2–3% of the population all over the world (82). Most often, it manifests as erythematous cutaneous lesions covered with silvery scales. The disease is incurable and although it is not life threatening on its own, patients suffering from psoriasis exhibit a higher risk of developing comorbidities, such as other autoimmune disorders, metabolic syndrome, and cardiovascular disease (83, 84). On a cellular level, psoriasis is characterized by dysfunctional keratinocyte proliferation and differentiation, as well as inflammation elicited by abundant immune cells that are rare or absent in healthy skin (85). Among them are plasmacytoid dendritic cells (pDCs) and neutrophils. Although emerging data indicate that chronic inflammation in this disease is mediated primarily by Th17 cells and their signature product, IL17 (86), the pathological events underlying the initiation of the disease are much less understood (87). pDCs accumulate early in psoriatic skin lesions or pre-lesional skin adjacent to affected skin (85, 88), suggesting that pDCs are well-placed to contribute to early skin alterations. These cells are considered key producers of interferon type I (IFNI) (89). pDCs and IFNI were implicated in the pathogenesis of psoriasis and other autoimmune diseases, such as SLE (90, 91). The diseases exhibit broad activation of IFNI pathways (1). Direct evidence for a pathogenic role for pDC-derived IFNI in psoriasis is provided by a human/mouse skin xenograft model. In this model, the spontaneous conversion of the transplanted human uninvolved (normal appearing) skin of psoriasis donors into psoriatic skin lesions is prevented by blocking IFNI signaling or inhibiting pDC production of IFNI (92). As one of the key cell types involved in antiviral immunity, pDCs are well equipped with intracellular sensors of nucleic acids, such as TLR7 and TLR9, which recognize single-stranded RNA and DNA, respectively (89). However, pDCs can be activated in a TLR-dependent manner, not only by foreign but also by self RNA and DNA. In each case, pDC stimulation can lead to the production of IFNI and IFNI-driven immunity (93, 94). To be functional ligands for TLRs, host nucleic acids need to be of mitochondrial origin and/or form complexes with specific proteins, such as antimicrobial LL37 (93, 94) or NE together with SLPI (80). Notably, activated neutrophils are a likely source of these IFNI-triggering factors because they extrude both oxidized mitochondrial nucleoids (95, 96) and nuclear DNA decorated with the proteins (3). Mitochondria, along with the phagosome-localized NADPH oxidase complex, are major sites of ROS production, and mitochondrial DNA is highly susceptible to oxidation (97). This modification is required for the potent interferogenic potential of mitochondrial DNA (95). Although mitochondrial DNA can be extruded from cells without subsequent cell death and release of nuclear DNA (98), it is likely that neutrophils triggered by a suitable stimulus co-release oxidized mitochondrial and chromosomal DNA. This possibility is supported by recent findings that demonstrate that NETs are enriched in oxidized mitochondrial DNA when stimulated with ribonucleoprotein immune complexes (96). Therefore, unique structural or functional assets of host DNA, such as levels of oxidized mitochondrial DNA in the NET structure, and/or other NET DNA modifications, possibly resulting from the specific assembly of DNA with neutrophil proteins, may allow pDCs to recognize self DNA.

In psoriatic skin, induction of IFN γ synthesis by neutrophil-derived self DNA in skin-infiltrating pDCs may depend on SLPI. Some insights into this came from the observation that NET-like structures, consisting of DNA, NE, or CatG, and SLPI were present in the affected skin of patients with psoriasis (80, 81). Moreover, pDCs were found to locate in close proximity to neutrophils and NETs (80), indicating that pDCs and SLPI-decorated NETs might be linked in controlling skin pathophysiology. Although not effective on its own, SLPI in complex with neutrophil DNA and its cognate enzymes, NE or CatG, induced a marked increase in the production of IFN γ by pDCs (80, 81). This response was mediated by TLR9, suggesting that recognition of self DNA by intracellular TLR9 and/or activation of the receptor is facilitated by SLPI. Other inhibitors exposed on NETs can potentially also be involved in the stimulation of IFN γ production in pDCs. Although the role of SerpinB1 in this process remains unknown, SLPI appears to have the selective capacity to induce the expression of IFN γ in pDCs. In contrast to SLPI, neither the main plasma inhibitor of NE, α 1-proteinase inhibitor (99, 100), were effective at stimulating IFN γ production by pDCs (80, 81). Therefore, regulation of the catalytic activity of serine proteases may not be a uniting or adequate property for an inhibitor to enable pDCs to produce IFN γ . This possibility was further supported by the finding that SLPI lacking potent anti-NE activity was still equipped with pDC-stimulating functions. By contrast, the proimmunogenic ability of SLPI together with DNA and NE required enzymatically active NE because NE inhibited by a small synthetic inhibitor, azurocidin, did not stimulate pDCs to produce IFN γ (80). The proimmunogenic properties of SLPI may primarily be related to its cationic nature because cationic peptides, such as LL37, display strong capacity to activate pDCs (101). However, cationicity alone does not appear to be sufficient for the stimulation of IFN γ production in pDCs because the complex of SLPI and neutrophil DNA was unable to potently trigger IFN γ production by pDCs (80).

A role for SLPI in psoriasis was initially suggested by the observation that SLPI is markedly upregulated in the epidermis of psoriasis patients, particularly in keratinocytes (102). As described previously, neutrophils are a potential but not necessarily unique source of SLPI on NETs in psoriatic skin. Given the ability of SLPI to bind to DNA (103), it can be envisaged that SLPI produced by keratinocytes might dock to NETs deposited in psoriatic skin *via* skin-infiltrating neutrophils (Figure 1). In principle, NETs enriched in SLPI might be particularly suitable to prime pDC responses. NETs different in composition or protein levels were described earlier. Although the protein constitution in NETs might be influenced directly by the triggering stimulus (32), it is also likely that the stimulatory power of NETs may depend on the tissue context, by recruiting additional tissue-specific components to the externalized nuclear/mitochondrial nucleic acids.

The functional significance of SLPI on NETs in psoriasis remains to be determined. However, SLPI might be involved in several levels of NET regulation in inflamed skin, each potentially leading to different outcomes. As a NET component,

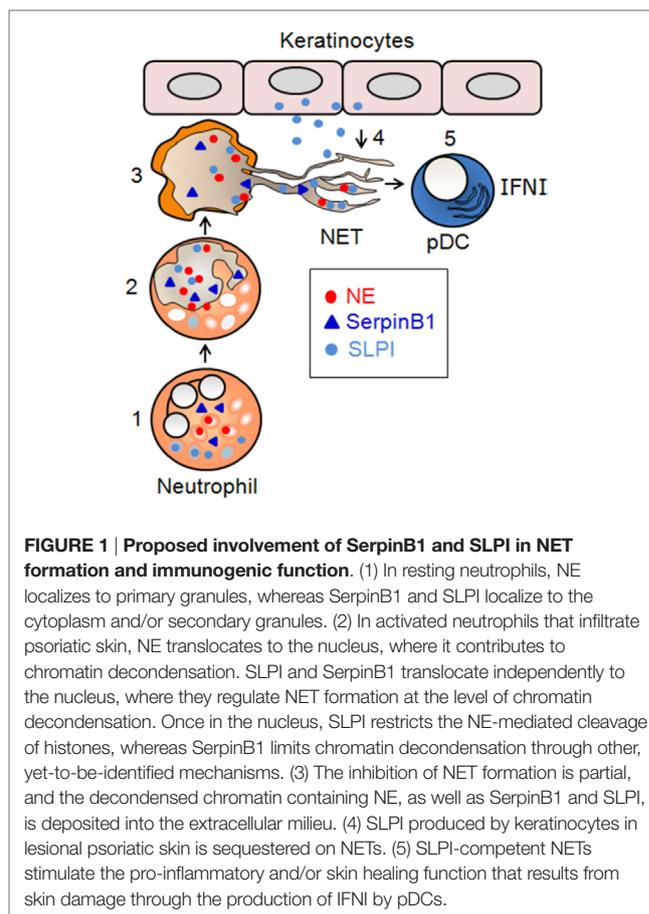


FIGURE 1 | Proposed involvement of SerpinB1 and SLPI in NET formation and immunogenic function. (1) In resting neutrophils, NE localizes to primary granules, whereas SerpinB1 and SLPI localize to the cytoplasm and/or secondary granules. (2) In activated neutrophils that infiltrate psoriatic skin, NE translocates to the nucleus, where it contributes to chromatin decondensation. SLPI and SerpinB1 translocate independently to the nucleus, where they regulate NET formation at the level of chromatin decondensation. Once in the nucleus, SLPI restricts the NE-mediated cleavage of histones, whereas SerpinB1 limits chromatin decondensation through other, yet-to-be-identified mechanisms. (3) The inhibition of NET formation is partial, and the decondensed chromatin containing NE, as well as SerpinB1 and SLPI, is deposited into the extracellular milieu. (4) SLPI produced by keratinocytes in lesional psoriatic skin is sequestered on NETs. (5) SLPI-competent NETs stimulate the pro-inflammatory and/or skin healing function that results from skin damage through the production of IFN γ by pDCs.

SLPI is likely to have an impact on the production of IFN γ by pDCs, facilitating IFN γ -mediated immune and skin healing responses (67). The flipside is the generation of a potentially harmful stimulus (SLPI-decorated NETs) that can increase the risk of autoimmune inflammation. On the other hand, the ability of SLPI to inhibit NETosis in neutrophils makes it ideally suited for serving as a safeguard against the harmful effects of NETs. Either way, SLPI emerges as an important participant in innate immunity *via* the regulation of NET generation and immunogenic function (Figure 1).

AUTHOR CONTRIBUTIONS

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

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The Protective Effect against Extracellular Histones Afforded by Long-Pentraxin PTX3 as a Regulator of NETs

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Pentraxin 3 (PTX3) is a soluble pattern recognition molecule that plays critical roles in innate immunity. Its fundamental functions include recognition of microbes, activation of complement cascades, and opsonization. The findings that PTX3 is one of the component proteins in neutrophil extracellular traps (NETs) and binds with other NET proteins imply the importance of PTX3 in the NET-mediated trapping and killing of bacteria. As NETs play certain critically important host-protective roles, aberrant NET production results in tissue damage. Extracellular histones, the main source of which is considered to be NETs, are mediators of septic death due to their cytotoxicity toward endothelial cells. PTX3 protects against extracellular histones-mediated cytotoxicity through coaggregation. In addition to the anti-bacterial roles performed in coordination with other NET proteins, PTX3 appears to mitigate the detrimental effect of over-activated NETs. A better understanding of the role of the PTX3 complexes in NETs would be expected to lead to new strategies for maintaining a healthy balance between the helpful bactericidal and undesirable detrimental activities of NETs.

Keywords: pentraxins, extracellular histones, cytotoxicity, coaggregation, sepsis

INTRODUCTION

The innate immune system serves as the first line of defense against pathogen invasion and consists of cellular and humoral arm. The innate immune response is triggered by pattern recognition molecules (PRMs) upon the recognition of pathogen-associated molecular patterns (PAMPs), which are structural patterns conserved across a broad spectrum of microbes (1, 2). In addition to PAMPs, PRMs recognize damage-associated molecular patterns (DAMPs) that are secreted from damaged cells as a “warning signal from the host” (2). Like the innate immune system, PRMs are classified into cellular and humoral components. The cellular arm of PRMs includes toll-like receptors (TLRs), C-type lectin receptors (CLRs), scavenger receptors, retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and NOD-like receptors (NLRs) with diverse patterns of localization, ligand recognition, and signal transduction. The humoral arm of PRMs includes complements, collectins, ficolins, and pentraxins that share a number of fundamental mechanisms against infection, such as complement activation, agglutination, neutralization, and opsonization (3, 4).

Pentraxin 3 (PTX3) was the first long pentraxin to be identified (5). The PTX3 gene is highly conserved across species (6). It is a circular multimeric glycoprotein that recognizes certain microbes and eliminates them through complement activation and opsonization. In addition to its activity in the innate immune system, PTX3 exerts effects in inflammation and matrix regulation (6). PTX3 expression includes hematopoietic and stromal cells by means of pro-inflammatory signals, while the characteristic expression mode of PTX3 production is that neutrophils store PTX3 in granules and release them in a “ready-to-release” manner (7). It is, thus, not surprising that PTX3 is one of the components of neutrophil extracellular traps (NETs) in which PTX3 serves as an anti-fungal activity. In addition to their host-protective activity, NETs also exert detrimental effects against the host. Extracellular histones, the major NET components and one of the DAMPs, afford a good example of the double-edged sword effect of NET components. They have bactericidal activity, but have also been shown to exert a cytotoxic effect on endothelial cells in sepsis. PTX3 has an ability to attenuate extracellular histone-mediated cytotoxicity through coaggregation (8). This implies that the role of PTX3 in NETs is not only to combat bacteria but also to mitigate the detrimental effects of NETs. Here, we describe our recent findings on the role of PTX3 as a regulator of NETs in relation to histone cytotoxicity.

PENTRAXIN 3: GENERAL DESCRIPTION AND ROLE IN NETs

Pentraxins

The pentraxins comprise an evolutionarily conserved multimeric protein family in which its members share the pentraxin domain [~200 amino acids (a.a.) long] in its C-terminal domain with a characteristic pentraxin signature (His-x-Cys-x-Ser/Thr-Trp-x-Ser, where x is any amino acid residue) (6, 9). The pentraxins are classified into two subfamilies based upon the N-terminal length, i.e., the short pentraxin and long pentraxin. C-reactive protein (CRP) and serum amyloid P component (SAP) are the prototypical short pentraxins, which are broadly known as acute phase proteins (10, 11). Neuronal pentraxin 1 (NPTX1) (12), neuronal pentraxin 2 (NPTX2) (13), neuronal pentraxin receptor (NPTXR) (14), and PTX4 (15) are the long pentraxins that have been reported in addition to PTX3.

General Background of PTX3 (Genome, Expression, Structure, and Function) Genome and Expression Pattern

The human PTX3 gene locates on chromosome 3q band 25, and consists of three exons and two introns. It consists of 1861 base pairs and is translated into 381 amino acids. The first and second exons encode the signal peptide (1–17 a.a.) and N-terminal domain (18–178 a.a.), and the last and third exon encodes the C-terminal pentraxin domain (179–381 a.a.), respectively. The promoter region of PTX3 contains PU-1, AP1, NF- κ B, SP1, and NF-IL6, and the expression of PTX3 is triggered by certain primary inflammatory signals, such as TLR agonists, IL-1 β , and TNF α . PTX3 is expressed several types of cells, including myeloid

dendritic cells, peripheral blood leukocytes, macrophages, mononuclear phagocytes, vascular endothelial cells, smooth muscle cells, fibroblasts, adipocytes, glial cells, cumulus oophorus cells, mesangial cells, synovial cells, epithelial cells, and uroepithelial cells. Please refer to the cited reviews for the gene structure (3, 9) and expression pattern of PTX3 (3, 6) in more detail. In addition to the cells described above, lymphatic endothelial cells (16) and polymorphonuclear neutrophils (7) have distinct expression patterns. The former cells constitutively express PTX3, while the latter cells store the PTX3 protein in a “ready-to-release” manner, the details of which will be discussed below.

Protein Structure and Ligand Binding

After the processing of its signal sequence, PTX3 protein has an N-linked glycosylation of its Asn220 site (17, 18). The glycosidic moiety is important for the fine-tuning of the interaction with C1q and complement activation (17), the stabilization of Factor H binding (19), the interaction with M-ficolin (20), the interaction with P-selectin for attenuating neutrophil recruitment at sites of inflammation (21), and the blocking of the binding site of the influenza virus hemagglutinin (22).

It is considered that, like the case of the short pentraxins, the PTX3 C-terminal pentraxin domain forms two antiparallel β sheets with a “jellyroll” topology (18, 23). As opposed to the PTX3 C-terminal domain, which is homologous among the pentraxins, the PTX3 N-terminal domain is unrelated to other known proteins. Presta et al. predicted four α -helix regions connected by short loops in the N-terminal domain using the PredictProtein server (24). They pointed out a heptad repeat motif (*abcdefg*) spanning a.a. residues 85–91, where *a* and *d* are non-polar residues and *e* and *g* are charged residues, together with hydrophobic residues repeated with a period of one every three to six a.a. in their helical regions (23) (**Figure 1A**). These motifs confer on PTX3 N-terminal domain a propensity for forming a coiled-coil structure. The signal of the α -helix was detected by circular dichroic (CD) spectroscopy (8). Inforzato et al. attributed the multimer formation of PTX3 to the inter- and intra-molecular disulfide bonds in the organization of the matrix (25) and proposed an asymmetric octamer structure for PTX3 based on biophysical analysis (26). PTX3 forms a circular-tetramer in the N-terminal domain, while the C-terminal domain forms octameric structure by stacking the two-tetramers. The N-terminal domain of one tetramer forms three intra-molecular helical coiled-coils, while the other, extended form of the tetramer consists of the inter-molecular interaction of the α -helices (**Figure 1B**). These two types of tetramer consist of an octamer structure, thus rendering an asymmetry on PTX3. This multimerization is considered to be important for the interaction of a variety of PTX3 ligands (27) and the recognition of pathogens (28). The anti-PTX3 monoclonal antibody MNB4 that recognizes 87–99 a.a. of PTX3 inhibits PTX3-inter-alpha-trypsin inhibitor (α 1) interaction (29) and/or PTX3-FGF2 interaction. The minimal PTX3 N-terminal peptide required for interaction with FGF2 is 97–110 a.a. (24) (**Figure 1B**). Tetramer formation is required for FGF2 binding, and both types of tetramer can bind to FGF2 (26). We elucidated the tertiary structure of PTX3 by secondary structure prediction re-calculated with PSIPRED (30) and SPIDER2

PTX3 were found to be recapitulated by N-terminal domain PTX3 (41, 42), and the interaction of fibrinogen/fibrin and plasminogen with PTX3 was limited under an acidic condition (42). In contrast to the inhibitory activity of SAP in fibrosis, PTX3 promotes fibrocyte differentiation through FcγRI in fibrotic lesions (43). A detailed at the molecular level of these observations will result in a better understanding of matrix formation and the process of tissue remodeling supported by PTX3.

Expression and Role of PTX3 in Neutrophils

PTX3 expression is observed in neutrophil precursors but not matured neutrophils, while the PTX3 protein can be detected in both (7). No PTX3 expression is observed in eosinophils and basophils. PTX3 is stored mainly in specific granules, and partially in azurophilic and gelatinase granules (7, 44). In response to stimuli, such as microorganisms and TLR agonists, PTX3 is released into the extracellular space and localized in NETs. NETs comprise a mesh-like structure that consists mainly of DNA and histones (45). Some of the proteins derived from neutrophils are localized in NETs and are active in the trapping and killing of bacteria (46, 47). The PTX3 in NETs co-localizes with microorganisms and other NET component proteins (7, 44, 48). As it is expected that the complex formation of PTX3 with other proteins in NETs exerts synergistic antimicrobial effects, further investigation will be needed to fully understand the activities of the NET component proteins.

CYTOTOXIC EFFECT OF EXTRACELLULAR HISTONES ON THE ENDOTHELIUM IN SEPSIS

The Extracellular Histones: A Double-Edged Sword in NETs

Histones are highly basic proteins that bind to DNA in the nucleus. Nuclear DNA becomes tightly condensed with the help of histones and ultimately forms chromosomes. Histones consist of five classes; H2A, H2B, H3, and H4 are the core histones, while H1 and H5 are linker histones (49). As well as the crucial functions of histones in the intracellular space, the extracellular histones also play certain roles, especially of the toxic sort. The first extracellular role of histones to be reported was that they are toxic to microbes (50). The toxic activity of purified calf thymus histones against various types of microorganisms was reported, including *Escherichia coli* K-12, *Klebsiella pneumoniae* and strains of *Shigella*, *Salmonella typhimurium*, and *Pseudomonas*. The microbicidal effect of each member of the histones was reported by subsequently. Histone H4 was identified from human sebocyte extract as an antimicrobial protein candidate and was shown to exert antimicrobial activity against *Staphylococcus aureus* and *Propionibacterium acnes* (51). The histones H2A and H2B exert a lethal effect on *Leishmania amazonensis*, but histone H1 does not exert any leishmanicidal effect (52). However, in contrast to the report by Wang et al., histone H1 has been identified as a potential antimicrobial agent (53). Although as yet not fully described, these reports imply that histone members have different types of toxic activities against a variety of microorganisms.

In contrast to the host-defense role of the extracellular histones, only detrimental effects have been reported to date. Extracellular histones are also toxic to host cells (54). The cytotoxicity of extracellular histones toward a variety of cell types and organs has been reported (55). Similar to the toxic effect against microbes, different histone members-dependent toxicity to each cell type has been reported. In the case of histone-mediated cytotoxicity to endothelial cells, histone H3 and H4 are the major components involved (56). As opposed to endothelial cells, histone H1 exerts cytotoxic effect against leukemia cells by causing severe plasma membrane damage while it does not affect normal peripheral blood mononuclear cells and bone marrow cells (57), suggesting that histone H1 exerts its cytotoxicity through leukemia cell-specific membrane components. In line with the report by Class et al., only histone H1, not core histones, is toxic to cortical neurons (58). In accordance with the reports above, the contribution of extracellular histones to certain disease models [reviewed by Allam and colleagues (55)] is mostly related to tissue injury.

In addition to the toxic effect of extracellular histones, signaling pathway activation and platelet aggregation have been reported. In sterile inflammation and cellular injury models, extracellular histones are released, and these are protected by an anti-histone antibody or as observed in TLR2- and TLR4-knockout mice (59), suggesting that TLR2 and TLR4 act as extracellular histone receptors. Similarly, the extracellular histone-TLR9-MyD88 pathway has been also reported in a hepatic ischemia/reperfusion injury model (60), as shown by the inhibitory effect of an anti-histone antibody and as observed in TLR9- and MyD88-knockout mice. Additionally, histone-mediated NLRP3 inflammasome activation has been confirmed both *in vitro* and *in vivo* (61). Extracellular histones bind to platelets and induce platelet aggregation. Histones activate platelets, and the TLR2 and TLR4 pathways are involved (62). Histone induces platelet aggregation, some part of which is mediated through fibrinogen and αIIb3-integrin. Interestingly, similar to the cytotoxic effect, histone H3 and H4 display higher levels of platelet activation and aggregation. Consistent with the reports above, an *in vivo* analysis revealed that histone infusion resulted in thrombocytopenia (63).

Considering the fact that histones are the most abundant NET components (46), the histones in NETs would be expected to exert a lethal effect on the microbes captured. In sepsis, NETs prevent the dissemination of microbes in order to be able to capture them (64). However, growing evidence suggests that NETs also inflict tissue damage. Indeed, NETs contribute to the pathogenesis of a number of diseases (65, 66), including sepsis (65, 67). Histone blockade has been shown to be effective in protecting against histone-delivered/histone-mediated cytotoxic effect (68) and in an acute lung injury model in which NETs contribute (69).

Relevance of the Extracellular Histones to Sepsis

Sepsis is a life-threatening organ dysfunction caused by a dysregulated host response to infection (70–72). The innate immune response participates in the pathogenesis of sepsis. In the

initiation of sepsis, PRMs recognize the PAMPs that are derived from invading microorganisms. Upon PAMP recognition, PRM signal transduction triggers the secretion of pro-inflammatory mediators from innate immune cells (73–75). On the progression of sepsis, the innate immune system becomes over-reactive. This leads to hypercytokinemia and the recruitment of neutrophils into infectious regions, eventually resulting in multi-organ failure (75). In addition to PAMPs, DAMPs contribute to the pathogenesis of sepsis (73). DAMPs also trigger PRM-mediated signaling in systemic inflammatory response syndrome (SIRS), including trauma, burns, ischemia, and hemorrhage (73, 74, 76). As one of the DAMPs, the extracellular histone-mediated cytotoxicity toward endothelial cells has emerged as one of the features of the pathogenesis of sepsis (55, 56). Extracellular histones are present in the plasma of patients with sepsis (77). Histone administration *in vivo* results in neutrophil margination, vacuolated endothelium, intra-alveolar hemorrhage, and macro- and micro-vascular thrombosis, all of which are similar to the events that take place in the pathogenesis of sepsis (56).

PROTECTIVE ROLE OF PTX3 AGAINST EXTRACELLULAR HISTONES: IMPLICATIONS FOR THE MAINTENANCE OF A GOOD BALANCE OF NETS

Extracellular histones are considered as a major factor in the severity of sepsis that results in organ failure and, thus, are targets in the treatment of sepsis. Certain inhibitors of the extracellular histone-mediated detrimental effects have been reported, such as activated protein C (APC), heparin, albumin, CRP, recombinant thrombomodulin (rTM), and I α I. The inhibitory effect against extracellular histones differs for each factor (Table 1). Extracellular histones were identified in a proteomic analysis of circulating PTX3 complexes in patients with sepsis (48). Considering the report that PTX3-transgenic mice are resistant to death from sepsis (78), the complex formation of PTX3 and histones is considered to have a host-protective in sepsis by attenuating extracellular histone-mediated detrimental effects. In the effort to understand the molecular mechanisms of PTX3–histone complex formation, both direct and high-affinity binding between PTX3 and histones has been reported. Of note, it was found that the binding induced coaggregation of PTX3

with histones due to a disorder of the PTX3 secondary structure (Figures 2A,B) (8). A cell-based assay revealed that PTX3 blocks histone-mediated cytotoxic activity toward endothelial cells. This blockade induced by PTX3 has been confirmed in all of the histone members. An *in vivo* analysis performed to investigate the function of PTX3–histone complex formation showed that PTX3 protects against histone-mediated endothelial cell cytotoxicity. The N-terminal domain of PTX3 was shown to be sufficient for both aggregate formation with histones and protection against histone cytotoxicity. This suggests the possibility of using the N-terminal PTX3 domain protein in sepsis treatment; indeed, *in vivo* administration resulted in resistance to septic lethality. Interestingly, however, the *in vivo* administration of PTX3 attenuated extracellular histones-mediated cytotoxicity, but did not suppress histone-mediated thrombocytopenia (Figures 2C,D). This result suggests that PTX3 has a distinct protective mechanism against histone-mediated detrimental effects because, among the factors reported, PTX3 is the only molecule that does not also protect against thrombocytopenia (Table 1). As it is considered that NETs are the source of extracellular histones, the protective activity of PTX3 against histone-mediated endothelial cell cytotoxicity implies that PTX3 participates in the regulation of NETs by attenuating the detrimental effects of NETs exerted by extracellular histones (Figure 3).

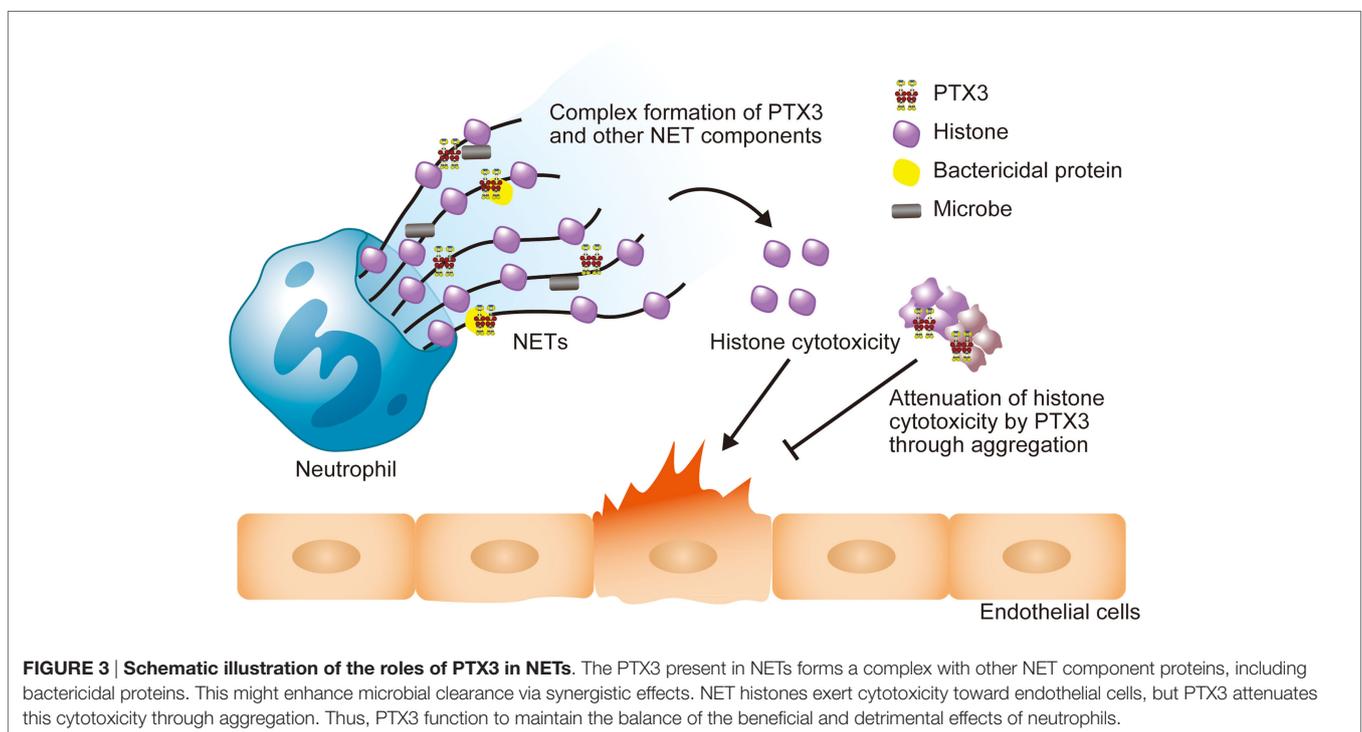
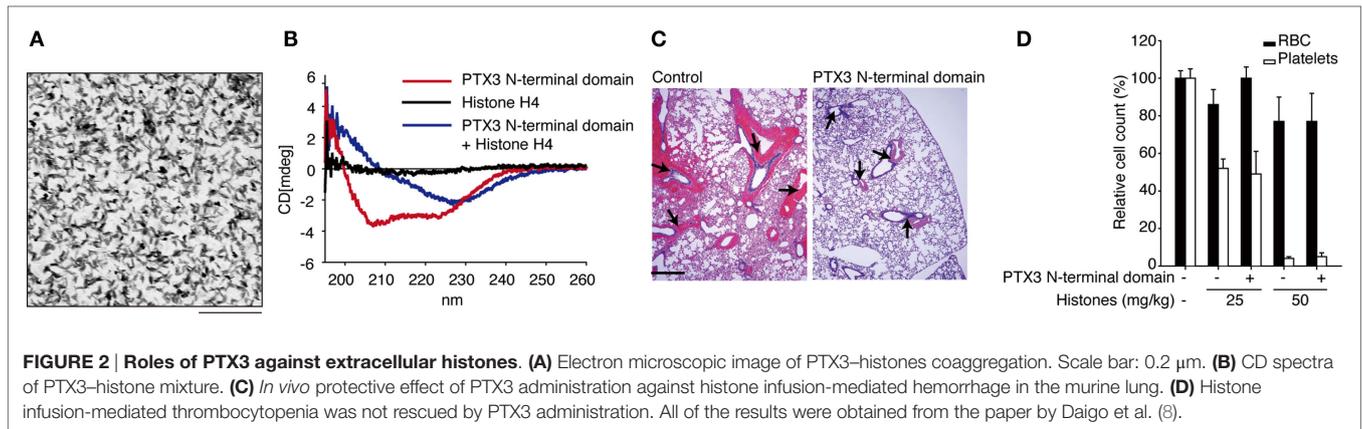
PERSPECTIVES AND CONCLUDING REMARKS

Pentraxin 3 in NETs plays a variety of antimicrobial roles through pathogen recognition, complement regulation, and complex formation with other NET component proteins, including histones. Growing evidence supports the notion that PTX3 has a role in the regulation of extracellular histones, which are considered to be both diagnostic and therapeutic targets in certain severe diseases, including sepsis, due to their cytotoxicity and DAMP activity. PTX3 exerts a host-protective role against the histone-mediated detrimental effects that occur in sepsis. It is also expected that an elucidation of the detail of PTX3–histone aggregate formation will lead to new strategies for sepsis treatment. It is noteworthy that the matrix formation, tissue remodeling and aggregate formation induced by PTX3 are mainly associated with the N-terminal domain of PTX3. The N-terminal domain

TABLE 1 | Inhibitors of extracellular histones.

Inhibitors	Inhibitory effects on the pathogenic effects of extracellular histones					Reference
	Endothelium cytotoxicity	Platelet aggregation	Thrombocytopenia	Lung injury	Acute death	
APC	Inhibit	–	–	Inhibit	Inhibit	(56)
Heparin	Inhibit	Inhibit	Inhibit	Inhibit	Inhibit	(63, 79)
Albumin	Inhibit	Inhibit	–	–	–	(80, 81)
CRP	Inhibit	Inhibit	Inhibit	Inhibit	Inhibit	(82)
rTM	–	Inhibit	Inhibit	Inhibit	Inhibit	(83)
I α I	Inhibit	Inhibit	Inhibit	Inhibit	–	(84)
PTX3	Inhibit	–	No inhibitory effect	Inhibit	Inhibit	(8, 81)

APC, activated protein C; CRP, c-reactive protein; rTM, recombinant thrombomodulin; I α I, inter-alpha-trypsin inhibitor; PTX3, pentraxin.3.



of PTX3 has the capacity to form a coiled-coil structure through heptad repeat motif with repeated hydrophobic residues. As a result of its activity of inter-molecular disulfide bond formation, PTX3 forms large complexes that interact with many different proteins. Such super-molecular formation with the assistance of PTX3 might be the ancestor of host-protective reactions. Further investigations are needed to elucidate the molecular mechanism of PTX3 complex interactions with proteins in NETs, especially histones.

AUTHOR CONTRIBUTIONS

KD, YT, and TH wrote the manuscript. YT performed secondary structure prediction and 3D structure modeling.

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NETosis as Source of Autoantigens in Rheumatoid Arthritis

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In neutrophils (but also in eosinophils and in mast cells), different inflammatory stimuli induce histone deimination, chromatin decondensation, and NET formation. These web-like structures that trap and kill microbes contain DNA, cationic granule proteins, and antimicrobial peptides, but the most abundant proteins are core histones. Histones contained in NETs have been deiminated, and arginines are converted in citrullines. While deimination is a physiological process amplified in inflammatory conditions, only individuals carrying genetic predisposition to develop rheumatoid arthritis (RA) make antibodies to deiminated proteins. These antibodies, collectively identified as anti-citrullinated proteins/peptides antibodies (ACPA), react with different deiminated proteins and display partially overlapping specificities. In this paper, we will summarize current evidence supporting the role of NETosis as critical mechanism in the breach of tolerance to self-antigens and in supporting expansion and differentiation of autoreactive cells. In fact, several lines of evidence connect NETosis with RA: RA unstimulated synovial fluid neutrophils display enhanced NETosis; sera from RA patients with Felty's syndrome bind deiminated H3 and NETs; a high number of RA sera bind deiminated H4 contained in NETs; human monoclonal antibodies generated from RA synovial B cells decorate NETs and bind deiminated histones. In RA, NETs represent on one side an important source of autoantigens bearing posttranslational modifications and fueling the production of ACPA. On the other side, NETs deliver signals that maintain an inflammatory milieu and contribute to the expansion and differentiation of ACPA-producing B cells.

Keywords: rheumatoid arthritis, neutrophils extracellular traps, histones, ectopic lymphoid structures, autoantigens, autoantibodies

INTRODUCTION

NETosis was discovered as a new function of neutrophils and thoroughly investigated as an important mechanism in the protection against bacterial, fungal, and parasitic infections (1). When the size of microorganisms is excessive for phagocytosis (2), neutrophils activate an alternative pathway leading to the extrusion of decondensed chromatin fibers containing histones as well as antimicrobial granular and cytoplasmic proteins (3). NETs are released during a form of cell death, distinct from necrosis and apoptosis, which requires reactive oxygen species (ROS) produced by NADPH oxidase.

Recent data however challenge the prevalent view of NETosis as a cellular suicide. An early NETosis has been described, which occurs rapidly after exposure to microbial specific molecular

patterns (e.g., within 60 min following *Staphylococcus aureus* stimulus), acts by a NADPH oxidase-independent pathway, and leads to the release of NET after nuclear envelope blebbing and vesicle formation, thereby preserving plasma membrane integrity. During this vital NETosis, cells are still able of some typical functions, such as chemotaxis and phagocytosis.

Slowly released from dying neutrophils or budding from live cells, NET fibers entrap microorganisms and represent a scaffold for enzymes, antimicrobial peptides, and ion chelators. These substances reach locally high concentrations and are thus able to cleave virulence factors and kill microorganisms (4).

Since the original description, it soon became apparent that both a defective and an excessive NET formation could have important consequences in human diseases, suggesting that a tight regulation of NETosis is critical to control pathogens while minimizing host damage.

When NET formation is impaired, as a result of NADPH oxidase or myeloperoxidase (MPO) deficiency (5), an immunodeficiency condition ensues, i.e., in chronic granulomatous disease, due to defective NADPH oxidase, restoration of NET formation by gene therapy allowed the control of severe fungal infection (6).

Conversely, a subset of neutrophils, identified for their lower density on gradients, is more abundantly represented in systemic lupus erythematosus (SLE) patients and is more prone to NETosis.

Netting neutrophils have not only been identified in nephritic kidneys in systemic lupus but also in ANCA-associated vasculitides (AAV), suggesting that NET constituents may be involved in the induction of severe manifestations of these systemic inflammatory disorders.

NET may also contribute to the pathogenesis of human diseases in a more subtle way, making potential autoantigens accessible to the immune system and creating the milieu where an autoimmune response may be triggered and fueled.

In this review, we shall summarize the current knowledge accumulated in recent years that point toward an important contribution of NET to the breach of immunological tolerance and the maintenance of autoimmunity and chronic inflammation in rheumatoid arthritis (RA).

NEUTROPHILS, CITRULLINATION, AND NETosis IN RA

Neutrophils are the most abundant cells in the synovial fluid of RA patients although they appear a less important component of the chronic synovial inflammatory infiltrate where neutrophils are believed to only transiently populate the synovial tissue. In RA, circulating but especially tissue-infiltrating and synovial fluid neutrophils have all the features of activated cells, characterized by a prolonged survival and by the ability to secrete a wide range of inflammatory mediators including chemokines and cytokines (7). Neutrophil contribution to arthritis has been directly addressed in animal models such as antibody-induced arthritis (i.e., anti-collagen antibody-induced arthritis) or the transgenic KBxN mouse model. In these models, neutrophil depletion or interference with key signaling receptors (leukotriene B4 receptors, C5aR,

CXCR1, and CXCR2) renders the mice resistant to disease induction. In RA, immune complexes engaging FcγRs activate neutrophils and trigger the release of ROS and proteases and the production of chemokines and cytokines. By means of these mediators, neutrophils recruit and modulate the function of other cell types, such as monocytes, dendritic cells, natural killer (NK), and lymphocytes, thus bridging innate and adaptive immunity (7).

A number of autoantibodies have so far been described in RA, but only anti-citrullinated proteins/peptides antibodies (ACPA) can be considered specific disease markers with sufficient specificity and sensitivity to be used as diagnostic tests (8). ACPA are a partially overlapping family of antibodies specific for protein sequences containing the amino acid citrulline, the deiminated form of arginine residues (9, 10). ACPA display extensive genetic diversity and are characterized by somatic hypermutation in their variable Ig domains, suggestive of an antigen-driven response (11).

Indeed, it appears that the immune response to citrullinated epitopes is initially restricted but expands with time from the preclinical, immune phase of the disease to the clinical onset (12). Specifically, in the pre-disease stage of RA, the breach of immune tolerance to citrullinated antigens appear to be triggered in genetically predisposed individuals by protein citrullination at putative extra-articular sites, such as the periodontal tissue during *Porphyromonas gingivalis*-induced periodontitis or in the lung of smokers (13–15), which gives rise to a restricted ACPA repertoire. However, with the progression to clinical disease onset, epitope spreading and further affinity maturation of ACPA occurs (16).

In established RA patients, the targets of ACPA include autoantigens (i.e., filaggrin, fibrinogen, vimentin, collagen II, and histones) as well as exogenous antigens (i.e., alpha-enolase, EBNA-1, and EBNA-2 proteins). All these proteins become target of ACPA after deimination (or citrullination), a post-translational modification (PTM) catalyzed by the calcium-dependent enzyme peptidyl arginine deiminase (PAD). PADs are inactive at intracellular calcium concentrations but can be activated by Ca²⁺ influx due to different stimuli: ionophore-induced macrophage apoptosis (extracellular calcium influx) (17) or lipopolysaccharide treatment of neutrophils (intracellular calcium mobilization). In neutrophils, PAD activation due to calcium influx takes place in the absence of caspase activation and triggering of apoptosis (18).

Furthermore, PADs can be released from the cell and become activated, as a result of the extracellular Ca²⁺ concentration (19).

Citrullination is a physiological process that regulates the homeostasis of several organs but is strongly amplified during inflammation. In RA, multiple proteins are citrullinated, especially in target organs of the disease, primarily the synovium but also in the lungs (20) and in myocardial tissue (21). In the RA joints, citrullinated fibrin is not only particularly abundant (22) but also several other citrullinated proteins including vimentin and aggrecan (23) are detectable. Citrullination is not an RA-specific process, and citrullinated proteins are present in several other inflammatory processes including the inflamed skeletal muscle tissue in myositis (24) and the synovium of spondyloarthritis

patients (25). Currently, the existence of a citrullination profile typical of RA is still an unsettled issue.

Neutrophils contribute to protein citrullination in RA in several ways. Cells contained in synovial fluid (mainly neutrophils and monocytes) are characterized by the citrullination of a wide variety of proteins. Neutrophil exposed to a variety of stimuli (cytokines, TLR ligands) contain deiminated histones (18). Moreover, both perforin and complement membranolytic pathways lead to pore formation in the membranes, augmenting the intracellular calcium concentration and favoring the activity of PAD enzymes (26). Thus, granzyme B/perforin and complement activation with membrane attack complex (MAC) formation are able to induce in synovial fluid neutrophils an extensive protein citrullination.

Although a large spectrum of citrullinated proteins is produced by neutrophils in RA joints, the immune response detected in RA sera is relatively restricted (**Figure 1**).

A major contribution to the generation of citrullinated proteins comes from the propensity of RA neutrophils from peripheral blood or synovial fluid to form NET, either spontaneously or after LPS stimulation (27). Indirect evidence for the higher spontaneous NETosis of RA neutrophils comes from the observation of Dwivedi et al. who detected higher deiminated H3 content in RA as compared with controls (28). Moreover, exposure to RA immunoglobulins or purified ACPA induces NET formation, as already observed with autoantibodies of other specificities (see ANCA in AAV) (29), and netting neutrophils

can be detected in synovial tissue and rheumatoid nodules from RA patients (27).

In synovial fluid, netting neutrophils release enzymatically active PAD2 and PAD4 that under the local conditions of inflamed joints may citrullinate extracellular proteins. Both soluble and NET-associated PAD can be detected, thus suggesting that NET may act as a molecular scaffold for protein citrullination (19).

Several recent works have evaluated the mechanisms behind NET regulation by PAD4. A single nucleotide polymorphism (SNP) at position 1858 (C1858T) in the DNA encoding a protein tyrosine phosphatase (PTPN22), which results in the conversion of an arginine (R620) to a tryptophan (W620), has risen interest due to its strong connection with RA (30). Interestingly, Chang et al. (31) have investigated the correlation between C1858T and PAD4 to test the hypothesis that PTPN22 might negatively regulate protein citrullination independently from its phosphatase activity. They observed that PTPN22 is a strong inhibitor of PAD4 and that the presence of R620 is required for this inhibitory mechanism, which is lost in the presence of C1858T modification, thus resulting in an expansion of the pool of citrullinated antigens and possibly in an increase in NET formation. Despite their results, Chang et al. also highlighted that this SNP is not disease specific. Indeed, C1858T modification is present in other autoimmune diseases such as SLE or type 1 diabetes. Furthermore, only a subset of RA patients appears to carry this SNP.

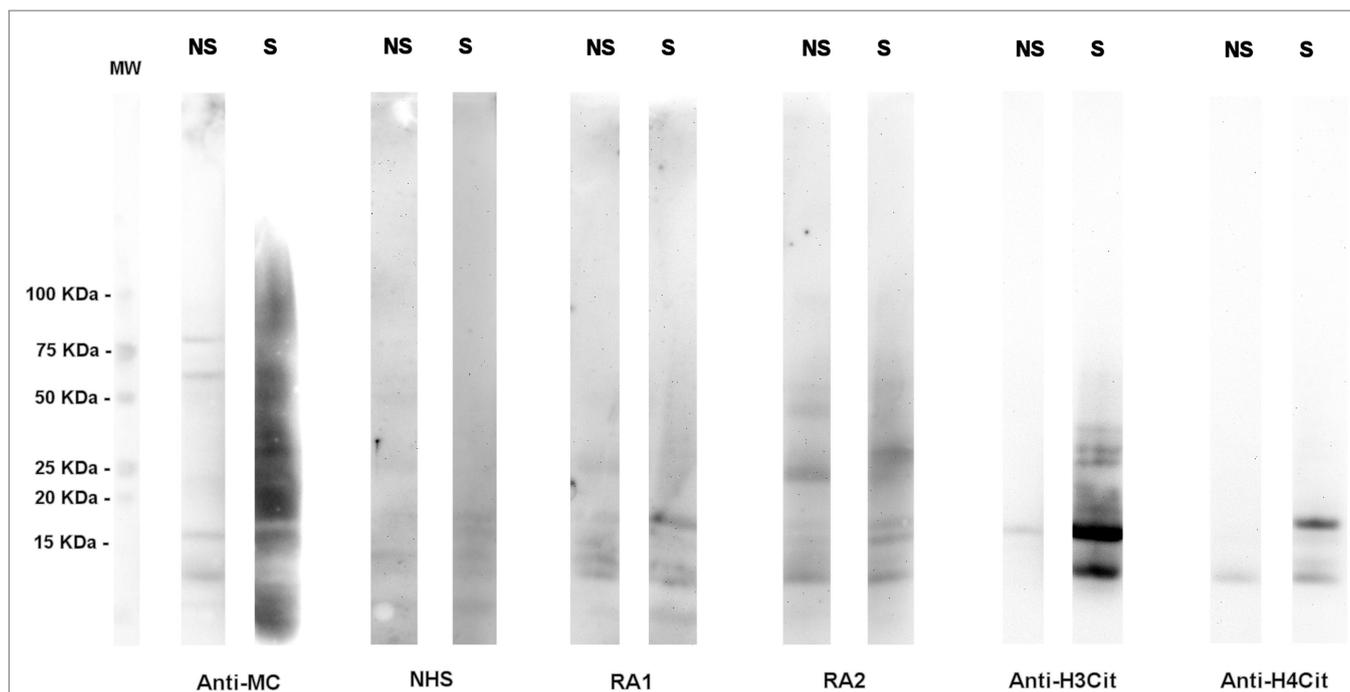


FIGURE 1 | A high number of citrullinated proteins is produced in neutrophils upon stimulation. Total proteins from A23187-stimulated (S) or unstimulated (NS) neutrophils were subjected to SDS-PAGE and western blotting and probed with an anti-modified citrulline human monoclonal antibody (anti-MC), NHS or RA sera, anti-citrullinated H3 (anti-H3cit), or anti-citrullinated H4 (anti-H4cit) polyclonal antibodies. Anti-MC h-mAb decorates a high number of proteins in stimulated neutrophils, part of which are recognized by RA sera. Proteins of 10–15 KDa are bound by anti-MC h-mAb, by RA sera, and by anti-H4cit or anti-H3cit antisera, thus suggesting that in stimulated neutrophils, H3 and H4 are citrullinated and become target of RA immune response, as previously showed (57, with the permission of BMJ Publishing Group, N° 3976570562524).

NET from RA patients is also able to activate synoviocytes, upregulating the production of pro-inflammatory cytokines and amplifying joint inflammation.

Thus, neutrophils have an active role in the inflammatory process of RA not only regulating the function of other immune or structural cells (32) but also being the source of and posttranslationally modified autoantigens (33, 34).

An increased NETosis has also been observed in SLE. A peculiar subset of neutrophils, identified by density gradients as low density granulocytes (LDGs) and more frequently detectable in active SLE, has a pro-inflammatory phenotype (35) and forms spontaneously NET (36). On the other hand, it has been demonstrated that SLE patients have a decreased ability to degrade NET (37). Upon exposure to NET or NET components, activated caspase-1 is produced in macrophages, leading to the production of active IL-1 β and IL-18 (38), while plasmacytoid dendritic cells are activated and release IFN α (39). Antimicrobial peptides like LL37 (40) and acetylated histones (41) are important mediators in these processes. Thus, NET represent on one side an important source of autoantigens fueling the production of anti-chromatin antibodies, on the other, a critical mechanism of disease induction, affecting several cell types and influencing the disease phenotype.

Increased NETosis has not only been described in type 1 diabetes, correlated with autoantibody titers and beta cell damage (42), but also in type 2 patients, and a direct role of hyperglycemia in increased NETosis has been shown (43).

More recently, an increased number of netting neutrophils has been reported in type 1 and type 2 diabetes, and their direct role in retarding wound healing has been demonstrated (44, 45).

HISTONE DEIMINATION IN NEUTROPHILS

The core nucleosome, comprising an H3–H4 tetramer and two H2A/H2B dimers is not a static DNA packaging structure, but on the contrary is a dynamic complex, and the modulation of its structure is an important component of transcriptional regulation.

Modifications in the conformation of histones, highly conserved in eukaryotic cells, from yeast to humans, are widely used in the dynamic modulation of chromatin structure and function. Indeed, evolutionary PTMs are more useful than amino acid substitutions.

So far, 20 types of histone PTMs have been described, which are able to modulate chromatin function by either altering the amino acid charge and consequently the inter-nucleosomal interactions or by enabling/inhibiting interactions with specific binding proteins external to nucleosomes but nonetheless essential for DNA regulation. Among all, one of the latest described is the deimination of arginine.

The first description of histone deimination was reported by the Yamada's group (46). They observed that when HL60-derived granulocytes and peripheral blood granulocytes are stimulated with A23187 (a mobile ion-carrier known as calcium ionophore), their cytoplasmic PAD V deiminates histone H2A, H3, and H4 (other than nucleophosmin/B23). The percentage of deiminated

histones detected in these studies was 10% of the total histone content.

In 2004, Cuthbert et al. (47) and Wang et al. (48) reported that PAD4 (correspondent to PAD V described by Yamada) deiminates histone H3 and H4 and has an impact on gene transcription by fine tuning the chromatin structure.

In particular, the group of Koutzarides (47) showed that PAD is activated when it is bound intracellularly by estrogen receptor. PAD deiminates histone H3 and H4 in different arginine located preferably in the N-terminal tail and increases the affinity of estrogen receptor for its target genes, thus resulting in a decrease of gene expression under the control of estrogen and thyroid hormones.

When PAD4 activity is inhibited by Cl-amidine, an increase in the expression of p53 and p53-related genes is observed as described by the group of Coonrod (48).

PAD4 is not the only PAD isoform involved in chromatin regulation. Indeed, Zhang et al. (49) suggested that stimulation of ER α -positive cells with 17 β -estradiol (E2) promotes global citrullination of histone H3 arginine 26 (H3R26) on chromatin, catalyzed by PAD2 and not by PAD4, which instead deiminates H4R3.

Importantly, deimination may involve arginine but also methylarginine on H4 and H3 induced by PRMT1 and CARM1, respectively, thus dubbing PAD4 as a demethylating enzyme, thereby reverting the epigenetic modification of arginine methylation.

Moreover, deimination of the H2A/H2B dimer, probably involving three arginines (given the mass increase of 2.7 Da) stabilizes the dimer, making it less susceptible to harsh conditions than the native complex, as demonstrated by mass spectrometry analysis with increasing concentration of ammonium acetate (50).

Like core histones, extranucleosomal linker histones can also be the target of PAD activity. Christophorou et al. (51) recently demonstrated that H1 can also be citrullinated. In pluripotent stem cells, the presence of citrullinated H1 is highly correlated with the adoption of a more open state of chromatin and with a high level of transcription of pluripotency genes. Conversely, inhibition of PAD4 activity and consequently of H1 citrullination leads to a more compact state of chromatin and to higher transcription of differentiation genes. Dwivedi et al. demonstrated that H1 is an additional substrate for PAD4, providing evidence that during NETosis a variety of linker H1 can be deiminated on multiple arginines. Notably, H1.2 is deiminated on arginine 53, and the neo-formed epitope is thus recognized by specific anti-citrulline antibodies present in a small percentage of SLE and SS patients but not in RA (28).

Nevertheless the topic of histone deimination is still a tangled issue, given the high number of PTM co-expressed on histones and the limitations of the chemical and biological methods presently available for citrulline detection, which are not fully citrulline specific (52).

For instance, the antibody-detecting citrulline after chemical modification with antipyrine and 2,3-butanedione, the so called "Senshuo reagent," also recognizes carbamylated proteins (53).

This lack of citrulline complete specificity is also a characteristic of some anti-unmodified citrulline antibodies commercially available.

To overcome this problem, recently Bicker et al. (54) suggested a rhodamine tagged phenylglyoxal derivative that can be used to directly visualize protein citrullination in a simple and highly sensitive quantitative method.

Besides technologies like mass spectrometry that allow determination of site-specific citrulline on proteins with high sensitivity but at high costs, the development of simple but nonetheless reliable and specific chemical or biological tools is a field still open to innovation.

Taken together, these results show that histone citrullination is a key regulatory mechanism for cell life, but in particular cells (neutrophils, eosinophils, mast cells, monocytes) histone deimination may lead to decondensation of the entire cell chromatin, thus affecting in an irreversible way the cell life and leading to ETosis (extracellular traps formation).

DEIMINATED HISTONES AND AUTOANTIBODIES IN RA

Core histones are the most abundant proteins in NET (55), and several reports indicate that deiminated histones are a target of antibodies in RA. Specifically, sera from RA patients decorate NET, co-localizing on chromatin with anti-deiminated histone H3 antibodies. Moreover, sera from patients with Felty's syndrome (characterized by RA, splenomegaly, and neutropenia) display a preferential binding to deiminated histones by ELISA, which was further identified to be directed against deiminated H3 using SDS-PAGE fractionated histones (56).

We have recently shown that RA sera, tested by immunoblot on acid-extracted proteins from calcium ionophore-stimulated neutrophils, frequently react with a band of 11 kDa. Its identity with deiminated H4 has been suggested by specific antibody recognition and demonstrated by MALDI/TOF analysis. The recognition of deiminated H4 has been confirmed by ELISA using either the entire molecule or citrullinated peptides corresponding to H4 sequences. When RA sera are tested with proteins contained in NET, the reactivity with a band identified as H4 is again detected. Moreover, by derivatization of citrulline residues, it has been shown that H4 contained in NET and recognized by RA sera is deiminated on arginine 23.

Antibodies specific for H4-derived citrullinated peptides (HCP1 – H4₁₄₋₃₄ and HCP2 – H4₃₁₋₅₀) are present in 67 and 63% of established RA (57). Their frequency is lower in early RA (37.3 and 48.5%, respectively), but they can be detected years before disease onset. As reported for other ACPA subtypes, anti-citrullinated histone antibodies precede symptom onset and predict disease development (58).

Similarly, antibodies against citrullinated sequences of H2A and H2B have been detected in healthy subjects that later develop RA. An increase in antibody frequency, together with the production of inflammatory cytokines, predicts the imminent development of clinically active RA (16).

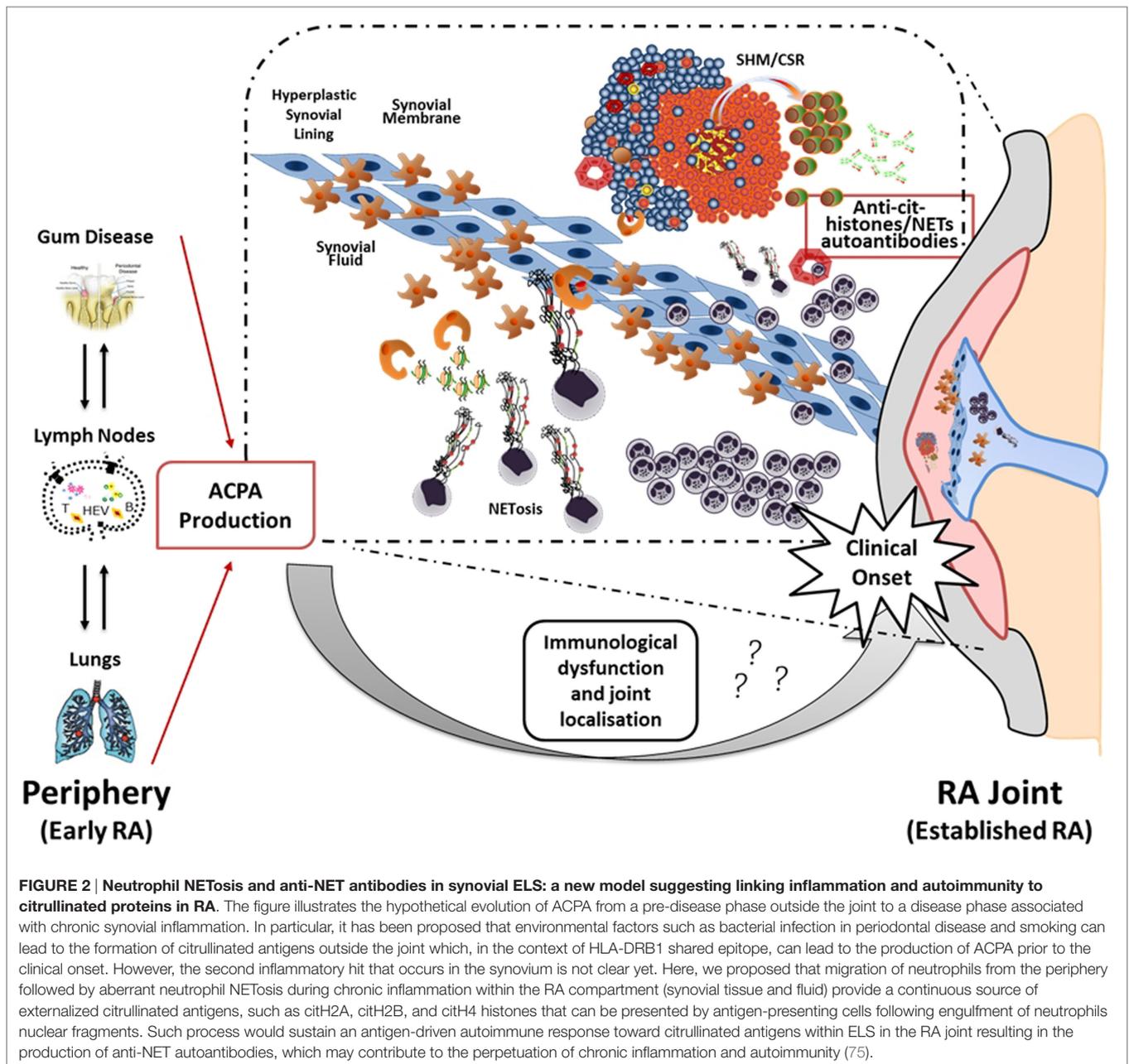
Recently, citrullinated H2B has been detected as a target of autoantibodies in a high number of patients with established RA (59). RA synovial fluids contain high levels of citH2B and its immune complex, which have pro-inflammatory and immunostimulatory capacity.

Most importantly, Sohn et al. (59) demonstrated the arthritogenic potential of citH2B by immunization in a mouse model, although it was necessary to generate a low-grade articular inflammation to observe this peculiar effect.

On the whole, the definition of “true ACPA” and their pathogenic role in the initiation of arthritis is still a matter of debate. Production of citrulline-specific autoantibodies was non-detected in MRL-lpr/lpr and (NZB × B6)F1-hbcl-2-transgenic mice (60), and any arthritogenic role in Lewis and Brown-Norway rats was excluded (61). It has been later shown that immunization with citrullinated antigens like collagen II can enhance tissue injury and stimulate ACPA production in experimental arthritis (62, 63), and that administration of anti-citrullinated fibrinogen in collagen-induced arthritis enhances tissue injury (62). Genuine ACPA, which is ACPA non-reactive with the correspondent non-deiminated antigen, are actually detectable in mice, but the production of these antibodies is highly dependent on the mouse strain, the antigen used for immunization and ACPA detection, and the immunization protocol (64). Further support for a pathogenic role of the immune response to citrullinated antigens derives from a recent report on the immunomodulatory potential of synthetic citrullinated antigens. In rats, a tolerogenic injection protocol using synthetic multiepitopes derived from common citrullinated proteins ameliorates adjuvant-induced arthritis, inducing an expansion of regulatory T cells and a reduction of Th17 cells (65).

ECTOPIC LYMPHOID STRUCTURES AS A SOURCE OF ANTI-NET ANTIBODIES IN RA

Approximately 50% of patients with RA are characterized by the presence of clusters of infiltrating lymphomononuclear cells in the RA joint synovium forming ectopic lymphoid structures (ELS). Synovial ELS not only resemble secondary lymphoid organs (SLO) but they can also support a germinal center (GC) response. In particular, ELS are characterized by segregation of T and B lymphocytes, differentiation of high endothelial venules (HEVs), and networks of stromal follicular dendritic cells (FDC) (66, 67). Moreover, ELS are functional structures supporting the affinity maturation and clonal selection of autoreactive B cells bringing to the differentiation of plasma cells producing antibodies toward citrullinated antigens (68). Thus, ELS in the RA synovium can directly sustain autoimmunity by not excluding self-reactive B cells to affinity mature into the ectopic GC. This process is also antigen and disease specific. In RA, ectopic GC support the production of antibodies against citrullinated proteins (68–70), in other autoimmune diseases, they can support the production of antibodies targeting other autoantigens, i.e., in Sjögren's syndrome ribonucleoprotein Ro/La (71, 72), in Hashimoto's thyroiditis thyroglobulin and thyroperoxidase (73), and in myasthenia gravis acetylcholine receptor (74).



Of relevance, we have recently demonstrated that up to 40% of recombinant monoclonal antibodies derived from single CD19+ synovial tissue cells (RA-syn-rmAbs) obtained from ACPA+ RA patients with functional ectopic GC display reactivity toward citrullinated histones (75). In particular, in this work we not only showed a strong reactivity of the RA-syn-rmAbs toward citrullinated histone H2A and H2B but also citrullinated vimentin and fibrinogen. Importantly, the reactivity against histones was confirmed in a cell-based NETs co-localization assay using either RA synovial fluid or circulating neutrophils as cell substrate. These antibodies were thus defined as anti-NETs antibodies. Moreover, the anti-NETs immunoreactivity was shown to be

acquired within the synovial microenvironment in the ectopic GC through affinity maturation and intra-synovial diversification and was lost when the Ig H and L variable regions were reverted to their germline sequences (75).

As discussed above, NETs formation is critically dependent on histone citrullination, and citrullinated histones comprise around 70% of all NETs proteins. A proteomic analysis of NETs derived from healthy control neutrophils has identified at least 25 different proteins that decorate these chromatin structures, such as citrullinated vimentin and α -enolase, which are also targets of ACPA, as discussed above (27). Therefore, a delay in the clearance of NET could form a reservoir of citrullinated and

non-citrullinated antigens in the extracellular space of the RA joint and may contribute to the autoimmune response in RA. Indeed, defects in NET clearance have been already associated with other autoimmune diseases like lupus nephritis. In particular, defects in DNase1, which is responsible to degrade NET, have been observed in SLE patients (37).

As discussed in the Section “Introduction,” although the local release of NET in the RA joint represents an important source of citrullinated autoantigens, additional sources of NET-related autoantigens should also be considered during the generation of ACPA, particularly in the preclinical immune phase of RA. Environmental factors such as bacterial infection during periodontal disease (i.e., *P. gingivalis*) (76, 77) and smoking (78) can bring to the formation of citrullinated antigens outside the joint and can lead to ACPA production before the clinical onset of the disease. Of interest, *P. gingivalis* is the only known pathogen expressing PAD, which can citrullinate both the endogenous and host proteins, thus supporting ACPA formation (79, 80).

However, periodontitis is also characterized by an increased production of antibodies toward the non-citrullinated form of RA-associated antigens, suggesting that in some patients periodontal disease could break the tolerance to non-modified autoantigens in the preclinical phase of RA (81).

In periodontal disease (82, 83), NETosis is increased, and NETs are also involved in acute and chronic lung inflammation (84). In the subset of ACPA-positive RA patients, lung abnormalities are detectable by HCRT early in the disease course, associated with the presence of citrullinated proteins in the lungs and ACPA in the bronchoalveolar lavage fluid (85). The level of ACPA and the production of ACPA of different specificity are both predictive

of the development of interstitial lung disease (86). Bronchial biopsies from early RA patients show an inflammatory infiltrate containing T cells, B cells, and plasma cells and occasionally GC-like structures (87). In early RA, synovia, periodontal tissue, or the lung share an increased content of citrullinated proteins, evidence of NETosis, and presence of ectopic GCs. It is conceivable that elevated concentration of NET-associated proteins due to an increase in NETs production or NETs removal impairment could locally provide a source of citrullinated autoantigens able to trigger an autoimmune response.

NETs expose citrullinated proteins together with danger signals like cathelicidin or HMGB1 that promote the activation and maturation of professional antigen-presenting cells. NETs uptake and processing may allow the expansion of T cells able to support the affinity maturation and clonal diversification of B cells, leading to ACPA production.

Thus, the current hypothesis is that the local release of citrullinated histones and probably other citrullinated and non-citrullinated antigens during an excessive NETosis can sustain the antigen-driven generation of high affinity anti-NETs antibodies within ELS with functional ectopic GC (Figure 2).

AUTHOR CONTRIBUTIONS

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

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NETosis in Alzheimer's Disease

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Alzheimer's disease (AD) is a neurodegenerative disorder characterized by the progressive deterioration of cognitive functions. Its neuropathological features include amyloid- β ($A\beta$) accumulation, the formation of neurofibrillary tangles, and the loss of neurons and synapses. Neuroinflammation is a well-established feature of AD pathogenesis, and a better understanding of its mechanisms could facilitate the development of new therapeutic approaches. Recent studies in transgenic mouse models of AD have shown that neutrophils adhere to blood vessels and migrate inside the parenchyma. Moreover, studies in human AD subjects have also shown that neutrophils adhere and spread inside brain vessels and invade the parenchyma, suggesting these cells play a role in AD pathogenesis. Indeed, neutrophil depletion and the therapeutic inhibition of neutrophil trafficking, achieved by blocking LFA-1 integrin in AD mouse models, significantly reduced memory loss and the neuropathological features of AD. We observed that neutrophils release neutrophil extracellular traps (NETs) inside blood vessels and in the parenchyma of AD mice, potentially harming the blood-brain barrier and neural cells. Furthermore, confocal microscopy confirmed the presence of NETs inside the cortical vessels and parenchyma of subjects with AD, providing more evidence that neutrophils and NETs play a role in AD-related tissue destruction. The discovery of NETs inside the AD brain suggests that these formations may exacerbate neuro-inflammatory processes, promoting vascular and parenchymal damage during AD. The inhibition of NET formation has achieved therapeutic benefits in several models of chronic inflammatory diseases, including autoimmune diseases affecting the brain. Therefore, the targeting of NETs may delay AD pathogenesis and offer a novel approach for the treatment of this increasingly prevalent disease.

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INTRODUCTION

The formation of neutrophil extracellular traps (NETs) is a defense mechanism used by neutrophils to trap and efficiently limit the damage caused by a wide range of microbial targets (1). NET production is associated with dramatic changes in cellular morphology, including the extrusion of decondensed chromatin into the extracellular space to form web-like structures decorated with histones and granular antimicrobial proteins, such as neutrophil elastase (NE), myeloperoxidase (MPO), proteinase 3, cathepsin G, lactoferrin, matrix metalloproteinase 9 (MMP-9), peptidoglycan-recognition proteins, pentraxin, and LL-37 (1–4). The sequential molecular events that generate NETs are the production of reactive oxygen species (ROS), the migration of NE protease and later MPO from granules to the nucleus, the processing of histones, and the rupture of the cell (5). NETs provide a key defense mechanism against pathogens to prevent their systemic dissemination during infection (1, 6). However, NETs are also major effectors involved during sterile inflammation,

autoimmune diseases, such as systemic lupus erythematosus (SLE), and atherosclerosis, and they may also promote metastasis (1, 7). In addition, we have recently shown the release of NETs in Alzheimer's disease (AD), suggesting that NETs may also play a role in AD pathology (8).

Alzheimer's disease, one of the most devastating neurodegenerative disorders, is characterized by progressive memory decline and cognitive deficits. The main neuropathological features of AD include neuritic plaques formed by deposits of amyloid- β (A β), the abnormal accumulation of hyper-phosphorylated tau protein in the neuronal soma, which manifests as neurofibrillary tangles (NFTs), synaptic dysfunction, and neuronal loss (9). AD is also characterized by cerebral amyloid angiopathy due to A β deposits in the cerebral vasculature, which lead to luminal stenosis, endothelial damage, basement membrane thickening, thrombosis, loss of autoregulation, and vasospasm (10). Chronic neuroinflammation is thought to play a role in AD pathology, and recent studies have identified several inflammation pathway genes associated with the risk of AD (11, 12). Microglial activation precedes neuronal loss in AD patients, and microglia-mediated neuro-inflammatory responses may promote the neurodegeneration observed in AD (9, 13). Moreover, in response to A β or NFTs, microglial cells produce pro-inflammatory cytokines, chemokines, and complement peptides, which can amplify the neuroinflammation in AD (13). A β and tau deposits cause detrimental effects in the neuronal milieu, due to the excessive release of cytotoxic factors, including the interleukins IL-1 β and IL-6, tumor necrosis factor α (TNF α), and free radicals, enhancing neuroinflammation and neuronal damage (8, 11–14). Epidemiological studies indicate that non-steroidal anti-inflammatory drugs (NSAIDs) reduce the risk of AD, providing further evidence that inflammation mechanisms play a role in this disease (15, 16). However, the lack of efficacy of NSAIDs against AD in clinical trials suggests that more specific inflammation mechanisms must be identified to inhibit AD-related neuroinflammation (11, 16, 17).

Compelling evidence indicates that AD-related inflammation develops in two different but interconnected compartments: the blood and the brain. In this context, systemic inflammation can lead to "brain activation," whereas cerebral inflammation may in turn influence the peripheral system through the release of danger signals and other inflammatory mediators (12, 18–21). The blood–brain barrier (BBB) is a connection point between blood and circulating leukocytes on one side and the brain parenchyma on the other. It is a highly specialized endothelial cell membrane that regulates the passage of essential nutrients and leukocytes into the central nervous system (CNS) and facilitates the clearance of potentially neurotoxic molecules from the brain to the blood (18–21). The BBB together with vascular cells (pericytes and vascular smooth muscle cells), glial cells, and neurons, constitutes the neurovascular unit (NVU) (20, 21). AD is characterized by the loss of BBB integrity, which disrupts the clearance of A β and thus promotes A β accumulation in the brain, leading to neuronal injury and cognitive decline (20). The accumulation of A β in the brain and in the vessel walls also induces the expression of adhesion molecules on brain endothelial cells and the release of inflammatory mediators, such

as cytokines, chemokines, and complement system peptides, potentially facilitating the adhesion and subsequent transmigration of leukocytes. Previous studies have shown that tau may also contribute to BBB deterioration *in vitro*, and BBB dysfunction correlates with the appearance of perivascular tau around major hippocampal blood vessels *in vivo* (22–24). Both tau and A β may, therefore, induce BBB dysfunction, contributing to brain inflammation and neurodegeneration.

The role of circulating immune system cells in AD has not been investigated in detail, but the migration of cells related to both innate and adaptive immunity has been observed in the AD brain (21, 25, 26). For example, monocytes migrate through the brain endothelium into the AD brain in a CCR2-dependent manner, and previous studies suggest they may promote A β clearance (27). However, the replacement of brain-resident myeloid cells with circulating peripheral monocytes in AD mouse models showed that monocyte repopulation does not modify the amyloid load, challenging the idea that peripheral monocytes play a role in A β clearance (28, 29). Lymphocytes can also enter the AD brain, and both CD4⁺ and CD8⁺ T cells in AD patients were shown to adhere inside the cerebral blood vessels or to migrate into the parenchyma (21, 26). Nevertheless, the role of these cells remains unclear because recent studies indicate they may play either a positive or negative role in AD models, probably depending on the specific cell subset and disease phase (21, 26). Unexpectedly, neutrophils were also found inside the brain vessels and parenchyma of AD subjects, and the capacity of neutrophils to invade the AD brain has recently been confirmed (8, 30–32). Moreover, our recent data reveal that neutrophils adhere to blood vessels and infiltrate inside the brain parenchyma in two transgenic animal models of AD, inducing cognitive deficit and neuropathological changes (8). Neutrophil depletion reduced the neuropathological hallmarks of AD and improved memory functions in these models, suggesting that neutrophils play a key role in AD pathogenesis (8).

Recently, we have observed NETs within the cerebral vasculature and parenchyma of animal AD models and individuals with AD, suggesting that NETs can potentially harm the BBB and neural cells (8). In this review, we discuss the involvement of NETs in AD as a novel mechanism for neutrophil-mediated neurotoxicity and neurodegeneration and suggest that the inhibition of NETs may offer a new pharmacological approach to slow down the progression of this disease.

NEUTROPHILS IN AD

Circulating neutrophils are the most abundant leukocytes in the peripheral blood and they provide the first line of defense in the innate immune system. Neutrophils are short-lived cells with circulating half-lives of approximately 1.5 h in mice and 8 h in humans, although this was recently challenged and longer survival times of up to several days were reported in humans (1). Nevertheless, neutrophils are activated during inflammation and their longevity increases, allowing them to carry out more complex activities, potentially causing bystander cell injury. Neutrophils are thought to be the main protagonists in the first line of defense during acute inflammation, when many of these

cells migrate into tissues and can easily be identified using conventional histology techniques. However, neutrophils have attracted more attention recently in the context of chronic inflammation, e.g., atherosclerosis, rheumatoid arthritis, SLE, anti-neutrophil cytoplasmic antibody-related vasculitis, deep vein thrombosis, chronic obstructive pulmonary disease, cystic fibrosis, and animal models of multiple sclerosis (33–35). Neutrophils are now thought to be key players that directly affect the pathogenesis of chronic inflammatory diseases. For example, they were recently shown to play a prominent role in chronic low-grade adipose tissue inflammation and insulin resistance mediated by the secretion of elastase (36, 37).

Neutrophil recruitment in the CNS is a central process during the pathogenesis of several neuro-inflammatory disorders, ranging from bacterial and viral encephalitis to non-infectious conditions, such as cerebral ischemia, trauma, and demyelinating syndromes (25, 35, 38, 39). Previous studies have shown that neutrophils transmigrated in the CNS acquire a toxic phenotype and approach neuronal cells, where they release harmful molecules and can compromise neuronal functions (40). Therefore, limiting neutrophil migration and/or functions can positively influence the outcome of neuronal injuries (8, 21, 35, 39).

The first evidence that neutrophils accumulate in the CNS of AD patients was the detection of cells expressing the neutrophil-specific protease cathepsin G within the AD brain parenchyma and cerebral blood vessels, often associated with A β deposits (30). This was followed by the detection of CAP37, an inflammatory mediator constitutively expressed in neutrophils, in the cerebral microvasculature of AD patients but not in age-matched controls or patients with other neuropathological conditions, such as Pick's disease, Parkinson's disease, Binswanger's disease, or supranuclear palsy (31, 41). Initially, the expression of CAP37 in the AD brain was not linked with the presence of neutrophils, but was instead associated with endothelial activation and neuronal cells, and was thought to be induced by A β (31, 41, 42). More recently, we identified MPO⁺ cells in areas with A β deposits, further supporting the presence of neutrophils in the AD brain (8). We found that intraparenchymal MPO⁺ cells were mainly localized to within 50 μ m of A β plaques, and their distribution was non-random, suggesting that A β may act as a chemoattractant by creating a favorable microenvironment for the accumulation of neutrophils inside the brain, thus promoting their pro-inflammatory activities (8). We also used (i) hematoxylin and eosin staining to confirm the presence of polysegmented nuclei in cells that have migrated perivascularly or within the parenchyma (8), (ii) naphthol AS-D chloroacetate esterase staining in brain sections to confirm the presence of cells of the granulocytic lineage specifically in AD brains (43, 44), and (iii) staining for the neutrophil-specific marker, CD66b, which likewise confirmed that neutrophils were present specifically in the brains of AD subjects but not age-matched controls (8). These neuropathological studies are supported by recent clinical data revealing increased numbers of neutrophils or a higher neutrophil/lymphocyte ratio associated with AD, suggesting that changes in the neutrophil population could be used as markers of AD-related peripheral inflammation (45–47). The amyloid protein precursor (APP) is also expressed more

strongly in the granulocytes of AD patients compared to controls, whereas there was no statistically significant difference in the lymphocyte and mononuclear cell populations, suggesting that the strong expression of APP in peripheral mononuclear cells could be used for the early diagnosis of AD (48). Other studies have also revealed differences in neutrophil functions and changes in granulocyte density in AD patients, further suggesting that the analysis of blood neutrophils may offer new AD biomarkers (49, 50).

In agreement with the data from AD subjects, we have recently shown the presence of Gr-1⁺ cells in 3xTg-AD mice during the early phases of AD, and more recent data obtained in the 5xFAD transgenic AD model revealed Gr1⁺ cells infiltrating the brain parenchyma and migrating toward A β plaques (32, 51). Our data in 5xFAD and 3xTg-AD mice confirmed these results, showing infiltrating neutrophils within the brain parenchyma at the onset of memory deficit, especially in the cortex and hippocampus, highlighting the role of these cells in AD pathogenesis (8). In addition, our two-photon laser-scanning microscopy (TPLSM) studies revealed neutrophil extravasation inside the cerebral parenchyma during the early phases of AD, preferentially in zones adjacent to vascular and intraparenchymal A β deposits, suggesting that A β may play an important role in neutrophil recruitment and movement inside the brain parenchyma (8). As stated above, A β may act as a chemoattractant for neutrophils and may represent an FPR1-binding “end-target” chemoattractant prevailing over “intermediate” chemokines, potentially contributing to the directional bias observed for a significant proportion of extravasated neutrophils in the brains of AD mouse models (8, 52, 53). FPR1 and LFA-1 may, therefore, promote neutrophil deep tissue penetration and thus contribute to widespread tissue damage.

In our studies, LFA-1 integrin controlled not only the intraparenchymal motility of extravasated neutrophils but also their intravascular adhesion in the cerebral microcirculation of transgenic AD mice (8). By blocking LFA-1 integrin in AD mice during the early phases of AD, neutrophil adhesion and extravasation were prevented and the neuropathological hallmarks of AD were clearly reduced, thus restoring cognitive functions. Notably, a transient therapeutic blockade of LFA-1 integrin during the early stages of disease also provided a long-term beneficial effect on cognition in older mice, suggesting that the therapeutic reduction of neutrophil trafficking during the early phases of AD may have prolonged beneficial effects in AD patients. Moreover, 3xTg-AD mice lacking LFA-1 integrin showed improved memory functions in behavioral tests compared to wild-type control mice, and the severity of microgliosis was reduced (8). The role of neutrophils in AD has been defined only recently, so the mechanisms controlling neutrophil trafficking and interactions with CNS-resident cells must be investigated in more detail.

In vivo experiments have shown that fluorescence-labeled neutrophils start to infiltrate the brain parenchyma of 5xFAD mice 12–18 h after cell injection, continue to migrate and reach a peak at 24 h post-injection, and then become undetectable at 48 h post-injection, suggesting that the half-life of the migrating neutrophils in the brain is approximately 12 h. Neutrophils

are highly reactive cells, releasing ROS, enzymes, NETs, and cytokines, and can thus cause chronic collateral tissue damage even in the absence of substantial accumulation within tissues during low-grade chronic sterile inflammation. Furthermore, previous results from our group and others have shown that neutrophils do not necessarily need to accumulate in high numbers in order to induce tissue damage: intravascular adhesion *per se* without transmigration is sufficient to induce endothelial injury and the resulting tissue damage (54, 55). This is supported by our data showing that blocking LFA-1 integrin, which controls the intravascular adhesion of neutrophils, reduces cognitive damage and neuropathological lesions in AD models. Our recent results also demonstrate that transgenic AD mouse models treated with a neutrophil-depleting antibody show a reduction in both microglial cell density and their activation state, suggesting that neutrophils promote the activation of glial cells, fueling an inflammatory loop that may promote neuronal injury and memory decline (8).

INTRAVASCULAR NETs IN AD

The release of intravascular NETs by adherent neutrophils has been observed in several diseases, including sepsis, atherosclerosis, autoimmune pathologies, such as autoimmune small-vessel vasculitis, experimental deep vein thrombosis, transfusion-related acute lung injury, and cancer (1, 4, 56–59). Intravascular NETosis can be triggered by different stimuli including microbes, pro-inflammatory cytokines, activated platelets, and antibody–antigen complexes (1, 2, 56, 60, 61).

The intravascular neutrophil adhesion cascade that causes neutrophils to leave the blood circulation begins with the capture of these cells on the endothelium followed by their rolling and firm arrest on activated endothelial cells (1, 25). Adhesion receptors specialized for the arrest of intravascular neutrophils are heterodimeric transmembrane proteins known as integrins. In order to mediate firm adhesion, integrins undergo an activation process induced by chemoattractants *via* G-protein-coupled receptors. The activation of the intracellular pathways leading to increased ligand binding affinity (“integrin activation”) and the clustering of integrins in the membrane, which together allow cell attachment, is defined as “inside–out signaling” (62). Furthermore, the signaling steps that occur following the ligand-induced clustering of leukocyte integrins are described as the “outside–in pathway” (62). Indeed, $\beta 2$ integrin engagement stabilizes neutrophil adhesion to the inflamed endothelium and induces neutrophils to release ROS, cytotoxic enzymes, arachidonic acid derivatives, cytokines, and chemoattractants, which may have a detrimental effect on the vessel wall (53, 55). Previous studies including our own have also shown that neutrophil adhesion on the vessel wall without transmigration is sufficient to induce endothelial injury, suggesting that intravascular adhesion *per se* may trigger NET formation and consequent endothelial injury, compromising BBB integrity (54, 55, 63). The adhesion-dependent production of ROS may also trigger the machinery involved in the final extrusion of fibers containing DNA and granule proteins (5). The adhesion of neutrophils to the endothelium induced by the

engagement of $\alpha M\beta 2$ integrin (Mac-1) promotes the release of NETs by neutrophils in the presence of lipopolysaccharide (64). In addition, the activation of $\alpha M\beta 2$ integrin induces changes in the neutrophil cytoskeleton that facilitate the breakdown of nuclear and plasma membranes, favoring the release of NETs (64). The engagement of LFA-1, another $\beta 2$ integrin ($\alpha L\beta 2$ heterodimer) expressed by neutrophils, triggers NET formation by activated platelets *in vitro* and *in vivo* (Figure 1) (59). During inflammation, Mac-1 and LFA-1 mediate interactions with vascular ICAM-1, and microvascular endothelial cells produce higher levels of ICAM-1 in both transgenic AD mouse models and human AD patients (8, 25). Interestingly, we have recently reported the expression of adhesion molecules in areas burdened by A β plaques and rich in migrated leukocytes in animal models of AD (8). Accordingly, *in vitro* studies have demonstrated that A β peptides induce the expression of endothelial adhesion molecules, including ICAM-1 in mouse and human brain endothelial cells, suggesting that A β may play a role in endothelial activation and intravascular neutrophil adhesion in AD (8, 65). Our recent data indicate that both oligomeric and fibrillary A $\beta 1$ –42 trigger the rapid, integrin-dependent adhesion of human and mouse neutrophils on fibrinogen and ICAM-1, a ligand for LFA-1 integrin (8). Moreover, we have shown that A $\beta 1$ –42 induces both the intermediate-affinity and high-affinity states of LFA-1, potentially providing stop signals for neutrophils. In addition, our recent data indicate that neutrophils migrate into the brains of AD mouse models by engaging LFA-1 integrin and that blocking this $\beta 2$ integrin prevents neutrophil adhesion in cortical venules and subsequent extravasation, suggesting intravascular neutrophil adhesion through $\beta 2$ integrins may trigger the formation of NETs in AD (8). We also have recently confirmed the formation of intravascular NETs in 5xFAD and 3xTg-AD mice and proposed as a mechanism for neutrophil-dependent damage in AD (Figure 1) (8). Furthermore, we have documented intravascular neutrophil adhesion and the release of NETs in the brains of human AD subjects, suggesting that intravascular NETs may also contribute to CNS damage in humans.

Previous studies have shown that pro-inflammatory cytokines, such TNF α , IL-1 β , and IL-8, can be released by activated endothelial cells and may, therefore, trigger intravascular NETs (60, 66–68). The cerebral vasculature in human AD subjects is strongly activated and produces cytokines, suggesting that intravascular cytokines may favor the formation of NETs in this context. AD brain microvessels release significantly higher levels of thrombin, TNF α , IL-1 β , and IL-8 than age-matched controls, indicating that such endothelial molecules may promote the formation of NETs by adherent neutrophils (69–71). Thrombin in particular becomes more abundant in the cerebral capillaries of AD brains, and induces the release of IL-1 β and IL-8, which may in turn contribute to intravascular NETosis (60, 70–73). Interestingly, subjects with mild cognitive impairment have higher serum IL-1 β levels than controls, suggesting this cytokine may trigger the release of NETs and contribute to the onset of AD (74). Previous studies have also shown elevated levels of IL-1 β and TNF α in the serum of AD patients (74–76). *In vitro* studies of brain endothelial cells indicate that exposure

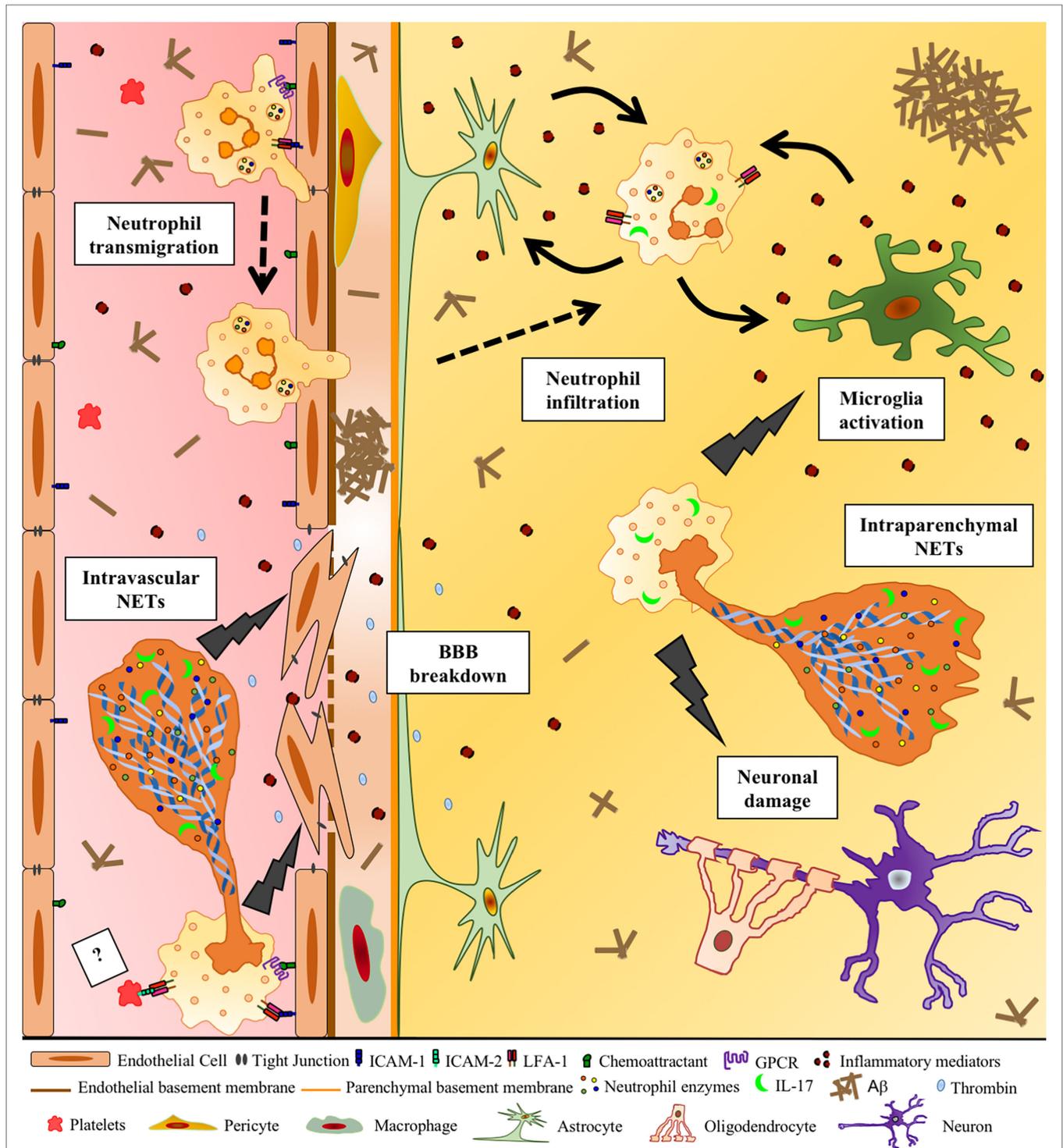


FIGURE 1 | Potential deleterious effects of neutrophil extracellular traps (NETs) in Alzheimer's disease (AD). Aβ and other DAMPs and pro-inflammatory factors lead to the activation of cerebral endothelial cells, inducing the upregulation of adhesion molecules and chemoattractants on these cells. This allows circulating neutrophils to adhere intravascularly via LFA-1 β2 integrin-ICAM-1 binding and then extravasate into the brain. Chemoattractants on the surface of the endothelium may activate β2 integrins via inside-out signaling triggered by G-protein-coupled seven-pass transmembrane receptors (GPCRs), allowing neutrophil arrest. Neutrophils that adhere to the vessel wall produce intravascular NETs, potentially with the contribution of activated platelets, probably through the binding of neutrophil LFA-1 with platelet ICAM-2 (?). DNA, histones, IL-17, active proteases, such as MPO, neutrophil elastase, and cathepsin G, released during NET formation, induce the production of pro-inflammatory cytokines and thrombin, contributing to the loss of blood-brain barrier integrity. Neutrophils inside the cerebral parenchyma are activated by inflammatory mediators potentially released by glial cells and produce NETs, which may further activate glial cells and harm surrounding neurons. NETs could, therefore, represent a mechanism of neutrophil-mediated intravascular and intraparenchymal tissue damage in AD.

to A β peptides increases the expression of cytokine genes, including the gene encoding IL-1 β , which induces NETosis (77). A β may further contribute to intravascular NETosis through its interaction with the receptor for advanced glycation end-products (RAGE) on brain endothelial cells, promoting the generation of ROS and the secretion of pro-inflammatory cytokines (19, 65, 78).

Several studies have shown that platelets exist in a pre-activated state in the blood of an AD mouse model (APP23) and in human AD patients, showing strongly enhanced responses upon stimulation and potentially offering biomarkers for the early diagnosis of AD (79, 80). The exposure of platelets to A β induces platelet activation with the further production of A β and ROS, initiating a vicious circle that enhances vascular inflammation (81, 82). Activated platelets can also trigger the production of NETs, and *in vitro* studies have demonstrated that adding platelets stimulated with agonists, such as ADP, collagen, thrombin, LTB₄, or arachidonic acid to neutrophils causes NET formation (58, 83, 84). Intravascular NETs may be induced by activated platelets interacting with neutrophils *via* Toll-like receptor 4 (TLR4) or LFA-1 integrin, and we speculate that the release of intravascular NETs found in AD mouse models and human AD subjects could be promoted by activated platelets interacting with adherent neutrophils (56, 59, 85). Interestingly, blocking LFA-1 integrin inhibits neutrophil adhesion in the brain microvasculature of AD mice, and LFA-1 deficiency reduces the cognitive deficit and neuropathological changes in animal models of AD (8). However, it is unclear whether intravascular NETosis is less severe in AD mice lacking LFA-1 integrin, and further studies are required to address this issue. Following activation, platelets present the high mobility group box 1 (HMGB1) protein to neutrophils, causing them to produce NETs (83). HMGB1 is a damage-associated molecular pattern (DAMP) released during apoptosis and is involved in leukocyte recruitment and local activation (86). It migrates from the cytoplasm to the surface following platelet activation and interacts with several receptors on the neutrophil surface including RAGE (87). RAGE has been shown to play an essential role in the production of NETs and the treatment of neutrophils with anti-RAGE antibodies prevents NET formation induced either by activated platelets or by recombinant HMGB1, suggesting that A β or HMGB1 inside vessels may interact with neutrophil RAGE leading to NET formation in AD (83). HMGB1 activates neutrophils, induces the production of pro-inflammatory cytokines and upregulates the expression of VCAM-1 and selectins on endothelial cells, potentially amplifying the inflammatory responses in AD (87–90). HMGB1 also potentiates further NET formation by interacting with TLR4 in a ROS-independent manner, contributing to tissue damage during sterile inflammation (91). We, therefore, hypothesize that the release of HMGB1 during the formation of NETs may exacerbate neuroinflammation and that HMGB1-targeted therapy may, therefore, be beneficial in neutrophil-associated inflammatory conditions, such that blocking the activity of this protein may also offer a new therapeutic approach to AD.

Intravascular NETosis promotes blood clotting, and NET release by activated neutrophils triggers both thrombin

formation through the induction of prothrombinase activity and the aggregation of platelets (57, 92, 93). Indeed, during severe sepsis in liver sinusoids, intravascular NETs induce thrombus formation and the partial or total occlusion of capillaries (59). Thrombin expressed by endothelial cells enhances platelet activation, amplifying chronic inflammation and thrombus formation (94). Thrombin levels are elevated in the vessel walls and senile plaques of AD patients, and thrombin inhibitors can reduce vascular inflammation by limiting the cerebrovascular expression of inflammatory proteins and ameliorating cognitive functions in transgenic animal models of AD (71, 95). Thrombin formation triggered by NETosis may, therefore, exacerbate vascular inflammation and neuronal injury in AD. Furthermore, NETs and IL-17 are important constituents of the fresh and lytic thrombus after acute myocardial infarction, and their specific colocalization suggests that they may play a role during thrombus stabilization and growth (96). Platelets express the receptor for IL-17A and IL17-F (IL-17RA), and the incubation of platelets with IL-17A promotes their aggregation (97). Notably, elevated IL-17 serum levels have been reported in a cohort of Chinese AD patients, suggesting it may contribute to platelet activation during AD (98). Altogether, these findings suggest that the formation of NETs together with IL-17 release by neutrophils may activate platelets and exacerbate brain microvessel pathology, contributing to the reduced brain perfusion and NVU alterations observed in AD (99).

Intravascular NETs can also damage the endothelial wall by releasing a mixture of nuclear proteins and proteases, NE, cathepsin G, and metalloproteinases (MMPs). Indeed, NE and MMPs may destroy tight junction components to promote endothelial cell injury (100). As recently reported in patients with SLE, the MMPs normally contained within neutrophil granules are externalized in NETs and can damage the integrity of the vascular wall, and MMP-9 in particular can activate endothelial MMP-2 and trigger apoptosis (101). In addition, NE increases endothelial permeability and the expression of ICAM-1 on endothelial cells and thus can damage the BBB (102). The most abundant proteins in NETs are MPO and histones, and these can also induce endothelial cell death (103, 104). These vasculopathic effects of NETs have been demonstrated *in vivo* by using protein arginine deiminase (PAD) inhibitors to prevent the formation of NETs, which protects against vascular damage and ameliorates the phenotype of SLE (105, 106). In AD mouse models, the expression of MMPs is induced whereas the expression of tight junction proteins is suppressed in microvessels near A β plaques in the brain (107). Furthermore, the treatment of the BBB with oligomeric A β 1–42 *in vitro* increased its permeability and reduced the availability of tight junction scaffold proteins (108). MMP-2 and MMP-9 released by endothelial cells stimulated with A β 1–42 contribute to A β -induced BBB leakage, so the MMPs externalized during NET formation may exacerbate tight junction damage and changes in BBB permeability induced by A β (108).

INTRAPARENCHYMAL NETosis IN AD

Neutrophils invade the brain parenchyma of AD mouse models at the early stage of AD and contribute to the induction of

memory deficit (8, 32). We have recently shown that intraparenchymal migrating neutrophils produce NETs, showing the presence of cells releasing MPO, NE, and citrullinated histone H3 in the parenchyma of mouse models of AD (8). Furthermore, we have also confirmed the formation of NETs in human AD subjects by the co-localization of MPO and citrullinated histones, and of MPO and NE (8). These data suggest that NETs may represent a neutrophil-dependent disease mechanism in patients with AD (**Figure 1**).

Our recent TPLSM data indicate that a significant proportion of the intraparenchymal neutrophils are fully arrested, suggesting the presence of activating stop signals for neutrophils inside the brains of mouse AD models. Neutrophils migrate inside the parenchyma in areas with A β plaques and less neuronal fluorescence, suggesting a role for A β in neutrophil migration inside the parenchyma and in providing stop signals for neutrophils. A β is included in the class of DAMP that are released following non-microbial tissue injury, alerting the innate immune system and activating a wide array of receptors and pro-inflammatory pathways (109).

A β promotes the generation of ROS by activating NADPH oxidase in both human and mouse neutrophils *in vitro*, and several reports, including our own, have demonstrated that ROS production is a necessary step in the formation of NETs (5, 8, 110). These data provide further support for the role of A β in intraparenchymal NET formation and neutrophil-dependent CNS damage during AD. Interestingly, a recent study demonstrated that NET formation in human neutrophils *in vitro* is also driven by the fibrillary form of amyloids from other sources, such as α -synuclein, Sup35, and transthyretin (111). In the same study, the presence of NETs was observed near amyloid deposits in patients with systemic amyloidosis, and NET-associated elastase was able to degrade amyloid fibrils into short toxic oligomeric species, suggesting that amyloid fibrils act as a reservoir of toxic peptides that may promote amyloid disease pathogenesis. We, therefore, hypothesize that A β may trigger NET formation in the AD brain by binding to FPR1 or FPR-like-1 receptors on neutrophils, and the NETs may in turn promote the release of toxic A β species from amyloid plaques, amplifying the inflammatory network in AD.

Intraparenchymal cytokines, such as TNF α , IL-1 β , and IL-8, produced by neural cells may also promote NET formation in extravasated neutrophils during AD (60). Indeed, our recent data suggest that interconnectivity between neutrophils and glial cells may create several feedback loops, amplifying and sustaining their reciprocal activation (**Figure 1**). Accordingly, activated astrocytes and microglia in AD patients secrete pro-inflammatory cytokines, such as IL-1 β , TNF α , and IL-8, as well as ROS into the surrounding brain tissue rich in A β deposits, thus potentially contributing to intraparenchymal NET formation and generating crosstalk with intraparenchymal neutrophils (**Figure 1**) (12, 13, 17). Both TNF α and IL-1 β have recently been implicated in NETosis in rheumatoid arthritis and gout, suggesting they may contribute to NET formation also in AD (112, 113). Moreover, treatment with anakinra (a recombinant IL-1 receptor antagonist) or a monoclonal antibody that blocks IL-1 β caused the partial inhibition of NET formation by neutrophils treated

with synovial fluid from patients with gout, further supporting a role for IL-1 β in the formation of NETs (112). Higher levels of IL-1 β and TNF α are found in the brain and cerebrospinal fluid (CSF) of AD patients, suggesting these molecules may contribute to neutrophil activation and NET formation in AD (114, 115). IL-8 is abundant in the CSF of patients in the prodromal stage of AD and in the brains of AD subjects, suggesting this cytokine may attract neutrophils and contribute to NET formation in the AD brain (116). Our recent data showed that migrating neutrophils produce IL-17 in the cortex and hippocampus of 3xTg-AD mice (8). IL-17 is a cytotoxic cytokine for neurons and may contribute to the loss of BBB integrity and the recruitment of neutrophils in other inflammatory CNS diseases (117, 118). Furthermore, recent studies show that IL-17 contributes to NETosis in rheumatoid arthritis and in a model of acute myocardial infarction, suggesting this cytokine may favor NET formation also in AD (96, 113). These combined data suggest that pro-inflammatory cytokines may act in concert with A β and ROS to promote intraparenchymal NETosis in AD.

Intraparenchymal NETosis may be harmful to neural cells in AD through several mechanisms (**Figure 1**). Indeed, during the generation of NETs, the azurophilic granules of neutrophils release MMPs, in particular MMP-9, and serine proteases such as NE, cathepsin G, and MPO, which can induce tissue damage and aggravate the inflammatory process. Neutrophils are equipped with high levels of MMP-9, stored as the inactive form pro-MMP-9. Recent data indicate that that A β _{25–35} induces the degranulation process following neutrophil activation and the massive secretion of the inactive pro-MMP-9 stored in cytoplasmic granules (119). After neutrophil stimulation, pro-MMP-9 can be converted into active MMP-9 by several of the proteases released by activated neutrophils (including NE), or by A β -stimulated brain cells (119). MMPs are involved in the proteolysis of the extracellular matrix and can thus damage the brain parenchyma (120). NE can also induce the degradation of tissues not only by cleaving extracellular matrix proteins, such as elastin, collagen, and proteoglycan but also by activating MMPs and inactivating the endogenous tissue inhibitors of MMPs (TIMPs) (121). MPO localized within NETs also inactivates TIMPs and thus indirectly enhances the local pathogenic activity of MMPs (122). TIMPs have been localized in neuritic senile plaques and NFTs in the hippocampus and cerebral cortex of human AD brains (123). Furthermore, MMP-9 is expressed in senile plaques, NFTs, and the vascular walls of human AD brains as well as in A β -stimulated astrocytes and activated microglia, and its inhibition is therapeutically beneficial in a transgenic mouse model of AD (124–127). The main constituents of NETs are histones (2). Each histone protein has an N-terminal tail with lysine and arginine residues that extend from the core. These residues can be modified by acetylation, methylation, and citrullination among others, and the latter is associated with PAD4, which plays a central role in the formation of NETs. In PAD4-knockout mice, the absence of histone citrullination prevents the decondensation of chromatin (64, 128). When translocated into the extracellular space, histones function as DAMP, amplifying the sterile inflammation state and showing toxicity to the surrounding cells by activating TLRs and

inflammasome pathways (129, 130). During neurodegeneration in particular, extracellular histones can stimulate the innate immune response and induce apoptosis in neuronal cells. Indeed, a recent study has shown that extracellular histone H1 induces a pro-inflammatory response in microglia and causes neuronal death by activating the mitochondrial apoptosis pathway (131). In AD brains, extracellular histone H1 has been found within amyloid plaques due to its capacity to bind APP and β -amyloid with high affinity (132, 133). The accumulation of extracellular histones may, therefore, accelerate neurodegeneration and perpetuate the inflammatory process in AD.

FUTURE DIRECTIONS

NETosis aggravates several inflammatory and autoimmune disorders. The unexpected recent discovery that neutrophils promote AD pathogenesis in mouse models opened a new area of investigation highlighting the prominent role of circulating immune system cells in AD. The mechanisms of neutrophil-dependent damage in AD are unclear, and the discovery of NETs in mouse models of AD and human AD patients indicates a potential mechanism that neutrophils may use to induce and exacerbate neuroinflammation, by promoting cerebral vasculature dysfunction and parenchymal damage. However, the role of NET components in the induction and perpetuation of neuroinflammation in AD needs to be determined in more detail in further studies.

Neutrophils are key regulators of the immune system because these cells communicate and interact with adaptive immune system cells during infections and chronic inflammatory and autoimmune diseases (33, 134). NETs activate plasmacytoid dendritic cells through TLR9 during viral infections and autoimmune diseases and can mediate the priming of T cells, which requires NET-T cell contacts and T-cell receptor signaling (7, 134, 135). Adaptive immune system cells are encountered in the AD brain, and they may play a role in AD pathogenesis, but it is unclear whether NETs link the innate and adaptive immune responses in AD (21). NET components provide a source of autoantigens, which promote the production of autoantibodies

and stimulate the immune system leading to tissue damage, but it is not known whether NETs are autoantibody targets and whether they generate AD biomarkers (4, 113). Citrullinated proteins in particular bind to autoantibodies, which in association with pro-inflammatory cytokines perpetuate the formation of NETs (113, 136). The presence of citrullinated vimentin and histone H3 has been detected in the hippocampus and cerebral vessels of AD patients, but the role of such proteins in AD is currently unclear (8, 137).

The inhibition of NET formation could offer a novel therapeutic approach to limit the extensive damage caused during AD. Evidence from animal models and human AD patients suggests that the targeting of NET components, such as NADPH oxidase, PAD, and DNase I, may help to prevent NET extrusion and limit tissue damage (138). Indeed, NET-targeted therapy has shown beneficial effects in animal models of diseases, such as SLE, atherosclerosis, and rheumatoid arthritis (105, 113, 138, 139). However, the effect of blocking NET formation in animal models of AD has not yet been demonstrated and further studies are required to determine whether this approach has merit. In conclusion, NETs represent a novel disease mechanism in AD, and targeting their effects during sterile inflammation may provide an additional therapeutic strategy for the treatment of this devastating disease.

AUTHOR CONTRIBUTIONS

The authors equally contributed to this work.

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NETosis in Cancer – Platelet–Neutrophil Crosstalk Promotes Tumor-Associated Pathology

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It has become increasingly clear that circulating immune cells in the body have a major impact on cancer development, progression, and outcome. The role of both platelets and neutrophils as independent regulators of various processes in cancer has been known for long, but it has quite recently emerged that the platelet–neutrophil interplay is yet a critical component to take into account during malignant disease. It was reported a few years ago that neutrophils in mice with cancer have increased propensity to form neutrophil extracellular traps (NETs) – web-like structures formed by externalized chromatin and secreted proteases. The initial finding describing this as a cell death-associated process has been followed by reports of additional mechanisms for NET formation (NETosis), and it has been shown that similar structures can be formed also without lysis and neutrophil cell death as a consequence. Furthermore, presence of NETs in humans with cancer has been verified in a few recent studies, indicating that tumor-induced NETosis is clinically relevant. Several reports have also described that NETs contribute to cancer-associated pathology, by promoting processes responsible for cancer-related death such as thrombosis, systemic inflammation, and relapse of the disease. This review summarizes current knowledge about NETosis in cancer, including the role of platelets as regulators of tumor-induced NETosis. It has been shown that platelets can serve as inducers of NETosis, and the platelet–neutrophil interface can therefore be an important issue to consider when designing therapies targeting cancer-associated pathology in the future.

Keywords: cancer, neutrophil extracellular traps, neutrophils, platelets

TUMOR-INDUCED MANIPULATION OF THE IMMUNE SYSTEM

Cancer development and progression is driven by complex interactions between neoplastic cells and non-malignant host cells. The tumor-promoting effects of the host cells often represent normal, or even essential, physiological functions that have been “hi-jacked” by the tumor microenvironment. A prominent example is platelet activation, which is required for wound healing and to prevent bleeding due to injury, while the same mechanism contributes to disease progression and mortality in individuals with cancer (1). Similarly, cells of the innate immune system that normally serve as an essential defense against infections can be modulated during malignancy to become promoters of disease. This phenomenon has been extensively studied especially for macrophages, where tumor progression is paralleled with a phenotypic switch from a tumor-suppressing classical M1-like subtype to a tumor-promoting M2-like macrophage. These M2 macrophages often represent

the majority of immune cells in a solid tumor and contribute to tumor progression by immunosuppressive and pro-angiogenic mechanisms (2). Novel data indicate that neutrophil function is altered in a similar way during malignant disease. Neutrophils have an indispensable role as a first-line defense to combat infectious disease, a function mediated by phagocytosis and secretion of antimicrobial peptides (3). However, in individuals with cancer, neutrophils may instead become prominent disease promoters, contributing to important steps during tumor progression such as angiogenesis and metastasis (4). In addition to the classical antimicrobial roles of neutrophils mentioned above, formation of neutrophil extracellular traps (NETs) was described approximately a decade ago as a novel defense mechanism during severe bacterial infections (5). NETs are formed when activated neutrophils externalize their chromatin and granular content and form a meshwork of DNA strands that function as a trap for microbes. In fact, a cell death process in neutrophils different from apoptosis and necrosis, and similar to what we today refer to as NETosis, was described already 1996 (6). The initial description of NET formation (NETosis) as a response to bacterial infections has now been followed by reports of NETs in infections caused by viruses and fungi (7–20) but also in sterile inflammation during conditions such as atherosclerosis, diabetes, and systemic lupus erythematosus (SLE) (21–24). Interestingly, NETosis was also detected in individuals with cancer for the first time a few years ago (25, 26), and the consequences are only beginning to emerge. Platelets have been found to play an essential role as inducers of intravascular NETosis in response to lipopolysaccharide (LPS) (27, 28). Conversely, NETs provide a strong activation signal for platelets due to the externalized DNA and associated histones, promoting platelet aggregation and thrombosis (29). This review describes mechanisms behind tumor-induced NETosis with a special focus on neutrophil–platelet interactions in an individual with cancer. Furthermore, consequences of tumor-induced NETosis and possible therapeutic approaches to target NETs in cancer patients will be discussed.

MECHANISMS OF NETosis

During NETosis, activated neutrophils release their chromatin and granular content and form a web-like structure from strands of DNA that functions as a trap for infectious agents in the circulation (30). Secretion of neutrophil-derived proteases, such as neutrophil elastase (NE) and myeloperoxidase (MPO), contributes to a locally elevated concentration of antimicrobial substances and hence enables efficient destruction of pathogens. Both nucleic acids and the associated histones are potent inducers of platelet activation and therefore exert a prothrombotic effect with platelet aggregation and fibrin deposition as a result. So how can neutrophils form these extracellular traps? Current knowledge suggests that NETosis can occur either as a cell death-associated mechanism or in a vesicular-dependent manner where the neutrophil survives and continues to function after NET formation (referred to as “vital NETosis”). In the case where NETosis results in neutrophil death, the suggested process is dependent on chromatin decondensation, degradation of the nuclear membrane, and cellular lysis with associated release of

chromatin and granular contents into the extracellular space. Nuclear decondensation is initiated by epigenetic modifications of histones, citrullination (i.e., arginine converted into citrulline), mediated by the enzyme peptidyl arginine deiminase 4 (PAD4). PAD4 has proven to be required for NETosis to be initiated and neutrophils in PAD4-deficient mice lack ability to form NETs (31, 32). Degradation of the nuclear membrane is driven by NE, which has to be translocated to the nucleus for this purpose (33). Furthermore, isolated neutrophils deficient in MPO fail to form NETs, suggesting that MPO is required for NETosis (34). In contrast to vital NETosis, which has been described as a quick event, the process of lytic NETosis takes several hours to complete (30). In addition, a recently identified process of programmed cell death, necroptosis, was earlier this year implicated as an additional mechanism for NETosis (35). Necroptosis is associated with inflammation and has been suggested to be involved in inflammatory conditions such as Crohn’s disease (36). The mechanism for necroptosis-associated NETosis was shown to depend on activation of the mixed lineage kinase domain-like protein (MLKL) for membrane degradation and subsequent cell death (35). The process of vital NETosis was first described a few years ago (30, 37). During infectious conditions, vital NETosis occurs upon stimulation of TLRs by both gram-negative and gram-positive bacteria, and involves nuclear envelope blebbing and vesicular trafficking of DNA to the extracellular space (37). The process leaves the cell membrane intact and allows the neutrophil to continuously exert its classical function *via* protease release and phagocytosis. Whether both cell death-associated and vital NETosis occur in individuals with cancer is still not clear.

It has been reported that only a fraction of all neutrophils are capable of forming NETs (30). How to distinguish these specific neutrophils with capacity for NETosis is still not clear. It has been suggested that the ability to form NETs is related to aging of the neutrophil, a process paralleled with upregulation of CXCR4 on the cell surface (38). Interestingly, the same study demonstrates that the aged neutrophil population is expanded under pathological conditions. It was recently suggested that the lifespan of a neutrophil may be significantly longer than previously reported and that the average human neutrophil remains in the circulation for more than 5 days (39). Therefore, the population of neutrophils that form NETs may be larger than previously expected. However, the finding of an extended lifespan of neutrophils beyond 1 or 2 days has been questioned (40). A vast amount of studies further support that neutrophils indeed are more heterogeneous than earlier presumed. For example, migration of neutrophils has previously been described as a one-way transfer from the circulation into the tissue. However, several publications now report observations of reversed migration of tissue-resident neutrophils back into the vasculature (41–43). Furthermore, a polarization similar to that of macrophages has been suggested for neutrophils with a division into antitumorigenic neutrophils (N1) and protumorigenic neutrophils (N2) (44). Sagiv and colleagues recently demonstrated that cancer is associated with a switch in neutrophil phenotype towards a low-density neutrophil type with more immature appearance and less lobulated nuclei (45). This subpopulation of neutrophils was suggested to be protumorigenic, as compared to high-density neutrophils with

an antitumorigenic function. How this relates to NETosis was not discussed in the paper. However, low-density neutrophils have previously been isolated from patient with SLE, an autoimmune disease characterized by NETosis (46, 47). These cells were further verified to be highly prone to undergo NET formation (48), suggesting that the protumorigenic neutrophils identified by Sagiv and colleagues are indeed a potential source of NETs. Studies of NETosis in autoimmune disease have suggested a role for proteinase 3 (PR3) in NET induction (49). In small-vessel vasculitis, antineutrophil cytoplasmic antibodies (ANCA), and specifically those directed against PR3, were demonstrated to induce NETosis (50). Whether PR3 stimulation mediates NETosis also in malignant disease is not yet known.

THE ROLE OF PLATELETS IN NETosis

LPS, a component of the cell wall in bacteria, is an inducer of NETosis during infectious disease (27). However, bacteria-derived LPS is not a general cause of NETosis in individuals with cancer, unless the patient suffers from bacterial infection.

So how can a tumor induce NETosis? While some mechanisms for tumor-induced NETosis have been described, there are possibly others that remain to be identified. A summary of identified mechanisms can be found in **Figure 1**. The first report of NETs in cancer appeared a few years ago and demonstrated that presence of a tumor primed neutrophils to undergo NETosis (25). The authors suggested that G-CSF was a critical factor for tumor-induced NETosis in mice with cancer. The importance of G-CSF was recently confirmed in another study, where tumors expressing high levels of G-CSF were demonstrated as more powerful inducers of NETosis than tumors expressing low levels of G-CSF (51). Furthermore, inhibition of G-CSF by injection of anti-G-CSF antibodies efficiently suppressed NET-induced vascular dysfunction in distant organs of mice with mammary carcinoma. It is however likely that additional factors are involved in induction of tumor-associated NETosis. The cytokine IL-8, frequently expressed by various tumor cells, has, for example, been described as a NET-inducing factor and was recently demonstrated to be crucial for tumor-induced NETosis (5, 52).

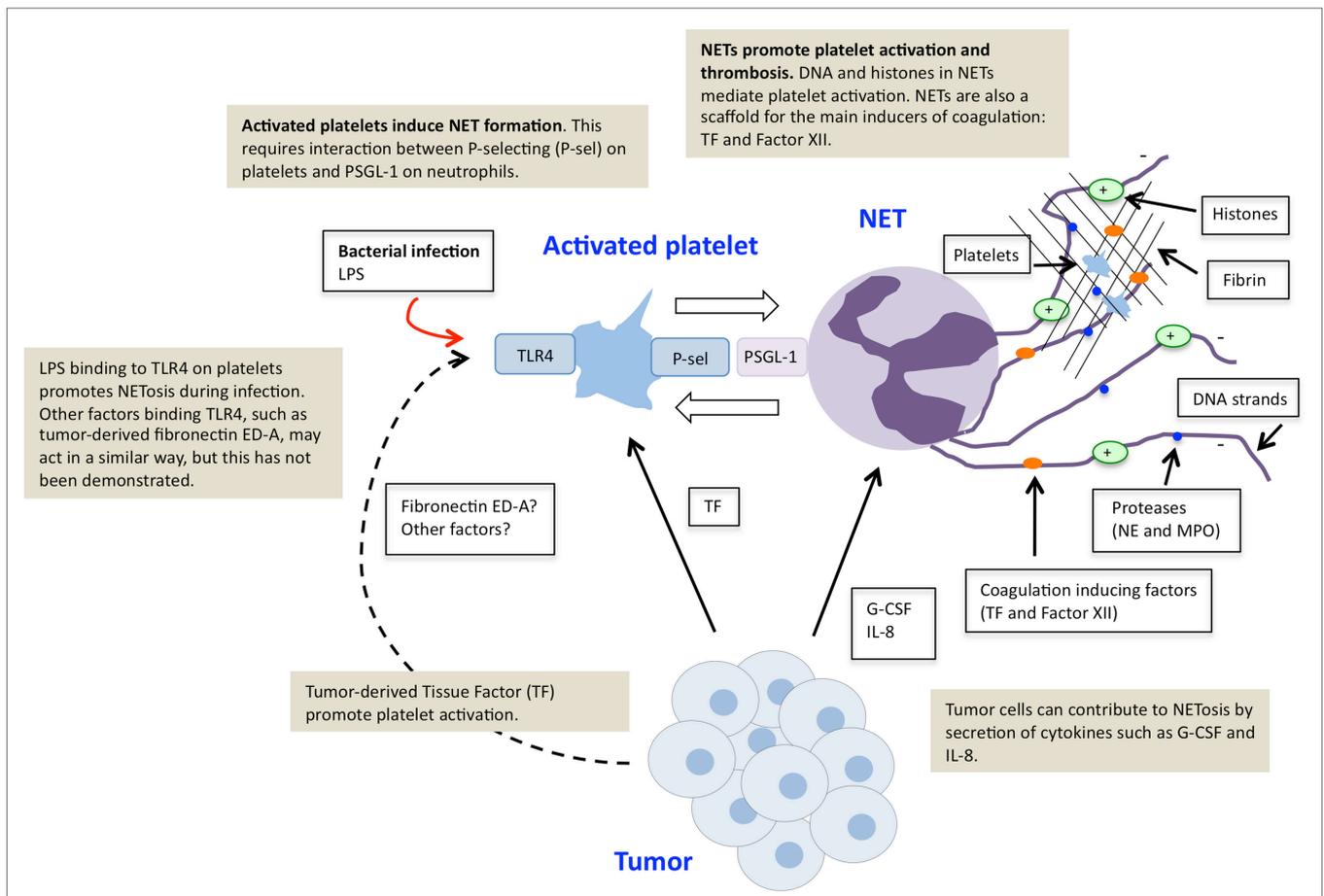


FIGURE 1 | Platelet–neutrophil crosstalk in tumor-induced NETosis. Tumor cells can directly induce NETosis in an individual with cancer by secretion of factors such as G-CSF and IL-8. Tumors furthermore promote platelet activation, for example, by production of tissue factor (TF). Activated platelets function as inducers of NETosis. This effect is mediated via direct binding of P-selectin on activated platelets and PSGL-1 on neutrophils. Stimulation of platelets via toll-like receptor 4 (TLR4), by LPS during infectious disease or tumor-derived factors, possibly fibronectin ED-A, may further contribute to platelet-induced NETosis. NETs further stimulate platelet activation and thrombosis due to externalized chromatin and localization of TF and factor XII.

As mentioned, activated platelets can regulate NET induction, although NETosis can also occur independent of platelet interaction, as exemplified by PMA stimulation. An important interaction for this effect seems to be the binding of P-selectin on activated platelets to PSGL-1 on neutrophils. It was recently demonstrated that platelets from P-selectin-deficient mice failed to induce NETosis, while platelets from mice with increased P-selectin levels were more prone to induce NETs upon co-culture with neutrophils (53). Platelets have previously been described as sensors during infectious disease for the severity of an infection, where LPS binding to TLR4 on the platelet surface is essential to determine whether NETosis should be initiated (27). Although LPS-induced TLR4 activation does not take place as a result of a tumor, it is possible that other tumor-derived factors can activate platelets *via* TLR4. For example, it has been shown that a tumor-associated splice variant of fibronectin, extradomain A (ED-A), can bind to TLR4 (54). Interestingly, studies in mice recently demonstrated that signaling *via* TLR4 on platelets by fibronectin ED-A promotes platelet aggregation and arterial thrombosis (55). Whether this effect is mediated *via* NETosis was not addressed, but the possibility is briefly discussed in the paper. Furthermore, these studies were not performed in a cancer setting, and the relevance of fibronectin ED-A for cancer-associated thrombosis still remains to be determined.

The importance of platelets for NET induction is obviously not a tumor-specific phenomenon. However, it has been known for more than a century that individuals with cancer suffer from increased risk for thrombotic disease – a fatal consequence of enhanced platelet activation (56, 57). The hyperactive state of platelets in malignant disease has been attributed to the fact that many tumors express Tissue Factor (TF), which leads to thrombin formation, coagulation, and platelet activation (58). Enhanced platelet activation in cancer patients does not only contribute to thrombosis but also to malignant progression by promoting processes such as tumor angiogenesis and metastasis (59). The increased platelet activation in cancer patients could therefore be a contributing factor to enhanced NETosis during malignant disease.

OTHER TYPES OF PLATELET-NEUTROPHIL INTERACTIONS

While the specific interplay between platelets and neutrophils in formation of NETs was quite recently discovered, interactions between platelets and neutrophils were described much earlier. Already 50 years ago, the phenomenon of platelets adhering to neutrophils was described and referred to as platelet satellitism (60–64). These platelet–neutrophil complexes were observed in a number of pathological conditions, but their contribution to disease was not clear. Interestingly, a case study from 1975 described platelet–neutrophil aggregation in a patient with invasive prostate cancer, but the cause or significance of the finding was not further explored (65). Today, complex formation between platelets and neutrophils are known to occur and contribute to a wide variety of pathological conditions, such as asthma, ulcerative colitis, sepsis, rheumatoid arthritis, and acute coronary syndrome (66–75). By

which mechanism do platelet–neutrophil complexes form? Initial platelet–neutrophil aggregation is mediated mainly by binding of the surface receptor P-selectin on activated platelets to neutrophil PSGL-1 and results in activation of the neutrophil (76–79). Thereafter, integrin receptors are important for continuous platelet–neutrophil interactions. For example, Gp1b-IX-V and alpha-IIb-beta-3 (GpIIb/IIIa), *via* fibrinogen, mediate binding to integrin alpha-M-beta-2 (Mac-1) on the neutrophil, while integrin alpha-L-beta-2 (LFA-1) on neutrophils can adhere to platelets *via* ICAM-2 (80–83). Platelets also facilitate leukocyte adherence to the endothelium *via* the same interactions, for example, upon damage to the vessel wall when direct adherence of leukocytes to endothelial cells is compromised and platelets function as a bridging factor (81). The ability of platelets to regulate neutrophil function is not limited to NETosis. It has been demonstrated that platelets promote initiation of inflammation by regulating neutrophil crawling, an effect dependent on signaling *via* PSGL-1 (84). Activated platelets can also promote neutrophil degranulation and phagocytosis (27, 85). Moreover, platelet-derived soluble CD40L promotes formation of reactive oxygen species (ROS) in neutrophils, which contributes further to the antimicrobial effect (86). There are also evidence showing that interaction between platelets and neutrophils promote metastasis by formation of an early metastatic niche (87). This study by Labelle et al. demonstrated that granulocyte recruitment to the metastatic site is mediated by platelet-derived CXCL5 induced by contact with tumor cells and signaling *via* the CXCR2 receptor on granulocytes. If the interaction between platelets and granulocytes is blocked with a CXCR2 antibody, metastatic seeding is significantly impaired. This study further highlights the importance of platelets as critical regulators of neutrophil function.

CONSEQUENCES OF TUMOR-INDUCED NETosis

What are the consequences of NETosis in individuals with cancer? The data presented so far suggest that tumor-induced NETosis may be a promoter of cancer-associated pathology. A couple of studies show that NETs may directly contribute to malignant progression. For example, Cools-Lartigue and colleagues showed that infection-induced NETs contribute to metastasis by sequestration of tumor cells in the circulation of mice with cancer (88). This suggests an increased risk for metastasis if cancer patients are affected by infectious disease. Recently, direct cancer-promoting effects were further demonstrated in a study where NETs were suggested to contribute to tumor relapse after surgery in patients with metastatic colorectal cancer. While this study did not address tumor-induced NETosis directly but rather NETosis induced by surgical stress, it still highlights the possibility that NETs could contribute to tumor progression and relapse (89). This finding is in line with an earlier study, suggesting that presence of NETs in tumor biopsies correlated with relapse in patients with Ewing sarcoma (26). Besides direct effects on malignant progression, tumor-induced NETosis further contributes to systemic pathological effects of cancer. For example, NETs have been suggested to promote cancer-associated deep

vein thrombosis (25). Hence, the interaction between neutrophils and platelets in NETosis is not limited to platelet-induced NET formation, but NETs can also stimulate platelet activation – adding yet an important aspect to the complex interplay between platelets and neutrophils. The procoagulant effect of NETs is primarily mediated *via* the negatively charged DNA inducing the intrinsic pathway of coagulation (90) and by histones contributing to thrombin formation (91). Moreover, both TF and factor XII, inducers of the extrinsic and intrinsic coagulation pathways, respectively, can be found in NETs (92–94). Furthermore, it was recently demonstrated that NETosis contributes to impaired vascular function and systemic inflammation in organs that are not sites for tumor growth, such as heart and kidneys, in mice with mammary carcinoma and insulinoma (51). When mice were treated with DNase I to dissolve NETs, vascular function was restored and inflammation abolished. Hypoperfusion of the renal vasculature and associated inflammation are indicators of renal insufficiency – a frequent issue in cancer patients with mortal consequences (95–97). Whether suppression of NETosis could prevent renal insufficiency in individuals with cancer remains to be explored. NETosis was connected to both thrombosis and vascular dysfunction in a study published earlier this year (98). Analysis of blood and post-mortem tissues from ischemic stroke patients revealed that a high number was affected by known or occult cancer and that this could be associated with formation of arterial microthrombi with presence of NETs in various organs. Altogether, these studies suggest that tumor-induced NETosis is connected to poor prognosis in cancer patients. It is however likely that the consequences of tumor-induced NETosis are not limited to those described today, but more reports on this phenomenon should be expected.

THERAPEUTIC TARGETING OF NETosis IN INDIVIDUALS WITH CANCER – WHAT ARE THE OPTIONS?

The role of tumor-induced NETs as potential promoters of malignancy and associated complications, such as thrombosis and systemic inflammation, suggests that therapeutic approaches to suppress NETosis might be beneficial for cancer patients. Several potential strategies could be considered for this purpose. Treatment with DNase I, a strategy to degrade extracellular DNA strands, would be an option to dissolve already formed NETs.

DNase I is already in clinical use for treatment of patients with cystic fibrosis, which indicates its safety as a drug (99). Another option would be to prevent NETosis by inhibition of PAD4, an enzyme required for initiation of NETosis (32). Specific PAD4 inhibitors, with capacity to prevent formation of NETs from both human and murine neutrophils, were recently developed (100). A third alternative approach would be treatment with heparin, which function to destabilize NETs by extraction of histones (29). Heparin has long been used in the clinic for its anticoagulative effects and is therefore well established as a therapeutic method. Based on current knowledge about NET induction described in this review, intervening with the P-selectin/PSGL-1 interaction could be yet a potential therapeutic strategy. An important issue to address to enable clinical use of NET targeting approaches is whether there are risks with NET inhibition. A few studies performed in mice lacking PAD4 and hence unable to form NETs have been published but with various results. While increased susceptibility to bacterial infection was described as a consequence of PAD4 deficiency in one study, other studies reported that mice lacking PAD4 are not more sensitive to infections. Instead, it was suggested that PAD4-deficient mice are protected against septic shock (31, 32, 101). Further research is needed to fully explore the potential risks with therapeutic approaches targeting NETs.

The existing data on tumor-induced NETosis strongly indicate that targeting NETs could be beneficial for cancer patients. NETs, originally identified as a defense against severe infectious disease, seem rather to have a negative influence during malignant disease by promoting mortal processes such as thrombosis, systemic inflammation, and cancer relapse. With this in mind, NETs could provide excellent targets for future anticancer therapies, with capacity to suppress processes contributing to the absolute majority of cancer-related deaths.

AUTHOR CONTRIBUTIONS

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Neutrophil Extracellular Traps in Pulmonary Diseases: Too Much of a Good Thing?

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Neutrophil extracellular traps (NETs) arise from the release of granular and nuclear contents of neutrophils in the extracellular space in response to different classes of microorganisms, soluble factors, and host molecules. NETs are composed by decondensed chromatin fibers coated with antimicrobial granular and cytoplasmic proteins, such as myeloperoxidase, neutrophil elastase (NE), and α -defensins. Besides being expressed on NET fibers, NE and MPO also regulate NET formation. Furthermore, histone deimination by peptidylarginine deiminase 4 (PAD4) is a central step to NET formation. NET formation has been widely demonstrated to be an effective mechanism to fight against invading microorganisms, as deficiency in NET release or dismantling NET backbone by bacterial DNases renders the host susceptible to infections. Therefore, the primary role of NETs is to prevent microbial dissemination, avoiding overwhelming infections. However, an excess of NET formation has a dark side. The pathogenic role of NETs has been described for many human diseases, infectious and non-infectious. The detrimental effect of excessive NET release is particularly important to lung diseases, because NETs can expand more easily in the pulmonary alveoli, causing lung injury. Moreover, NETs and its associated molecules are able to directly induce epithelial and endothelial cell death. In this regard, massive NET formation has been reported in several pulmonary diseases, including asthma, chronic obstructive pulmonary disease, cystic fibrosis, respiratory syncytial virus bronchiolitis, influenza, bacterial pneumonia, and tuberculosis, among others. Thus, NET formation must be tightly regulated in order to avoid NET-mediated tissue damage. Recent development of therapies targeting NETs in pulmonary diseases includes DNA disintegration with recombinant human DNase, neutralization of NET proteins, with anti-histone antibodies and protease inhibitors. In this review, we summarize the recent knowledge on the pathophysiological role of NETs in pulmonary diseases as well as some experimental and clinical approaches to modulate their detrimental effects.

Keywords: neutrophil, neutrophil extracellular traps, NETs, pulmonary diseases, lung infection, respiratory infection, bacteria, viruses

INTRODUCTION

Neutrophils are key players in microbial killing, being the first immune cells to achieve the site of injury or infection (1). Therefore, neutrophils act as the first line of defense against microorganisms through phagocytosis, release of reactive oxygen species (ROS), and degranulation (2). Aside from these traditional mechanisms, neutrophils are also able to extrude DNA lattices,

called neutrophil extracellular traps (NETs), which entrap and facilitate the killing of bacteria, fungi, protozoa, and even viruses (3–8). NETs are composed of decondensed chromatin fibers coated with antimicrobial proteins, such as histones, neutrophil elastase (NE), myeloperoxidase (MPO), and α -defensins (3, 7). Besides being expressed on NET fibers, NE and MPO also regulate NET formation (9). Differently, the participation of NADPH oxidase-derived ROS in NET release seems to be a matter of time of stimulation. While ROS are required to NET generation in time points beyond 1 h after stimulation (10, 11), a very rapid process (5–30 min) of NET extrusion has been reported to be ROS-independent in response to *Staphylococcus aureus* and *Candida albicans* (12, 13). Furthermore, histone deimination by peptidylarginine deiminase 4 (PAD4) is a central step to NET formation (14). Additionally, the release of these DNA threads requires autophagy and activation of p38 MAPK and the Raf-MEK-ERK signaling pathways (15–17). However, it is important to keep in mind that the specific cell components and signaling cascades may vary depending on the stimulus (18).

The primary role of NETs is to prevent microbial dissemination because of its stringy structure, and to kill pathogens due to the high local concentrations of antimicrobial molecules (19). However, these attributes make NETs potentially detrimental to the host. The pathogenic role of NETs has been described for many human diseases, infectious and non-infectious (20), being particularly important to lung diseases. Netting neutrophils in the lung tissue are able to disturb microcirculation and NETs produced in the pulmonary alveoli can expand easily, filling the lungs, as is the case for cystic fibrosis (CF) (19, 21). Therefore, NET formation must be tightly regulated. In this review, we summarize the recent knowledge on the pathophysiological role of NETs in pulmonary diseases as well as some experimental and clinical approaches to modulate their detrimental effects.

CYSTIC FIBROSIS

Cystic fibrosis is a fatal hereditary disorder resulting from mutations in the CF transmembrane conductance regulator (CFTR) anion channel (22). This anion channel is responsible for the transport of chloride ions across the epithelial layer of the airways, which is necessary for the production of thin, freely flowing mucus. Therefore, the lungs of CF patients produce large amounts of thick mucus, leading to an obstruction of the airways and colonization by bacteria (23). Typically, CF infants are rapidly colonized by *Haemophilus influenzae* or *S. aureus*, or both. Over time, *Pseudomonas aeruginosa* represents the main bacterial pathogen infecting CF lungs (23, 24). Due to these frequent infections, there is a massive neutrophil infiltration to the lungs and development of chronic inflammation (25, 26). The chronic and progressive lung disease accounts for morbidity and mortality of CF patients (25).

Cystic fibrosis sputum constituents include DNA, NE, MPO, and other neutrophil proteins (27), as it has been shown that bronchoalveolar lavage fluid (BAL) from CF infants presented high concentrations of DNA, which correlated with neutrophil numbers in BAL (28). However, the great amounts of extracellular

DNA in CF sputum were considered to be from necrotic neutrophils and lung tissues (29). More recently, several studies have demonstrated that NETs and NET-associated proteins are present in CF sputum (30–35). Marcos and coworkers quantified free DNA levels in airway fluid from CF patients and found that those patients with poor pulmonary function presented higher levels of extracellular DNA compared to patients with mild lung disease (36), indicating that the accumulation of NET–DNA in the airways contributes to airflow obstruction in CF. Moreover, analysis of CF sputum samples revealed that elevated levels of macrophage migration inhibitory factor (MIF), a potent pro-inflammatory cytokine, correlated with poor pulmonary function, and MIF was able to induce NET formation (33). Although many of the microorganisms that colonize CF airways have been shown to induce NET formation directly (4, 6, 12, 37, 38), pro-inflammatory cytokines and neutrophil chemokines present in CF lungs are also able to stimulate NET release (30, 33), thus perpetuating the inflammation.

Neutrophil recruitment and NET production in the lungs would be key events to fight against invading microorganisms, but their mission accomplishment is profoundly compromised in CF airways as patients often suffer chronic infections. Together with the failure in killing the bacteria, the excessive release of extracellular DNA accounts for biofilm formation by *P. aeruginosa*, and NETs act as a proinflammatory component of biofilms (39). Furthermore, over the time of infection in CF airways, *P. aeruginosa* is able to acquire resistance to NET-mediated killing (38), probably due to its hypermutability, a well-described mechanism for *P. aeruginosa* adaptation within CF lungs (40–42). In addition, it has been recently demonstrated that sub-inhibitory concentrations of LL-37, a NET component, triggers *P. aeruginosa* mutagenesis in chronic infections (43). Interestingly, *P. aeruginosa* triggers the release of the eicosanoid heptaxilin A3 by lung epithelial cells, which induces neutrophil transepithelial migration and is a natural inducer of NET formation (44, 45). Thus, the excessive release of NETs coated with proteases, together with the colonizing bacteria may worsen pulmonary inflammation and dysfunction. Besides NETs being able to directly induce endothelial and epithelial cell death *in vitro* through histones (46), MPO and NE expressed on NET fibers could exacerbate lung pathology through the destruction of connective tissue and degradation of endothelial cell matrix heparan sulfate proteoglycan (47, 48). Moreover, it has been shown that NE cleaves host proteins at the site of inflammation (49). Additionally, histones are highly cytotoxic to endothelial cells *in vitro* and are lethal in mice (50). Altogether, these findings highlight the need to target the massive NET release in CF.

The current therapy to improve CF symptoms is the administration of recombinant human DNase I (pulmozyme/dornase alpha) (51). DNase inhalation is one of the successful treatments for CF, as it improves lung function and reduces infectious exacerbations (52); however, it is not effective for all CF patients. Therefore, alternative therapeutic options are desired. Dubois and colleagues have demonstrated that DNase administration to CF sputum dramatically increased its elastase activity (53). Thus, the combined administration of DNase and an elastase inhibitor could be useful to avoid the devastating effects of

excessive proteases in CF lungs. There are also candidate drugs to inhibit NET release, such as chloroquine and PAD4 inhibitors, however neither of these molecules has been evaluated in animal models of CF.

ASTHMA

Asthma is a chronic heterogeneous inflammatory disorder of the airways characterized by airway inflammation and reversible airflow obstruction (54–56). Asthmatic subjects present periods of stable condition that alternate with severe episodes of exacerbations, leading to the impairment of lung function (57). Asthma symptoms include recurrent wheezing, coughing, and shortness of breath (55). This very complex disease is caused by multiple environmental factors that act in combination with hundreds of susceptibility genes (55). Asthma has been seen for a long time as an eosinophilic disease (56); however, in recent years, it has become evident that some asthmatics have a prominent neutrophilic inflammation in the lungs (58). Patients with neutrophilic asthma usually present a severe form of the disease that does not respond to the classical treatment with glucocorticoids (59, 60). In addition, glucocorticoid administration to neutrophilic asthmatics could aggravate lung inflammation, since glucocorticoids can prolong neutrophil survival (61). It has been described that neutrophils recruited to the lungs of atopic asthmatic patients generated NETs colocalized with elastase (62). In some patients, the number of neutrophils and NET-releasing neutrophils exceeded the number of eosinophils in the lungs. In this study, Dworski and colleagues also demonstrated that eosinophils infiltrating the airways of atopic asthmatics were able to release eosinophil extracellular traps (EETs), which colocalized with eosinophil granule proteins, such as major basic protein (MBP) and eosinophil cationic protein (ECP). Similar to the first study reporting the release of EETs from viable eosinophils (63), the DNA actively released by eosinophils in asthmatic lungs was from mitochondrial origin, and not nuclear (62). Interestingly, allergen challenge did not increase EET or NET formation in the airways of asthmatic subjects (62). Thus, what would be the role of EETs and/or NETs in the pathogenesis of asthma? And what would be the cause of EET/NET release in asthma? Taking into consideration the high concentrations of proteases anchored in extracellular DNA traps, one can assume that these enzymes could contribute to epithelial and endothelial cell damage, a hallmark of asthma. On the other hand, the formation of DNA lattices could protect the host against possible infections secondary to cell damage. Currently, these and many other questions regarding DNA traps formation in allergic diseases are still open for debate. More recently, it has been demonstrated that eosinophils from asthmatic mice release EETs decorated with eosinophil peroxidase (EPO) with no signs of cell death (64), indicating that DNA release is an active process. In addition, recombinant human DNase treatment of asthmatic mice improves lung resistance and decreases oxidative stress in the lungs, providing a potential antioxidant effect on asthma (65, 66). Accordingly, the combined use of recombinant human DNase therapy together with the current treatments (such as inhaled glucocorticoids) for severe acute asthma may prove effective in

decreasing sputum viscosity, as it has been shown in specific case reports (67, 68).

CHRONIC OBSTRUCTIVE PULMONARY DISEASE

Chronic obstructive pulmonary disease (COPD) is a progressive disorder of the airways characterized by persistent neutrophilic inflammation (69, 70). The disease develops following long-term exposure to external stresses, such as inhaled tobacco smoke (71–73). COPD patients are affected by recurrent bacterial and respiratory viral infections, which represent the main causes of exacerbations in these subjects. Exacerbations are associated with increased upper and lower airway and systemic inflammation (74). Patients with severe COPD present large amounts of airway neutrophils when stable, and these numbers further increase during exacerbations, which may be due to the high expression of neutrophil chemokines and chemokine receptors in airway mucosa (75). Furthermore, NE is expressed in the airway mucosa of COPD patients during severe exacerbations (75) and has a proinflammatory role by inducing the secretion of IL-8 in COPD (76). It is noteworthy that IL-8 is a potent NET inducer (3, 77). These features make COPD lungs more likely to be filled by NETs. Indeed, confocal microscopy analysis has shown that sputum from exacerbated COPD patients presents extracellular DNA, frequently entangled with bacteria (78), characterizing NETs. Moreover, NETs are present not only during COPD acute exacerbations, but also in the lungs of patients with stable disease (79–81). There is a clear correlation between the abundance of NETs in the sputum of COPD patients and disease severity – over 90% of exacerbated COPD subjects presented large amounts of NETs in their sputum compared to 45% of stable COPD subjects. In addition, the very large quantities of NETs directly correlate with the severity of airflow limitation in these patients (79). Why NETs are produced in excess in COPD and what would be the trigger for NET release are unsolved issues. Under physiological conditions, NETs would be degraded by endogenous nucleases and cleared by alveolar macrophages (82). However, COPD subjects present lower numbers of alveolar macrophages (81) and these macrophages are defective in phagocytosis (83), which may explain the persistence of NETs in the airways. Nonetheless, a recent interesting study has shown that the outcome of the interaction of macrophages and netting neutrophils depend on macrophage phenotype. M2 macrophages in contact with netting neutrophils helped to perpetuate an inflammatory response, while M1 macrophages initially released extracellular DNA and thereafter degraded DNA in a caspase-activated DNase-dependent manner (84). These findings highlight a phenotype-dependent mechanism of macrophage regulation of NET release, which reinforces the argument that a prolonged exposure to NETs may favor the development of autoimmunity. The exact role of NETs in COPD pathogenesis is uncertain, but the need for developing novel diagnostic and therapeutic strategies is clear. The treatment for COPD is very difficult, as anti-inflammatory drugs are ineffective. The most successful current treatment for COPD is long-acting bronchodilators, but no therapy reduces the progression or inhibits the inflammation (85). As NETs were implicated

in disease worsening, selective inhibitors of NET formation or NET-associated proteins (such as NE, MPO, histones) may prove valuable in improving the clinical picture of the disease.

TUBERCULOSIS

Tuberculosis (TB) remains a major health problem for humankind. Annually, there are approximately nine million new cases and 1.5 million deaths caused by the disease (86). This chronic bacterial infection is caused by *Mycobacterium tuberculosis* and affects the lungs, promoting huge morbidity and mortality rates (86, 87). *M. tuberculosis* is usually transmitted by tiny droplets from cough or sneeze of an infected subject. Once in the lungs, the bacilli is phagocytosed and killed by alveolar macrophages. However, *M. tuberculosis* developed strategies to survive inside the macrophages. Therefore, the infection develops as a latent infection, inducing granuloma formation in the lung parenchyma. Consequently, the subject remains healthy while harboring dormant bacteria (87, 88). The key factor for the maintenance of latent TB infection is the equilibrium between the bacteria and the host immune response. TB reactivation is achieved when the immune response decreases and cannot restraint bacteria growth, inducing cell death and an increase in granulomatous lesions, as a result of inflammatory cell recruitment (88). Clinical symptoms of TB are caused by a severe impairment of lung function and by substantial morphological alterations in the lung parenchyma (87).

Although macrophages are generally viewed as the main cells involved in harboring *M. tuberculosis*, a growing body of evidence shows that neutrophils are rapidly recruited to infected lungs and can serve as bacterial reservoirs. Additionally, neutrophils were identified as the main immune cell type in sputum and BAL from active TB patients (89). Furthermore, human neutrophils are able to phagocytose *M. tuberculosis in vitro*, but fail to kill the bacilli (90). Neutrophils have been assigned to play both protective and pathological roles during active TB (91–93). As a part of their role in TB pathogenesis, neutrophils have been shown to release NETs coated with NE and histones when stimulated by two genotypes of *M. tuberculosis* (H37Rv and *M. canettii*). NETs were able to trap mycobacteria but not to kill them (94). This lack of killing ability of NETs may favor lung destruction in active TB. Another study has found matrix metalloproteinase-8 (MMP-8) expressed on NET fibers induced by *M. tuberculosis in vitro*. In addition, induced sputum from TB patients had increased amounts of NETs compared to healthy subjects and MMP-8 secretion correlated to lung tissue destruction in these patients (95). The effect of *M. tuberculosis* on NET induction might be mediated by the early secretory antigen-6 (ESAT-6), a protein secreted by *M. tuberculosis*, responsible for the escape of mycobacteria from phagosome to cytoplasm of cells (96), as ESAT-6 induces the production of NETs colocalized with MPO (97). ESAT-6 is also secreted in large quantities in the extracellular space and therefore can interact with immune cells to stimulate them and facilitate the maintenance of chronic inflammation in the lungs of TB patients (98). Importantly, neutrophils release high levels of calprotectin (S100A8/A9) within lung granulomas of patients with active TB (99), which are constituents of NETs

(100). The release of calprotectin in TB could be related to NET formation, as neutrophil cytoplasmic proteins can attach to DNA fibers before being released. Urban and coworkers have shown that calprotectin can be released from neutrophils in two ways: bound to NETs and unbound (100). This could be the case for calprotectin release in the lungs of TB subjects; however whether *M. tuberculosis* induces the formation of NETs expressing calprotectin remains to be determined.

Tuberculosis is a curable disease, although the treatment is difficult, since it can take several months (6–9 months) and has different drug regimens. Currently, the first line anti-TB drugs include isoniazid, rifampin, ethambutol, and pyrazinamide, among others when necessary, according to CDC (Centers for Disease Control and Prevention – <http://www.cdc.gov/tb/topic/treatment/>). Moreover, new therapies aiming to improve the treatment outcomes, shorten the duration of treatment, and reduce lung pathology in TB patients were described (101). However, no therapeutic approach aimed to specifically regulate the deleterious effects of NETs in TB lungs was reported.

BACTERIAL PNEUMONIA

The most common type of bacterial pneumonia is community-acquired pneumonia (CAP). CAP remains a burden worldwide, being responsible for approximately 3.5 million deaths annually (102). A total of 20–60% of CAP patients require hospitalization due to disease severity, including children under age 5 years (102, 103). The etiology of CAP is variable, depending partly on the diagnostic tools used in the population studied. Among all bacteria, *Streptococcus pneumoniae* (*S. pneumoniae*) is the most frequently identified cause of CAP, with high morbidity and mortality rates, but *H. influenzae* is also an important etiologic agent of CAP (102, 104).

Once bacterial infection is established in the lungs, neutrophils are massively recruited to the infection site, inducing a prominent inflammatory response. The clinical outcome in CAP depends on the balance between the inflammatory response and pathogen clearance (102). In this sense, neutrophils actively producing NETs during CAP might lead to potential collateral damage to the lungs. Indeed, three different strains of *S. pneumoniae* (serotypes 3, 4, and 19F) were able to induce pulmonary NET formation in mice, which correlated with the histopathologic severity. In addition, the pneumococcal capsule directly contributes to excessive NET release that paralleled with pneumonia severity in mice (105). The mechanism of NET induction by *S. pneumoniae* seems to be mediated by the pneumococcal protein α -enolase, which binds to myoblast antigen 24.1D5 on neutrophil surface and stimulates NET generation (106). However, *S. pneumoniae* appears to have evolved strategies to counteract NET-mediated killing. In an elegant study, Beiter and colleagues have demonstrated that *S. pneumoniae* expresses EndA, a membrane-localized endonuclease able to degrade NETs *in vitro* and to promote spreading of bacteria from the upper airways to the lungs and from the lungs to the bloodstream of mice. Additionally, mutant bacteria lacking EndA infect the upper airways but fail to disseminate to the lungs and bloodstream (107). Moreover, EndA is secreted into the culture medium during pneumococcal cell

growth and rapidly dismantle DNA in NETs, being required for full virulence of *S. pneumoniae* during lung infection (108). Corroborating with these studies, streptococcal endonuclease has been previously implicated in disease progression (109). Besides EndA, streptococcal cells hold other important mechanisms to protect them from NET trapping and killing, such as a positive charge on their surfaces as a result of capsule expression and lipoteichoic acid D-alanylation (110). Thus, it seems that NETs released during *S. pneumoniae* infection function only to damage lung tissue, instead of having a bactericidal activity. The evidence that NETs released in response to bacterial infections can trap and inactivate viruses (8, 111) points out the utmost importance of NETs during co-infections *in vivo*. On the other hand, secondary pneumococcal infection following primary influenza intensified NET formation, but NETs did not show any bactericidal activity, only worsening lung pathogenesis (112). Altogether, these findings suggest that the nature of NET trigger is fundamental to the clearance of subsequent infections.

Non-typeable *H. influenzae* (which lacks a capsule) is an important cause of pneumonia, mainly in subjects with chronic bronchitis and COPD (113), and the persistence of NETs could worsen lung inflammation in these subjects. Viable and heat-killed *H. influenzae* induces NET release *in vitro*, in a mechanism possibly mediated by lipooligosaccharide binding to TLR-4 and Myeloid Differentiation Primary Response (MyD)-88, an adaptor protein necessary to TLR-4 signaling. Interestingly, bacteria are not killed by NET proteins and survive within NETs (114). Accordingly, it has been recently demonstrated that these bacteria evolved to express specific molecules, peroxiredoxin–glutaredoxin and catalase, which allow them to resist to host oxidants and to survive within NETs *in vivo* (115). In addition, non-typeable *H. influenzae* populations survive in biofilm communities in the airway surface, and NETs constitute an integral part of these biofilms (116). Astoundingly, it has been reported a fatal case of non-typeable *H. influenzae* infection with severe pneumonia and bacteremia in an adult found to have large amounts of NETs expressing NE and histone H3 in his sputum (117). This case highlights the association between excessive NET generation and severe respiratory infection and sepsis. More recently, it has been shown that besides NETs, non-typeable *H. influenzae* is also able to induce macrophage extracellular traps (METs) expressing MMP-12 (118). MMP-12 has been implicated as a key factor for protease imbalance and emphysema. Therefore, the release of METs together with NETs may have a detrimental role during emphysema, pneumonia, and COPD. Importantly, DNase was effective to dismantle non-typeable *H. influenzae*-induced MET and NET formation (118), which could be used as a short-term adjunctive therapy to avoid the injurious effects of these extracellular traps and associated proteases during pneumonia and other lung diseases.

RESPIRATORY SYNCYTIAL VIRUS BRONCHIOLITIS

Respiratory Syncytial Virus (RSV) is the leading cause of acute bronchiolitis in children under age 2 years (119). Throughout

the winter, RSV causes a significant number of hospitalizations, resulting in a huge burden to communities worldwide (119, 120). Due to the high infectivity of RSV, almost 70% of all children are infected with the virus during the first year of life, and by age 3, practically all children will have experienced at least one infection with this virus (121, 122). The clinical symptoms of RSV bronchiolitis include labored breathing, coughing, and wheezing (123). Microscopically, there is a massive neutrophil recruitment to the airways of infected children – these cells comprise for approximately 80% of infiltrated cells (124–127). Once in the airways, RSV is able to activate neutrophils, inducing degranulation and IL-8 secretion (128), and also to inhibit neutrophil apoptosis, through phosphoinositide 3-kinase (PI3K) and nuclear factor- κ B (NF- κ B)-dependent mechanisms (129). This body of evidence suggests that neutrophils may play a significant role in disease pathogenesis.

Aside from the mechanisms mentioned above, we have recently demonstrated that RSV particles and one of its membrane-bound glycoproteins are capable of inducing NET formation by human neutrophils (130). RSV Fusion protein mediates the fusion of virus with the host cell and it is essential for viral replication both *in vivo* and *in vitro* (131), being considered the primary target for vaccine and antiviral drug development. RSV F protein induces the release of NETs coated with MPO and NE through Toll-like receptor (TLR)-4 activation. Moreover, F protein stimulates ROS generation and MAPK phosphorylation, and these signaling pathways are necessary to F protein-induced NET formation (130). Data in the literature regarding the role of NETs in viral diseases are conflicting (132). We hypothesized that the excessive production of NETs could fill the lungs and impair lung function, worsening inflammation in young children and babies affected by RSV infection. Indeed, analysis of bronchoalveolar fluid cytology samples from children with severe RSV lower respiratory tract infection revealed the presence of NETs expressing NE and citrullinated histone 3 (citH3) (133). Furthermore, the infection of calves with bovine RSV induced an extensive release of NETs colocalized with dense cellular plugs containing shed epithelial cells and large amounts of neutrophils, which obstructed the airways (133). These recent studies indicate that NETs contribute to the airway obstruction and immunopathology observed in children and animals infected with RSV.

Despite extensive research efforts, there is no RSV vaccine currently available. Nevertheless, monoclonal antibodies targeting the RSV fusion protein have been developed and they passively protect against RSV challenge in an animal model and reduce the severity of infection in premature and newborn babies (134, 135). However, the humanized monoclonal antibody against RSV F protein is only used in high-risk groups, such as preterm infants and those suffering from cardiovascular diseases or immunosuppression (134). In addition, ribavirin is an antiviral drug used to treat severe RSV bronchiolitis due to its anti-replicative activity, but it presents a high cost and is administered only to high-risk infants (136). Moreover, the use of recombinant human DNase in the management of severe RSV bronchiolitis has been previously reported. The administration of nebulized DNase to young babies with complicated bronchiolitis was able to immediately improve

the clinical signs and chest radiograph, and even led to the resolution of atelectasis (137, 138). In contrast, in infants with mild RSV bronchiolitis, recombinant DNase therapy did not reduce the length of hospital stay or the duration of supplemental oxygen (139). Thus, DNase seems to be a useful therapeutic option in the treatment of infants who develop atelectasis due to severe RSV bronchiolitis.

INFLUENZA VIRUS INFECTION

Influenza A virus is responsible for regular outbreaks, whose severity may vary among the population. While the influenza pandemic that started with the Spanish flu in 1918 killed approximately 50 million people worldwide, the pandemic influenza A H1N1 2009 virus has affected more than 214 countries and caused nearly 18,449 deaths (140, 141). The clinical features of influenza infection include fever and upper respiratory symptoms, such as cough, runny nose, and sore throat (141). To date, there is little information about clinical complications of influenza A infection, but they appear to be similar to those of seasonal influenza, including sinusitis, otitis media, pneumonia, bronchiolitis, seizures, toxic shock syndrome, and secondary bacterial pneumonia with or without sepsis. Among subjects with high risk for complications are those at extremes of age and those with pre-existing medical conditions (141).

The characteristic feature of acute lung inflammation following influenza virus infection is the excessive infiltration of neutrophils in the lungs (142, 143), and CXCR2 seems to be the major receptor mediating neutrophil recruitment during this infection (144). Neutrophils have been demonstrated to play both protective and detrimental roles during influenza virus infection (143, 145, 146). Among the harmful roles played by neutrophils is the excessive production of NETs in the lungs of animals infected with influenza A H1N1 virus. NETs expressing histones and MMP-9 were found entangled with alveoli, causing increased alveolar capillary damage and obstruction of the small airways, thus confirming the link of these DNA lattices with lung damage (146). Furthermore, NET formation stimulated by influenza A infection is dependent on histone deimination by PAD4 (147). In addition, NET release induced by influenza virus is potentiated by the cathelicidin LL-37 (148), which has been shown to facilitate the formation of NETs (149). Paradoxically, the antimicrobial protein expressed on NETs, α -defensin-1, is able to directly inhibit influenza replication through the inhibition of protein kinase C (PKC) in infected cells (150); however the expression of α -defensins on NETs induced by this virus has yet to be demonstrated. The expression of α -defensins on NETs could inactivate the virions sequestered in NET fibers and consequently prevent them from reaching the target cells in the lungs. Thus, although antimicrobial proteins expressed on NETs have the ability to inactivate the virus and to prevent spreading, they are also able to inflict damage to host cells and tissues due to their cytotoxic properties.

Currently, influenza treatment relies on the administration of two groups of antiviral drugs, the adamantanes and neuraminidase inhibitors. Zanamivir and oseltamivir are neuraminidase

inhibitors active against both influenza A and B, and are approved for the prevention and treatment of influenza in the United States. Supportive care of uncomplicated cases of influenza includes administration of fluids and rest (141). To date, there is no study describing the effect of DNase treatment on the outcome of influenza infection in animal models.

TRANSFUSION-RELATED ACUTE LUNG INJURY

Transfusion-related acute lung injury (TRALI) is a serious complication of blood transfusion (whole blood or blood components) that develops within 6 h of transfusion and is characterized by hypoxemia, respiratory distress, and pulmonary infiltrates (151, 152). Currently, TRALI is the most important cause of transfusion-related morbidity and mortality (152). Histological analysis revealed lung edema, capillary leucostasis, and massive neutrophil infiltration (153). TRALI development requires the presence of antileukocyte antibodies in the transfused product, and antineutrophil antibodies have been linked to the most severe cases of TRALI (154). These antibodies activate recipient's neutrophils, inducing their sequestration in the pulmonary capillaries and consequently tissue injury (155).

In an elegant study, Thomas and coworkers have found NET biomarkers (DNA, nucleosomes and MPO) in the serum of patients with documented TRALI (156). In addition, in a fatal case of TRALI neutrophils with decondensed nuclei were detected in lung vessels together with abundant extracellular histones and MPO (157). In a mouse model of TRALI, DNA streaks colocalizing with citrullinated histone H3 were found in alveoli outside blood vessels (156). Moreover, platelets also accumulate in the lungs of mice with TRALI, being required for injury development (158). In this model, platelets were shown to induce NET formation during TRALI (157). As a vicious cycle, histones expressed on NETs may activate platelets (159), which in turn induce further NET release, promoting coagulation and thrombi formation in the lungs. Accordingly, the pretreatment of mice with a histone-blocking antibody decreased lung edema, lung vascular permeability, and even mortality. This treatment also reduced NET generation detected in plasma, indicating that extracellular histones may help to spread NETs in the body (157). Furthermore, intranasal administration of DNase provided several benefits to mice undergoing TRALI, such as improvement of blood oxygenation, reduction in lung edema and vascular permeability, impairment of NET formation, and platelet sequestration in the lungs (156, 157). These studies support the argument that NETs are formed and play a critical role in the pathogenesis of TRALI and may be a promising target for therapeutic approaches.

MECHANICAL VENTILATION

Mechanical ventilation is a supportive intervention and a key feature of intensive care for patients with acute respiratory failure, including those with severe RSV bronchiolitis, pneumonia, or influenza infection (160, 161). However, it can be potentially

injurious to the ventilated lung, inducing the so-called ventilator-associated lung injury (VALI), which contributes to morbidity and mortality in those patients (162). Furthermore, animal models of acute lung injury have been developed and characterize an experimental insult to a normal lung and therefore were named ventilator-induced lung injury (VILI) (162).

Neutrophils have been implicated as central cells in the pathogenesis of both VALI and VILI. It has been described that the early phase of VILI involves the release of several pro-inflammatory cytokines and chemokines, whereas the late phase is characterized by the infiltration of a lung-margined neutrophil pool (163, 164). However, more recently Choudhury and coworkers demonstrated that injurious mechanical ventilation induced a prominent neutrophil recruitment to the lung at the very early stage of VILI, before the development of physiological signs of lung injury. The infiltration of neutrophils in the course of VILI was dependent on L-selectin engagement but independent of CD18 (165), indicating that immune mechanisms mediate neutrophil recruitment and activation during mechanical ventilation. Moreover, lung-derived soluble mediators appear to have a pathogenic role in an isolated perfused lung model of VILI (166). In line with this evidence, the chemokine receptor CXCR2 and its ligands, CXCL1 (KC) and CXCL2/3 (MIP-2), were shown to play a significant role in mediating neutrophil recruitment and promoting lung inflammation in VILI (167). Accordingly, short periods of mechanical ventilation in preterm infants induce an overproduction of the pro-inflammatory cytokines TNF and IL-1 β , neutrophil chemokines IL-8 and MCP-1, and MMP-9 (168, 169). These inflammatory mediators may work together to induce a massive neutrophil infiltration to ventilated lungs and to stimulate NET release in response to mechanical ventilation in those patients. So far, IL-8, TNF, and IL-1 β were shown to promote NET release in different experimental settings (10, 170, 171). In fact, excessive NET formation has been recently implicated in the pathogenesis of VILI. A double-hit model of intratracheal LPS challenge followed by high tidal mechanical ventilation induced a prominent lung injury in mice, with high amounts of NETs, decreased lung compliance and release of pro-inflammatory cytokines (172). The mechanism of NET formation during VILI seems to rely on the simultaneous engagement of G protein-coupled receptors (GPCR) and Mac-1 (CD11b), by the platelet-derived CCL5/CXCL4 heterodimer and a β 2-integrin ligand, respectively (173). Surprisingly, these two studies showed opposing results regarding the role of NETs during VILI. Rossaint and coworkers found that DNase treatment of mice after induction of VILI was protective, as treated mice showed an improved gas exchange and reduced NET markers in the blood; whereas Yildiz and colleagues did not find a significant impact of DNase treatment on lung injury induced by VILI. There is at least one possible explanation for these differences: in the study of Yildiz and colleagues, the lungs of mice were already filled with neutrophils at the early stage of VILI due to LPS instillation, which could not be counteracted by DNase. Whereas in the study of Rossaint and coworkers, neutrophils infiltrated the lungs in the course of VILI, in this case a sterile inflammation. Although the outcome of DNase treatment in VILI is an issue for debate, there is no

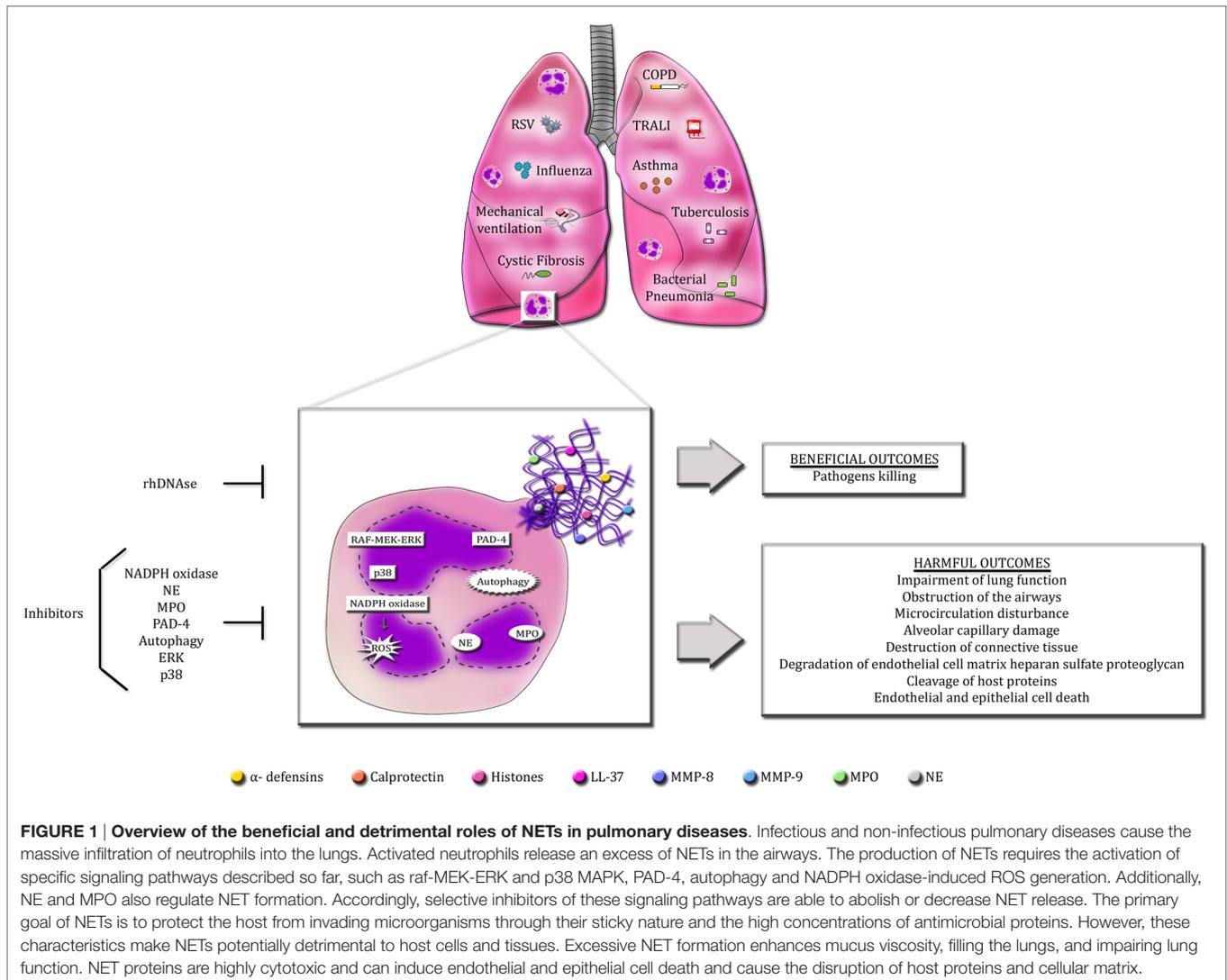
doubt that excessive NET formation accounts for the pathogenesis of acute lung injury.

OTHER PULMONARY DISEASES AND NETS

Besides the pulmonary diseases aforementioned, there are other disorders or syndromes affecting the lungs, in which NETs may play harmful roles as well.

Acute lung injury following severe sepsis is a common clinical consequence with significant morbidity and mortality rates (174), as the lung is the most sensitive target organ during systemic inflammation (175). Czaikoski and collaborators have recently shown that NETs are produced systemically in mice with cecal ligation and puncture (CLP) model of sepsis. The excessive release of NETs was directly correlated to heart, liver, and lung injury, as rhDNase plus antibiotics treatment of septic mice drastically decreased organ damage (176). Additionally to extracellular DNA measurement, NETs were observed in alveolar spaces and pulmonary capillaries of septic mice (177). Furthermore, higher concentrations of cell-free NETs were present in the serum of septic patients who developed severe acute respiratory distress syndrome (ARDS) compared to healthy controls (176), extending the experimental observations in mice to the clinical setting. Mechanistically, platelet TLR-4 is essential for NET induction within hepatic sinusoids and pulmonary capillaries of septic mice (178). Interestingly, NETs retained their integrity under flow conditions and were able to trap bacteria in septic blood. Therefore, platelets may serve as a platform for neutrophil activation and NET production, which can trap and kill pathogens but also induce disseminated organ injury during severe sepsis (178).

Another lung disorder featuring neutrophil-induced injury is interstitial lung disease (ILD). Actually, ILD are a group of diffuse parenchymal lung disorders characterized by pulmonary fibrosis. ILD can be frequently associated with a specific environmental exposure or an underlying connective tissue disease (179). Activated neutrophils were found increased in BAL from patients with idiopathic pulmonary fibrosis and were associated with early mortality (180). Interestingly, patients with ILD complications due to autoimmunity showed elevated levels of circulating cell-free NETs and plasma LL-37 (a NET component), together with a decreased DNase activity (181), suggesting that the prolonged exposure to NETs is involved in the pathogenesis of ILD. *In vitro*, NETs have been demonstrated to promote the activation of lung fibroblasts and differentiation into myofibroblast phenotype. Moreover, these fibrotic effects were significantly decreased after degradation of NETs with DNase (182). Consistently, these findings were supported by the detection of NETs in close proximity to alpha-smooth muscle actin-expressing fibroblasts in biopsies from patients with fibrotic ILD (182). This effect is very likely to be mediated by NE, since NE directed both lung fibroblast proliferation and myofibroblast differentiation *in vitro* (183). In addition, a NE inhibitor attenuated pulmonary fibrosis induced by bleomycin in mice via inhibition of TGF- β 1 and inflammatory cell recruitment to the lungs (184). Altogether, these studies point to a key role of NETs in the development of ILD of different etiologies.



CONCLUSION

Neutrophil extracellular traps formation by activated neutrophils has a crucial role in host defense against microorganisms, as deficiency in NET release or dismantling NET backbone by bacterial DNases render the host susceptible to disseminated and lethal infections (107, 185). Moreover, aggregated NETs have been shown to limit sterile inflammation by degrading cytokines and chemokines via serine proteases (186). However, an excess or persistence of NET release is potentially injurious to host organs and cells, leading to worsening or perpetuation of many diseases. The pathogenic effects of excessive NET production is especially important in pulmonary diseases due to lung architecture itself, which may favor the spreading of DNA fibers, consequently enhancing tissue damage and impairing lung function (Figure 1). The mechanisms underlying NET production and the boundaries between the beneficial and detrimental effects of NETs during disease state are still to be unveiled. To date, recombinant human DNase is the only treatment targeting

NETs approved for a small number of pulmonary disorders. Nevertheless, a long-term DNase therapy presents side effects to patients. Hence, the quest for an ideal therapy targeting NETs and its associated proteins continues to be a challenge for scientists around the globe.

AUTHOR CONTRIBUTIONS

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

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The Emerging Role of NETs in Venous Thrombosis and Immunothrombosis

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Venous thrombosis (VT), a leading cause of morbidity and mortality worldwide, has recently been linked to neutrophil activation and release of neutrophil extracellular traps (NETs) *via* a process called NETosis. The use of various *in vivo* thrombosis models and genetically modified mice has more precisely defined the exact role of NETosis in the pathogenesis of VT. Translational large animal VT models and human studies have confirmed the presence of NETs in pathologic VT. Activation of neutrophils, with subsequent NETosis, has also been linked to acute infection. This innate immune response, while effective for bacterial clearance from the host by formation of an intravascular bactericidal “net,” also triggers thrombosis. Intravascular thrombosis related to such innate immune mechanisms has been coined immunothrombosis. Dysregulated immunothrombosis has been proposed as a mechanism of pathologic micro- and macrovascular thrombosis in sepsis and autoimmune disease. In this focused review, we will address the dual role of NETs in the pathogenesis of VT and immunothrombosis.

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INTRODUCTION

Neutrophils (PMNs) have frequently been touted as the *pawns* of the immune system. As our knowledge of immune system grows and as our techniques for evaluating dynamic cell populations improve – we are learning that this could not be further from the truth. While a PMNs principle function remains as a key player in the front line of innate immunity and host defense against bacteria, they are proving to have a multifaceted role in coagulation and have also been implicated as major contributors in the pathophysiology of many systemic illnesses.

Until the early 2000s, the associations between PMN activation and systemic disease had not been well understood; but in March of 2004, Brinkmann et al. published a landmark study in *Science* (1), where they described a fragile fibrillar material extruded from PMNs in the presence of lipopolysaccharide (LPS) by transmission electron microscopy (TEM). In actuality, these fragile fibers were decondensed chromatin and DNA, as they stained strongly for DNA and histones, they were resistant to proteases, and they disappeared upon instillation of DNase. Bacteria were found to colocalize with the extruded DNA *in vivo* in a rabbit model of shigellosis and in human specimens of acute appendicitis. In summation, they demonstrated that these large webs of DNA trap bacteria and allow adjacent or connected PMNs to drive bactericidal activity with proteases and reactive oxygen species. Brinkmann et al. coined these nuclear extrusions “neutrophil extracellular traps” or NETs.

Since that time, there has been a flurry of exciting new work in the field of NET formation (NETosis). NETosis has been demonstrated to be a distinct form of cell death outside of necrosis and apoptosis (2). Also, more interestingly, NETs have been indicted in the pathophysiology of many systemic diseases, including venous thrombosis (VT) (3), sepsis (4, 5), trauma (6), cancer-related thrombosis (7), and autoimmune diseases (8–12). Despite the apparent widespread influence of NETs on disease, there remains a common theme throughout that NETs drive micro- or macrovascular thrombosis leading to ischemia and further injury (13, 14).

In this article, we will review the role of NETs in pathologic thrombosis. Specifically, we will review the findings of NET pathophysiology in murine models of VT, NETs in primate models and human studies of VT, and NETs in immunothrombosis.

NETs IN MURINE VT MODELS

Murine models have been essential to our understanding of the role of NETosis in the pathophysiology of thrombosis. PMNs were first shown to be essential for immune-mediated microvascular thrombosis in a murine model of glomerulonephritis, in which CD11b^{-/-} or PMN-depleted mice were resistant to glomeruli thrombosis and renal failure (15). At that time, it was not widely recognized that NETs contributed to thrombosis; however, this changed in 2010, when Fuchs et al. showed that NETs caused platelet adhesion, activation, and aggregation (3). Stimulation of platelets with purified histones was sufficient for aggregation, and interestingly, DNase and heparin dismantled the NET scaffold and prevented thrombus formation. Brill et al. later demonstrated that NETs are principle effectors in an IVC stenosis model (16). In mice with uninterrupted IVC side-branches, levels of extracellular DNA increased in plasma 6 h after thrombus initiation. Citrullinated histone H3 (CitH3), an element of NETs' structure, was present in thrombi and was frequently associated with the Gr-1 antigen. Furthermore, immunofluorescent staining of thrombi showed proximity of extracellular CitH3 and von Willebrand factor (vWF), a platelet adhesion molecule crucial for thrombus development in this particular model.

Neutrophils, monocytes, and NETs have also been found to affect the clotting cascade in murine models of thrombosis (17–20). For example, myeloid cells roll along the venous endothelium in a P-selectin-dependent manner and produce thrombogenic tissue factor (TF) in the IVC stenosis model (17). TF, then contributes to thrombin generation and extensive fibrin deposition along the vein wall. Despite this finding, TF alone was inadequate for thrombus propagation. Neutropenia, genetic ablation of Factor XII, and disintegration of NETs were all protective against thrombus propagation. Later, activated PMNs within the fibrin matrix were found to produce NETs that associate with secreted Factor XII, activate the intrinsic pathway, and lead to thrombus extension (17, 21). However, these conclusions were questioned, as during the same year, TF was also found to be secreted by PMNs in an inflammatory signaling and autophagy-dependent manner, with adherence to extruded NETs, activation of the extrinsic pathway, and eventual propagation of the thrombus

(18, 22). Regardless, the presence of PMNs and/or monocytes at the endothelial interface has long been assumed to have a major role in VT and now appears unquestionable (17, 18, 23, 24).

Many proteins have been implicated in contributing to NETosis and thrombosis. In 2013, Martinod et al. demonstrated that the enzyme peptidyl arginine deiminase 4 (PAD4) – an enzyme essential for the citrullination and decondensation of chromatin was – not only essential for NET formation but also important for thrombus formation in the IVC stenosis model of murine VT (25). PAD4^{-/-} mice formed IVC thrombus in less than 10% of cases of IVC stenosis at 48 h compared to 90% in C57BL/6 controls (25). This antithrombotic tendency was rescued in the PAD4^{-/-} mice with adoptive transfer of wild-type PMNs. Conversely, in a stasis IVC ligation model of VT, either preemptive administration of DNase to wild-type mice or PAD4^{-/-} mice did not have an effect on thrombogenesis, suggesting a model-dependent effect (19).

Other proteins that have been implicated in NET formation and NET-associated thrombosis include cathepsin G, serine proteases, PMN elastase, P-selectin, high-mobility group box protein 1 (HMGB-1), platelet glycoprotein Ib, integrin beta-2, vWF, and platelet factor 4. Pharmacological inhibition or genetic deletion of cathepsin G increases mouse bleeding time, decreases platelet activation, and decreases circulating neutrophil–platelet conjugates (26). While the serine proteases and PMN elastase have been implicated in pathologic thrombosis and have been found to colocalize with NETs (27), it was recently demonstrated that PMN elastase is neither necessary for NET formation in mice nor essential for IVC thrombosis in murine IVC stenosis model (28). Indeed, it is likely that the prothrombotic effect of the serine proteases is better explained by their inhibition of tissue factor pathway inhibitor (TFPI) (29). Signaling through P-selectin by P-selectin glycoprotein ligand 1 (PSGL-1) is known to assist in PMN migration to sites of injury or inflammation (30), but beyond that, the P-selectin axis has now been postulated to promote NETosis in the setting of activated platelets (24). However, this conclusion was recently challenged in a study that demonstrated that HMGB-1 expressed by activated platelets was individually capable of activating PMNs *via* the receptor for advanced glycosylation end-products (RAGE) to induce NETosis (31). Similarly, in 2016, an *in vitro* study demonstrated activated platelets binding to vWF through glycoprotein Ib, in turn, linked to PMNs *via* integrin beta-2 and stimulated by platelet factor 4 led to NETosis independent of P-selectin (32). While our knowledge of NET formation continues to grow, the specific proteomic cascade that leads to NETosis remains contested. These findings also underline the heterogeneity and limitations of model systems as well.

Given that thrombi consist of significant quantities of DNA combined with proteins, thrombosis resolution and associated inflammation becomes less straight forward. We have explored this topic in our laboratory by examining toll-like receptor 9 (TLR9) function in VT resolution. TLR9 is a conserved pathogen-associated molecular pattern (PAMP) and damage-associated molecular pattern (DAMP) receptor that recognizes CpG DNA repeats and alerts the immune system to invading pathogens or

local damage. In an IVC ligation model of VT (complete stasis), TLR9^{-/-} mice had significantly increased thrombus size at 2 and 8 days despite increased numbers of PMNs and monocyte/macrophages (33). Further, TLR9^{-/-} mice had increased apoptosis, citrullinated histones, PAD4, and neutrophil elastase; and reduced TFPI (19), suggesting that TLR9 is important for normal thrombogenesis and thrombosis resolution. Lastly, M1-like (CCR2+) monocyte/macrophages were decreased in TLR9^{-/-} thrombi, consistent with impaired inflammatory cell influx, and this divergence was corrected with adoptive transfer of TLR9^{+/+} bone marrow-derived monocytes with normalization of thrombus size (20). The new found composition of DNA-rich thrombi will likely have long-standing implications for future research in VT resolution.

NETs IN PRIMATE AND HUMAN VT STUDIES

Although first thought to only occur in pathologic states, NETosis with release of extracellular DNA has been shown to occur in healthy individuals (34). Following exhaustive treadmill or cycling exercise, circulating levels of cell-free DNA (cfDNA) and myeloperoxidase (MPO) rise, and isolated PMNs from the circulation develop swollen nuclei and emanating DNA. A concomitant rise in circulating DNase occurs, suggesting that similar to the ongoing processes of fibrin formation and fibrinolysis, NETosis may be a tightly regulated and constantly ongoing homeostatic process. Dysregulation of NETosis and its relationship to thrombosis has been recognized in a variety of clinical scenarios: NETs are present in fresh thrombi from individuals with acute myocardial infarction (31, 35), they are found in high circulating levels in patients with severe trauma and microvascular thrombosis with acute lung injury (6); and in patients with thrombotic microangiopathies (TMAs) (8). Circulating cfDNA rises 24 h following chemotherapy in breast cancer patients, corresponding to peak in thrombin-antithrombin levels (36). This has been proposed as a potential causative mechanism for the high rate of thrombotic events experienced by individuals undergoing chemotherapy.

Ex vivo platelet activation studies with recombinant human histones demonstrate that not all histones are created equal in NETosis: only histone 3 (H3) and histone 4 (H4) induce functional platelet response (37). Recent advances in techniques of PMN isolation (38) and recognition of NETs in human pathologic thrombus specimens have improved the ability to study NETosis in humans (39).

The link between human venous thromboembolism (VTE) events and NETosis has been established in a handful of studies to date. Analysis of balloon occlusion-induced iliac thrombosis in a baboon model of VT demonstrated increased circulating NETs at 48 h post-thrombosis and persisting through 6 days (3). Consistently, extracellular DNA markers were present in the experimental thrombus. In 2013, a human study composed of healthy controls and symptomatic patients (swelling and leg pain) with and without VT examined circulating NET markers (40). Extracellular DNA and MPO were significantly elevated in symptomatic patients with VT compared to both groups of non-thrombosed patients. A direct correlation was also seen

between common predictors of thrombosis including D-dimer, Wells score, and plasma DNA, suggesting that NETs may be useful in diagnostic evaluation. Development of more accurate methods to diagnose VT is of particular interest as the current “gold standard,” duplex ultrasound, is often limited in availability and in evaluation of central veins (41). In the same year, a similar case-control study of 195 individuals with and without VT examined nucleosomes and α -1 antitrypsin elastase (as a PMN activation marker) in relation to thrombosis. Levels of nucleosomes and PMN activation above the 80th percentile were associated with a threefold risk of VT (42).

The precise role of NETs in VT initiation, formation, and propagation, as well as optimal area to intervene in human VT remains relatively unknown. In a study evaluating unorganized, organizing, and organized thrombi from patients with either VT or PE, CD11b, and MPO positive cells were seen in organizing thrombi along with intra- and extracellular CitH3 and PAD4 (43). Unorganized and organized thrombi failed to demonstrate the similar histopathology, suggesting the predominant role of NETs during the inflammatory response and thrombus organization (43). Another study of 29 VT patients and controls demonstrated increased neutrophil adhesion and inflammatory cytokine profile among patients with residual vein obstruction and elevated D-dimer (44). *Ex vivo* studies of NETs related hypercoagulability in inflammatory bowel disease (IBD), and TMA patients have demonstrated efficacy of DNase I in decreasing the thrombotic response, although this has yet to be shown in human VT (11, 12). To date, no clinical trial has targeted NETs in humans as a mechanism to prevent or treat VT.

NETs IN IMMUNOTHROMBOSIS

While the ability to form thrombus in the face of vessel injury has long been known to be essential for the maintenance of hemostasis, until recently, it had not been recognized as an intravascular mechanism of immune defense. Engemann and Massberg recently reviewed the mechanism of NET-mediated microvascular thrombosis and proposed it as a potential biological adjunct for containing uncontrolled infection, coining this process “Immunothrombosis” (45). In their review of the topic, they proposed four mechanisms by which immunothrombosis prevents the spread of infection (1) it captures and ensnares pathogens in the microvasculature and prevents dissemination, (2) it prevents distant tissue invasion by forming microthrombi in microvessels, (3) it concentrates pathogens in one area for bactericidal killing by innate immune cells, and (4) it recruits other immune cells to the site of inflammation for further bacterial killing. Indeed, coagulation has been previously postulated to play a role in host defense against bacteria (46, 47), but if left unchecked, immunothrombosis may contribute to significant systemic pathology.

Severe sepsis accounts for 10–40% of ICU admissions in the United States and carries a mortality rate of 20–50% (48, 49). NET-related immunothrombosis, cfDNA, and histones have been implicated in the morbidity and mortality of sepsis. In *in vitro* and *in vivo* models of sepsis, LPS has been shown to activate platelets and PMNs *via* toll-like receptor 4 (TLR4) to

induce NETosis (31, 50, 51). Intravascular NETs can be digested by DNase to release cfDNA and histones, and further, cfDNA obtained from human specimens has been shown to be at ~150–300 bp sizes consistent with nucleosome units (52). In the same study, cfDNA levels in peripheral blood of patients with severe sepsis were found to be highly predictive of ICU mortality. In 2009, Xu et al. demonstrated that extracellular histones led to endothelial dysfunction, organ failure, and death in animal models of sepsis (4). Specifically, histone administration to mice resulted in neutrophil activation, endothelial toxicity, acute lung injury, and microvascular thrombosis. Interestingly, antihistone antibodies and activated protein C (APC) improved mortality rates in sepsis models in these mice. In addition, NETosis has also been linked to prothrombotic activity because NET-associated enzymes break down TFPI (29), while also enhancing antifibrinolytic activity because cfDNA inhibits plasmin-mediated fibrin degradation (13, 14, 28). Further, H3 levels were found to correlate with ICU mortality and were inversely correlated with antithrombin and platelet levels (53). Structurally, NET-induced immunothrombosis leads to more sturdy thrombi with less permeability and decreased susceptibility to lysis, although this may be overcome with DNase (54). In a murine cecal ligation and puncture model of sepsis (CLP), DNase given at 6 h after injury reduced organ damage and mortality (55). Lastly, bacterial resistance to NET trapping has been demonstrated *in vitro* and has been postulated to contribute to widespread immunothrombosis in the septic host leading to disseminated intravascular coagulation (DIC) (56, 57).

In addition to its inflammatory role in sepsis, NETs and immunothrombosis have also been implicated in autoimmune disease. Phosphatidylserine (PS) and TF-bearing microparticles (MP) were found to be markedly increased in the sera of IBD patients (11). Increased TF-bearing MPs correlated with increased NETosis, markedly shortened coagulation times, and increased levels of fibrin, thrombin, and factor Xa. Similarly, in an *in vitro* study of antineutrophil cytoplasmic antibody-associated vasculitis (ANCA-AV or AAV), C5a-primed PMNs treated with ANCA IgG-released NETs and TF-bearing MPs contributing to a prothrombotic state, and interestingly, this was remedied partially by DNase treatment (58). TMAs, more commonly known as thrombotic thrombocytopenic purpura (TTP), hemolytic uremic syndrome (HUS), and DIC, are characterized by microvascular thrombosis and coagulopathy. *In vitro* studies of TMAs demonstrate that the sera of TMA patients are incapable of degrading NETs due to a deficiency of DNase I and this may contribute to widespread microvascular thrombosis and organ dysfunction (12). Intriguingly, supplementation of TMA sera with recombinant human DNase I reestablished NET degradation capability. Lastly, PMNs from patients with antiphospholipid antibody syndrome (APS) were inclined for spontaneous NETosis and thrombin generation, and further, APS sera and APS-isolated IgG stimulated NETosis in control PMNs (59). These findings highlight the prothrombotic role of NETs in autoimmune-related immunothrombosis.

Finally, NETs have also been implicated in multiple organ failure related to severe trauma. In 2013, Abrams et al. examined

a cohort of 52 patients with severe non-thoracic blunt trauma and correlated circulating histone levels with acute lung injury and sequential organ failure assessment scores (SOFA) (6). Circulating histone levels increased dramatically immediately following trauma and high levels correlated with acute lung injury, SOFA scores, endothelial injury, and coagulation activation. In the same study, with translational animal models of blunt trauma, circulating histone levels corresponded with organ edema, hemorrhage, microvascular thrombosis, and neutrophil congestion. Multiple organ dysfunction syndrome (MODS) is a leading cause of delayed mortality following major trauma and has been correlated with circulating cfDNA levels (52). Inherently, future research should look to address NETosis and immunothrombosis as potential therapeutic targets for preventing immune-mediated MODS after severe injury.

SUMMARY, INTEGRATION, AND POTENTIAL TRANSLATION

Over the past 12 years, since its discovery, NETosis has been catapulted to the forefront of innate immunity research. Nowhere is its effect more relevant in human disease than in its implication in immune-mediated micro- and macrovascular thrombosis (Figure 1). In this review and others, NETs have been shown to have a significant role in pathogenic thrombosis through platelet and PMN recruitment to the endothelial wall, subsequent activation and NETosis, and proteomic activation of the intrinsic and extrinsic coagulation cascades, ultimately leading to thrombosis (3, 13, 14, 16, 17, 31, 32, 45, 50, 60, 61).

This unique development in the etiology of immune-mediated thrombosis affords novel targets for the prevention of pathologic thrombosis in a susceptible patient (Table 1) (4, 25, 55, 62–70). One obvious target for the prevention of NET-mediated VTE is PAD4 inhibition (25, 60). Enzymatic inhibition of PAD4 would prevent NETosis, and hopefully, its prothrombotic effects as well; however, this may also leave the host susceptible to bacterial infection (71). DAMP-mediated NETosis by HMGB-1 is also a potential therapeutic target. HMGB-1 is released from damaged cells and expressed on the surface of activated platelets and leads to immune system activation *via* RAGE, TLR2, and TLR4 (5, 72, 73). HMGB-1-mediated PMN activation subsequently contributes to microvascular thrombosis and NETosis (31, 74). HMGB-1 is cleaved by thrombomodulin–thrombin complexes *in vivo*, and recombinant thrombomodulin is presently approved for the treatment of DIC in Japan and is in phase III trials here in the United States (5, 70).

Other targets include the components of PMN and platelet recruitment to the endothelium. Specifically, blockade of platelet alpha-granule release or endothelial Weibel–Palade body release would decrease P-selectin and vWF-mediated platelet and PMN recruitment to the endothelium decreasing NETosis (24, 32, 75, 76). Similarly, blockade of P-selectin (24), vWF A1 domain (16), glycoprotein Ib, or integrin beta-2 may reach a similar end, however, more specifically (32). Lastly, the vWF degradation enzyme, ADAMTS13 (a disintegrin and metalloproteinase with thrombospondin type 1 motif – member 13), may

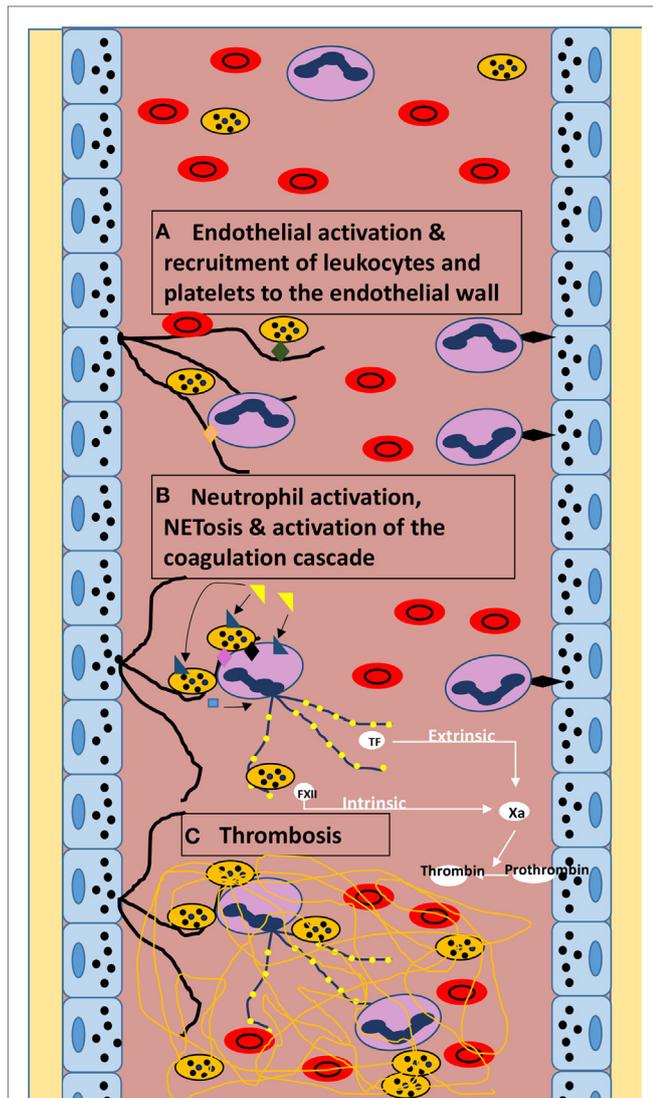


FIGURE 1 | Diagram of theoretical mechanism of NET-mediated microvascular thrombosis in sepsis. (A) Sepsis-induced endothelial activation leading to the release of vWF (black lines) from endothelial cell Weibel–Palade bodies provoking the recruitment of leukocytes and platelets to the endothelial wall via vWF A1 domain–beta-2 integrin interaction (vWF–PMN interaction; orange diamond) and vWF A1 domain–glycoprotein Ib interaction (vWF–platelet interaction; green diamond). PMNs are also recruited to the endothelium via P-selectin-to-PSGL-1 interaction (black diamond) inducing neutrophil rolling. **(B)** Neutrophil and platelet activation by LPS (yellow triangle) via TLR4 (blue triangle). Platelet activation leading to the release of PF4 (blue square) and expression of P-selectin (black diamond) and HMGB-1 (pink diamond) further stimulate NETosis [purple lines of decondensed chromatin with attached histones (yellow circles)]. NETosis also leads to autophagy-induced release of TF, and NETs further stimulate FXII activation, inciting both the intrinsic and extrinsic coagulation cascades. **(C)** Thrombin activation by factor Xa leads to the conversion of fibrinogen to fibrin (yellow lines) culminating in microvascular thrombosis.

be given in a recombinant form (rADAMTS13) to degrade vWF and prevent PMN recruitment with subsequent NETosis (77). Although, each of the above-listed countermeasures may result in mild immunodeficiency, if given in a dose-dependent manner,

TABLE 1 | Targets for translation in the prevention of NET-mediated thrombosis (4, 25, 55, 62–70).

Scenario	Target	Treatment
Sepsis thrombotic microangiopathies	Histones	Antihistone Antibodies Activated protein C
	cfDNA	DNase
Endothelial activation and thrombosis (stasis, endothelial injury, coagulopathy, sepsis, trauma, and transplant rejection)	Weibel–Palade body release	Inducers of inducible nitrous oxide synthase (iNOS)
	Platelet α -granule release	Aspirin and clopidogrel thromboxane A2 inhibitors
	vWF	rADAMTS13
	vWF A1 domain	vWF A1 domain aptamer
	Glycoprotein Ib	Inhibitors of glycoprotein Ib–vWF interaction
	Integrin β 2	Inhibitors of integrin β 2–vWF interaction
	P-selectin	P-selectin inhibitors and clopidogrel
	HMGB-1 PAD4	Thrombomodulin PAD4 inhibitors

they may abrogate the pathologic immune-mediated thrombosis without sacrificing immune competence.

Despite the relatively recent discovery of NETs as a contributor to VTE, some long-standing traditional VTE therapies already affect NETs. Polyanionic heparin, long considered the gold standard therapy for prevention and treatment of VTE, has a secondary effect of displacing histones from chromatin (78, 79). This allows increased accessibility of nucleases to the exposed chromatin, further permitting degradation of NETs (78, 79). Similarly, aspirin, recently shown to decrease risk of recurrent VTE (80), inhibits NETosis *in vitro* by inhibition of thromboxane A2 synthesis (63, 81). Finally, clopidogrel has also been shown to decrease inflammation and platelet-mediated expression of soluble P-selectin, further decreasing PMN–platelet interactions and hence NETosis (68). It is currently unknown as to the effects of the new oral anticoagulants on NETs, although this may represent an important area for future investigation.

Thrombolysis has become a key weapon in the arsenal against pathologic thrombosis; however, not all thrombotic events are susceptible to thrombolysis with tissue plasminogen activator (tPA) (82). Indeed, the addition of DNA and histones to a fibrin matrix has been shown to make artificial thrombus more stable, more rigid, and more resistant to tPA, and this is partially remedied by DNase (83). Preliminary data from murine models of VT demonstrate inhibition of thrombus formation with DNase instillation prior to and during IVC stenosis (16, 17). In another study of limb ischemia-reperfusion injury, DNase instillation decreased tissue NETs but did not effect end tissue damage or inflammatory infiltrate (84). In spite of this, more recent studies have shown decreased inflammation, increased tissue perfusion, and improved survival with (1) late DNase treatment in cecal ligation puncture (CLP) model of murine sepsis (55), (2) concurrent DNase and tPA treatment in a rat model of myocardial ischemia

(85), and (3) preemptive DNase treatment in a rat model of renal ischemia-reperfusion injury (86). Combination tPA and DNase therapy for thrombolysis of acute ischemic events has yet to be studied in human patients but will likely constitute a major area of research in the near future.

There are still many unanswered questions in the field of NETosis. What are the exact proteomic mechanisms that lead to neutrophil activation and subsequent NET formation? What is the role of RAGE, and does this have implications for diabetics? What is the intracellular cascade that leads to PAD4 induction? After NETosis and thrombosis, how are NETs naturally removed from the resolving thrombus? Do NETs play a role in the post-thrombotic syndrome? Will the addition of DNase to tPA broaden the spectrum of patients that can be treated with thrombolysis as opposed to surgery? These questions and more will be answered with time and continued diligent, meticulous, and conscientious research. We look forward to the future with great expectations for upcoming discoveries in the field of NETosis.

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AUTHOR CONTRIBUTIONS

Dr. Andrew S. Kimball did the literature review, wrote the paper, and approved of the submission. Drs. Andrea T. Obi and Jose A. Diaz did a literature review, wrote sections of the paper, and approved of the submission. Dr. Peter K. Henke wrote and edited the paper, approved of the submission, and is responsible for the content.

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Neutrophil Extracellular Traps in ANCA-Associated Vasculitis

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A group of pauci-immune vasculitides, characterized by neutrophil-rich necrotizing inflammation of small vessels and the presence of antineutrophil cytoplasmic antibodies (ANCA), is referred to as ANCA-associated vasculitis (AAV). ANCAs against proteinase 3 (PR3) (PR3-ANCA) or myeloperoxidase (MPO) (MPO-ANCA) are found in over 90% of patients with active disease, and these ANCAs are implicated in the pathogenesis of AAV. Dying neutrophils surrounding the walls of small vessels are a histological hallmark of AAV. Traditionally, it has been assumed that these neutrophils die by necrosis, but neutrophil extracellular traps (NETs) have recently been visualized at the sites of vasculitic lesions. AAV patients also possess elevated levels of NETs in the circulation. ANCAs are capable of inducing NETosis in neutrophils, and their potential to do so has been shown to be affinity dependent and to correlate with disease activity. Neutrophils from AAV patients are also more prone to release NETs spontaneously than neutrophils from healthy blood donors. NETs contain proinflammatory proteins and are thought to contribute to vessel inflammation directly by damaging endothelial cells and by activating the complement system and indirectly by acting as a link between the innate and adaptive immune system through the generation of PR3- and MPO-ANCA. Injection of NET-loaded myeloid dendritic cells into mice results in circulating PR3- and MPO-ANCA and the development of AAV-like disease. NETs have also been shown to be essential in a rodent model of drug-induced vasculitis. NETs induced by propylthiouracil could not be degraded by DNaseI, implying that disordered NETs might be important for the generation of ANCAs. NET degradation was also highlighted in another study showing that AAV patients have reduced DNaseI activity resulting in less NET degradation. With this in mind, it might be that prolonged exposure to proteins in the NETs due to the overproduction of NETs and/or reduced clearance of NETs is important in AAV. However, not all ANCAs are pathogenic and some might possibly also aid in the clearance of NETs. A dual role for ANCAs in relation to circulating NET levels has been proposed because a negative correlation was observed between PR3-ANCA and NET remnants in patients in remission.

Keywords: neutrophil extracellular traps, ANCA-associated vasculitis, ANCA, NET, small-vessel vasculitis, NET remnants

ANTINEUTROPHIL CYTOPLASMIC ANTIBODY-ASSOCIATED VASCULITIS

Vasculitides are inflammations in the walls of blood vessels, and they can affect any organ system in the body. They are divided into broad groups based on the size of the vessels predominantly being affected. A subgroup of small-vessel vasculitides is characterized by a scarcity of immune depositions (pauci-immune) and the presence of antineutrophil cytoplasmic antibodies (ANCA) and is referred to as ANCA-associated vasculitis (AAV) (1). AAV comprise three diseases, including granulomatosis with polyangiitis [GPA, previously known as Wegener's granulomatosis (2)], microscopic polyangiitis (MPA), and eosinophilic granulomatosis with polyangiitis (EGPA, previously known as Churg–Strauss syndrome) (3). GPA and EGPA share the feature of necrotizing granulomatous inflammation of the lower respiratory tract, whereas MPA is characterized by the absence of this component. Also, GPA often affects the upper respiratory tract and can result in rhinitis, otitis, and cartilage destruction, while eosinophilia and asthma are defining features of EGPA. Renal involvement is observed in as many as 90% of the patients with MPA, compared to 80% of the patients with GPA and 45% in EGPA. All three diseases affect the skin, joints, eyes, and nerves to various extents (1, 4). There is also an increased incidence of venous thromboembolism in AAV patients, especially during active disease (5, 6). AAVs are relapsing–remitting diseases, and 50% of the patients have a relapse within 5 years of successful treatment. The mortality rate is around 80% at 1 year when left untreated, but with current treatments, the mortality rate is reduced to 25% within 5 years (7).

Autoantibodies specific for proteinase 3 (PR3) (PR3-ANCA) or myeloperoxidase (MPO) (MPO-ANCA) are found in over 90% of patients with active disease (8), and these are important as diagnostic tools. The association between PR3- and MPO-ANCAs and active disease in AAV suggests a pathogenic role for the autoantibodies, and such a role is supported by results from

animal models (9, 10) and *in vitro* studies showing that PR3- and MPO-ANCAs can activate neutrophils to produce reactive oxygen species (ROS) and proteolytic enzymes (11). ANCA-induced neutrophil activation also leads to increased adhesion of the neutrophils (12) and the activation of the alternative complement pathway (13) with the generation of C5a. C5a in turn potentiates the inflammatory response by priming neutrophils and acting as a chemoattractant to recruit more neutrophils to the inflammatory site (14). However, ANCA levels do not conclusively predict relapses (15, 16), and there is an unmet need for biomarkers for this purpose.

NEUTROPHIL EXTRACELLULAR TRAPS

Neutrophil extracellular traps (NETs) were first described in 2004 as a means for neutrophils to trap and kill bacteria (17) and are released as a result of a programmed cell death mechanism referred to as NETosis (18, 19). NETs consist of a DNA backbone and various proteins with proinflammatory characteristics, such as histones, high-mobility group box 1 (HMGB1), LL37, neutrophil elastase (NE), calprotectin (S100A8/S100A9, MRP8/14), and, interestingly, MPO (Figure 1) and PR3 (20, 21). All described ANCA antigens are components of NETs. NETosis depends on a cascade of events that lead to the mixing of nuclear, cytoplasmic, and granular components before the NETs are released into the surrounding matrix (18). NETosis has been shown to depend on NADPH oxidase and ROS production as well as on autophagy and histone citrullination. Peptidyl arginine deiminase 4 (PAD4), NE, and MPO have been shown to play important roles in this signaling pathway (18, 22, 23). More recently, other forms of NETosis have also been described, including NETosis with the release of mitochondrial DNA (mtDNA ETs) (24) instead of nuclear DNA and ROS-independent NETosis (25–27). Interestingly, when releasing mtDNA ETs, the neutrophils can also remain viable (24). In addition to their role as antimicrobial agents, NETs of both nuclear and mitochondrial origin have also been connected to various autoinflammatory and autoimmune diseases (28–33).

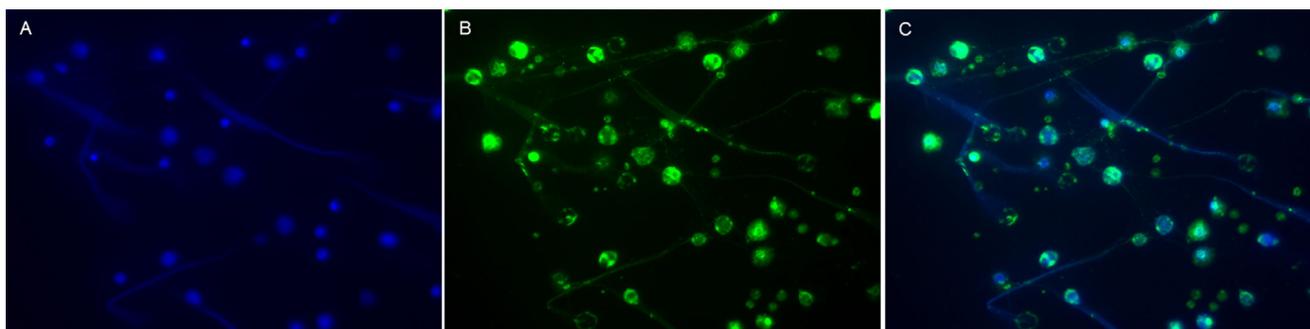


FIGURE 1 | Visualization of MPO in NETs from human neutrophils. Neutrophils isolated from human peripheral whole blood were cultured for 4 h at 37°C with 25 nM PMA. NETs were then visualized by immunofluorescence microscopy using a 40× objective. **(A)** DNA, the backbone of NETs, was labeled with DAPI (blue). **(B)** MPO (clone 2B11), a granule protein within the NETs (17), was labeled with a Dylight 488-conjugated antibody (green). **(C)** DNA and MPO (merged) co-localized in the NETs. NETs, neutrophil extracellular traps; PMA, phorbol-12-myristate-13-acetate. Blood samples were collected after obtaining informed consent in accordance with the declaration of Helsinki, and the study was approved by the Regional Ethical Review Board in Linköping. This figure is not intended to be quantitative, but only to serve as a representative image of common prior knowledge regarding the presence of MPO in NETs (17).

NETs ARE PRESENT IN GLOMERULI, SKIN LESIONS, AND THROMBI OF AAV PATIENTS

Dying neutrophils surrounding the walls of small vessels are a histological hallmark of AAV. Traditionally, it has been assumed that these neutrophils die by necrosis, but in 2009, Kessenbrock et al. showed that NETs were present in the glomeruli in kidney biopsies from AAV patients (34). They reported the presence of NETs as co-localizations of DNA, histones, and the granule proteins PR3, LL37, NE, and MPO in various combinations (34). This phenomenon was later confirmed by others (35–38). Although the method for detecting NETs in glomeruli was rather similar in these studies, i.e., visualization of DNA and histones (although some looked at citrullinated histones) in combination with the granule proteins already described – one study also reported the presence of PAD4 in the NETs, which is necessary for histone citrullination (35), and another study detected LAMP2, which is also an ANCA antigen (36, 39).

Neutrophil extracellular traps have also been shown to be present in skin lesions (40, 41) and thrombi from AAV patients (38, 42). In the studies investigating NETs in skin lesions, the presence of NETs was based on extracellular MPO (40, 41) or on DNA in combination with MPO (41). The presence of NETs in thrombi was defined not only as co-localizations of DNA and MPO but also as citrullinated histones alone (38). Another study also defined NETs based only on the presence of citrullinated histones (42).

INCREASED LEVELS OF NETs AND NET-ASSOCIATED PROTEINS IN THE CIRCULATION OF AAV PATIENTS

In addition to the presence of NETs in various lesions from AAV patients, it has been shown that these patients also have elevated levels of NETs in the circulation (34, 43–46) (Table 1). In these studies, NETs were defined as nucleosome/MPO complexes (34, 43, 46), total DNA or DNA/MPO or citrullinated histone 3 (H3) complexes (45), DNA/histone complexes (46), or as nuclear DNA or mtDNA (44). There are also several observations regarding circulating neutrophil components that are the main constituents of NETs. Important examples are HMGB1, calprotectin (S100A8/S100A9, MRP8/14), PR3, MPO, and NE (46–55) (Table 1). The study measuring calprotectin used longitudinally collected samples from the NORAM trial and found that calprotectin levels correlated with disease activity (47), and the studies measuring NE observed a correlation between NE and Birmingham Vasculitis Activity Score (i.e., disease activity) (51). However, the presence of these proteins in the circulation does not reveal whether they are released as a result of NETosis or by other mechanisms, but it was recently shown that the levels of MPO and NE correlate with the levels of DNA/MPO complexes in the circulation (46). The capability of using NETs as a biomarker to monitor disease activity in AAV has not been evident in previous studies. No study has so far measured the levels of NETs longitudinally in patients at multiple time points.

TABLE 1 | NET-associated proteins and structures present in the circulation of AAV patients.

Protein/structure	Method	AAV vs. HC	Correlation with disease activity
Nucleosome + MPO complexes	ELISA	+ (34, 43, 46)	Yes (34, 43, 46)
DNA + MPO or citrullinated histone 3 complexes	ELISA	+ (45)	No (45)
DNA + histone complexes	ELISA	+ (46)	No (46)
DNA	PicoGreen	+ (45)	No (45)
mtDNA	qPCR	+ (44)	Yes (44)
Nuclear DNA	qPCR	+ (44)	No (44)
PR3	ELISA/ Luminex	+ (46, 49, 52, 53, 55)	No (46)
MPO	ELISA	+ (53)	Yes (46)
HMGB1	Western blot/ELISA	+ (48, 50, 54)	Yes (48, 50, 54)
Calprotectin	ELISA	+ (47)	Yes (47)
NE	ELISA/ Luminex	+ (46, 51)	Yes (46, 51)

Numbers in parenthesis indicate referenced publication.

AAV ANCA-associated vasculitis; HC, healthy blood donors; +, increased levels; nd, not determined; PR3, proteinase 3; HMGB1, high-mobility group box 1 protein; MPO, myeloperoxidase; mtDNA, mitochondrial DNA; nDNA, nuclear DNA; NE, neutrophil elastase.

In some cross-sectional studies, the levels of NETs have been measured in patients during both remission and active disease, but with inconclusive results regarding their correlation with disease activity (43, 45).

PROINFLAMMATORY ASPECTS OF NETs IN AAV

Neutrophil extracellular traps have previously been described as double-edged swords of innate immunity (56), considering that they are involved in both fighting pathogens and in contributing to autoinflammatory and autoimmune conditions. Various proinflammatory aspects of NETs in general might also be important in the pathogenesis of AAV. For example, NETs can cause endothelial damage (57–59) and can activate the alternative complement pathway (60), which, as already mentioned, plays an important role in amplifying the inflammatory process in AAV. Further, anti-histone antibodies have been shown to ameliorate experimental glomerulonephritis, emphasizing the proinflammatory aspect of histones in the NETs (61). It has also been shown that the presence of histones in NETs can contribute to thrombus formation (62) and that the presence of tissue factor (63, 64) in NETs can contribute to the generation of thrombin. In turn, it has been demonstrated that activated platelets can stimulate neutrophils to release NETs and that platelet-induced NETs propagate deep vein thrombosis in mice (65). Others have shown that HMGB1 expressed on platelets mediate the formation of platelet-induced NETs and that this process is dependent on autophagy (66), and in mice, it has been shown that platelet-derived p-selectin can induce NETosis (67). Increased levels of platelet-neutrophil aggregates and soluble P-selectin have been

observed in the circulation of AAV patients during active disease and to correlate with disease activity (46). Additionally, HMGB1 has also been shown to potentiate the effect of ANCAs on NET formation (68). Oxidized mtDNA ETs released from neutrophils in systemic lupus erythematosus (SLE) have been shown to possess proinflammatory characteristics (33), and the role of mtDNA in general as a danger-associated molecular pattern has been extensively described (69).

SPONTANEOUS NET FORMATION *IN VITRO*

Earlier studies have shown that neutrophils from AAV patients are less prone to undergo apoptosis (70), suggesting that these neutrophils are more prone to other forms of cell death. Indeed, *in vitro* studies have shown that neutrophils from AAV patients are more prone to release NETs spontaneously than neutrophils from healthy blood donors (36, 43, 71). A subpopulation of neutrophils, referred to as low-density granulocytes (LDGs), have been shown to spontaneously release NETs significantly more than normal-density neutrophils, and these LDGs have been proposed to be the major source of NETs in AAV (71). However, the same study also showed that normal-density neutrophils from AAV patients spontaneously released more NETs than normal-density neutrophils from healthy blood donors (71). More detailed studies of LDGs in SLE have revealed that LDGs express increased levels of mRNA of various immunostimulatory bactericidal proteins and alarmins compared to normal-density neutrophils (59). It is important to note that during the various isolation procedures normally used to obtain neutrophils from peripheral whole blood, LDGs will not be included because they will be found in the fraction of peripheral blood mononuclear cells. This is important to consider in future *in vitro* studies of neutrophils and NET formation.

ANCAs AS MEDIATORS OF NETosis

In addition to the effects already ascribed to PR3- and MPO-ANCA in terms of neutrophil activation, they are also capable of inducing NETosis (Figure 2) (34). Although the exact mechanism for neutrophil activation by ANCAs is not clear, full activation requires binding of autoantibodies to both Fc-receptors and to PR3/MPO on the surface of neutrophils (72). It has been suggested that neutrophil activation, in this case evaluated as ROS production by MPO-ANCA, is epitope-specific, that epitope specificity varies with disease activity and that ANCAs activate neutrophils more robustly during active disease (73). Furthermore, *in vitro* studies have shown that neutrophils from patients are more easily activated (they produce more ROS) by ANCAs (in this case PR3-ANCA) than neutrophils from healthy blood donors (74). It has previously been shown that neutrophils from AAV patients possess increased membrane expression of PR3 (75, 76), which could possibly be explained by disrupted epigenetic silencing of the PR3 and MPO gene in these patients (77). However, in the study by Ohlsson et al. the results could not be explained by increased PR3 expression on the cell surface of neutrophils

from patients or the ANCA levels (74). Rather, epitope specificity and affinity seemed to be of importance for the antibodies' ability to activate neutrophils (74). It has also been shown that MPO-ANCA has higher affinity for MPO during active disease and that MPO-ANCA induces more NETs during active disease (78), and the observation that the affinity for MPO-ANCA is important for the ability to induce NETs was recently confirmed by another group (79). In summary, it seems that both epitope specificity and affinity are important for neutrophil activation by ANCAs and that at least the affinity is important for their ability to induce NETs.

NETs: BRIDGING INNATE AND ADAPTIVE IMMUNITY

It has been shown using NETotic neutrophils from mice that MPO and PR3 can be taken up from the NETs by myeloid dendritic cells (mDCs) and that injection of NET-loaded mDCs into mice results in circulating MPO- and PR3-ANCA and development of AAV-like disease (41). The addition of DNaseI to the *in vitro* cultures prevented PR3 and MPO uptake by the mDCs from the NETs, and when mice were injected with those mDCs, the mice did not develop disease (41). In the same study, injection of mDCs cocultured with apoptotic neutrophils into mice also caused autoantibody production, but those mice did not develop AAV-like disease. These experiments indicate that NETs show higher immunogenicity than apoptotic cells and that the structural integrity of the NETs is important for transferring NET-antigens to mDCs and the subsequent production of pathogenic autoantibodies. This is in line with a previous study showing that rats immunized with apoptotic neutrophils do develop ANCAs, but not disease (82). In another study, rats were immunized with NETs induced by phorbol-12-myristate-13-acetate (PMA) and propylthiouracil (PTU) (which together induced abnormal NETs that could not be degraded by DNaseI) or were given PTU orally in combination with PMA (intraperitoneal injection), and these rats developed MPO-ANCA and pulmonary capillaritis or glomerulonephritis and pulmonary capillaritis, respectively (80). This resembles the situation in humans, where over 20% of patients with Graves' disease treated with PTU develop MPO-ANCA and some also AAV-like disease (83, 84).

NET FORMATION VS. CLEARANCE: THE IMPORTANCE OF BALANCE

The studies described earlier imply that NETs can act as a link between the innate and adaptive immune system with the production of pathogenic ANCAs. With this in mind, it might be that prolonged exposure to the proteins in the NETs due to the overproduction of NETs and/or reduced clearance of NETs is important in AAV. In line with this, it has been shown that AAV patients have reduced capacity to degrade NETs *in vitro* (78). This could possibly be due to the reduced DNaseI activity observed in these patients compared to healthy blood donors, although DNaseI activity did not correlate with disease activity. Thus, the elevated levels of NETs in the circulation of AAV patients might

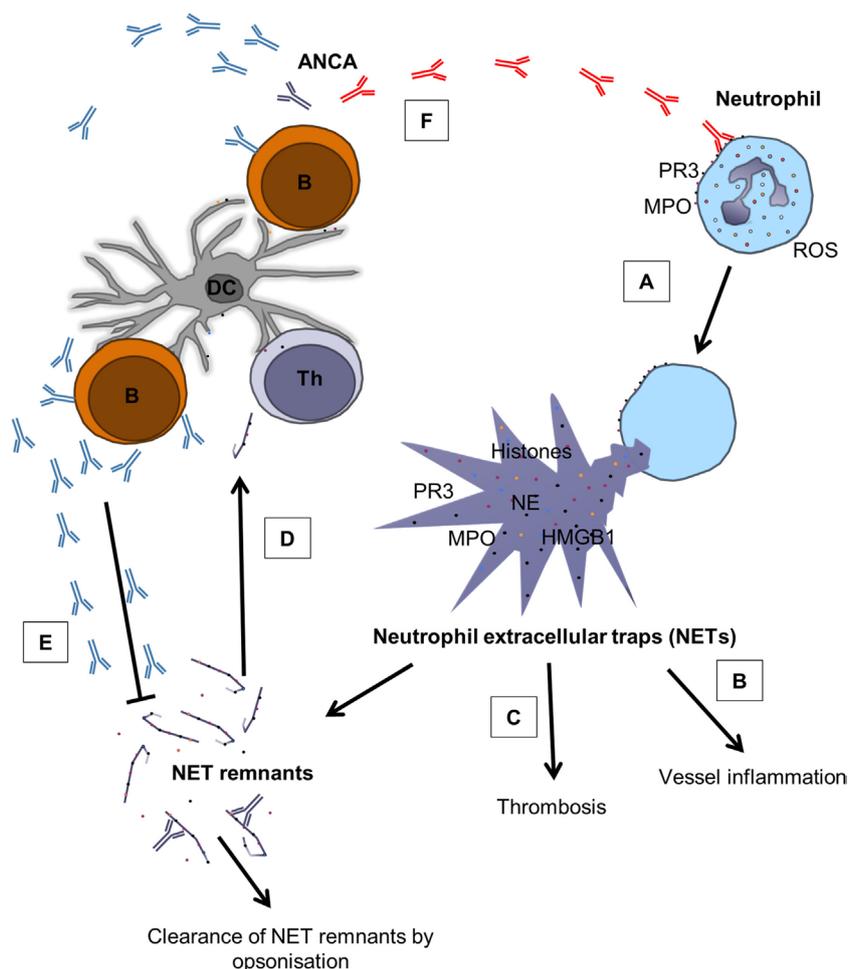


FIGURE 2 | The role of NETs in AAV and the complex relation between ANCAs and NETs. (A) Pathogenic ANCAs (red) reacting with PR3 and MPO on the surface of neutrophils cause ROS production and the release of NETs through NETosis (34, 78, 79). **(B)** NETs contain various proinflammatory mediators, such as histones, HMGB1, PR3, MPO, and NE (17, 20, 21), and contribute to vessel inflammation by damaging endothelial cells (57–59) and by activating the complement system (60). **(C)** NETs do also promote thrombosis through the expression of histones (62) and tissue factor (63, 64). **(D)** NETs can also act as a link between the innate and adaptive immune system through the generation of ANCAs (41, 80). **(E)** ANCAs seem to belong to repertoire of “natural” antibodies (81), indicating that not all ANCAs are pathogenic, and it has been proposed that ANCAs can aid in clearance of circulating NET remnants (43). **(F)** However, under unfavorable circumstances, pathogenic ANCAs (red) are produced, creating a vicious circle that promotes inflammation. B, B cell; Th, T helper cell; DC, dendritic cell. Modified from Ref. (43) with permission from Oxford University Press.

also be explained by the reduced capacity to clear the NETs from the circulation. Interestingly, low levels of both PR3-ANCA and MPO-ANCA can be found in the circulation of healthy individuals (81), indicating that the presence of ANCAs does not necessarily lead to disease development. Rather, ANCAs might be part of the repertoire of natural antibodies that are important for maintaining homeostasis (85). In line with this, a dual role for ANCAs was recently suggested, where the autoantibodies in addition to inducing NET formation can also aid in the clearance of NETs (**Figure 2**) (43), possibly through opsonization and the formation of immune complexes. This hypothesis was proposed because a negative correlation was observed between PR3-ANCA and circulating NET remnants in AAV patients in remission (43). As others have shown that the pathogenicity of ANCAs seems to vary with both epitope specificity (73) and affinity (78) and

that these parameters change with disease activity, it appears that ANCAs might play different roles at different stages of AAV. Together, these studies might suggest how and why all individuals can possess ANCAs but only some develop AAV.

INFECTIONS AND ANCAs

Antineutrophil cytoplasmic antibodies are common in chronic infections, such as *Pseudomonas aeruginosa* infections, in patients with cystic fibrosis, tuberculosis, HIV, and infective endocarditis (86–89). Infections are also implicated in the pathogenesis of AAV and as a trigger of relapses. Molecular mimicry, either directly (90) or indirectly through autoantigen complementarity (91), is the traditional way to explain the relationship between AAV and infection. However, infections lead to neutrophil

activation, which triggers NETosis. Lipopolysaccharide-activated platelets can also activate neutrophils to release NETs (92), and this suggests an indirect way in which bacteria can contribute to NETosis as well as to the coagulation cascade and thrombosis formation discussed earlier. In sepsis, the liver sinusoids are filled with neutrophils undergoing NETosis (93), and in infective endocarditis, a role for NETs has also been described (94). ANCAs are found in up to 20% of patients with endocarditis (95), and many of these patients have symptoms resembling vasculitis, such as fever, increased CRP, weight loss, malaise, multifocal skin lesions, and renal involvement (1, 96–98).

CONCLUDING REMARKS/DISCUSSION

This review has outlined the role of NETs in the pathogenesis of AAV. There is compelling evidence that NETs contribute to vessel inflammation directly by damaging endothelial cells and by activating the complement system and indirectly by acting as a link between the innate and adaptive immune system through the generation of PR3-ANCA and MPO-ANCA. This can lead to a vicious circle because ANCAs can activate neutrophils. However, ANCA pathogenicity is dependent on both affinity

and epitope specificity, and there also seem to be ANCAs that are non-pathogenic and even beneficial. NETs are most probably formed at a constant rate in healthy individuals, but NET formation can become highly elevated by infections, certain drugs, and possibly by epigenetic changes as one ages. Increased NET formation must be balanced by clearance mechanisms, which seem to include DNaseI and possibly autoantibodies with ANCA specificity. We hypothesize that under unfavorable circumstances some individuals (partly depending on genetics) develop pathogenic autoantibodies that can activate neutrophils thus creating a vicious circle resulting in widespread vessel wall inflammation.

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Neutrophil Extracellular Traps Open the Pandora's Box in Severe Malaria

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INTRODUCTION

Malaria is transmitted by mosquitoes and kills 2,000 humans in sub-Saharan Africa each day (1). Most of the victims are children younger than 5 years of age. Out of the five *plasmodium* species causing malaria, *Plasmodium falciparum* is responsible for most of the severe and fatal infections (2). Upon feeding, the female *Anopheles* mosquito inoculates sporozoites that seek out the hosts' liver within minutes. After a first replication cycle in hepatocytes for 5–8 days, rupture releases the parasite into circulation where erythrocytes are infected. During the second replication cycle *plasmodium* consumes the contents and energy reserves of the erythrocytes, changes the membrane to enable adherence to the vessel walls (3), and produces waste, including crystalline urate (MSU) and hemozoin (4). After 24, 48, or 72 h, depending on the species, the infected erythrocytes synchronously burst and release their content into the circulation and cause first clinical symptoms (Figure 1). The key pathogenic processes that cause severe malaria include rapid increase of infected erythrocytes, destruction of both infected and uninfected erythrocytes, acute inflammation, and microvascular obstruction. The final outcome is a reduced tissue perfusion that leads to downstream events compromising the cellular metabolism (5). We hypothesize that intravascular formation of neutrophil extracellular traps (NETs) contributes to the vasculopathy, driving severe malaria. NETs are web-like structures of highly modified chromatin and antimicrobial peptides released by activated neutrophils (6). In this article, we discuss the evidence supporting the role of NET formation in the pathogenesis of malaria and propose potential therapeutic interventions.

NETs CAUSE VASCULOPATHY IN MALARIA

NETosis has evolved as an important innate strategy for killing extracellular pathogens (7). The sticky chromatin fibers of NETs immobilize pathogens and inhibit their spread. Uric acid is to be found in infected erythrocytes as a precipitate together with high concentrations of hypoxanthine and the insoluble hemoglobin metabolite hemozoin. During rupture of infected erythrocytes, these compounds are abruptly released into the circulation. In this scenario, insoluble uric acid and hemozoin encounters the immune system as crystalline matter (4) and the former crystallizes in the sodium-rich plasma as MSU (8). These crystals are potent inducers of NETs formation, even in the presence of plasma proteins (8). Hemozoin activates leukocytes through an inflammasome-dependent pathway (9). Indeed, it has been described that NETs with trapped parasites circulate in children infected with *P. falciparum* (10).

In flow chambers perfused with blood, NETs promote fibrin deposition and bind fibronectin and von Willebrand factor, important for platelet adhesion and thrombus propagation (11). NET fibers contain further procoagulant factors. Neutrophil elastase in NETs may proteolytically inactivate tissue factor pathway inhibitor (12). Furthermore, tissue factor can be deposited on extracellular

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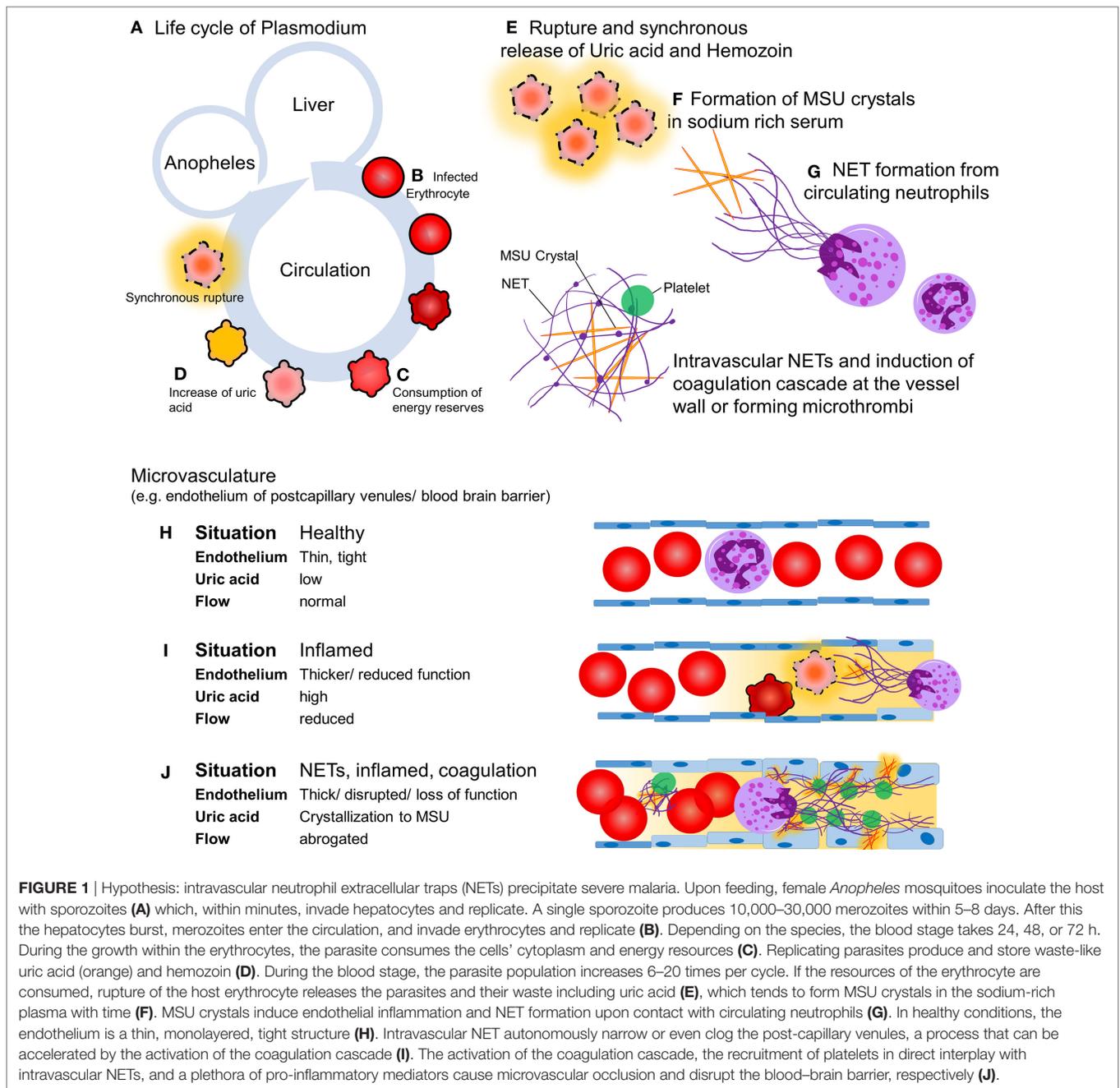


FIGURE 1 | Hypothesis: intravascular neutrophil extracellular traps (NETs) precipitate severe malaria. Upon feeding, female *Anopheles* mosquitoes inoculate the host with sporozoites (**A**) which, within minutes, invade hepatocytes and replicate. A single sporozoite produces 10,000–30,000 merozoites within 5–8 days. After this the hepatocytes burst, merozoites enter the circulation, and invade erythrocytes and replicate (**B**). Depending on the species, the blood stage takes 24, 48, or 72 h. During the growth within the erythrocytes, the parasite consumes the cells' cytoplasm and energy resources (**C**). Replicating parasites produce and store waste-like uric acid (orange) and hemozoin (**D**). During the blood stage, the parasite population increases 6–20 times per cycle. If the resources of the erythrocyte are consumed, rupture of the host erythrocyte releases the parasites and their waste including uric acid (**E**), which tends to form MSU crystals in the sodium-rich plasma with time (**F**). MSU crystals induce endothelial inflammation and NET formation upon contact with circulating neutrophils (**G**). In healthy conditions, the endothelium is a thin, monolayered, tight structure (**H**). Intravascular NET autonomously narrow or even clog the post-capillary venules, a process that can be accelerated by the activation of the coagulation cascade (**I**). The activation of the coagulation cascade, the recruitment of platelets in direct interplay with intravascular NETs, and a plethora of pro-inflammatory mediators cause microvascular occlusion and disrupt the blood–brain barrier, respectively (**J**).

traps, especially those derived from eosinophils, and factor XII is reportedly present and active on NETs (13). The negatively charged DNA in NETs may provide a scaffold for factor XII activation, which is aided by platelets, but the mechanism is still elusive. Fibrin and NETs synergize to augment the microbial defense in a process referred to as immunothrombosis (14). We hypothesize that the formation of NETs and the activation of the coagulation cascade is a double-edged sword. On the one side, they form a barrier on the endothelial surface protecting it from damage by MSU crystals (15) and hemozoin. On the other side, their occurrence limits the bore diameter of capillaries and postcapillary venules. This is prone to restrict perfusion of the end

organs. In worst case, intravascular NETs autonomously clog the vessels or initiate disseminated intravascular coagulation (DIC), a condition involving hemorrhage and microthrombosis that synergize in the abolishment of tissue perfusion (**Figure 1**). The inflammatory mediators released during the coupled processes of NET formation and coagulation cause opening of the neuro-immunological blood–brain barrier and precipitate the clinical disease as, often fatal, cerebral malaria.

The clinical aspects of malaria vary with age, geography, epidemiology, and immunity. In endemic areas, young children and pregnant women are at highest risk to develop severe forms of malaria. Here, we want to interlace NET formation in plasmodium

BOX 1 | Hypotheses for neutrophil extracellular traps (NETs) as pathogenomic factor for severe malaria.

1. *Impaired consciousness*: Intravascular NET formation and subsequent reduction of capillary bore diameters impair brain perfusion. In addition, toxic metabolites, e.g., ammonium, accumulate due to liver and kidney deficiency.
2. *Prostration*: Lack of perfusion of skeletal muscles due flow restriction in their capillaries. Flooding the patient with cytokines like IL1 β .
3. *Multiple convulsions*: NET-driven disseminated intravascular coagulation (DIC) and microthrombi cause multiple focal brain lesions and convulsions.
4. *Acidosis*: NET formation and acidosis are mutually influencing each other. Reduced blood flow causes hypoxia and drop in the plasma pH. However, acidic conditions interfere with NET formation (17).
5. *Hypoglycemia*: Impaired perfusion and high consumption of glucose by *Plasmodium* deprives host tissues from glucose and precipitates end organ damage.
6. *Renal impairment*: Kidney is damaged due to hypoperfusion and glomerular obstruction. Reduced renal uric acid secretion causes hyperuricemia and promotes further NET formation.
7. *Hemorrhages*: NET-driven DIC contributes to the consumption of coagulation factors.
8. *P. falciparum parasitemia (>10%)*: Infected as well as ruptured erythrocytes are trapped by intravascular NETs, escaping sequestration in liver and spleen.
9. *Severe malarial anemia, jaundice, pulmonary edema, and shock*: There is no obvious specific contribution of NETs, besides the fact that these symptoms are the consequence of decreased end organ perfusion.

infections as key factor for the development of severe clinical manifestations (**Box 1**) (2, 16).

DISCUSSION

Each year, 240 million people develop symptomatic malaria infections, placing malaria as one of the most serious diseases of mankind. Treatment options in affected areas are challenging and casualties remain high. Here, we propose NET formation as key event determining the severity of disease progression. Parasites within erythrocytes reportedly produce uric acid, which is released as MSU crystals together with crystalline hemozoin synchronously upon rupture of the infected cells. MSU crystals may induce the formation of intravascular NETs, and hemozoin pigments activate neutrophils, immobilizing parasites and crystals at the endothelium. We discussed three possible outcomes (**Figure 1**). (i) The NETs shield the endothelium, dampening the pro-inflammatory effects of MSU (15) and hemozoin (9). (ii) The NETs and the activation of the coagulation cascade form a barrier on the surface of endothelium, reducing the vessels' inner diameter and, thereby, the perfusion to virtually all organs. (iii) NETs autonomously, as well as the activation of the coagulation cascade by the negatively charged DNA strands promote DIC. As consequence, hemorrhages and microthrombi may impact microcirculation and promote end organ ischemia. The interplay between intravascular NETs and the coagulation cascade is still elusive. However, the formation of a scaffold in the vasculature by NETs allows the recruitment of coagulation factors, inflammatory mediators, immune cells, and platelets—quite possible being able to disrupt the blood-brain barrier and promote cerebral malaria (18).

Intriguingly, reports indicate a decrease in the counts of circulating granulocytes, monocytes, and lymphocytes in the face of

Plasmodium infections, especially during gestation. This includes a significant reduction in peripheral neutrophil count (19). We speculate this might be partly due to the formation of NETs in the course of the disease. However, there is no direct evidence correlating the neutrophil count and the release of parasites into the circulation. An abnormal capacity to execute an oxidative burst was described for neutrophils of children suffering from *P. falciparum* malaria (20). The authors show that the reduced oxidative burst capacity in neutrophils occurred after the infection. In a murine model for malaria, this effect was attributed to the induction of the cytoprotective heme oxygenase 1 in neutrophil progenitors of the bone marrow (20). Although NET formation is closely associated with oxidative burst, the aforementioned study did not address NET formation. Therefore, we prompt researchers to include NET formation in their repertoire for functional evaluation of neutrophils. A number of studies argue that antimalarial drugs might influence the capacity of neutrophils to undergo oxidative burst or alter their effector functions (21, 22). Resulting limitation of neutrophils to form NETs might be beneficial in the context of malaria.

The occurrence of anti-neutrophil cytoplasmic antibodies (ANCA) in 50% of patients with malaria supports the role of NETs in the etiopathogenesis of this, often fatal, disease (23, 24). The detection of these autoantibodies is used in the diagnosis of systemic vasculitides, e.g., granulomatosis with polyangiitis. ANCA are classified into C- and P-ANCA according to their antigen specificity for proteinase 3 and myeloperoxidase, respectively. While C- and P-ANCA bind to intact neutrophils, NETs contain only antigens of P-ANCA (25). Malaria patients primarily develop P-ANCA (23), supporting the notion that NETs may serve as an autoantigen in this disease.

The involvement of NETs in severe malaria has not been studied in detail. Utilizing NET formation, MSU, and the coagulation cascade as targets of new therapies harbors the potential to reduce the cases with mortality outcomes and alleviate severe forms of malaria infection.

AUTHOR CONTRIBUTIONS

SB wrote the manuscript. LM, MH, and TF supervised the project and wrote the manuscript. All the authors read and approved the manuscript.

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Antimicrobial Activity of Mast Cells: Role and Relevance of Extracellular DNA Traps

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Mast cells (MCs) have been shown to release their nuclear DNA and subsequently form mast cell extracellular traps (MCETs) comparable to neutrophil extracellular traps, which are able to entrap and kill various microbes. The formation of extracellular traps is associated with the disruption of the nuclear membrane, which leads to mixing of nuclear compounds with granule components and causes the death of the cell, a process called ETosis. The question arises why do MCs release MCETs although they are very well known as multifunctional long-living sentinel cells? MCs are known to play a role during allergic reactions and certain parasitic infections. Nonetheless, they are also critical components of the early host innate immune response to bacterial and fungal pathogens: MCs contribute to the initiation of the early immune response by recruiting effector cells including neutrophils and macrophages by locally releasing inflammatory mediators, such as TNF- α . Moreover, various studies demonstrate that MCs are able to eliminate microbes through intracellular as well as extracellular antimicrobial mechanisms, including MCET formation similar to that of professional phagocytes. Recent literature leads to the suggestion that MCET formation is not the result of a passive release of DNA and granule proteins during cellular disintegration, but rather an active and controlled process in response to specific stimulation, which contributes to the innate host defense. This review will discuss the different known aspects of the antimicrobial activities of MCs with a special focus on MCETs, and their role and relevance during infection and inflammation.

Keywords: MCET, extracellular traps, mast cell, neutrophil, innate immunity, antimicrobial activity, phagocytosis, degranulation

INTRODUCTION

Mast cells (MCs) have become famous for their role in type I hypersensitivity reactions. Better known as IgE-mediated allergic reactions, this MC response is induced after multivalent cross-linkage of antigens with antigen-specific IgE, which then bind to high-affinity IgE receptors (Fc ϵ RI) on the cellular surface (1–3). For a long time, MCs have been underestimated and mainly known for their role as mediators in the early and acute phases of allergic reactions as well as their activation during certain parasitic infections (4). Indeed, they hold a multitude of very important functions in the innate and adaptive host immune responses against bacterial and fungal pathogens (5, 6) (see **Table 1**).

TABLE 1 | Interaction of MCs with selected pathogens.

Pathogen	Mast cell type	Phagocytosis	MCETs	Degranulation	Reference
<i>Staphylococcus aureus</i>	CBHMC	√ no		√	(7, 10)
	HMC-1		√		(8, 9)
	BMMCs		√		(8)
<i>Streptococcus pyogenes</i>	BMMC	no	√	√	(9)
	HMC-1	no	√		(9,11)
<i>Pseudomonas aeruginosa</i>	Murine skin mast cells			√	(12)
	HMC-1		√		(9)
<i>Citrobacter freundii</i>	CBHMC	√			(7)
<i>Klebsiella pneumoniae</i>	CBHMC	√			(7)
	Mouse lung mast cells <i>in vivo</i>			√	(13)
<i>Escherichia coli</i>	CBHMC	√			(7)
	Mouse lung mast cells <i>in vivo</i>			√	(13,14)
<i>Streptococcus faecium</i>	CBHMC	√			(7)
<i>Citrobacter rodentium</i>	BMMC			AMP	(14)
<i>Enterococcus faecalis</i>	BMMC		√	√	(15)
<i>Candida albicans</i>	HMC-1, CBHMC		√	√	(16)
<i>Listeria monocytogenes</i>	BMMC	no		√	(17)
<i>Salmonella typhimurium</i>	BMMC	no		√	(17)
<i>Trichinella spiralis</i>	RBL-2H3, BMMC			√	(18)
<i>Leishmania major</i>	<i>in vivo</i>			√	(19)
<i>Helicobacter pylori</i>	BMMC, RBL-2H3 cells			√	(20)

MCs derive from hematopoietic progenitor cells and circulate in the blood until they reach their destination in the tissues, where they differentiate under the influence of growth factors and cytokines that ultimately determine their mature, long-living phenotype (3). Different subsets of mature MCs have been described on the basis of functional, structural, and biochemical characteristics. Consequently, they are classified into at least two subgroups: mucosal MCs (MMCs) and connective tissue-type MCs (CTMCs) (3, 21). CTMCs typically reside in the skin and the peritoneal cavity. In contrast, MMCs are predominant in the mucosal layer of the intestine, where their numbers expand dramatically during e.g. parasitic infections (22, 23). Considering their long life span and phenotypic plasticity in the tissues, MCs contribution in chronic or acute infections is not fully understood (24). They are largely distributed near interfaces and potential entry sites of pathogens, such as the skin, the respiratory, and intestinal mucosa, and in close proximity to blood vessels and nerve cells (25, 26); therefore, MCs belong to the first immune cells, which come in contact with intruders. Since they orchestrate the immune response by releasing various mediators, these long-living sentinel cells are crucial for the early recruitment of effector cells (24).

The main function of MCs is the release of inflammatory mediators such as proteases, cytokines, and chemokines by degranulation into the surrounding environment (22, 27). MCs are the only cell type known to pre-store TNF- α in their secretory granules, which can be released immediately upon activation e.g., by pathogens to initiate the early phase of the inflammatory response (13, 28). Rocha-de-Souza et al. (10) showed that both alive and dead *Staphylococcus* (*S.*) *aureus* trigger TNF- α and IL-8 release from cord-blood-derived MCs in a time-dependent manner. Nakamura et al. (29) published that culture supernatants of *S. aureus* contain potent MC degranulation

activators. Biochemical analysis identified δ -toxin as the MC degranulation-inducing factor produced by *S. aureus* (30). Importantly, skin colonization with *S. aureus*, but not a mutant deficient in δ -toxin, promoted IgE and IL-4 production as well as skin diseases. Dietrich and colleagues showed that, in response to toll-like receptor (TLR) activation by the Gram-positive and -negative bacteria *Listeria* (*L.*) *monocytogenes* and *Salmonella* (*S.*) *typhimurium*, respectively, MCs elicit proinflammatory, but not type I IFN responses. In contrast, the response to viral infection is type I IFN dependent. Type I IFN signaling attenuates mast cell-dependent neutrophil recruitment that is required for bacterial clearance. Thus, the fact that MCs are equipped with the ability to release type I IFNs, but mount proinflammatory responses only upon TLR activation by bacteria, illustrates how MCs adjust their responses for optimal antibacterial and antiviral host defenses (17).

MCs are highly efficient effector cells that do not only release inflammatory mediators but also different antimicrobial peptides (AMPs), such as cathelicidins (31). These peptides have cationic and amphipathic properties that promote interactions with biological membranes and selectively kill a wide spectrum of microbes including bacteria, fungi, enveloped viruses, and protozoa (31). *In vivo* evidence from MC- and cathelicidin-deficient mouse models indicates that MC cathelicidins modulate tissue responsiveness to bacterial infection (32). The authors suggested that cathelicidins act as a natural antibiotic in MCs and may protect the skin from invasive group A *Streptococcus* (GAS) and *S. aureus* infection by direct bacterial killing. Moreover, the presence of cathelicidin in MCs may act to facilitate recruitment of neutrophils, thus indirectly providing enhanced protection against infection.

Despite the fact that the MCs release key inflammatory compounds to modulate the immune response and to fight pathogens

with AMPs, the cells are additionally discussed to be able to eliminate bacteria through an intracellular bactericidal mechanism similar to that of professional phagocytes (33). This mechanism seems to involve the endosome–lysosome pathway, in which the bacteria are killed through a combination of oxidative and non-oxidative killing systems (33). Arock and coworkers showed that human cord blood MCs (CBHMCs) are able to phagocytose and kill *S. aureus*, *Streptococcus faecium*, *Klebsiella pneumoniae*, *Citrobacter freundii*, and *Escherichia (E.) coli* by scanning and transmission electron microscopy and by quantifying bacterial survival in the presence of MCs compared with human umbilical vein endothelial cells (HUVECs) (7). However, although the authors compared *in vitro* results generated by different techniques with distinct pathogens, the data still remain short of a definitive proof in respect to the phagocytic response, since only total killing of bacteria in the presence of MCs was measured, and not a specific intracellular killing as normally performed by a gentamicin-protection assay. Finally, in contrast to the above hypothesis that MCs are able to phagocytose, Dietrich et al. found only an exclusive extracellular interaction of MCs with *L. monocytogenes* or *S. typhimurium* using transmission electron microscopy (17).

Interestingly, Abel et al. (34) showed that MCs can internalize *S. aureus* without subsequent intracellular killing: the *S. aureus* strain SH100 was shown to be internalized *in vitro* by murine and human MCs (primary murine bone marrow-derived MCs “BMMCs,” and human carcinoma MC line “HMC-1”) and by skin MCs during *in vivo* infections. MCs are utilized as a vehicle and a safe intracellular niche providing protection against other immune cells. Since the internalization efficiency depends on bacterial viability, the authors hypothesized that *S. aureus* may actively induce its uptake into MCs (34). In a recent follow-up publication, Goldmann and colleagues demonstrated enhanced *S. aureus* internalization by MCs based on the interaction of staphylococcal fibronectin-binding protein with host cellular integrin $\beta 1$ (35). However, aside from the fact that *S. aureus* mediates its own uptake into MCs to evade immune cell killing, MCs have been shown by several authors to exert a direct antimicrobial activity against this and other pathogens (7, 9, 34). Thus, it may be assumed that although MCs may act as a long-term staphylococcal reservoir supporting persistence and chronic carriage, their activation can help to limit the early pathogen burden in the infected host.

In summary, MCs do not only orchestrate the early innate immune response through the release of mediators but they can also act antimicrobially in a pathogen-specific manner: extracellular by the release of antimicrobial products such as the cathelicidin-related AMPs (36) or intracellular by a phagocytic process. Finally, in 2008, an additional antimicrobial strategy was described for MCs, which was already known for neutrophils: the formation of antimicrobial mast cell extracellular traps (MCETs) (9).

MAST CELL EXTRACELLULAR TRAPS

Similar to the formation of neutrophil extracellular traps (NETs), MCs have also been shown to release their nuclear DNA and

subsequently form antimicrobial MCETs that resemble extracellular dendritic extensions (9). Interestingly, already in 1989, Trotter and colleagues mentioned that “superficial MCs have a smaller size and may be dendritic, with relatively few granules [...]” (37). However, the first experimental study on MCs and the formation of antimicrobial extracellular structures that strongly resembled the recently described NETs was published by von Köckritz-Blickwede et al. (9). The authors showed that even though MCs are unable to phagocytose *Streptococcus (S.) pyogenes*, they still can efficiently inhibit the growth by the release of MCETs. MCETs were found to support the extracellular killing of clumped bacteria that were not efficiently phagocytosed. Those MCET fibers are composed of DNA, histones, the MCs-specific protease tryptase, and AMPs such as the cathelicidin AMP LL-37 (9).

Detailed information about the specific trigger that initiates MCET formation and the mechanisms regulating the removal of MCETs is still missing. However, literature clearly demonstrates that MCET formation is not the result of a passive release of DNA and granule proteins during cellular disintegration but, rather, an active and controlled process similar to that described for NETs (9). Similar to the observations of Fuchs et al., who has implicated the production of reactive oxygen radicals and induction of cell death in the production of NETs, the formation of MCETs also strongly depends on the production of reactive oxygen species (ROS) and results in the death of the MC. Examination of stimulated MCs by electron and fluorescent microscopy confirmed that MCs undergo a similar mechanism of cell death as described for neutrophils when releasing NETs: MCET formation is associated with the disruption of the nuclear membrane before nuclear and granular components mix causing the death of the cell. Although ROS had been previously associated with the induction of neutrophil apoptosis, Fuchs et al. showed that the process accounting for NET formation is neither typical apoptosis nor necrosis, but rather a new form of ROS-dependent cell death recently termed “NETosis” (38). Since this is also true for the formation of MCETs, this cell death was also named “ETosis” (39). The formation of MCETs can be greatly increased after stimulation of MCs with phorbol-12-myristate-13-acetate (PMA), similar to NETs (40) or with the H₂O₂-producing enzyme glucose oxidase, which is another indicator for the ROS-dependent MCET formation (9). Nevertheless, a key question still needs to be answered: why do MCs release MCETs although they are very well known as multifunctional long-living sentinel cells – what is worth dying for?

Several publications show that the formation of MCETs represents a novel mechanism by which MCs contribute to host defenses against bacterial and fungal pathogens. Interestingly, a diffused gradient of extracellular tryptase staining was often observed in areas with large numbers of bacteria during *in vivo* infections, which may indicate a massive release of this enzyme and possibly the not clearly visible but occurring formation of MCETs at the site of infection. The first specific bacterial protein identified to promote MCET production was the *S. pyogenes* surface M1 protein (11). In quantitative assays, loss of M1 protein in the *S. pyogenes* Δ M1 mutant resulted in a significant decrease in the stimulation of NET as well as MCET release. Despite its

role in inducing ET formation, the authors found M1 protein promoting extracellular bacterial survival, at least in part due to resistance to the human AMP LL-37, an important effector of bacterial killing associated with extracellular traps. LL-37 and its murine analog CRAMP are stored in MC granules; its expression is upregulated by LPS and found within the MCET structures where it contributes to the antimicrobial activity (9, 31). It has already been shown for neutrophils that LL-37 significantly facilitated NET formation by primary human blood-derived neutrophils alone, in the presence of the classical chemical NET inducer PMA or in the presence of *S. aureus* (41). Nonetheless, the role of LL-37 in MCETs still needs to be investigated. Interestingly, Scheb-Wetzel et al. recently showed that MC release extracellular traps in response to *Enterococcus (E.) faecalis*, and the sensitivity of this pathogen to the antimicrobial effect of cathelicidin LL-37 indicated a potential major role for this AMP in the antimicrobial activity of MCs against *E. faecalis* (15). However, the level of MCET formation was not as pronounced as it has been reported for other pathogens such as *S. pyogenes* (9); therefore, killing of *E. faecalis* by MCETs cannot fully account for the antimicrobial effect of MCs observed in this study. This was further confirmed by the diminished, but still significant, antimicrobial effect of MCs after dismantling of the MCETs by nuclease treatment. The reason why only a certain percentage/population of MCs in the cultures released MCETs is not yet clear; eventually, this phenomenon may reflect heterogeneity in the physiological status of the MCs in culture; a similar phenomenon has been reported for extracellular trap formation by neutrophils (38).

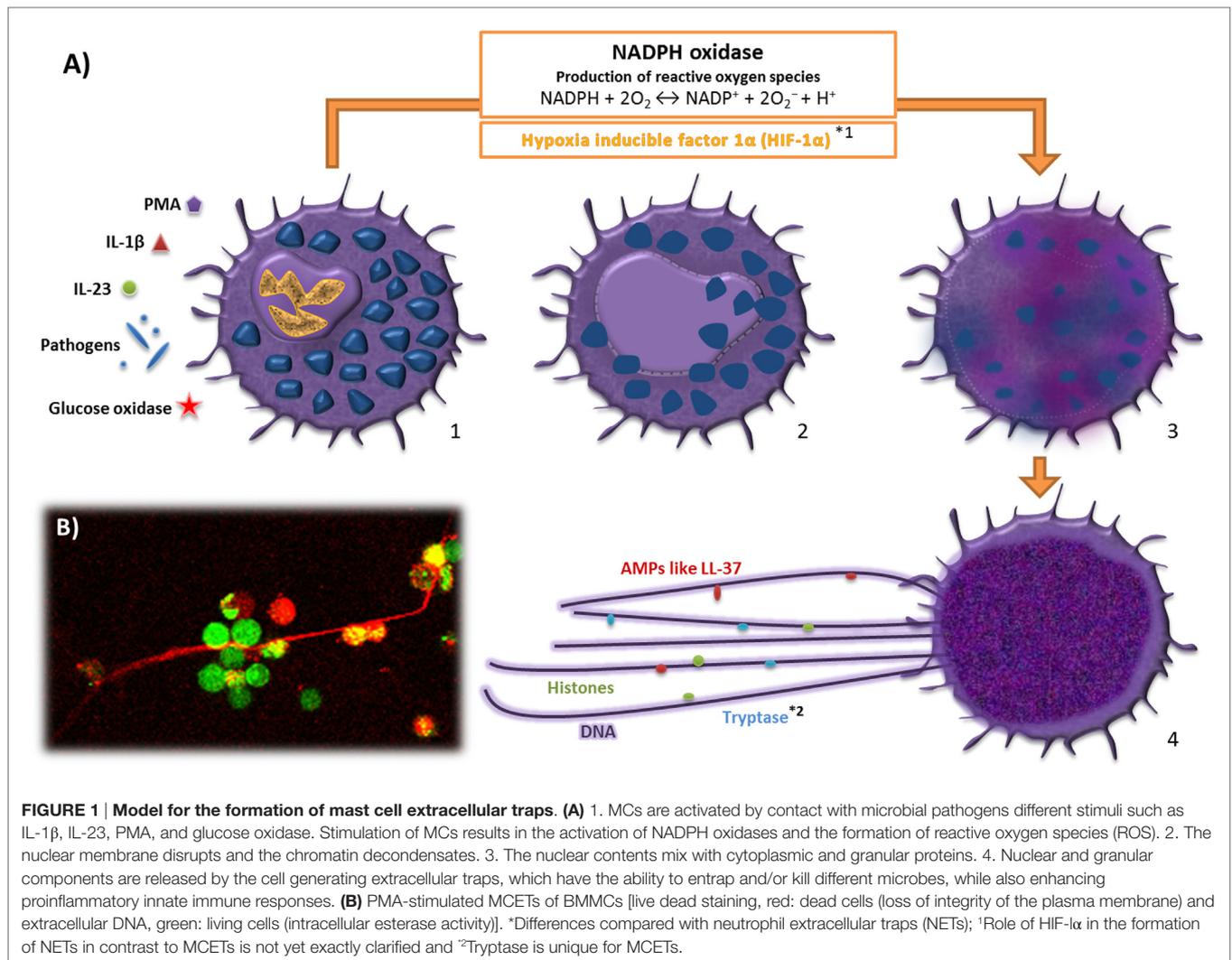
Importantly, Lopes et al. (16) showed that MCs reveal an antimicrobial activity against higher eukaryotes, namely the fungi *Candida (C.) albicans*. MCET release appears to be a mechanism of immune defense present in the MC toolbox against fungal pathogens, as both primary cells and HMC-1 release MCETs upon *C. albicans* stimulation. Interestingly, in contrast to bacteria (8, 9, 11, 15), *C. albicans* viability was not affected by the MC-derived DNA fibers and thus MCETs rather contribute to physical restriction of this fungal pathogens. Finally, the exact mechanism and the *in vivo* relevance of *C. albicans*-induced MC death need to be determined in further studies.

Similar to what has been shown for NETs, the formation of MCETs also seems to be associated with detrimental effects during health and diseases: a novel mechanistic stimulus for the release of extracellular traps in psoriasis lesions was described by Lin et al. (42). It was demonstrated that IL-23 and IL-1 β increased the numbers of extracellular trap forming cells e.g., neutrophils and MCs contributing to the release of the pathogenic cytokine IL-17. Nevertheless, the precise signaling mechanisms regulating this process remain to be defined. These observations support a model in which MCs and neutrophils play significant roles in the pathophysiology of psoriasis and potentially other autoinflammatory diseases driven by the IL-23-IL-17 axis. The authors suggested that a modulation of MC and neutrophil ETosis and release of IL-17 could be used as a novel therapeutic mechanism of action for drugs targeting IL-23.

REGULATION OF THE FORMATION OF MCETs: COMPARISON WITH NETs

It is still not entirely clear how the formation of MCETs is transcriptionally regulated. One factor identified to contribute to formation of MCETs is the central transcriptional regulator of the cellular response to hypoxic stress, namely the hypoxia-inducible factor 1 α (HIF-1 α). Oxygen stress or hypoxia occurs in tissues during an infection, mostly, due to overconsumption of oxygen by pathogens and recruited immune cells. Importantly, HIF-1 α activation or stabilization has been shown to support myeloid cell production of defense factors and improved bactericidal capacity of immune cells (43, 44). In good correlation, Branitzki-Heinemann et al. (8) showed that MCET release was enhanced after MCs were treated with AKB-4924, a HIF-1 α stabilizing agent. These MCETs were able to entrap and immobilize *S. aureus*. Inhibition of phagocytosis did not alter the antimicrobial activity of MCs, whether or not HIF-1 α activity was boosted with AKB-4924. Augmentation of HIF-1 α -activity resulted in a boosting of the antimicrobial activity of human and murine MCs by inducing MCET formation. The results show for the first time that the extracellular antimicrobial activity of MCs is transcriptionally regulated and support the assumption that the transcription factor HIF-1 α is not only a global player in the cellular response to low oxygen stress but also may, furthermore, act as a key regulator of the antimicrobial response of several immune cells including MCs (43–47). However, in contrast to MCs, the role of HIF-1 α in the formation of extracellular traps produced by neutrophils remains to be elucidated. It has been shown that some well-known HIF-1 α agonists including mimosine and desferrioxamine (DFO) have an impact in neutrophil function. Mimosine has been proven to boost the bacterial killing by neutrophils. This effect was eliminated after DNase treatment suggesting the involvement of NETs in the mimosine-mediated neutrophil killing activity (48). In good correlation to these data, DFO was recently described as a positive stimulus for NET formation (49). The authors hypothesized that stabilization of HIF-1 α with agonists, such as DFO or mimosine, might facilitate the formation of NETs, which confirmed results from McInturff and coworkers showing that HIF-1 α contributes to rapamycin-induced NET formation in human neutrophils (50).

Although, MCETs and NETs share common characteristics, there are several cell type-specific differences. The formation of MCETs can be greatly increased after stimulation of MCs with PMA prior to infection (9), similar to NETs (40). But interestingly, MCs release less MCETs stimulated with the same stimuli in comparison to neutrophils when studying the respective literature: whereas more than 90% of neutrophils undergo ETosis within 3 h upon stimulation with PMA (38), approximately only 40% of MCs undergo ETosis after 6 h of stimulation with the same stimulus and concentration (9). Another important difference between MCs and neutrophils are their components, which are embedded in the DNA structures: elastase and MPO are essentially involved in the formation of NETs by degradation of histones and subsequent decondensation of chromatin; later both enzymes perform an antimicrobial role in NETs (51, 52).



In MCs, elastase and myeloperoxidase are not even expressed (53). Until now, it is unclear if, for example, MC-specific tryptase, which has also been shown as a component of MCETs (9), has similar functions in MCs.

In neutrophils, it has been additionally demonstrated that these cells can release ETs in response to infection, while remaining in a viable status, confirmed *in vivo* in a murine *S. aureus* skin infection model (54, 55). Formation of extracellular traps by viable eosinophils and basophils was also shown in response to *E. coli* and *S. aureus* (56, 57) and subsequent release of mitochondrial DNA (54). However, if a similar phenomenon also occurs in MCs is still unknown. Interestingly, in **Figure 1**, we can identify viable MCs in close contact to MCETs after treatment of MCs with PMA for 3 h. Thus, further investigations are needed to determine if MCs are also able to release extracellular traps in a viable status.

CONCLUSION

In summary, the actual literature assumes that MCET formation is not the result of passive release of DNA and granular proteins

during cellular disintegration, but rather an active and controlled process in response to specific stimulation. The extracellular structures act antimicrobially through a combination of direct killing of the entrapped pathogen or by its physical immobilization that enables the recruited effector cells to eliminate the pathogen. Both aspects may have a significant impact on the disease outcome. The fact that aggregated NETs limit inflammation by degrading cytokines and chemokines has already been shown (58). If MCET formation may decrease uncontrolled mast cell degranulation and subsequent dissemination of chemokines and cytokines during an acute overwhelming infection or autoimmune disease, which could lead to tissue damage, inflammation, and nerve cell activation with a potentially negative impact on the organism needs further investigation (59, 60). In any case, a plausible answer to the question “why do these long-living cells form MCETs” could be: MCETs are of particular importance in the direct and indirect antimicrobial activity against various pathogens; additionally, MCET formation may help to avoid or minimize affliction of the host by restricting the inflammatory responses.

AUTHOR CONTRIBUTIONS

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

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Neutrophil Extracellular Traps Go Viral

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Neutrophils are the most numerous immune cells. Their importance as the first line of defense against bacterial and fungal pathogens is well described. In contrast, the role of neutrophils in controlling viral infections is less clear. Bacterial and fungal pathogens can stimulate neutrophils extracellular traps (NETs) in a process called NETosis. Although NETosis has previously been described as a special form of programmed cell death, there are forms of NET production that do not end with the demise of neutrophils. As an end result of NETosis, genomic DNA complexed with microbicidal proteins is expelled from neutrophils. These structures can kill pathogens or at least prevent their local spread within host tissue. On the other hand, disproportionate NET formation can cause local or systemic damage. Only recently, it was recognized that viruses can also induce NETosis. In this review, we discuss the mechanisms by which NETs are produced in the context of viral infection and how this may contribute to both antiviral immunity and immunopathology. Finally, we shed light on viral immune evasion mechanisms targeting NETs.

Keywords: neutrophil extracellular traps, immunopathogenesis, neutrophils, viruses, viral immune evasion

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INTRODUCTION

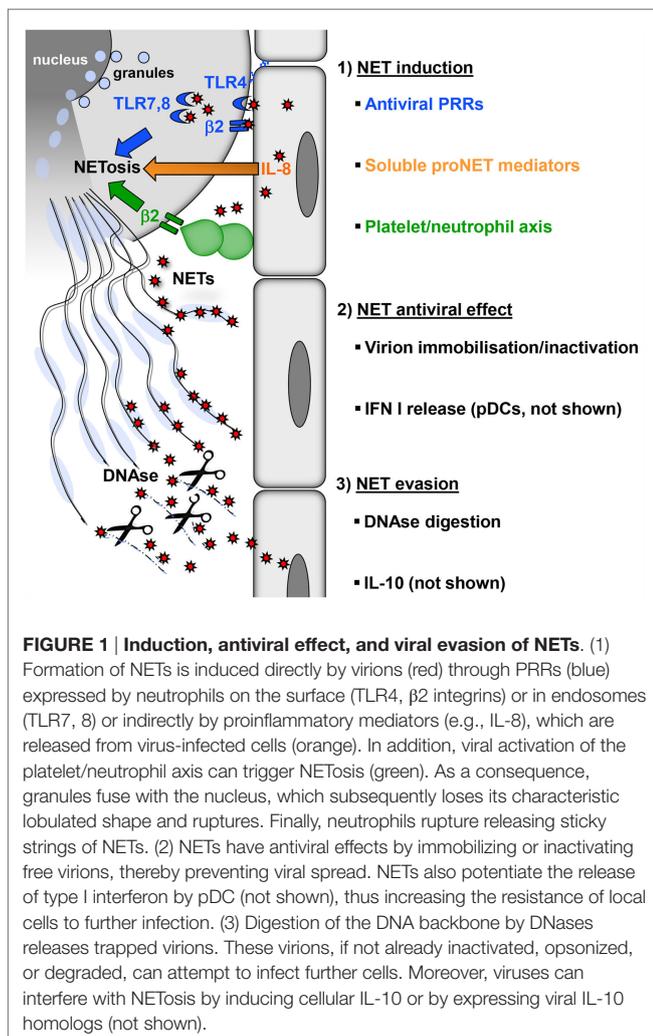
As the first line of defense against invading pathogens, neutrophils have a broad arsenal of antimicrobial functions (1). For example, activated neutrophils release granules containing antimicrobial molecules and produce reactive oxygen species (ROS) by oxidative burst. An alternative antimicrobial function of neutrophils is based on a special type of programmed cell death called NETosis that is distinct from apoptosis and necrosis (2, 3). During NETosis, the nuclei of neutrophils lose their characteristic shape, and chromatin decondensation takes place (4). Subsequently, the membranes of the nucleus and the granules disintegrate, allowing the mixing of their content. Finally, neutrophils release neutrophil extracellular traps (NETs). NETs are net-like structures that are composed of chromatin and endowed with granule proteins. They bind to, entrap, and often kill certain pathogens. NETs are released particularly in response to large microbial structures that cannot be easily phagocytosed such as *Candida albicans* hyphae and *Mycobacterium bovis* aggregates (5).

Classical NETosis requires the generation of ROS by NADPH oxidase. However, mitochondrial ROS production in the absence of a functional NADPH oxidase is sufficient to trigger NETosis (6). Moreover, a very rapid and ROS-independent form of NETosis is triggered by *Staphylococcus aureus* (7). Thus, depending on the stimulus NADPH is not always required for NET formation (8). Similar to necrosis and apoptosis, there are different forms of NETosis (9, 10). For example, it has been observed that NET formation can occur without concomitant neutrophil death (7, 11–14). The physiological and pathological meanings of these different NETosis forms still have to be elucidated.

Only recently, it was recognized that NETs are also generated during viral infection (15–17). Evidence is accumulating that neutrophils play a role in antiviral immune responses (18). These virus-induced NETs can both control the virus and damage the host (19). In this review, we focus our attention on the physiological and pathological relevance of virus-induced NETosis.

VIRAL NET INDUCTION

Many viruses stimulate neutrophils *in vitro* directly to produce NETs at low levels (20). Some of these viruses can be detected inside neutrophils, but there is no direct evidence that they establish productive infection in this cell type (20–23). This suggests that pattern recognition receptors (PRRs) expressed on the surface or in endosomes of neutrophils play an essential role in NETosis (Figure 1). For example, neutrophils sense HIV-1 by endosomal PRRs that detect viral nucleic acids, i.e., toll-like receptor (TLR) 7 and TLR8, and subsequently undergo NETosis (17). The fusion protein of respiratory syncytial virus (RSV) induces NETosis through TLR4 (24). NET formation induced by hantaviruses is mediated by signaling through $\beta 2$ integrins (20).



Influenza virus A can also stimulate neutrophils directly to release NETs; however, the molecules involved have not been defined (25). Surprisingly, influenza A virus-induced NETs do not protect against secondary bacterial infection (26). Thus, virus-induced NETs differ structurally and functionally from those generated during bacterial infection. In line with this view, the protein content of NETs depends on the type of NET-inducing stimulus (27).

In the context of viral infection, neutrophils can switch on antiviral effector programs other than NETosis, such as release of antiviral agents or phagocytosis, and can even become apoptotic (18). At the moment, it is unclear how neutrophils decide between these different responses. Possibly, not a single PRR but rather as-yet undefined combinations of neutrophilic PRRs determine the antiviral mode of action of neutrophils. Moreover, only a proportion of cells undergo NETosis, suggesting that only a special neutrophil subtype or maturation stage is susceptible to NETosis induction (4).

Viruses also induce NETosis indirectly without engaging PRRs expressed by neutrophils (Figure 1). The inflammatory milieu created by virus-infected endothelial and epithelial cells contains cytokines and chemokines such as interleukin-8 (IL-8) that trigger NETosis (3, 28, 29). In addition, type I interferon (IFN) is produced in large amounts during viral infections and primes neutrophils for NET formation (30). There is also evidence that platelets play an important role in antiviral defense (31). Platelet activation is frequently observed during viral infections. For example, single-stranded RNA viruses from the family *Picornaviridae* activate platelets through TLR7. This is important for reducing viral titers and increasing the survival of the host (32, 33). Activated platelets form aggregates with neutrophils and in this process stimulate NETosis (34) (Figure 1). On the molecular level, this NET-inducing aggregation has been attributed to surface molecules: CD41 on activated platelets interacts with CD11b, a $\beta 2$ integrin, on neutrophils. Other infection models have also shown that platelet–neutrophil interactions through $\beta 2$ integrins induce NET formation (11, 35, 36). Massive activation of the platelet/neutrophil axis and subsequent NET-based clearance mechanisms may represent an emergency strategy of the host in the face of systemically multiplying viruses. This reaction is followed by a drop in platelet counts, which is observed in many viral infections, e.g., viral hemorrhagic fever (VHF) caused by hantaviruses (37, 38). In fact, the degree of platelet loss correlates with the severity of virus-induced disease and determines the clinical outcome (39–41).

ANTIVIRAL ACTIVITY OF NETS

Although virus induction of NET formation is now well established, it is less clear how NETs contribute to antiviral immunity. In a mouse model of poxvirus infection, induction of NETs with LPS prior to infection strongly reduced the number of virus-infected liver cells and this protective effect was reversed by DNase treatment (34). There are direct mechanisms by which NETs develop antiviral activity (Figure 1). First of all, the web-like chromatin backbone of NETs can bind to and immobilize viral particles, in part by electrostatic attraction, thereby mechanically preventing virus spreading (17). Histones are enriched in positively charged amino acids and can attach to negatively

charged viral envelope. For example, the core histones H3 and H4 induce aggregation of seasonal influenza A particles and may inactivate HIV-1 (17, 42). Intriguingly, extracellular histones also reduce HIV-1 transcription (43). Finally, histone H1 binds to noroviruses, the most common cause of viral gastroenteritis and prevents their attachment to intestinal cells (44). Second, attached to the chromatin backbone of NETs are antimicrobial molecules such as myeloperoxidase (MPO), cathelicidins, and α -defensin. They have a proven antiviral activity against both enveloped and non-enveloped viruses and can inactivate viral particles (45).

NETs components also indirectly contribute to antiviral immunity by stimulating antiviral effector mechanisms executed by other immune cells. For example, histones and high mobility group box-1 (HMGB1) proteins act as danger-associated molecular patterns (DAMPs) that trigger release of proinflammatory cytokines and chemokines by other immune cells (46). This process is self-limiting as under high neutrophil densities NETs build aggregates that in turn degrade cytokines and chemokines (47). NETs also activate plasmacytoid dendritic cells (pDCs) through TLRs (48–50). pDCs have a key function in antiviral immunity by releasing high amounts of type I IFN (51). In fact, NETs can be enriched in oxidized mitochondrial DNA which is very efficient in inducing a type I IFN response (52). Finally, NETs could increase antiviral adaptive immunity by reducing the activation threshold of T lymphocytes (53).

VIRAL NET EVASION

Viruses are known for their extraordinary capacity to evade immune control mechanisms. There are also viral mechanisms that counteract NET formation (**Figure 1**). For example, HIV-1 envelope glycoprotein stimulates DCs to produce cellular IL-10 through DC-SIGN (17). IL-10 is an immunosuppressive cytokine that also inhibits TLR-induced ROS production (54). It is quite often produced in the context of viral infections suggesting that more viruses exploit IL-10 as a means of NET evasion (55, 56). In the genome of several large DNA viruses IL-10, homologs have been found including ubiquitous human pathogens such as human cytomegalovirus (HCMV) and Epstein–Barr virus (EBV) (57, 58). As these virus-encoded IL-10 molecules shape the function and cell death of immune cells, they may also modulate NETosis similar to cellular IL-10 (59, 60). Dengue virus (DENV) serotype-2 can arrest NET formation at a ROS-independent late stage by interfering with glucose uptake (61, 62). Finally, latency-associated nuclear antigen 1 encoded by Kaposi's sarcoma-associated herpesvirus (KSHV) impairs expression of NET-stimulating cellular IL-8 (63).

Some bacteria, such as streptococci, express DNase to degrade NETs (64–66). Herpesviruses also encode proteins that have DNase activity. These viral molecules process and package the replicated viral genome into the capsid (67). If released from virus-infected cells, they could degrade NETs, thereby remobilizing NET-entrapped virions.

Taken together, virus-induced NETs help to control viral dissemination by several direct and indirect mechanisms, whereas at the same time viral evasion mechanisms target NET formation to minimize the antiviral NET effect and immunopathology.

ROLE OF NETs IN VIRAL PATHOGENESIS

As for all effective immune responses against pathogens, NETosis may also result in immunopathology. Unbalanced NET formation is associated with pathological conditions such as respiratory distress, autoimmune disease, and thrombosis (68). NETs are directly cytotoxic to epithelial and endothelial cells (69, 70) as well as hepatocytes (71). They contain several components such as histones that are antimicrobial but at the same time can cause tissue damage and other pathological abnormalities including thrombosis (72). Moreover, NETs can occlude secretory ducts or small airways, thereby driving inflammation (73, 74). Other components of NETs such as HMGB1 may also play a detrimental role in virus-associated disease (75).

There is evidence supporting the concept that local NET deposits contribute to viral immunopathology. NETs have been detected in bronchoalveolar lavage fluid from children with severe RSV infection of the lower respiratory tract (76). Dense plugs occluding the small airways in RSV-infected calves contain NETs (76). Moreover, in a mouse model of influenza pneumonia, NET formation was observed in areas of alveolar-capillary damage in the lung (16). On the other hand, mice deficient in peptidylarginine deiminase 4 (PAD4) were as efficient in controlling influenza virus and showed similar survival as wild-type mice (77). This result suggests that NETs do not play an important role in individual antiviral immunity and virus-induced pathology because PAD4 deiminates histone H3 and H4 and is required for NET formation. The different outcomes of these studies may be due to different virus and mouse strains used. In line with this view, neutrophils from different mouse strains undergo NETosis with different efficiency (78). Furthermore, the influence of NETs on viral dissemination was not addressed in these studies. If virus-induced NET deposits represent an important pathogenic factor treatments that alleviate NET-induced pathological manifestations such as DNase should ease symptoms of virus-associated disease (79). Clinical or radiological improvement after DNase treatment of infants with virus-associated bronchiolitis was observed in some clinical trials (80, 81) but not in others (82). Thus, further studies have to elucidate the precise pathogenic role of virus-induced NET deposits in the lung and explore the efficiency of anti-NET treatment.

NETs start to circulate in detectable amounts in the serum if the NET degradation and clearance machinery of the host is overwhelmed. This systemic NET overflow has severe direct and indirect adverse effects. First, NETs can damage directly endothelial cells lining the interior face of the blood vessels cells (69, 70). Second, NET overflow drives autodestructive processes as components of NETs act as neo self-antigens and induce autoantibodies. In fact, a number of molecules that have been identified as important targets in autoimmune diseases (e.g., dsDNA, histones, MPO, vimentin, and enolase) are actually NET components. Accordingly, NETs have been connected to systemic pathology associated with disease entities such as small vessel-vasculitis, systemic lupus erythematosus (SLE), disseminated intravascular coagulation, rheumatoid arthritis, and preeclampsia (83, 84).

Systemic NET overflow may result from clearance deficiency or increased NET production. For example, sera from a

subpopulation of SLE patients show decreased DNase I activity and NET degradation (85, 86). Another enzyme that could prevent systemic NET overflow is DNASE1L3. It is released by DCs and macrophages and digests microparticle-associated chromatin, thereby preventing SLE (87). In those individuals who are deficient in NET-degrading enzymes even viruses with a relatively weak NETs-stimulatory capacity could drive NET-associated systemic pathology (Figure 2). NET formation represents a plausible link between viruses and systemic autoimmune disease. Supporting this idea, viral infections are associated with transient autoantibody production and are known to mimic SLE, induce SLE onset, or trigger lupus flares (88–90).

Transient systemic NET overflow due to increased NET formation without noticeable deficiency in DNase activity can occur during infection with hantaviruses (20) (Figure 2). Neutrophils play an antiviral role during VHF caused by hantaviruses (92–94). These zoonotic pathogens belong to the family *Bunyaviridae* and infect humans after transmission *via* inhalation of aerosolized urine, saliva, and feces from chronically infected rodents, their natural hosts. In humans, they can induce severe pulmonary and renal dysfunction as well as intravascular coagulation and hemorrhagic shock (95). Hantaviruses replicate in endothelial cells, their main target cells, without causing programmed cell death *in vitro*. This suggests that immunopathological mechanisms such as those driven by NETs contribute to Hantavirus-associated pathogenesis

(94, 96). In hantavirus-infected patients, high levels of circulating NETs are detected (20). In accordance, increased amounts of cell-free DNA (97) and histones (98) are found in the circulation of hantavirus-infected individuals. The cytotoxic effects of NETs may significantly contribute to hantavirus-associated pathology. In line with this view, histones have been shown to increase thrombin generation and intravascular coagulation (99, 100). They also upregulate the permeability of the endothelial barrier (101). Finally, NETs can induce the formation of autoantibodies that may contribute to the systemic pathology observed during hantavirus-associated disease (20).

Another form of VHF is caused by DENV. DENV is transmitted between humans by *Aedes* mosquitoes and poses a threat to roughly two billion people (102). There is no evidence as yet for a strong direct NET-stimulatory effect of DENV particles *in vitro* (61). Nevertheless, *in vivo* DENV-infected cells could stimulate NETosis indirectly by secreting the viral non-structural protein 1 (NS1). NS1 activates uninfected cells including endothelial cells *via* TLR4 (103, 104). Subsequently, activated endothelial cells could drive neutrophils into NETosis (69, 79). Moreover, NS1 could activate platelets *via* TLR4 which in turn stimulate neutrophils to undergo NETosis (105). Finally, IL-8 is produced by human endothelial cells in response to DENV (29) and is known to drive NETosis (3). In accordance, high levels of IL-8 and elastase, a key component of NETs, are found in DENV patients and correlate with disease severity (106).

These pathological effects explain why NET formation as part of an antiviral defense strategy is a double-edged sword. The host may benefit from NETs deposited precisely in the area of infection, thereby immobilizing or even neutralizing virus and killing virus-infected cells. This benefit may turn into disaster if NET formation is too widespread creating NET deposits in healthy tissue. As a consequence, too many uninfected host cells in the neighborhood of the infected areal may come under “friendly fire” resulting in considerable collateral tissue damage. Local NET-associated pathology may become systemic, if the NET degradation machinery (DNase activity) is impaired, or if the viral NET-stimulatory capacity is too strong. Such an unbalanced NET formation results in NET overflow. Under this condition, autoimmune phenomena are triggered that could result in systemic pathology (Figure 2).

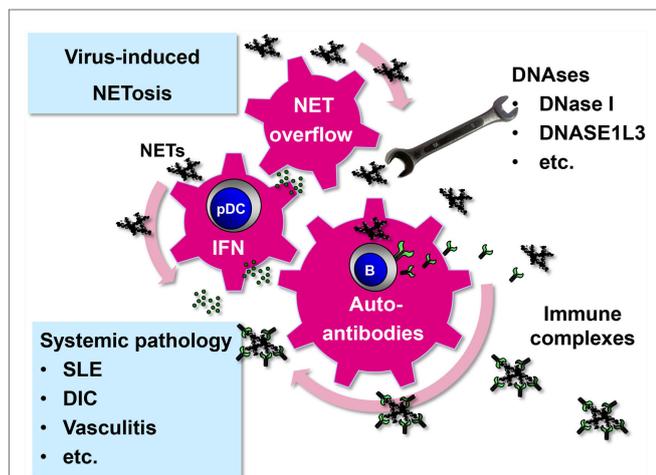


FIGURE 2 | Systemic pathology driven by virus-induced NET

formation. Virus-induced NETs may start to circulate and become systemic under certain circumstances. First, systemic infection with viruses that have a strong NET-stimulatory capacity, such as hantaviruses, may overwhelm intact NET-degrading function of DNases (20). Second, persistent viruses with low NET-inducing capacity, such as herpesviruses, may produce systemic NET excess if DNase activity is compromised. As a result of NET overflow, self-reactive memory B cells are stimulated to release autoantibodies after binding and internalizing NET components through their B cell receptor (91). NETs are enriched in oxidized mitochondrial DNA inducing a strong inflammatory response (52). NETs stimulate pDCs to release type I IFN that adds momentum to the vicious cycle by further activating and expanding autoreactive B cells (48–50). Immune complexes are formed which not only cause systemic pathology as observed in several disease entities such as SLE but also promote the autoimmune process by driving a positive feedback loop.

CONCLUDING REMARKS

It is now evident that most pathogens, including viruses, can stimulate neutrophils to undergo NETosis. Although much smaller than bacteria, fungi, or parasites, viral particles do not seem to slip through NETs but rather become immobilized. Whether these viral particles are inactivated as well is a moot point, as long as they are ensnared by NETs, they represent no threat. However, an increasing number of studies indicate that a disproportionate virus-induced NET release can contribute to damage, locally as well as systemically. It will be important to explore the mechanisms that control NET formation in the context of viral infections. On the basis of this knowledge, it could be possible to prevent NET-assisted control of viruses becoming a Pyrrhic victory.

AUTHOR CONTRIBUTIONS

Both authors contributed to the conception, writing, and editing of this review.

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A Critical Reappraisal of Neutrophil Extracellular Traps and NETosis Mimics Based on Differential Requirements for Protein Citrullination

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NETosis, an antimicrobial form of neutrophil cell death, is considered a primary source of citrullinated autoantigens in rheumatoid arthritis (RA) and immunogenic DNA in systemic lupus erythematosus (SLE). Activation of the citrullinating enzyme peptidylarginine deiminase type 4 (PAD4) is believed to be essential for neutrophil extracellular trap (NET) formation and NETosis. PAD4 is therefore viewed as a promising therapeutic target to inhibit the formation of NETs in both diseases. In this review, we examine the evidence for PAD4 activation during NETosis and provide experimental data to suggest that protein citrullination is not a universal feature of NETs. We delineate two distinct biological processes, leukotoxic hypercitrullination (LTH) and defective mitophagy, which have been erroneously classified as “NETosis.” While these NETosis mimics share morphological similarities with NETosis (i.e., extracellular DNA release), they are biologically distinct. As such, these processes can be readily classified by their stimuli, activation of distinct biochemical pathways, the presence of hypercitrullination, and antimicrobial effector function. NETosis is an antimicrobial form of cell death that is NADPH oxidase-dependent and not associated with hypercitrullination. In contrast, LTH is NADPH oxidase-independent and not bactericidal. Rather, LTH represents a bacterial strategy to achieve immune evasion. It is triggered by pore-forming pathways and equivalent signals that cumulate in calcium-dependent hyperactivation of PADs, protein hypercitrullination, and neutrophil death. The generation of citrullinated autoantigens in RA is likely driven by LTH, but not NETosis. Mitochondrial DNA (mtDNA) expulsion, the result of a constitutive defect in mitophagy, represents a second NETosis mimic. In the presence of interferon- α and immune complexes, this process can generate highly interferogenic oxidized mtDNA, which has previously been mistaken for NETosis in SLE. Distinguishing NETosis from LTH and defective mitophagy is paramount to understanding the role of neutrophil damage in immunity and the pathogenesis of human diseases. This provides a framework to design specific inhibitors of these distinct biological processes in human disease.

Keywords: NETosis, leukotoxic hypercitrullination, citrullination, rheumatoid arthritis, systemic lupus erythematosus, NADPH oxidase, peptidylarginine deiminase, mitophagy

INTRODUCTION

The History of NETs: Discovery and Misconceptions

Following Elias Metchnikoff's initial proposal that wandering cells ("Wanderzellen"), known today as neutrophils, phagocytose and kill bacteria (1), it took an additional 117 years to elucidate a second mechanism by which neutrophils can entrap microbial agents (2). In 2004, Brinkmann and colleagues reported that neutrophils can release nuclear chromatin together with granule proteins to form an extracellular mesh that binds and kills bacteria while also degrading virulence factors (2). The term neutrophil extracellular traps (NETs) was introduced to describe these neutrophil-derived antimicrobial fibers (2). Since the extracellular release of chromatin has historically been linked to necrosis (3), these initial findings allowed for speculation as to whether NETs were released from necrotic cells or as an active function of live neutrophils (4). It was later shown that NETs are released as a consequence of a regulated form of cell death called NETosis, which is dependent on the generation of reactive oxygen species (ROS) by NADPH oxidase (5, 6). To date, NETs and NETosis are used synonymously.

The discovery of NETs has generated an avalanche of research over the past 10 years, providing important insights into novel antimicrobial pathways and a plethora of pathologies as diverse as sepsis and thrombosis, autoimmune and metabolic diseases, and immunodeficiency and cancer. At the same time, the definition of NETosis has evolved rapidly, suffering many modifications that allowed researchers to incorporate and potentially misclassify almost any biological process involving the extrusion of neutrophil DNA (nuclear or mitochondrial) (7). The term "extracellular trap" has moreover been applied to an increasing number of cell types that release chromatin (e.g., eosinophils, mast cells, and macrophages) (8).

While the initiation and execution components during NETosis are not yet fully understood, unique cellular changes and biochemical pathways that distinguish NETosis from other forms of cell death have been well characterized (5, 9–11). Despite this, the definition of NETs/NETosis still hinges on purely morphological descriptors, primarily the extrusion of DNA. This arguably broad definition has generated an ever-increasing list of stimuli and pathways reported to induce NETs, while NETosis has inevitably become an umbrella term that encompasses a diverse group of cellular processes with potentially fundamental differences in biology and significance for immunity and disease. While some NETs are part of a unique form of programmed cell death that evolved to fight pathogens (referred to in this review as NETosis), it is conceivable that many processes currently identified as "NETosis" may be the consequence of cellular damage or other forms of cell death (12). Distinguishing these different forms of NETs has critical implications. If NETs are driven by different biological processes, it is unlikely that all forms of DNA extrusion will have the same capacity to fight infection, drive various human diseases, or be inhibited by targeting a single biochemical pathway.

Our interest in NETs is related to the process of protein citrullination. To date, it is commonly accepted that citrullination of

histones by peptidylarginine deiminase type 4 (PAD4) is required for chromatin unfolding and the formation of NETs (13). This misconception has drawn attention to PAD4 as a promising cellular target to broadly inhibit NETosis (14). Additionally, this has led to the proposal that this form of neutrophil death represents a major source of citrullinated autoantigens in rheumatoid arthritis (RA) (15, 16), an autoimmune disease characterized by the appearance of anti-citrullinated protein antibodies (ACPAs) (17). Similarly, it has focused interest on PAD4 as a therapeutic target in systemic lupus erythematosus (SLE) in which NETosis is believed to play a pathogenic role (18).

In this review, we will revisit some aspects of citrullination during the process of "NETosis" that we consider controversial. We provide experimental evidence to highlight differences in protein citrullination induced by various NET-inducing stimuli and propose a framework to understand these disparities. The corollary of this work is that protein citrullination can distinguish at least two mutually exclusive mechanisms that generate extracellular DNA structures. One of these is NETosis, an antimicrobial process that is not associated with cellular hypercitrullination. The second mechanism, which merely mimics NETosis, is induced by calcium ionophores or membranolytic agents that cause calcium-dependent hyperactivation of PADs, cellular hypercitrullination, and as a consequence extrusion of DNA. This mechanism, which we call leukotoxic hypercitrullination (LTH), is not antimicrobial, but linked to the production of citrullinated antigens in RA. Moreover, it is utilized by some pathogenic bacteria to abrogate neutrophil activity. A third process that can readily be confused with NETosis is the extracellular release of mitochondrial DNA (mtDNA), which results from a constitutive defect in mitophagy in neutrophils (19). This process has not been associated with protein citrullination. However, it is linked to the production of extracellular oxidized mtDNA (ox-mtDNA), which is highly interferogenic and initially confounded with NETosis in SLE (19). The need for a biochemical definition of NETs and their distinction from other mechanisms of neutrophil damage based on their antimicrobial and pathogenic effects will be discussed.

NETs Released during Antimicrobial Programmed Cell Death (NETosis)

The capacity of neutrophils to die by ROS-dependent "autotoxicity" in response to phorbol myristate acetate (PMA), a cocarcinogen derived from oil of the *Croton* shrub (20), was initially described by Min-Fu Tsan in 1980 (21). It was later appreciated that this form of neutrophil death differs from apoptosis and necrosis. Early changes in nuclear morphology, specifically chromatin decondensation and rupture of the nuclear envelope, are followed by rupture of the plasma membrane and subsequent dispersal of cytoplasmic contents into the extracellular space (22). The significance of this novel form of cell death was not recognized until 2004, when Brinkmann and colleagues reported two major findings (2). First, they found that the material released from the dying neutrophils was chromatin coated with granular antimicrobial proteins, forming extracellular fibers with bacterial binding capacity (NETs). Second, they noted that these fibers have the capacity to degrade virulence factors and kill bacteria, suggesting a novel mechanism by which the

innate immune system can limit acute infections. Importantly, the production of NETs was reproduced with two physiological stimuli: interleukin-8 (IL-8) and lipopolysaccharide (LPS). The existence of NETs *in vivo* was confirmed in both experimental dysentery and spontaneous human appendicitis (2). These studies thus provided evidence that NETs may not simply be an artifact of PMA toxicity, but rather a process of potential physiological relevance.

Several features initially suggested that NETs were actively generated by neutrophils and not just a consequence of cellular rupture resulting from known forms of cell death (2). The concept that live neutrophils can actively weave extracellular traps using their own DNA created substantial excitement about this novel antibacterial process. Yet, in the absence of molecular mechanism, the argument that NETs were merely an artifact of necrosis remained (4). Subsequent studies demonstrated that NETs indeed emerge from dying neutrophils (5), but this process was neither related to neutrophil apoptosis nor necrosis. Instead, it was shown that PMA induces NETs through the unique form of cell death that was dependent on ROS production by NADPH oxidase (5, 21, 22). Acknowledging the non-accidental nature of committed cell death by NET formation distinct of necrosis, this process was named NETosis (in analogy to other forms of programmed cell death) (6). Parallel studies demonstrated that *Staphylococcus aureus* (*S. aureus*) is able to replicate the changes in neutrophil morphology seen with PMA, supporting the biological relevance of this process as an antibacterial strategy. However, bacterial growth conditions that avoided expression of staphylococcal toxins (toxin-free *S. aureus*) were required to reproduce NETosis (5). The significance of using toxin-free, rather than virulent bacteria in these experiments will be discussed later.

Morphological and Biochemical Features That Define NETosis

Neutrophil extracellular traps induced by PMA or “toxin-free” *S. aureus* exhibit unique features that support NETosis as a novel, regulated form of cell death (5). Morphologically, NETosis begins with the disappearance of nuclear lobules. This is followed by chromatin decondensation and disintegration of the nuclear envelope into small vesicles containing this decondensed chromatin. Subsequently, the membranes of these nuclear vesicles and cytoplasmic granules disintegrate, allowing for mixing of chromatin with cytoplasmic and granule contents. Finally, the plasma membrane ruptures and allows for the release of chromatin decorated with antimicrobial granule proteins into the extracellular space (the NET). While this process is distinct from necrosis and apoptosis (5), it is still uncertain whether NETosis may be mechanistically associated with other forms of regulated cell death (e.g., autophagy and necroptosis) (12, 23–27).

The production of ROS by NADPH oxidase is considered the biochemical hallmark in the process of NETosis (5). Much of the current understanding of the molecular mechanisms driving NETosis is based on studies using PMA, a phorbol ester that mimics diacylglycerol (20, 28). PMA directly activates protein kinase C (PKC) (28), which then phosphorylates the p40^{phox} and p47^{phox} components of NADPH oxidase. This induces the

production of superoxide (29–31). Several kinases have been implicated downstream of PKC, including c-Raf, MEK, Akt, and ERK (11, 32–34). However, the mechanistic role of these enzymes is not well understood. For example, ERK has been proposed as a major downstream kinase involved in NETosis *via* phosphorylation of p47^{phox} and activation of NADPH oxidase (11). However, conflicting evidence exists as to whether ERK is indeed activated upstream or downstream of ROS production (11, 34). Nevertheless, an additional contribution of ERK to this process may not be required as PKC can directly activate NADPH oxidase (29–31).

Not every neutrophil undergoes NETosis upon activation with PMA. The mechanism behind why some cells survive and others die remains unclear. In cells that commit to NETosis, oxidative burst triggers the dissociation and activation of neutrophil elastase (NE) from a membrane-associated complex called the “azurosome” in a myeloperoxidase (MPO)-dependent process (35). Once in the cytoplasm, NE first cleaves F-actin and subsequently translocates to the nucleus. Here, NE degrades histones, thereby promoting chromatin decondensation (9, 10, 35). The degradation and disassembly of the actin cytoskeleton may further facilitate the disruption of the cytoplasmic membrane, a requirement for NET release (35). Citrullination of histones, along with their proteolytic cleavage, is considered essential for the disassembly of chromatin and release of DNA during NETosis (36, 37). Evidence for the presumed citrullination of histones during NETosis is reviewed in the next sections.

PAD Activation and Cellular Hypercitrullination in Neutrophils

Citrulline is a non-essential amino acid that is generated in proteins by posttranslational enzymatic conversion of arginine residues to peptidylcitrulline. This modification, called either deimination or citrullination, is catalyzed by the PADs, a family of calcium-dependent enzymes (38–40). Among the five PAD enzymes encoded in humans (PAD1–PAD4 and PAD6) (41–45), PAD4 has received special attention in neutrophil biology. This enzyme, initially named PAD5 (PAD V), was cloned from a human myeloid leukemia cell line (HL-60 cells) induced to differentiate into granulocytes (44). Expression of PAD4 was later found in both human eosinophils and neutrophils (46). PAD4 shows nuclear localization, and histones were among the first PAD4 substrates to be identified (47, 48). To date, more than 70 putative PAD4 substrates have been described (49, 50). It was initially proposed that this enzyme may act as a transcriptional regulator by modifying the function of histones (51, 52), but PAD4-deficient mice demonstrated normal development, suggesting that, at least in mice, PAD4 has no essential role in steady-state neutrophil functions or development (37).

The mechanisms controlling PAD activation in cells are not yet fully understood. Since PADs are calcium-dependent enzymes (38), stimuli that potently increase intracellular calcium concentration have been used to identify cellular targets of protein citrullination in cells (48, 49, 53, 54). Ionophores are compounds that form lipid-soluble complexes with polar cations and act as vehicles in the transport of ions across biological membranes (55). A23187 (calcimycin) and ionomycin, both derived from

Streptomyces species (55–57), are divalent carboxylic ionophores with selectivity for calcium (calcium ionophores). Induction of calcium influx by either A23187 or ionomycin in neutrophils results in hyperactivation of PADs and citrullination of a myriad of proteins across all molecular weights (48, 54), which we termed cellular hypercitrullination (58). During this process, citrullinated proteins accumulate with time and plateau ~3 h after cell activation (**Figure 1**).

Citrullination results in a loss of positive charge per modified arginine residue, thus reducing the net charge of the modified protein and increasing its hydrophobicity. This can lead to protein unfolding and either gain or more likely loss of protein function (51, 52, 60–62). While the spectrum of citrullinated proteins (i.e., the citrullinome) generated by calcium ionophores in neutrophils has not been fully characterized, known targets include nuclear proteins (nucleophosmin and histones) (48), antimicrobial proteins (MPO, NE, azurocidin, and defensins) (63), and components of the cytoskeleton (vimentin and actin) (54, 64). Among these substrates, citrullination of histones and vimentin promote chromatin decondensation and the disassembly of intermediate filaments, respectively (36, 61). The effect of hypercitrullination on other cellular proteins is unknown, but the vast accumulation of citrullination-induced protein unfolding is likely to provoke functional and structural collapse of the cell. For example, overexpression and spontaneous activation of PAD4 in osteosarcoma U2OS cells, without any other element

required for NETosis (i.e., NE or MPO), is sufficient to induce cell disintegration and the production of NET-like structures (pseudo-NETosis) (65). This may also explain how neutrophils become dismantled and release their intracellular contents upon hyperactivation of PAD4 with calcium ionophores, including the release of genomic DNA (gDNA) that mimics NET formation (36). Although the mechanisms that drive cell damage and DNA extrusion by calcium ionophores are biochemically distinct from NETosis, as described in detail below (66), both PMA and calcium ionophores have been equivalently used to study this unique form of neutrophil cell death.

The Misconception That PAD4 Is Required for NETosis

The Race to Find Mechanisms of Chromatin Decondensation during NETosis

While early studies demonstrated that ROS production by NADPH oxidase was required to initiate NET formation (5), the discovery of a downstream molecular mechanism driving chromatin decondensation during NETosis, specifically the cleavage of histones by NE, lagged behind (10, 35). During this time, it was found that (1) hypercitrullination of histones by PAD4 promotes chromatin unfolding and (2) calcium ionophores, potent activators of PADs, induce extracellular release of DNA similar to NETosis (36). Assuming that the nature of NETs generated by calcium ionophores and PMA were the same, PAD4 activation and histone citrullination were proposed to be the missing link between ROS production and chromatin decondensation during NETosis (13, 36). In fact, the belief that PAD4 is activated by ROS during NETosis prevails to this day (67), although evidence to support this concept is lacking. Since a reducing environment is necessary to maintain the active site free thiol Cys645 required for PAD4 function (68, 69), we can anticipate that oxidation of PAD4 by ROS will instead inactivate the enzyme.

While a lack of biochemical insight initially suggested that NETs induced by calcium ionophores and PMA were identical, subsequent studies established that they are the consequence of biologically distinct processes. The formation of NET-like structures induced by calcium ionophores occurs more rapidly than those formed during NETosis. Additionally, this process is independent of ERK and NADPH oxidase activity (a hallmark of NETosis), and thus often referred to as NADPH oxidase-independent NET formation (32, 66). These calcium ionophore-induced structures are in part mediated by calcium-activated small conductance potassium (SK) channel member SK3 and mitochondrial ROS (mtROS) (32), and completely dependent on calcium influx, unlike those formed during PMA-induced NETosis (32, 66). No evidence exists indicating that NE is required for the induction of “NETs” or citrullination by calcium ionophores (70), nor that F-actin and/or histones are cleaved during this process, as required during NETosis (10, 35). Notably, the calcium requirement for calcium ionophore-induced “NET” formation is not surprising as PAD activation and cellular hypercitrullination are calcium-dependent processes. In contrast to calcium ionophores, however, NETosis induced by PMA proceeds without PAD4 activation and histone citrullination (32, 71). Moreover, PMA is not only unable to initiate protein citrullination in neutrophils

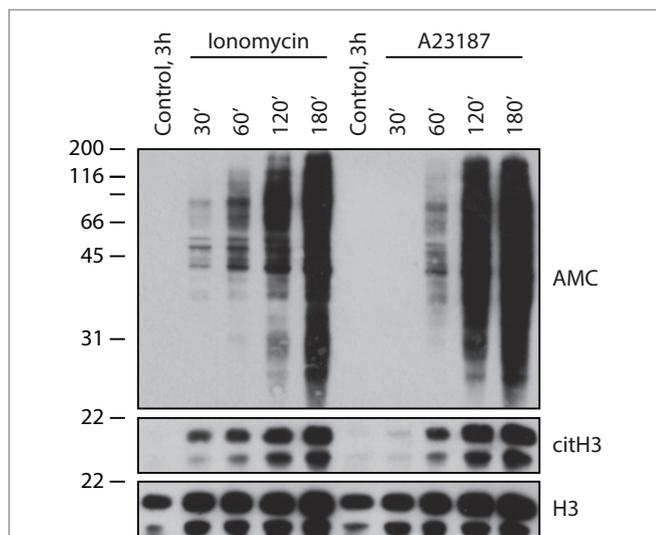


FIGURE 1 | Calcium ionophores induce cellular hypercitrullination in neutrophils. After informed consent, peripheral blood neutrophils were isolated from healthy donors as previously described (58), resuspended in HBSS/10 mM HEPES/1.5 mM CaCl₂, and incubated with 1 μM ionomycin or 5 μM A23187 at 37°C for 30–180 min. Neutrophils incubated for 3 h in the absence of stimulus were used as negative control. General protein citrullination was visualized by anti-modified citrulline (AMC) immunoblotting (59) (upper panel) and histone H3 citrullination (Cit-H3) using antibodies against citrullinated histone H3 (citrulline 2 + 8 + 17) (Abcam, Cat# ab5103) (middle panel). Histone H3 (H3) was detected as loading control (lower panel) (anti-histone H3, Abcam, Cat# ab1791). The experiments were performed on at least three separate occasions with similar results.

(Figure 2A), but also acts as a potent inhibitor of PAD4 in these cells (71). As such, PAD4 hyperactivation induced with calcium ionophores is significantly inhibited by PMA in a process that is dependent on PKC α (71). While the mechanism by which this kinase suppresses the activity of PAD4 is not elucidated, it is interesting that a different PKC isoform (i.e., PKC ζ) is required for PAD4 activation induced by calcium ionophores (71). This suggests that PAD4 is regulated *via* phosphorylation. By activating different kinases, PAD4 function may be stimulus dependent, thus avoiding unwanted effects of protein citrullination during NETosis and other biologically relevant processes.

Two major conclusions can be drawn from these data. First, calcium ionophores can induce cellular hypercitrullination and NET-like structures, but this process is not associated with NETosis (i.e., an antimicrobial form of cell death dependent on NADPH oxidase) (5). Second, NETosis is associated with a decrease in PAD4 activity and absence of cellular hypercitrullination (Figure 2A). Regrettably, the original misconception that hypercitrullination occurs during NETosis remains to date.

The Erroneous Use of Citrullinated Histones as Specific Markers of NETosis

The identification of biochemical markers associated with specific forms of cell death is critical to study their mechanisms and biological relevance both *in vitro* and *in vivo* (23). Different to other forms of programmed cell death, unique markers of NETosis have not yet been identified. Although cellular hypercitrullination

induced by calcium ionophores targets a broad range of proteins (48, 54), initial studies using these ionophores to suggest a role of citrullination during NETosis focused entirely on the analysis of histones (36). This erroneously created the idea that histones were the only targets of ionophore-induced citrullination in neutrophils, and that histone citrullination was the mechanism underlying dismantling of the cell in response to calcium influx. Not only did the misconception that calcium ionophores induce NETosis become accepted, but it also fueled dogmatic belief that histone citrullination can serve as a specific biomarker for this form of cell death. Given that PAD4 is actively inhibited during NETosis (71), the detection of citrullinated histones both *in vitro* and *in vivo* can be explained by other means.

First, PAD4 targets histones as a mechanism to regulate transcription upon cell activation (72–74). The detection of citrullinated histones alone *in vivo* may thus identify activated cells, rather than cells dying by NETosis (75). Second, histone citrullination is similarly observed in other forms of cell death such as apoptosis (58, 76), while shown to be inhibited during NETosis (71). Identifying extracellular citrullinated histones together with other neutrophil contents (DNA, MPO, PR3, and elastase, among others) is therefore not a suitable indicator of NETosis, but may rather indicate that neutrophils are dying by a form of cell death that is distinct from NETosis. Third, prominent citrullination of histones and extracellular release of DNA are rapidly induced as consequence of membranolytic damage of neutrophils (i.e., LTH). This distinct form of death can easily be confused with NETosis, as discussed in detail below. Finally, isolated neutrophils, in the absence of NETosis-inducing stimuli, show increased staining for citrullinated histones over time. This may represent spontaneous cell death by apoptosis or evolving activation/damage suffered during neutrophil purification. In the absence of appropriate negative controls, any stimulus, including PMA, will thus appear to induce neutrophil citrullination. Importantly, the use of PAD4-deficient cells or PAD inhibitors is not suitable negative controls to discard spontaneous activation of PAD4. In the opinion of these authors, a large number of studies that have reported citrullination in the setting of NETosis lack appropriate controls to delineate whether citrullination is induced upon stimulation or merely represents background staining. The absence of negative controls may thus explain why an unlikely number of stimuli tested have been shown to induce some degree of citrullination in neutrophils, and why the reproducibility of these findings remains poor.

The Haste of Linking NETosis and Citrullination

The excitement and expectations regarding citrullination and NETosis for systemic autoimmunity, the arbitrary use of PMA and calcium ionophores to induce NETs, and some overzealous data interpretation may have also contributed to keep the erroneous concepts about hypercitrullination in NETosis alive. For example, in a frequently cited review on PAD4 and NET formation (13), 16 references were included to establish that PMA induces PAD4 activation during NETosis. This includes the original paper by Brinkmann and colleagues, which was published 5 years prior to data suggesting that PAD4 is involved in NET formation (2, 9, 10, 27, 37, 77–87). In fact, only 1

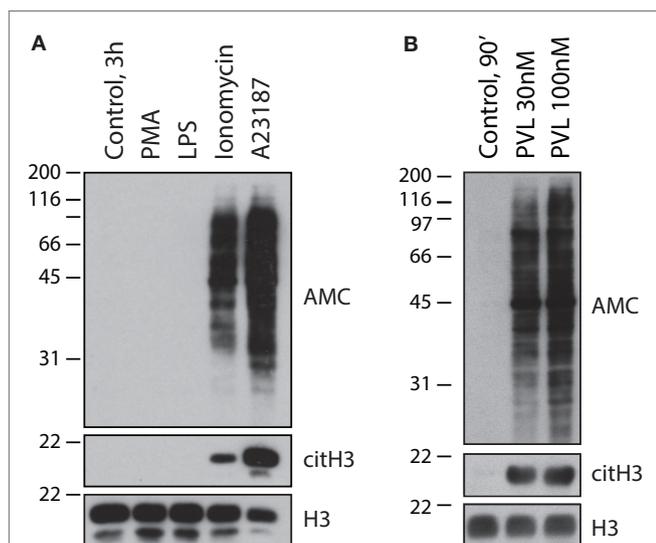


FIGURE 2 | Calcium ionophores and the pore-forming toxin PVL from *S. aureus*, but not NETosis-inducing stimuli, activate cellular hypercitrullination in neutrophils. Purified human neutrophils in HBSS/10 mM HEPES/1.5 mM CaCl₂ were incubated (A) in the absence or presence of 100 nM PMA, 500 ng/mL LPS, 1 μ M ionomycin, or 5 μ M A23187 at 37°C for 3 h, or (B) in the absence or presence of PLV from *S. aureus* (recombinant LukS and LukF from IBT BioServices) at 30 or 100 nM for 90 min at 37°C. Total citrullinated proteins (AMC), citrullinated histone H3 (citH3), and histone H3 (H3) (loading control) were detected by immunoblotting. The experiments were performed on at least two separate occasions with similar results.

of these 16 references included experiments involving protein citrullination. The majority of the referenced studies merely noted the presence of histones in NETs, and not histone citrullination in NETs.

The misconception that PMA induces hypercitrullination and that calcium ionophores drive NETosis is more than a semantic problem. For example, in a recent study, NETs induced with PMA were used to demonstrate that monoclonal antibodies generated from RA synovial B cells target citrullinated histones in NETs (88). Considering that PMA effectively blocks citrullination (71), it is much more likely that cross-reactivity of RA autoantibodies with native (uncitrullinated) histones or other NET components explains these findings. Nevertheless, the study embraced the misconception that NETosis is a source of citrullinated autoantigens in RA. Similarly, calcium ionophores are potent inducers of neutrophil hypercitrullination and citrullinated RA autoantigens (54, 63), which may be erroneously interpreted as the result of NETosis. In this context, the premature excitement and hastiness surrounding the rapidly growing field of NETosis has already misguided our understanding of etiology and pathogenesis of human diseases.

The Incomplete Understanding of the Role of PAD4 in the Immune System

Peptidylarginine deiminase type 4 plays a major role in epigenetic regulation *via* the citrullination of histones and transcription factors (72–74, 89–95). In the immune system, PAD4 promotes cytokine production by augmenting chromatin association of E2F-1 and decreasing HP1-mediated transcriptional repression of cytokine genes (74, 89). Moreover, PAD4 inhibitors were shown to decrease pro-inflammatory Th1 and Th17 responses and to increase anti-inflammatory Th2 responses both *in vitro* and *in vivo* (96). PAD inhibition also blocks the functional maturation of dendritic cells induced by toll-like receptor agonists (97). Despite this multifaceted role of PAD4 for immune function, the prominent misconception that PAD4 is required for NETosis has frequently led investigators to postulate that the anti-inflammatory effects of PAD inhibitors seen *in vivo* are best explained by blocking NET formation. Regardless of whether NETosis may have any role in disease pathogenesis, the observed benefit of PAD inhibition in experimental models of inflammation and autoimmunity is thus haphazardly attributed to limiting NET formation. This has importantly led to the current paradigm that NETs are pathogenic *in vivo*, confounding our understanding of disease pathogenesis.

For example, inhibition of NETosis by propylthiouracil (PTU) induces the production of anti-MPO antibodies and vasculitis in rats (98), suggesting that the normal process of NETosis is protective against this autoimmune process. However, the finding that PAD inhibition by Cl-amidine decreases PTU-induced autoantibody production in this model has been paradoxically interpreted as evidence that excessive formation of NETs is implicated in the production of these antibodies and vasculitis (75). The possibility that PAD inhibition may act through mechanisms different from NETosis was not considered. In the case of SLE, cumulative evidence now suggests that NETosis is not involved in the disease pathogenesis, as

discussed below (19, 99, 100). Thus, the demonstrated benefits of PAD inhibition in experimental models of lupus, attributed to a decreased burden of NETosis (18, 101), may indeed be mediated by alternative mechanisms, such as the transcriptional regulation of anti-inflammatory pathways by these inhibitors. The inhibition of PAD4 activity as direct evidence *in vivo* that citrullination and NETosis are linked to disease pathogenesis therefore requires critical evaluation.

PMA-Induced NETosis vs. NETs Induced by Calcium Ionophores: Biological Relevance and Proposed Physiological Counterparts

Phorbol myristate acetate and calcium ionophores have had a historical role in the functional understanding of the immune system. Since the early 1970s, these molecules have been used as surrogate stimuli that mimic signals induced by microbial and immune products, simplifying the study of mechanisms and pathways of immune activation (102, 103). In neutrophils, PMA potently activates ROS production *via* NADPH oxidase and induces degranulation (104–109). PMA has no effect on intracellular calcium during neutrophil activation (110, 111). This may also explain the inability of PMA to activate PAD4, in addition to the inhibition mediated by PKC α (71). In contrast, calcium ionophores have been used to study the role of calcium in neutrophil function (109, 112–114). While calcium ionophores also induce neutrophil degranulation (109, 114), these molecules are extremely weak stimulators of NADPH oxidase activity as compared to PMA (110, 115). Because of their functional divergence, PMA and calcium ionophores are commonly used in combination to achieve maximal activation of cellular responses that require elevated intracellular calcium and PKC activation (116, 117).

Although a large number of stimuli with potential biological relevance have been proposed to induce NETosis (13, 118), the underlying mechanisms for the majority of these stimuli have not been elucidated. Instead, studies have focused on understanding the pathways used by PMA and calcium ionophores to induce NETs, assuming that these surrogates are analogous to NETosis *in vivo*. The biochemical pathways activated by immune and microbial stimuli, however, may not completely replicate those induced by PMA or calcium ionophores. Stimuli such as IL-8 and LPS, initially used to demonstrate that NETs are not simply an artifact of PMA treatment, are neither efficient in driving ROS production (as seen with PMA) nor in increasing intracellular calcium (analogous to calcium ionophores) (119–123). The precise mechanism of NET induction with both IL-8 and LPS is unknown, and their ability to reliably reproduce NETosis is questionable (27, 124). Other soluble factors, such as tumor necrosis factor- α (TNF- α) (37, 79), have similar limitations in explaining the induction of NETs (125).

The potential importance of NETs in human biology is supported by the direct induction of NETosis by pathogens. The fungi *Candida albicans* (*C. albicans*) and *Aspergillus fumigatus* (*A. fumigatus*) appear to be ideal model pathogens

for consistently activating NETosis *via* NADPH oxidase, MPO, and NE, thus using a mechanism similar to PMA (9, 10, 35, 126–129). However, there is no evidence that citrullination or pathways activated by calcium ionophores are required by these fungi to induce NETosis.

The mechanisms of bacterial induction of NETs, however, are inconsistent. *S. aureus* has been used as a model organism to demonstrate bacterial NET formation by both NADPH oxidase-dependent (PMA-like) and NADPH oxidase-independent pathways (5, 81). *S. aureus* has also been reported to induce “vital” NET formation, a morphological description chosen to highlight that neutrophils remain “alive” (i.e., transiently functional and somewhat motile) after releasing NET-like structures (130, 131). Other bacterial species face similar caveats. For example, *Klebsiella pneumoniae* has been shown to require MPO and NE to induce NETosis in mice (PMA-like?) (10). However, the induction of NETs by *K. pneumoniae in vitro* was not reproducible (128). In the case of *Shigella flexneri* (*S. flexneri*) and group A *Streptococcus pyogenes* (*S. pyogenes*) deficient in an extracellular DNase (Sda1), used to support that PAD4 is required to induce NETs (37), the mechanism(s) that may drive PAD4 activation remain elusive. Indeed, it has been proposed that only large pathogens such as *C. albicans* hyphae and bacterial aggregates have the capacity to induce NETosis, questioning experiments that involve the induction of NETosis by single-cell suspensions of bacteria that are not aggregated (128).

The poor reproducibility of NET formation by some biologically relevant stimuli and the limited understanding of these discrepancies may explain why PMA and calcium ionophores remain primary tools in the study of NETs.

Synergistic Signals May Be Required to Efficiently Trigger NADPH Oxidase-Dependent NETosis

Several stimuli that were initially proposed to induce NETs are poor activators of NADPH oxidase (such as IL-8, LPS, and TNF- α) (119–123, 125). However, it is fascinating that these molecules can prime neutrophils to produce high amounts of ROS in response to a second signal, such as fMLP (119, 120, 125), a peptide that mimics bacterially derived signals by activating the formyl peptide receptor 1 (FPR1) (132). Similarly, neutrophil priming with interleukin-1 β (IL-1 β), TNF- α , granulocyte macrophage colony-stimulating factor (GM-CSF), and granulocyte colony-stimulating factor (G-CSF) enhances ROS production by IL-8 (121, 123). Although the ability of LPS itself to induce NETs is uncertain, the combination of platelets and LPS induces platelet aggregation and acts as a potent stimulus for NETosis (124). Therefore, it is conceivable that NETs are induced under synergistic circumstances where more than one inflammatory and/or bacterial stimulus may be present (e.g., infection), although these stimuli in isolation may not induce NETosis.

NET-Like Structures Induced by Calcium Ionophores Mimic Damage to the Cellular Membrane

Stimuli that induce NETs through physiological pathways mimicking the action of calcium ionophores are more difficult to elucidate. PADs are calcium-dependent enzymes that require millimolar amounts of calcium for *in vitro* activity (38). In cells,

however, PAD activation can be observed during biological processes (73) and in response to physiologic stimuli (51, 93, 94, 133) that do not increase intracellular calcium concentrations above the nanomolar range. Activation of PADs has therefore been posited to require cellular cofactors that modulate calcium sensitivity of the enzyme. Nonetheless, citrullination under these conditions represents a tightly controlled process. Protein citrullination appears limited to nuclear substrates involved in gene regulation such as histones and transcription factors, with no damage to the cell (51, 73, 93, 94, 133). In contrast, calcium ionophores induce hypercitrullination of proteins across multiple cellular compartments, including many likely “accidental” targets. These fundamental differences between limited protein citrullination induced by physiologic stimuli and hypercitrullination observed with calcium ionophores may be best explained by the kinetics and absolute amount of calcium influx.

Calcium ionophores induce rapid, prominent, and sustained increases in cytosolic calcium concentration in neutrophils (above 1 μ M) (110, 111). In contrast, even the most potent physiologic stimuli only induce transient increases in cytosolic calcium below 1 μ M. fMLP, likely one of the most potent activators of intracellular calcium signaling, increases cytosolic calcium to a transient peak of 500–800 nM that returns to baseline within 2–3 min (111, 134, 135). Other stimuli, such as IL-8 and C5a, generate similar transient increases in cytosolic calcium, but with much smaller effect sizes (~300–400 nM) (110, 123). Under these conditions, it is hard to envision that PAD4 could be hyperactivated by these stimuli. Indeed, fMLP, IL-8, and other stimuli that induce transient cytosolic calcium increases are unable to reproduce calcium ionophore-induced hypercitrullination (58). Moreover, fMLP fails to induce NETs (27), reinforcing that limited elevations in intracellular calcium are insufficient to generate NET-like structures. In contrast to calcium ionophores, which lead to decreased chemotaxis (likely because the cells disintegrate) (113, 136), intracellular calcium signals activated by fMLP, IL-8, C5a, and interferon (IFN)- γ are potent chemotactic factors (122, 137–139). This again highlights major physiological differences between signals that slightly increase cytosolic calcium and the effect of calcium ionophores.

Considering that no physiological stimulus is known to reproduce the effects of calcium ionophores on neutrophils, we propose that ROS production *via* NADPH oxidase is the primary mechanism for NETosis. In contrast, NET-like structures induced by calcium ionophores (a form of cell death distinct from NETosis) may be representative of pathological conditions that drive massive intracellular calcium influx and hypercitrullination as result of direct damage to the cellular membrane. To distinguish this form of neutrophil death from NETosis, we will use the term leukotoxic hypercitrullination.

Bacterial and Immune Pore-Forming Proteins Induce Neutrophil Death by Leukotoxic Hypercitrullination

Several unique features distinguish LTH from NETosis, which are as follows: (1) the NET-like structures are triggered by

prominent and sustained calcium influx and can be inhibited by chelation of extracellular calcium (66), (2) are generated independently of NADPH oxidase activity (66), (3) require PAD4 activity and are suppressed by PAD inhibitors (14, 36), (4) undergo rapid formation (within minutes) (36), and (5) protein citrullination is not limited to histones and transcription factors, but encompassing proteins across all molecular weights (54). Whether cellular membrane rupture is required for the release of nuclear content into the extracellular space during LTH is not known.

***S. aureus*, Staphylococcal Pore-Forming Toxins, and Leukotoxic Hypercitrullination during Bacterial Infection**

The idea that NET-like structures induced by calcium ionophores are representative of membranolytic pathways is derived from the studies of *S. aureus*, which induces NETs through a variety of mechanisms (5, 81, 130). To induce bactericidal (true) NETosis, *S. aureus* requires growth under conditions that preclude the expression of bacterial toxins (5). In contrast, when neutrophils are exposed to toxin-producing strains of *S. aureus*, rapid and NADPH oxidase-independent formation of NET-like structures was observed (81). Strikingly, the study also identified that this unique form of “NETosis” (we argue this actually represents LTH) was triggered by Pantón–Valentine leucocidin (PVL), a pore-forming toxin secreted by highly virulent *S. aureus* strains that induces a rapid, prominent, and sustained increase in intracellular calcium (140, 141). Since phagocytosis is a potent stimulus to activate NADPH oxidase (142), it is likely that, by avoiding the action of pore-forming toxins, the engulfment of *S. aureus* by neutrophils in the initial studies by Fuchs and colleagues allowed for ROS production and NETosis (5). However, LTH (toxin-induced neutrophil death without phagocytosis) is likely to predominate during human bacterial infection. This mirrors the initial morphological observations of dying neutrophils during human skin infection by Metchnikoff (1). Interestingly, different to NETosis that requires rupture of the cellular membrane to release DNA and NETs, NET formation induced by PVL appears to release chromatin *via* nuclear envelope blebbing and vesicular exportation, preserving the integrity of the cell membrane (81). In fact, “vital NETosis” described in mice infected with *S. aureus* may represent LTH induced by PVL or other staphylococcal pore-forming toxins (130, 131). In addition to PVL, *S. aureus* produces several other membranolytic toxins that can induce rapid formation of NET-like structures (143, 144). These proteins include N-terminal ArgD peptides and leukotoxin GH (LukGH), possibly among others that have not been studied in detail.

Finally, two additional features are important to support that membrane pore formation, calcium influx, and hypercitrullination are involved in the process of chromatin extrusion induced by *S. aureus*. First, it has been demonstrated that PAD4 inhibitors decrease the production of “NETs” by *S. aureus* (14). Second, we demonstrate here that PVL is a potent inducer of cellular hypercitrullination in human neutrophils (**Figure 2B**), which contrast with the absence of citrullination in neutrophils activated with PMA (**Figure 2A**). Thus, LTH, but not NETosis, is likely the

mechanism underlying NADPH oxidase-independent “NET” formation observed with *S. aureus*.

Bacterial Pore-Forming Toxins as Triggers of Leukotoxic Hypercitrullination – The Need for Standards in the Definition and Study of NETs

The model that bacterial pore-forming proteins are responsible for inducing LTH and secondary extrusion of nuclear material is not limited to *S. aureus*. Pore-forming toxins are potent virulence factors produced by many pathogenic bacteria carrying diverse cellular specificities (145–147). These protein toxins have the ability to change from a water-soluble state to a membrane-bound and membrane-inserted conformation. This large family of molecules includes both short peptides and large globular proteins and is expressed both by Gram-positive and Gram-negative bacterial species alike (145–147).

For the context of this review, the primary and unifying effect of bacterial pore-forming toxins is the ability to induce rapid changes in ion concentration in the cytosol of their respective target cells, specifically an increase in intracellular calcium (145, 148). Early studies on the mechanisms of cell damage by bacterial pore-forming toxins indeed suggested that these molecules act as calcium ionophores (149). The capacity of toxins secreted by bacteria to induce NET-like structures is not novel and has previously gained the attention of others (150). However, the potential link between bacterial pore formation, calcium influx, and hypercitrullination has not been considered as a driver of cellular damage and DNA extrusion. The production of pore-forming toxins therefore is a critical variable that must be considered to interpret mechanisms of NET formation used by pathogenic bacteria. It is thus important to communicate whether bacteria were cultured under conditions that allow for toxin production, whether the specific strains used produce or are deficient of toxins, the spectrum of toxin expression, and their concentration. Finally, future studies on NETosis should be required to disclose whether bacteria were opsonized to facilitate phagocytosis, as this may alter their ability to generate ROS and thus induce NETosis.

Inhibition of PAD4 Activity in NETosis and Leukotoxic Hypercitrullination

The use of PAD4-deficient mice and PAD4 inhibitors to block NET formation is frequently presented as evidence that citrullination is required for the induction of NETosis (14, 37). It is important to appreciate, however, that these studies employed conditions that promote PAD activation and DNA extrusion by calcium influx, not through stimuli that induce NETosis. In the case of PAD4 inhibitors (14), ionomycin and *S. aureus*, both inducers of calcium-dependent and NADPH oxidase-independent pathways, were used to demonstrate that PAD4 inhibition can block histone H3 citrullination and possibly the formation of “NETs” (albeit incorrectly quantified by histone H3 citrullination, not chromatin extrusion). The inhibition of “NETs” in a murine model of sepsis can be similarly explained by blocking LTH rather than NETosis (151).

In mice deficient in PAD4, *S. flexneri* and *S. pyogenes* were used to address the role of PAD4 activity during NETosis (37).

Interestingly, both bacteria produce virulence factors that can affect calcium homeostasis. *Shigella* has been shown to induce calcium responses that are dependent on the type III secretory apparatus. This allows for the insertion of a pore containing the IpaB and IpaC proteins into host cell membranes and acts in conjunction with the pore-forming toxin hemolysin E (HlyE) (152–154). *S. pyogenes* streptolysin O (SLO), a cholesterol-dependent cytolysin (CDC), forms large transmembrane pores evolutionary related to immune pore-forming proteins that permit extracellular calcium flux into the target cell cytosol (147, 155).

While the abnormal activation of PAD4 induced by ionomycin or bacterial toxins can easily explain the effect of PAD4 inhibitors and PAD4 deficiency in these studies, it remains uncertain how these inhibitors could block PMA-induced NETosis (18), which proceeds without PAD4 activation (32, 71). It is conceivable that molecules designed to block PADs exert additional functions that indirectly affect PMA-induced NETosis. We also cannot exclude that some residual activity of PAD4 is maintained in the presence of PMA, resulting in limited citrullination of histones that may contribute to chromatin decondensation, without affecting their antimicrobial properties (as discussed below). Indeed, trace citrullination of histone H3 has been reported in neutrophils in response to LPS and other stimuli (71, 156). However, some of these stimuli are highly unreliable means to induce NETosis (27, 124).

Since the expression of pore-forming toxins easily confounds the study of NETosis induced by bacterial species, the fungi *C. albicans* and *A. fumigatus* may represent a better model to interrogate the effect of PAD4 deficiency and inhibition in the induction of NETs.

Immune Pore-Forming Proteins – A Role for Complement and Perforin in Leukotoxic Hypercitrullination?

Pore-forming proteins are not exclusive to microbial species. Vertebrates have evolved similar systems to kill infectious agents [i.e., the complement membrane attack complex (MAC)], as well as infected or malignant cells (i.e., perforin) (157). Similar to other proteins that create transmembrane pores, MAC and perforin promote prominent calcium influx, PAD activation, and cellular hypercitrullination (58). Moreover, we found that perforin also induces the extracellular release of DNA (**Figure 3**), which may erroneously be attributed to NETosis in the future. We anticipate that killing of neutrophils by complement MAC would have a similar effect.

We believe that these data are consistent with the herein proposed model that calcium ionophores act analogous to pore-forming proteins, which drive cellular damage, hypercitrullination, and chromatin extrusion mimicking NETosis (i.e., LTH). Defining the independent role of toxins secreted by each pathogen shown to induce “NETosis” would be necessary to confirm or discard this hypothesis.

Does PAD2 Play a Role in Leukotoxic Hypercitrullination Induced by Pore-Forming Proteins?

PAD2 is an abundant enzyme in neutrophils and is potently activated in cells using calcium ionophores or perforin. Hypercitrullination induced by PAD2 generates a repertoire of citrullinated proteins that is even more prominent than the pattern observed with PAD4 (54, 58), with protein targets that are common and unique for both enzymes (50). Proteins of the cellular cytoskeleton, such as actin and vimentin, are prominently

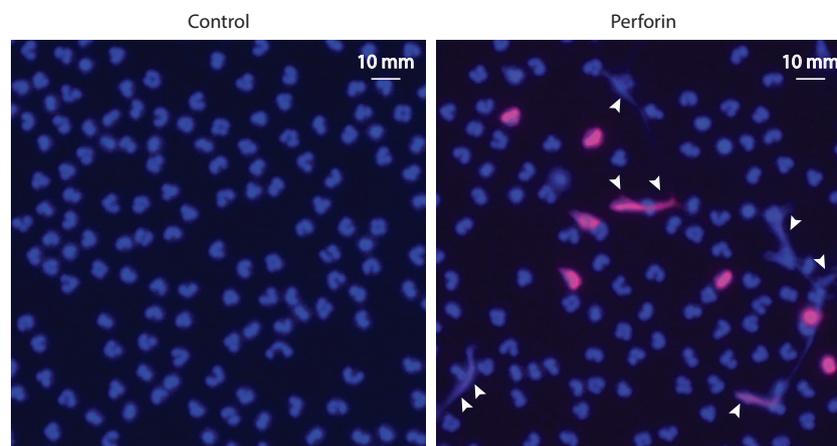


FIGURE 3 | Purified perforin induces PAD activation and the release of extracellular DNA in neutrophils. Human neutrophils (5×10^5 cells/50 μ L) in HBSS/10 mM HEPES/1.5 mM CaCl_2 were plated onto standard microscope slides coated with poly-D-lysine (Sigma) and incubated for 30 min at 37°C to allow for cell attachment. Neutrophils were then incubated in the absence or presence of sublytic amounts (500 ng/mL) of purified perforin (Enzo Life Science) for 1 h at 37°C, as previously described (58). Then, the cells were fixed, permeabilized, and stained with anti-histone H3 (citrulline 2 + 8 + 17) antibodies (H3cit) and Alexa Fluor 594 goat anti-rabbit F(ab')₂ fragment (Invitrogen, Cat# A-11072). Cells were mounted with ProLong Gold (Molecular Probes) plus DAPI. Samples were analyzed using a Zeiss axioScope microscope. Excitation filter: 510–560 nm for Alexa Fluor 594 and excitation filter: 300–390 nm for DAPI. Merged images of DAPI (blue) and H3cit (red) staining are shown. Perforin induces PAD activation (detected by citrullination of histone H3) and the extracellular release of neutrophil DNA (arrowheads). The experiments were performed on at least four separated occasions, with similar results.

citrullinated by PAD2 (50, 54). This may contribute to the dismantling of neutrophils during cellular hypercitrullination. Whether hyperactivation of PAD2 plays any direct role in this process, as demonstrated for PAD4 (65), is unknown. Elucidating a potential role of PAD2 during LTH is of great interest as targeted inhibition of PAD4 will likely have negligible effects on PAD2 hyperactivation and its consequences.

NETosis and Leukotoxic Hypercitrullination in Immunity: Protein Citrullination May Impair the Bactericidal Function of NETs

Based on our proposed model, neutrophils can suffer two biochemically distinct forms of cell death that can easily be confused morphologically. One is driven by signals that induce ROS production by NADPH oxidase (NETosis), the other involves bacterial toxins that generate transmembrane pores, abnormal calcium influx, neutrophil hypercitrullination, and the extracellular release of chromatin (LTH). Applying this model, what would be the biological significance of these distinct forms of neutrophil death?

In 1942, Miller and colleagues identified that histones have potent antimicrobial activity (158). It was later demonstrated that the arginine-rich histone fraction can effectively kill both Gram-positive and Gram-negative bacteria at nanomolar concentrations (159). More recently, the importance of histones as potent bactericidal factors was resurrected by the discovery that the antibacterial activity in NETs is largely dependent on histones (2). The mechanism by which histones kill bacteria is not fully understood, but has been attributed to their high content of arginine and lysine. These cationic residues are thought to allow for binding, disruption of the bacterial membrane, and translocation of small antibacterial molecules (160). Using histone-derived antimicrobial peptides, it was shown that arginine is required for antimicrobial activity, membrane embedding, and peptide translocation (160). Since histones are potent antimicrobial factors in NETs and their arginine content is critical for bactericidal function, it is not surprising that decreasing the number of arginine residues during histones citrullination reduces their potent antimicrobial activity (37). Similarly, citrullination of the cationic bactericidal protein LL37 attenuates its capacity to neutralize LPS and to protect against endotoxin-induced death in mice (161).

From an evolutionary perspective, we believe that the inactivation of PAD4 that occurs during NETosis is unlikely to be accidental (71). This inhibition may have evolved to avoid citrullination-mediated inactivation of critical antimicrobial factors (e.g., arginine-rich bactericidal proteins) released during NETosis. Although the spectrum of citrullinated proteins induced by bacterial toxins in neutrophils has not been described to date, antimicrobial proteins including MPO, NE, azurocidin, and defensins appear to be targets of citrullination induced by ionomycin (63). This suggests that antimicrobial components of NETs other than histones may be similarly inactivated by citrullination.

NETosis and toxin-mediated LTH may represent two fundamentally opposed strategies used by the innate immune system and microbial pathogens to kill each other (Figure 4). NETosis

defines a form of programmed, antimicrobial cell death initiated by signaling pathways that suppress PAD activation and induce the release of bactericidal NETs. This process is dependent on ROS production *via* NADPH oxidase. Stimuli that generate NETosis may include inflammatory cytokines acting synergistically, bacterial products, fungi, and opsonized bacteria that can potentially activate NADPH oxidase. In contrast, pathogens have evolved strategies that generate abnormal and sustained increases in cytosolic calcium of target cells through pore-forming toxins. This process hyperactivates calcium-dependent pathways (PADs), thus rapidly inactivating antimicrobial neutrophil factors and triggering disintegration of the target cell.

The deleterious effect of hyperactivation of PAD4 and LTH on the antimicrobial function of neutrophils is further supported by several findings, which are as follows: (1) NET-like structures induced by toxin-expressing strains of *S. aureus* have limited proteolytic activity; this loss of function is PVL-dependent (81), (2) NET-like structures induced by the *S. aureus* pore-forming toxin LukGH demonstrate no antimicrobial potential against this bacterium (144), in striking contrast to the potent bactericidal activity of PMA-induced NETs (2), (3) Group B *Streptococcus* induces neutrophil lysis, NET-like structures and resistance to NET-mediated bacterial killing via its hemolytic pigment/lipid toxin (162), (4) PAD4-deficient mice are partially protected from LPS-induced shock and demonstrate increased production of IL-10 (163), and (5) PAD inhibition increases survival in a murine model of sepsis (151), supporting that PAD4 activity negatively affects the host during infection.

Leukotoxic Hypercitrullination vs. NETosis in Rheumatoid Arthritis Pathogenesis

The induction of LTH by pore-forming proteins may not only be detrimental for the clearance of pathogens. The hypercitrullination of self-proteins that are not physiological targets of PADs may have a primary role in generating neoantigens. This cellular dysregulation may have important implications for the etiology and pathogenesis of RA, as citrullinated proteins are major targets of the autoimmune response in this disease (17). Immune-mediated membranolytic pathways (perforin and MAC formation) are activated in the rheumatoid joint and may drive the generation of citrullinated autoantigens in established disease (58). Similarly, chronic hypercitrullination induced by bacterial pore-forming toxins may represent a primary source of citrullinated autoantigens in susceptible individuals with the potential to initiate the ACPA response and RA.

The hypothesis that NETosis is the source of citrullinated autoantigens in RA, as proposed in recent years, contradicts experimental data showing that hypercitrullination does not occur during NETosis (58, 71). This contradiction may be explained by the following points. (1) Studies on RA pathogenesis frequently use calcium ionophores to induce citrullination in neutrophils, which mimic LTH induced by pore-forming pathways, but not NETosis (63, 164). It is likely that the arbitrary use of PMA and calcium ionophores may be a major confounder in the study of autoantigen citrullination in RA. (2) Neutrophils dying by LTH in the RA joint cannot be morphologically distinguished from

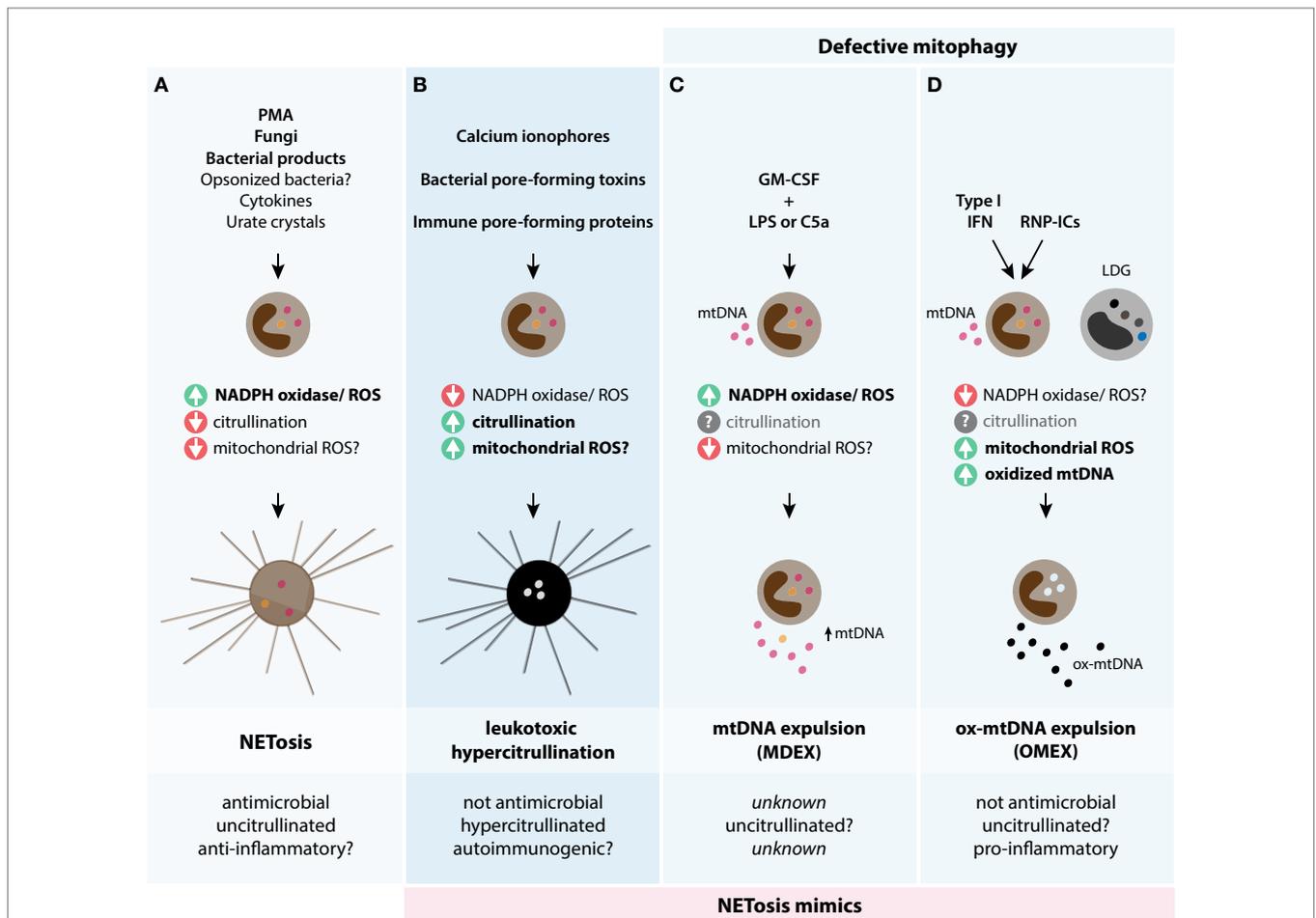


FIGURE 4 | Theoretical models of biological mechanisms of DNA release in neutrophils. The three models are depicted and illustrate their potential triggers, biochemical pathways, and biological consequences. **(A)** NETosis is initiated by stimuli that potently activate NADPH oxidase and ROS production, but decreases PAD4 activation and likely mtROS production. NETosis is antimicrobial, potentially anti-inflammatory, and has limited protein citrullination. **(B)** Leukotoxic hypercitrullination is triggered by stimuli that generate prominent and sustained increases in cytosolic calcium, such as ionophores and proteins that damage the cellular membrane (e.g., pore-forming proteins). PAD hyperactivation and protein hypercitrullination are characteristic of this process, but not ROS production by NADPH oxidase. While mtROS may have some role in the release of DNA by calcium ionophores in neutrophils (32), the relevance of this pathway in neutrophil damage induced by pore-forming proteins is unknown. Antimicrobial functions and the immunogenicity of self-antigens are affected as a result of cellular hypercitrullination. **(C,D)** To compensate a constitutive defect in mitophagy, neutrophils normally extrude mitochondrial components, including mtDNA, as a mechanism for mitochondria clearance. **(C)** The process of mtDNA expulsion (MDEX) may be enhanced by inflammatory signals such as LPS or C5a following neutrophil priming with GM-CSF and requires NADPH oxidase activity. **(D)** IFN-I and RNP-ICs alter the normal disposal of mitochondria in neutrophils and promote the expulsion of ox-mtDNA (OMEX). This process is dependent on mtROS, but not NADPH oxidase, and may be predisposed by factors that decrease NADPH oxidase activity. Different to mtDNA, ox-mtDNA is pro-inflammatory and highly interferogenic. LDGs from patients with SLE have spontaneous OMEX. The role of citrullination in **(C,D)** is unknown. The question marks (?) reflect pathways/functions that require further experimental confirmation.

NETosis. This may falsely suggest that NETosis is increased in RA and relevant to disease pathogenesis. (3) Citrullination in the cellular compartment of the rheumatoid joint is characterized by modification of a broad range of proteins and autoantigens (58, 165, 166) and can only be reproduced *in vitro* by membranolytic damage to the neutrophil (58). In contrast, *in vitro* studies that aimed to define a role of NETosis in autoantigen citrullination are less stringent and posit that citrullination of one or two proteins is sufficient evidence to link NETosis and RA. (4) The potential success of PAD4 deficiency and PAD inhibitors in treating experimental arthritis may be misinterpreted as *in vivo* evidence that NETosis is relevant for RA, while this effect may result from

transcriptional repression of immune effector functions or inhibition of LTH.

It is the opinion of these authors that the role of NETosis in autoantigen citrullination needs to be revisited. This reappraisal needs to consider the type of stimulus used, the spectrum of citrullinated autoantigens generated during NETosis, and whether this spectrum is representative of the citrullinome in the RA joint (58, 165, 166). It further requires the presence of appropriate negative controls to confirm the specificity of citrullinated protein detection.

Depending on the mechanism that inactivates PAD4 during NETosis (reversible vs. irreversible) (71), it is possible to assume

that PADs released during this process may promote citrullination of extracellular substrates (167). Nevertheless, neutrophils dying by necrosis or LTH are even more efficient than NETosis to induce citrullination of extracellular substrates (167), suggesting that NETosis itself may not be required for this process.

Other Forms of Cell Death That May Mimic NETosis

Defective Mitophagy in Neutrophils

Since mitochondrial dysfunction can have deleterious effects on cells, quality control mechanisms have evolved to eliminate damaged mitochondria (168). However, while most cells use an autophagic process called mitophagy to remove damaged mitochondria, recent evidence demonstrated that mitophagy is defective in neutrophils (19). To compensate this problem, neutrophils employ two complementary pathways to achieve mitochondrial clearance. (1) Mitochondrial contents including mtDNA in complex with transcription factor A mitochondria (TFAM) are expelled from the neutrophil. This likely happens through direct fusion of the mitochondria with the plasma membrane. The release of mtDNA–protein complexes into the extracellular space is therefore a normal process in neutrophil biology. (2) If mtDNA undergoes oxidation, it dissociates from TFAM and is redirected to lysosomes for degradation (19).

Interestingly, early studies had demonstrated that expulsion of mtDNA from neutrophils can be induced by LPS or complement C5a following priming with GM-CSF (7) (Figure 4). This may indicate physiologically increased clearance of mitochondria in response to inflammatory stimuli. Due to morphological similarities with NETosis (extracellular DNA), however, these structures have been termed mitochondrial NETs. Different to actual NETs, mtDNA is released from viable neutrophils and does not appear to contain histones, neutrophil granule proteins (e.g., MPO and PR3), or antimicrobial properties (7). The analogy with NETs is therefore unsuitable and creates confusion around the biology of NETosis. Importantly, no rationale exists to posit that citrullination may have a role in the release of mtDNA–protein complexes from neutrophils, reinforcing that PAD4 is not central to any process that releases DNA and so-called NETs.

Neutrophil Extracellular ox-mtDNA in SLE

In 2011, three different groups suggested distinct mechanisms to explain a possible increase of NETosis in patients with SLE (83, 169, 170). While no effort was made by other groups to support or discard any of these models, two recent studies stressed that NETosis may indeed play no role in SLE pathogenesis (19, 100). Instead, the release of ox-mtDNA from neutrophils, initially mistaken for NETosis, appears to be relevant to SLE (19). While data from either study can lead to similar conclusions, some differences deserve attention.

In previous studies, freshly isolated neutrophils from patients with SLE were shown to suffer NETosis when exposed to ribonucleoprotein-containing immune complexes (RNP-ICs) *via* TLR7 and FcγRIIa ligation. This unique process was reproduced in neutrophils from healthy controls when cells were primed with type I IFN (IFN-I) to induce TLR7 expression (170). Using a similar model, Caielli and colleagues challenged this paradigm

and demonstrated that defective mitophagy together with IFN priming, but not NETosis, is responsible for the release of pro-inflammatory ox-mtDNA in response to RNP-ICs (19). In this scenario, neutrophil exposure to IFN-I and RNP-ICs interferes with the disassembly of mtDNA–TFAM complexes, which are required for ox-mtDNA disposal by lysosomes. This results in ox-mtDNA accumulation in mitochondria and further expulsion as highly interferogenic ox-mtDNA–TFAM complexes (19). Different to NETosis, neutrophils remain alive while releasing ox-mtDNA in the extracellular space, gDNA is not released, and the process is dependent on mtROS, but not NADPH oxidase (19) (Figure 4). While Lood and colleagues reached similar conclusions in regard to the release of ox-mtDNA in response to RNP-ICs and the requirement of mtROS, but not NADPH oxidase, they found that IFN-I priming was not required to drive this process (100). They also described that a similar process spontaneously occurs in low-density granulocytes (LDGs), a subset of inflammatory granulocytes enriched in SLE (100).

While the studies by Caielli and Lood were performed using distinct populations of patients (pediatric vs. adult) (19, 100), it is unclear whether this may explain the difference in the requirement of type I IFN to induce TLR7, as RNP-ICs signal through this receptor (170).

Although the study by Lood and colleagues still uses the term NETosis (100), both studies are consistent with the evolving idea that this form of neutrophil death is not involved in the pathogenesis of SLE. Instead, mechanisms that enhance the production and release of ox-mtDNA may be responsible for generating critical inflammatory components that contribute to SLE pathogenesis. Importantly, these studies support the conclusions by Campbell and colleagues in lupus-prone mice deficient in Nox2 (the p91^{phox} component of the phagocyte NADPH oxidase), which demonstrated that NETosis does not contribute to lupus *in vivo*. Since Nox2 deficiency exacerbates lupus in these mice, NETosis, or some other activity linked to Nox2, instead acts to inhibit disease pathogenesis (99). This fascinating idea is further supported by the observation that deficiencies in NADPH oxidase activity in humans are associated with SLE, lupus-like disease, lupus autoantibodies, and a systemic IFN-I signature (100, 171–175). Neutrophils from these patients are resistant to NETosis, but have fully conserved mtROS activity (176), which together may predispose to SLE. Finally, individuals with NADPH oxidase deficiency have an increased risk of fungal and bacterial infections (176), underscoring that the sole expulsion of mtDNA or ox-mtDNA is not antimicrobial and should not be considered a form of NETosis. As NADPH oxidase appears to be protective for the development of SLE, it is possible that this enzyme complex regulates the production or clearance of neutrophil ox-mtDNA. Indeed, strong evidence supports an interplay between mitochondria and NADPH oxidase that may provide both feed-forward and feedback regulations in the production of ROS (177). Moreover, it is interesting to highlight that the extracellular release of mtDNA induced by GM-CSF and LPS/C5a is dependent on NADPH oxidase (7), supporting that this enzyme complex has a role in the maintenance of mitochondria in neutrophils. NETosis could moreover be protective for SLE by degrading cytokines and chemokines (178).

The finding that the inhibition of NETosis by PTU induces the production of anti-MPO antibodies and vasculitis in rats (98) further supports a protective role of NETosis in autoimmunity. Defining the role of NADPH oxidase in SLE has therefore implications for both disease pathogenesis and the development of therapeutic strategies.

CONCLUDING REMARKS

The biochemical and functional distinction of NETosis, LTH, and defective mitophagy is critical to understanding the distinct role of these processes for human immunity and autoimmunity. While the extracellular release of neutrophil DNA induced by various stimuli may appear morphologically similar, they seem to be consequences of opposing evolutionary forces. Here, we propose the existence of at least three distinct mechanisms that drive the active extrusion of neutrophil DNA (Figure 4), which are as follows: (1) NETosis, which represents a hardwired cellular program that likely evolved to kill pathogens, (2) LTH, which is the cellular consequence of successful strategies that evolved in specific pathogens to kill neutrophils (membranolysis), and (3) the expulsion of mtDNA, which represents a normal function in neutrophil biology to clear the cell of damaged mitochondrial material, but can generate ox-mtDNA in the SLE environment. While these processes can be easily studied by defining the effects of PMA, calcium ionophores and IFN- γ plus RNP-ICs on neutrophils, it is important to emphasize that these stimuli are artificial. They are most likely non-representative of the complexity of signals that converge in the neutrophil during infection or autoimmunity. We anticipate that variations of these mechanisms exist *in vivo*.

The attempt to derive a biochemical definition of the distinct processes that release DNA from neutrophils offers a unique opportunity to reevaluate the role of PAD4 and NETosis as drivers and potential targets in human diseases. Based on this model, the use of PAD inhibitors may be indicated to block the detrimental effects of LTH, but not NETosis. First, PAD inhibitors could act as “antibiotics” to prevent ongoing hypercitrullination induced by bacterial toxins, thus allowing bactericidal NETosis to occur. The finding that the PAD inhibitor Cl-amidine improves survival in a murine model of sepsis strongly supports this hypothesis (151). Second, they may decrease the burden of citrullinated

autoantigens in RA without affecting NETosis, which has been shown to have distinct anti-inflammatory activities (178). Given that ionomycin and perforin induce hyperactivation of PAD2 and PAD4 in neutrophils, it is conceivable that blocking LTH may require inhibition of both enzymes. Third, PAD4 inhibitors may have an indication to treat autoimmune diseases as transcriptional repressors of immune effector functions, but not under the rationale that these molecules act by blocking NETosis. In SLE, the use of PAD4 inhibitors to block the production and release of ox-mtDNA has no rationale, unless it is demonstrated that this enzyme is essential for this process. Blocking the production of mtROS, however, may be reasonable.

Besides infectious and autoimmune diseases (e.g., SLE) that have been associated with decreased production of NETosis (as consequence of NADPH oxidase deficiency) (100, 171–176), diseases related to an increased neutrophil propensity to undergo NETosis need to be carefully defined. A better biochemical definition of the diverse mechanisms that can affect neutrophil function should allow for the identification of novel biomarkers and help with the classification of disease entities that may benefit from targeting those pathways. There remains a need to clarify the true role of NETosis in human health and disease.

ETHICS STATEMENT

Informed consent was obtained from all individuals as approved by the Johns Hopkins Institutional Review Board.

AUTHOR CONTRIBUTIONS

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

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NET Confusion

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Neutrophils are arguably the most important white blood cell for defense against bacterial and fungal infections. These leukocytes are produced in high numbers on a daily basis in humans and are recruited rapidly to injured/infected tissues. Phagocytosis and subsequent intraphagosomal killing and digestion of microbes have historically been the accepted means by which neutrophils carry out their role in innate host defense. Indeed, neutrophils contain and produce numerous cytotoxic molecules, including antimicrobial peptides, proteases, and reactive oxygen species, that are highly effective at killing the vast majority of ingested microbes. On the other hand, it is these characteristics – high numbers and toxicity – that endow neutrophils with the potential to injure and destroy host tissues. This potential is borne out by many inflammatory processes and diseases. Therefore, it is not surprising that host mechanisms exist to control virtually all steps in the neutrophil activation process and to prevent unintended neutrophil activation and/or lysis during the resolution of inflammatory responses or during steady-state turnover. The notion that neutrophil extracellular traps (NETs) form by cytolysis as a *standard* host defense mechanism seems inconsistent with these aforementioned neutrophil “containment” processes. It is with this caveat in mind that we provide perspective on the role of NETs in human host defense and disease.

Keywords: host defense, extracellular trap, inflammation, inflammatory disorder, neutrophil

PRODUCTION OF NEUTROPHILS

Neutrophils are an essential component of the human innate immune response to bacterial and fungal infections. These leukocytes are among the first to be recruited to sites of inflammation and/or infection, and they are the most numerous white blood cell in humans. Under normal steady-state conditions, neutrophils develop from mitotic precursor cells (myeloblasts, promyelocytes, and myelocytes) in bone marrow for several days (~7.5 days) and then mature for 6–7 days as post-mitotic cells (metamyelocytes, band cells, and ultimately mature neutrophils) (1). Approximately 60% of the total nucleated cells in normal human bone marrow are granulocytes or granulocyte precursors (1). Cartwright et al. estimated the total granulocyte pool in bone marrow to be 1.86×10^{10} cells/kg body weight, of which 0.69×10^{10} cells/kg are mature neutrophils (2). A subsequent study by Dancey et al., which used a different method to label bone marrow granulocytes, reported the total number of bone marrow neutrophils as 0.77×10^{10} cells/kg body weight (3). With either method, it is clear that there is remarkable production of neutrophils in humans during steady-state conditions. Moreover, the production of granulocytes can be increased dramatically during severe infection – this process is known as emergency granulopoiesis (4).

The vast majority of granulocytes released from bone marrow into circulation are neutrophils (~95%), and these cells remain in circulation for a relatively short time (~12–18 h) (5). More

recently, Pillay et al reported that the human neutrophil life span in circulation is 5.4 days (6), although other interpretations of these data have been proposed (7, 8). Consistent with high production of neutrophils in bone marrow, neutrophils comprise ~60% of leukocytes in human blood. Athens and colleagues reported that the total blood granulocyte pool comprises circulating and marginal granulocytes, which collectively are estimated as 6.5×10^8 cells/kg body weight in total (3.2×10^8 and 3.3×10^8 cells/kg body weight for circulating and marginal granulocyte pools, respectively) (5). Several early landmark studies reported blood neutrophil turnover rate in humans as $0.87\text{--}1.63 \times 10^9$ cells/kg/day (2, 3, 9). Thus, the estimated granulocyte turnover rate in humans is enormous – on the order of $0.5\text{--}1 \times 10^{11}$ cells/day in a healthy adult (3, 5).

The continuous removal and replacement of neutrophils is critical for maintenance of immune system homeostasis and, importantly, the prevention of unintended damage to host tissues (10). Inasmuch as neutrophils contain and produce numerous cytotoxic molecules, which are highly effective at killing and degrading phagocytosed microbes, multiple (and redundant) mechanisms exist to prevent or limit host exposure to such molecules.

REGULATION OF NEUTROPHIL TURNOVER

Neutrophils traverse the vasculature in large numbers as an efficient means of readily disseminating to distal sites of host infection. Neutrophils are rapidly recruited to sites of infection by host and pathogen-derived molecules and have enormous pro-inflammatory capacity. The high rate of granulopoiesis frequently results in production of a superfluous number of neutrophils, and apoptosis is the predominant mechanism that regulates neutrophil turnover to maintain immune system homeostasis. In addition, neutrophils undergo apoptosis as a mechanism to limit pro-inflammatory capacity and to resolve infection. Neutrophil apoptosis is a highly regulated process mediated by several molecular mechanisms including intrinsic (intracellular) and extrinsic (extracellular) signaling pathways that lead to activation of caspase-3, and these pathways have been reviewed extensively (11–13). Constitutive (or spontaneous) neutrophil apoptosis is an example of the intrinsic pathway and governs removal of senescent cells, although the precise mechanism that triggers this process is incompletely defined. The intrinsic pathway is generally associated with cellular stress and mitochondrial damage and is mediated by members of the BCL-2 family of proteins. Extrinsic apoptosis is initiated by ligation of death receptors that bind FAS ligand, tumor necrosis factor (TNF)- α , or TNF-related apoptosis inducing ligand (TRAIL), and is largely at play within the context of complex inflammatory milieu.

Neutrophil apoptosis is a non-inflammatory process characterized by membrane blebbing, cell shrinkage, loss of cytoplasmic granules, cytoplasmic vacuolation, and nuclear chromatin condensation. Apoptosis is accompanied by DNA fragmentation into nucleosome-length fragments, exposure of phosphatidylserine on outer leaflets of the plasma membrane, and neutrophil outer

cell membrane integrity is maintained throughout the process (14). Moreover, spontaneous neutrophil apoptosis is associated with diminished capacity for chemotaxis, degranulation, reactive oxygen species (ROS) production, and phagocytosis (14). Importantly, apoptotic neutrophils are safely removed by macrophages through a process known as efferocytosis (15–17). Macrophage recognition of apoptotic neutrophils is facilitated by receptors for phosphatidylserine, $\alpha_v\beta_3$ integrin, and CD36 (18). Following recognition, macrophages phagocytose apoptotic neutrophils, and the process does not stimulate release of pro-inflammatory mediators (19).

Neutrophil lifespan is highly variable and can be influenced by many external factors capable of either prolonging survival or inducing apoptosis. A diversity of pro-inflammatory mediators, such as granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon (IFN)- γ , interleukin 1 β , C5a, and LPS, are known to delay neutrophil apoptosis (20, 21). Enhanced neutrophil survival presumably increases neutrophil numbers during early stages of inflammation and promotes clearance of bacterial pathogens from infected tissue. The process of phagocytosis significantly accelerates the rate of apoptosis in human neutrophils (22–24), and the increase occurs irrespective of any delay in cell fate imparted by cytokines or bacteria-derived factors (25). Effete neutrophils containing dead or partially digested microbes are cleared from infection sites by efferocytosis. Given that neutrophil apoptosis is accelerated by phagocytosis and apoptotic cells are at increased risk for necrotic lysis and/or leakage of cytotoxic molecules, efficient macrophage cell clearance is critical to prevent excessive damage to host tissue. Thus, the ability of pathogens to alter neutrophil fate by either promoting rapid lysis to eliminate neutrophils or interfering with efferocytosis is a plausible virulence strategy that can exacerbate acute inflammation. Indeed, bacterial pathogens such as *Streptococcus pyogenes* can additionally alter neutrophil apoptosis in a manner that ultimately results in rapid cell lysis (26) – a feature consistent with the ability of *S. pyogenes* to present clinically with necrotic lesions and gross inflammation (27). Moreover, it is known that some *Staphylococcus aureus* strains have the ability to promote rapid neutrophil lysis after phagocytosis (26, 28), and recent evidence indicates that the process occurs by programmed necrosis or necroptosis (29). Necroptosis is a pro-inflammatory form of cell death dependent on receptor interacting protein-1 kinase and leads to necrotic cell lysis. Thus, neutrophil apoptosis and efficient clearance by macrophages is essential for maintenance of host health, and pathogen-mediated deviations from this normal process that result in neutrophil lysis – irrespective of mechanism – contribute to pathogenesis.

CONTROL OF NEUTROPHIL ACTIVATION

The extraordinary ability of neutrophils to protect the host against a wide array of pathogens necessitates that these cells utilize highly toxic and damaging weapons to target pathogen incapacitation and/or destruction. Given the potential for collateral host tissue damage, it is essential that neutrophil activation is finely tuned to result in the appropriate level of response for any given situation. Indeed, neutrophils utilize a

variety of mechanisms to control activation and subsequent delivery of these toxic components. One of the first approaches to controlling activation seems to be a very tight control of activation initiation. Resting neutrophils are maintained in the blood in an essentially dormant state, expressing very few, if any, adhesion molecules and receptors for activating ligands (30). However, these cells seem to be exquisitely sensitive to the presence of a danger signal or mechanical perturbation and can immediately increase their responsiveness through the mobilization of secretory vesicles, leading to surface expression of adhesion molecules, chemoattractant receptors, and other functional proteins involved in neutrophil mobilization without releasing potentially harmful inflammatory molecules (31). This reversible process is known as priming and transforms these cells into a state of heightened sensitivity and ability to generate a maximal host defense response (32). Indeed, the level of neutrophil priming has been linked to the severity of disease and disease outcome, and several studies have suggested that priming may be a good indicator of clinical disease activity (33, 34). On the other hand, absence of an infection or inflammatory stimulus would result in reversal of the primed condition back to a quiescent state, again demonstrating exquisite control of the neutrophil and its state of activation.

The selective mobilization of secretory granules during priming illustrates a second key mechanism utilized by neutrophils to regulate the inflammatory response. Neutrophils, also known as granulocytes, contain a number of cytoplasmic granules/vesicles that act as readily mobilizable reservoirs of potent enzymes and toxic molecules, which are selectively mobilized based on a hierarchy that is not completely understood but seems to control the level and types of enzymes released to meet the needs of the host defense situation (31). For example, gelatinase granules require a higher neutrophil activation threshold for exocytosis than do secretory vesicles, an even higher threshold is required for mobilization of specific granules, whereas the highest mobilization threshold seems to be for azurophil granules (31). Thus, selective compartmentalization of toxic and potentially host-damaging enzymes allows neutrophils to adjust their response to the level needed to address the insult by not inflict excessive damage to host tissues. Selective mobilization of granules also results in appropriate changes in the array of neutrophil cell-surface molecules and, thereby, modulates the way in which neutrophil interact with their environment.

As discussed above, neutrophil activation leads to the differential release of cytoplasmic granules, which participate in various host defense processes. Neutrophil activation is also characterized by the production of ROS *via* the activation of a multiprotein enzyme complex, known as the NADPH oxidase. This process, also known as the respiratory burst, results in the initial generation of superoxide anion (O_2^-); however, subsequent biochemical and enzymatic events can convert O_2^- into more potent microbicidal products, including hydrogen peroxide (H_2O_2), a required substrate for the myeloperoxidase-halide system that generates hypochlorous acid (HOCl), hydroxyl radical (HO \cdot), and other reactive oxygen and nitrogen species (35). While the NADPH oxidase system is essential for host defense, its products can also damage host tissues and, when inappropriately

regulated, contribute to inflammatory disease (36). Thus, this system is also highly regulated through compartmentalization to avoid inappropriate activation and excessive host tissue damage. For example, the NADPH oxidase is composed of cytosolic and membrane-bound proteins that must assemble with each other through a complex sequence of signaling events, posttranslational modifications, and protein:protein binding interactions to finally achieve an active complex. Optimally, this complex assembles on the phagosomal membrane, where oxidants are targeted at high concentrations to a pathogen, but are also compartmentalized inside the cell to minimize host damage (37). Furthermore, neutrophil cytosol contains high levels of antioxidant enzymes, such as superoxide dismutase, catalase, and glutathione peroxidase to further limit release of toxic ROS into host tissues (38). Thus, it is clear that significant effort is devoted to the control of neutrophil activation and, thereby, unnecessary exposure of the host to damaging agents through regulated priming and activation, sequential mobilization of cytoplasmic granules, and compartmentalization of effector systems.

NEUTROPHILS AND INFLAMMATION

Inflammation is a host protective response against invading microbes or tissue injury. It consists of complex interactions between soluble mediators and host cells with hallmark features that include swelling, redness, pain, and heat. During acute inflammation, initial recognition of pathogen or damage-associated molecular patterns by pattern recognition receptors on tissue resident immune cells elicits production of immune mediators (39). Subsequently, these immune mediators (e.g., pro-inflammatory cytokines, chemokines, eicosanoids, and vasoactive amines) create a chemoattractant gradient that primes neutrophils and summons these cells to the site of injury or infection. This process is accompanied by vascular permeability and increased expression of selectins on activated endothelium, which, in turn, increases neutrophil adhesion and extravasation (40). Upon arrival in the infected tissues, neutrophils phagocytose and kill microbes using processes described above. Additionally, neutrophils secrete numerous pro-inflammatory molecules that amplify the immune response, and exocytosed granule proteases contribute to extracellular matrix degradation and tissue remodeling (41–43).

Non-phlogistic removal of effete and/or apoptotic neutrophils by mononuclear phagocytes is crucial to the resolution of inflammation and initiation of the tissue repair process (44–46). The overall importance of macrophages in tissue repair and restoration of homeostasis is perhaps exemplified by a mouse wound-healing model, in which depletion of these cells results in impaired angiogenesis, reduced granulation tissue formation and collagen deposition, decreased cell proliferation, and delayed re-epithelialization (47). Thus, under normal circumstances, acute inflammation is a self-limiting process that eliminates invading microbes and promotes tissue repair and return to homeostasis. Eicosanoids and other lipid molecules play a key role in the initiation and resolution of the inflammatory response (48). For example, leukotrienes and prostaglandins such as PGE_2 are essential for trafficking of neutrophils to sites of infection.

On the other hand, high concentrations of PGE₂ in inflammation exudate signals for host activation of the 15-lipoxygenase pathway and lipoxin production, which stop recruitment of neutrophils and promote the resolution of inflammation (49, 50). Lipoxins belong to a group of specialized pro-resolving mediators that includes resolvins, protectins, and maresins. These lipid mediators promote recruitment of monocytes, efferocytosis of apoptotic neutrophils, uptake of debris, resolution of inflammation, and tissue regeneration (51, 52). Interestingly, generation of resolution signals starts early during the inflammation process and often depends on cell–cell (e.g., neutrophil–endothelial cell) interaction (50, 53).

It is widely known that neutrophils play a key role in inflammatory diseases. When the intricate network of signals controlling inflammation becomes imbalanced or the acute inflammatory response fails to eliminate the source of tissue damage, it can transform into a chronic inflammatory state. During chronic inflammation, the majority of tissue damage is caused by macrophages, monocytes, and granulocytes (54–57). Rheumatoid arthritis (RA) is an example of a chronic inflammatory disease to which the contribution of neutrophils has been studied extensively. Interestingly, neutrophils isolated from patients with RA are primed for ROS production and resemble low-density granulocytes (LDGs) from lupus erythematosus patients (58, 59). Production of ROS and release of granule enzymes by neutrophils contribute directly to cartilage and joint damage and perpetuate the inflammatory response (60).

Host tissue damage can also be caused by neutrophils during the acute inflammatory response. For example, neutrophils are known to contribute directly to lung tissue damage during severe pneumonia caused by *Staphylococcus aureus* (61, 62). This severe tissue damage, which in humans can be fatal, is largely caused by cytotoxic molecules released from activated and lysed neutrophils (63, 64). Inasmuch as neutrophil-derived cytotoxins are central to the pathology of inflammatory diseases, it should not be surprising that neutrophil extracellular traps (NETs), which are largely reported to form from a cytolytic process, are associated with many diseases or pathologic conditions.

NETs AND DISEASE

Neutrophil extracellular traps are filamentous web-like structures that consist of extruded nuclear DNA and histones and are decorated with neutrophil granule enzymes, such as MPO, elastase, cathepsin G, and lactoferrin (65). They can be formed in response to infectious agents, inflammatory mediators, and/or under certain conditions, including non-specific osmotic cytolysis (**Figure 1**). NETs have been reported to entrap and kill numerous microorganisms (66–71). Many studies have investigated the molecular events leading to the formation of NETs. The first cell death mechanism proposed to explain formation of NETs was named NETosis (72), and the authors reported that it is RAF/MEK/ERK pathway dependent and requires ROS production (72). During NETosis, ROS trigger release of elastase from azurophilic granules into the cytoplasm, which then translocates to the nucleus and promotes decondensation of the chromatin through degradation of histones (72–76). This process is followed

by rupture of the plasma membrane and extrusion of the DNA granule–protein complexes into the extracellular milieu to form NETs. Recent studies have compared signal transduction events involved in necroptosis and PMA-induced NETosis, but the findings of two studies were discordant (77–79). Not all reported mechanisms of NET formation require NADPH oxidase or cell lysis. One of the NADPH oxidase-independent mechanisms for NET formation was reported to be a calcium-ionophore-mediated process that utilizes mitochondrial ROS (80). Yousefi et al. reported that NETs form by release of mitochondrial DNA and that this process is not associated with cell death or lysis (81). A similar phenomenon has been described for eosinophils (82, 83). Kubes and colleagues made the intriguing discovery that neutrophils form ETs by extrusion of nuclear DNA, while the cells remain intact and functional afterward (84, 85). This process has been called vital NETosis – although the term “vital NET release” is perhaps less confusing (86). Such a process would circumvent many of the potential issues associated with cytolytic NET formation. However, the vast majority of studies report NETs formed by cytolysis.

Although the process of NET formation *in vitro* is relatively well characterized, triggers for the process *in vivo* are incompletely understood. It is not clear whether neutrophils release ETs as a specific response to stimuli *in vivo*, or if the presence of NETs is simply the aftermath of these cells being overwhelmed with inflammatory signals or pathogen insult, and/or if the mechanism for clearance of effete neutrophils is overwhelmed. Nonetheless, the fundamental outcome of NET formation in most studies is lysis of neutrophils and accompanying extracellular release of cytotoxic molecules. This outcome seemingly defies the numerous aforementioned host systems that are in place to ensure safe neutrophil removal and minimize damage to surrounding host tissues. Moreover, cell-free DNA and DNA-binding proteins (e.g., histones or high mobility group box 1 protein) – all components of NETs – are classic damage-associated molecular pattern autoantigens. NETs have been reported to activate and perpetuate the immune response and, thereby, promote chronic inflammation. Indeed, NET-associated molecules have been shown to elicit inflammatory responses mediated by toll-like receptors (TLRs), which may, in turn, impact autoimmunity (88). This topic has been reviewed recently by Thieblemont and colleagues (89).

Extracellular traps have been detected in a growing number of inflammatory and autoimmune diseases, in which contribution of neutrophils, or more specifically, cytotoxic components released during neutrophil lysis, was previously reported (**Table 1**). In these pathologic conditions, NETs appear harmful and sustain inflammatory processes. For example, Kolaczowska et al. showed in an animal model of *S. aureus*-induced sepsis that extensive liver damage was primarily caused by neutrophil influx and presence of NETs within the liver vasculature (90). Necrotic liver damage was reduced significantly in mice deficient in neutrophil elastase or PAD4, as these mice had decreased ability to form NETs (90). NET components are also potent procoagulants that activate factor XII of the coagulation cascade and contribute to formation of both venous and arterial thrombi. Thus, NETs play an instrumental role in deep vein thromboses, atherosclerosis, or acute myocardial infarction (56, 91–97). In certain types

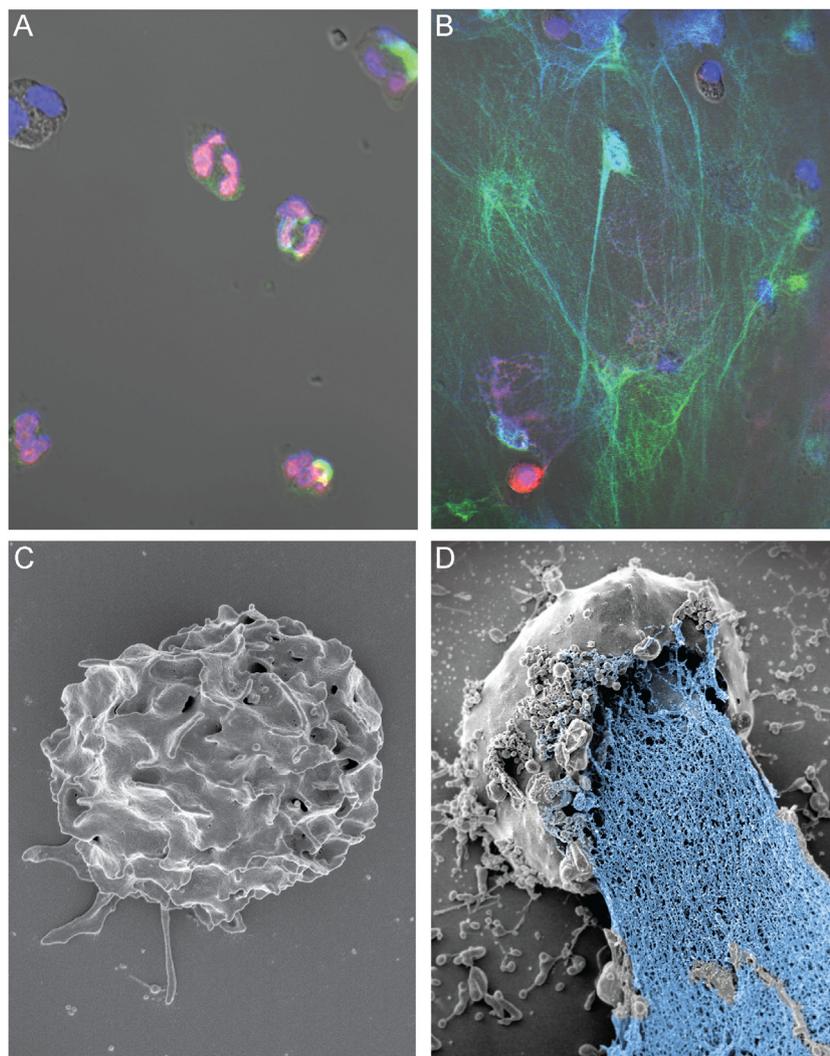


FIGURE 1 | NETs form during osmotic lysis of human neutrophils. (A) Immunofluorescence staining of freshly isolated human PMNs (histone 2A; red), MPO (green), and DNA (DAPI; blue). **(B)** NETs formed following electroporation (pulse of 800 V at 25 mF). Brightness and contrast of the images in **(A,B)** were adjusted in Adobe Photoshop CC2014 (Adobe Systems Inc., San Jose, CA, USA). **(C)** Scanning electron micrograph of a control neutrophil that was not electroporated, and **(D)** NET-forming human neutrophil following electroporation (pulse of 600 V at 10 mF). Studies with human neutrophils were performed according to a protocol approved by the Institutional Review Board for Human Subjects, US NIAID/NIH, as described elsewhere (87). All subjects gave written informed consent prior to participation in the study and in accordance with the Declaration of Helsinki. The image in **(A)** was originally published in Ref. (87). Copyright © (2013) The American Association of Immunologists, Inc.

of respiratory diseases, removal of NETs reduces some of the disease-associated symptoms. In lung diseases, in which NETs contribute to formation of obstructive “plugs,” human recombinant DNase I has been used to dismantle NETs (98–100). This treatment reduces the risk of disease exacerbation and improves overall outcome for the patient (98–100). Consistent with those findings, DNase treatment and removal of NETs has also been shown to improve lung function in murine asthma models (101).

CONCLUDING PERSPECTIVE

Formation of NETs is usually accompanied by neutrophil lysis, although there are notable exceptions (86). Here, we focus our

discussion solely on NETs that form following neutrophil lysis. A cytolytic process for NET formation exposes the host to toxic molecules that contribute to inflammation, tissue damage, and disease. Inasmuch as the potential for neutrophil lysis poses a significant threat to human health, neutrophil activation and turnover are highly regulated. Multiple host mechanisms exist to prevent neutrophil lysis and control release of cytotoxic granule components and ROS – and these regulatory processes are presumably circumvented by the formation of NETs. Therefore, it seems unlikely that the host immune system has evolved to use NETs as routine means for innate host defense against microbes. Rather, we suggest formation of NETs by cytolysis is an incidental phenomenon and not a *standard* or

TABLE 1 | Selected neutrophil-associated inflammatory diseases and contribution of NETs.

Syndrome/disease	Description/role of neutrophils	Contribution of NETs	Reference
Pulmonary disorders			
Cystic fibrosis lung disease	Neutrophils contribute to many of the pathological manifestations of CF, including vigorous inflammation, chronic bacterial infections, and a self-perpetuating cycle of airway obstruction	CXCR2-mediated and NADPH oxidase-independent NET release	(102)
Chronic obstructive pulmonary disease (COPD)	Aberrant inflammatory response to cigarette smoke or other particles; emphysema	NETs and NETotic neutrophils are present in COPD sputum NETs contribute to the severity of restricted airflow	(103, 104)
Respiratory syncytial virus disease (RSV)	Major cause of lower respiratory tract disease in children. Extensive neutrophil accumulation	Occlusion of small airways by DNA rich plugs. NETs have the ability to capture RSV particles	(105)
Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS)	Involves complement C5 activation, acute inflammatory response and neutrophil accumulation, alveolar hemorrhage, edema, and fibrin deposition	NETs induce toxicity in epithelial and endothelial cells Predominant role of histones in lung epithelial and endothelial cell death	(106, 107)
Vascular disorders			
Venous thromboembolism (VTE), including pulmonary embolism (PE) and deep vein thrombosis (DVT)	Inflammatory cells play a key role in thrombus formation; large numbers of neutrophils in early thrombus	NETs are present in the initial stage of thrombus formation	(56, 96, 97)
Disseminated intravascular coagulation (DIC)	Wide spread activation of coagulation; thrombotic occlusion of small and midsize vessels	NETs promote coagulation	(108)
Acute tubular necrosis, acute renal failure	Cell necrosis during initial inflammation, which amplifies the inflammatory response (renal necroinflammation)	NETs as a DAMP signal	(109)
Atherosclerosis	Chronic inflammation of the arterial wall. Neutrophil elastase-dependent secretion and activation of IL-1 β by endothelial cells; LL-37	NETs present in atherosclerotic plaques and contribute to endothelium dysfunction	(43, 94, 110)
Acute myocardial infarction	Rupture of coronary atherosclerotic plaque and subsequent thrombotic occlusion of the vessel	NETs and histones as a pro-coagulant	(95)
Acute thrombotic microangiopathies (TMA)	Excessive microvascular thrombosis	Decreased DNase I activity leads to impaired NET degradation	(111)
Transfusion-related acute lung injury (TRALI)	Presence of anti-neutrophil antibodies. Activation of neutrophils in lungs that leads to damage of the endothelium and capillary leakage	Abundance of NETs in affected alveoli	(112)
Primary systemic vasculitis: granulomatosis with polyangiitis (GPA) (Wegener's granulomatosis) and microscopic polyangiitis	Necrotizing vasculitis that affects small and medium size vessels – results in organ dysfunction; involvement of ANCA; neutrophilic inflammation; and formation of neutrophil granulomas	Not verified	(113, 114)
Others			
Systemic lupus erythematosus (SLE)	Systemic autoimmune disease characterized by production of autoantibodies against self-nuclear antigens; more apoptotic neutrophils in circulation	Patients develop antibodies against DNA and antimicrobial peptides present in NETs NETs increase the risk of venous and arterial thromboses An abnormal subset of neutrophils, called low-density granulocytes (LDGs), are present in SLE. These cells form NETs readily, but a direct contribution to SLE remains to be determined	(58, 115, 116)
Pancreatitis	Granulocytic epithelial lesions, formation of neutrophil rich aggregates and occlusion of pancreatic ducts	NET aggregates occlude pancreatic ducts and promote inflammation	(117)
Psoriasis	Immune-mediated genetic disorder; dysregulation between immune system and cutaneous cells, dendritic cells and lymphocytes are key players; characterized by hyperkeratotic plaques	Release of IL-17 during NET formation; subset of LDG similar to those in SLE; neutrophil elastase cleaves IL-36Ra, which is linked to psoriatic inflammation	(118–120)
Tumors (e.g., Ewing sarcoma, Lewis lung carcinoma; chronic myelogenous leukemia)	Not well defined; MMP-9 (gelatinase), cathepsin G, and neutrophil elastase contribute to tumor proliferation and angiogenesis	Primary tumors facilitate NET production from circulating neutrophils NETs can influence proliferation of B cells	(121–124)
Liver metastases after surgical stress	Activation of immune system after surgery, which enhances the risk of systemic metastases and tumor recurrences	Production of NETs activates TLR9 pathway to induce their pro-tumorigenic activity	(125)
Periodontitis	Chronic inflammation of periodontium that is triggered by bacterial infection and subsequent influx of neutrophils	NETs present	(126)

(Continued)

TABLE 1 | Continued

Syndrome/disease	Description/role of neutrophils	Contribution of NETs	Reference
Rheumatoid arthritis (RA)	Systemic autoimmune disease, which has genetic and environment risk factors; joint inflammation and damage mediated by influx of immune cells into synovial joint space. Cartilage destruction mediated by ROS production and secretion of proteases	Increased spontaneous NETosis NETs as targets for auto-antibody	(60, 127)
Inflammatory bowel diseases (IBD) includes Crohn's disease (CD) and ulcerative colitis (UC)	Chronic relapsing gastrointestinal inflammation	Possible induction of NETs through NOX2 (gp91phox)	(128, 129)
Chronic otitis media (COM)	Acute middle ear infection that can result in hearing loss; characterized by mucoid effusions	NETs play a central role in effusions	(130)
Gout (form of arthritis)	Precipitation of uric acid induces rapid onset of inflammation and influx of neutrophils into affected joint	Possibly anti-inflammatory mediators	(131)

traditional means used by neutrophils to eliminate invading microorganisms. Such a hypothesis is more consistent with neutrophil biology and function, including recent studies of phagocytosis (132), and has no bearing on NET function *per se*. In other words, NETs may simply be the remnants of dead neutrophils – however effective they may be at ensnaring and/or killing microbes. On the other hand, a mechanism of NET formation that leaves neutrophils intact – as with vital NET formation – avoids many of the caveats of a cytolytic process and merits further investigation.

AUTHOR CONTRIBUTIONS

NM, SK, MQ, and FD wrote and edited the manuscript.

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NETosis – Does It Really Represent Nature’s “Suicide Bomber”?

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NETosis is a term that evolved following publication of an original article supposedly describing a novel form of programmed neutrophil death that resulted in the formation of neutrophil extracellular traps (NETs) (1). NETosis was subsequently added to the cell death classifications, almost joining the ranks of other, better documented pathways, such as apoptosis, necroptosis, and autophagic cell death (2). Fuchs et al.’s (1) article seems to NETosis converts to be so seminal that reviewers deny publication to manuscripts in this area, which fail to reference it [Ref. (3); see Supporting Information; Peer Review Correspondence: URL: Link 1].

We have been puzzled by the ready acceptance of a proposed programmed cell death in this format. Let us examine the phenomenon of NETosis as it is cited in the recent literature and see why this concept seems inconsistent with the economy of nature. NETosis was described as a death process in which the plasma membrane ruptures, allowing chromatin release following the collapse of the nuclear membrane (1). This is supposed to happen in order to rescue and protect the affected environment, and this theory has been promoted by many reputable scientific journals, including *Nature* (videos: Link 2). However, thus far, no explanation has been offered as to how the remains of neutrophils that have undergone NETosis would be eliminated under *in vivo* conditions. This lack gives pause because such residue must be expected to be potentially harmful to the host. In fact, under physiological conditions in healthy individuals, nuclear DNA release following activation of neutrophils encountering microorganisms is still controversial and the question has, in fact, been raised whether a NETosis in this format would be at all beneficial to the host (4).

We argue that under physiological conditions, NETosis would be a destructive process. NETosis implies a waste of neutrophils, but more importantly, it would mean exacerbated inflammation. We consider that it is important for neutrophils to remain viable in order to exercise their useful skills, phagocytosis of invading microorganisms and extracellular killing of pathogens by the programmed release of (as we believe, mitochondrial) DNA together with granule proteins. Neutrophils can subsequently die through apoptosis (5) or, under inflammatory conditions, also by programmed necrosis (6). In both cases, recognition of the dying cells by phagocytes would assure disposal without unnecessary inflammation.

Mature neutrophils are terminally differentiated white blood cells that depend on glycolysis for ATP production; hence, they can afford to lose mitochondrial DNA (mtDNA) in response to invading microorganisms. In addition, mitochondria are evolutionary endosymbionts derived from bacteria, which carry bacterial molecular motifs (7), and are considered to be master regulators of danger signaling (8). Unmethylated mtDNA, such as bacterial DNA, is the most potent activator of plasmacytoid dendritic cells (pDCs) and the type I interferon (IFN) pathway [Ref. (9–11), and our own unpublished data]. In our view, the innate immune system attempts to overcome an infection primarily with a combination of mtDNA-containing NET formation and phagocytosis. This offers the advantage that, in case of persisting infection, the mtDNA will have boosted the adaptive immune response. Furthermore, no exaggerated inflammation caused by local cell lysis occurs. In fact, the clearance of NETs occurs in an immunologically silent manner (12).

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In the literature, the terms NETs and NETosis are often used indiscriminately, which is problematic. NET formation was first described by Brinkmann et al. (13). These authors observed the formation of extracellular traps consisting of DNA together with granule proteins of neutrophils, which were released upon brief stimulation with physiological agonists, such as interleukin-8 (IL-8) or lipopolysaccharide (LPS), Gram-positive and Gram-negative bacteria, and unphysiological stimuli, such as phorbol myristate acetate (PMA) that cause increases in cellular ROS levels. PMA at concentrations between 5 and 50 nM for 30 min (dose-response) and with 10 nM PMA for 10, 20, and 30 min (time course) were able to induce NET formation [Ref. (13), see Supporting Online Material]. The same was true for co-culture with bacteria, i.e., a 30-min incubation was sufficient to form NETs and kill bacteria extracellularly. The neutrophils were reported to remain viable, but the source of the released DNA was not identified at that time (13).

Owing to the apparent presence of histone reactivity in NETs, many investigators have assumed that NETs contain chromosomal DNA. This idea that NETs consist of chromosomal DNA, granule proteins, and histones has become cemented in the literature because it had already been shown that histones exhibit antibacterial activity. Thus, it just seemed to make sense. However, it is important to realize that the antibodies used to support this conclusion, i.e., that histones are present in NETs, are also known to detect DNA as well (14), especially at the high concentrations employed. In addition, the existence of extranuclear histones, namely pools of H1 and H3 in the cytoplasm, has also been reported (15).

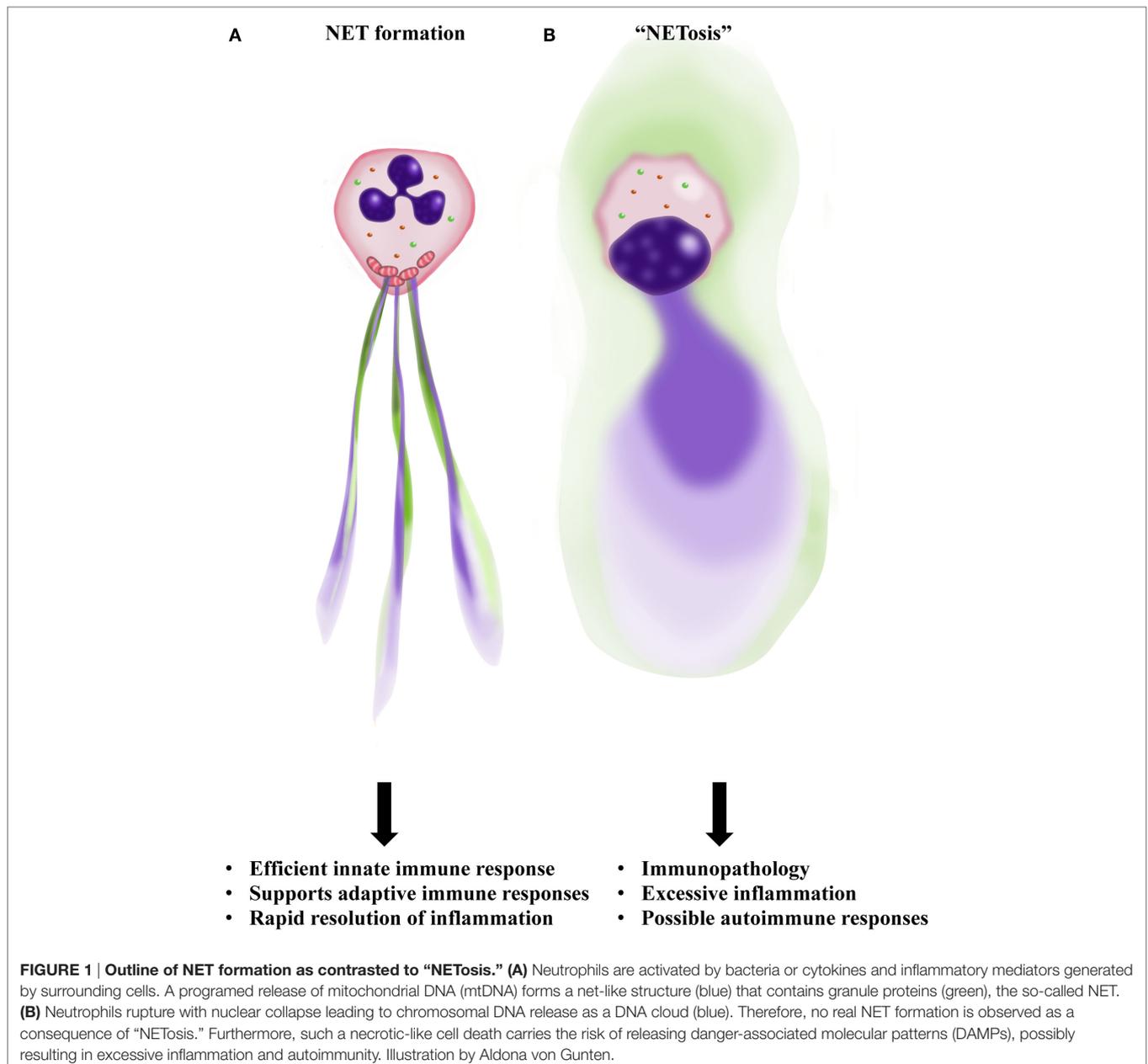
Moreover, one has argued that the presence of citrulline-containing proteins in extracellular proteins, and presumably in NETs, is an argument in favor of NETosis. Human primary neutrophils express not only protein arginine deiminase 4 (PAD4) but also PAD2 enzymes that catalyze citrulline modification of number of proteins, most importantly fibrinogen, collagen, vimentin, and platelet actin, as well as histones (16, 17). Current evidence suggests that protein citrullination may occur extracellularly and, therefore, substrate selection by the PADs would not be limited to their subcellular localizations (e.g., not just to histones in nucleus) (16). PAD2 lacks a nuclear localization signal (18) and is highly expressed in the cytoplasm of human neutrophils (17). Interestingly, the cytoplasmic concentration of PAD2 was dramatically reduced within 30 min after stimulation with PMA and, furthermore, enzymatically active PADs were detected in supernatants of cultured, activated neutrophils (17). This observation might explain the presence of citrullinated proteins in NETs upon physiological stimulation of neutrophils (e.g., activation by platelets) *in vitro* (19) and *in vivo* (20), considering that PAD2's main substrates are fibrin and platelets' actin, which would be present within entangled NET structures following platelet activation in the absence of cell death. PAD2 could also citrullinate extracellular histone H3 released owing to secondary necrosis (which might occur under *in vitro* as well as *in vivo* conditions), though with lower efficiency than with PAD4 (16). It is surprising, that so far no one has investigated the potential role of PAD2 for NET formation and the protein content of NETs, respectively. Perhaps

some of the discrepancies in the PAD4 knockout mouse model could be explained if we take into account the possible role of the PAD2 enzyme in extracellular protein citrullination. It was easy to pick PAD4 as the culprit owing to its nuclear localization, ignoring the fact that all PADs, including PAD4, can function extracellularly. Another reason researchers preferred PAD4 as a candidate is perhaps its specific expression in the myeloid lineage as compared to PAD2, which is ubiquitously expressed (21). This would make PAD2 less attractive as a potential commercial drug target.

Thus, considering these uncertainties, the argument that NETs contain chromosomal DNA is actually still unsubstantiated today. In fact, subsequent studies using DNA sequencing methods have established that NETs are generally composed of mtDNA [Yousefi et al. (22), McIlroy et al. (23), Wang et al. (9), and recently Lood et al. (10)]. We recognize that under certain conditions, neutrophils do release nuclear DNA. For instance, neutrophils can release nuclear DNA upon encountering bacteria capable of secreting pore-forming enzymes/toxins. This type of nuclear DNA release can occur as early as 5 min after bacterial contact (24–26). We also do not exclude the possibility that nuclear DNA originating from cells dying in the neighborhood of NETs, as a consequence of immunopathology, could subsequently bind to NETs *in vivo*. Moreover, under *in vitro* conditions, neutrophils stimulated with PMA may first form mtDNA-containing NETs before undergoing a subsequent necrotic cell death. As a consequence of necrotic cell death, nuclear DNA and histones could bind to already existing NETs. Furthermore, it should be noted that nuclear DNA released from dying neutrophils following PMA stimulation has been shown to generate a DNA cloud, rather than DNA fibers [Ref. (27); video: Link 3] (**Figure 1**).

Since the discovery of NETs, many groups have focused on finding the molecular mechanism and origin of the DNA released. For instance, elastase-deficient mouse neutrophils were reported to be unable to form NETs (28). By contrast, NET formation has recently been reported to occur in these mice in a model of deep vein thrombosis (29). We also have not found any defect in NET formation by elastase-deficient bone marrow-derived primary mouse neutrophils activated either physiologically or with brief stimulation using low concentrations of PMA (unpublished observation). Moreover, it has been reported that NETosis could actually represent a necroptosis (30). However, on the contrary, we obtained no evidence for the involvement of the RIPK3–MLKL pathway, as would be required for genuine necroptosis (3). The conditions for forming NETs have gradually evolved from 10 to 30 min of stimulation to 3–4 h, while PMA concentrations have skyrocketed from 10–25 to 100–800 nM (31). With mouse neutrophils, NET formation after 16 h of 100 nM PMA stimulation has been reported (32). It is worth noting that even low concentrations of PMA (25 nM) are known to induce cell death due to excessive intracellular ROS levels within 2–3 h (33).

Meanwhile, our group has reported that with physiological activation or low doses of PMA (25 nM), eosinophils (34), neutrophils (22), and basophils (35) all release mtDNA combined with granule proteins within <1 h, in every case without cell death (**Figure 1**).



The aim of this opinion article with our inflammatory title is to raise the awareness for this problem. For more honest scientific behavior, the reviewing process is ultimately where changes will have to be made to allow opposing ideas, as long as scientifically solid, to reach the overall scientific community and to receive critical scrutiny. An established opinion is not always correct; de-construction and re-construction of theories is a part of the scientific process. As an option for defusing such long-lasting scientific controversies, it would also be appropriate to deliberately arrange that proponents of opposing viewpoints present their work at international meetings. Furthermore, there should

be guidelines for the stimulation and the detection of NETs both *in vivo* and *in vitro*.

AUTHOR CONTRIBUTIONS

SY wrote the article. H-US corrected the article.

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Immunodetection of NETs in Paraffin-Embedded Tissue

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The pathogenic potential of neutrophil extracellular traps (NETs) was recently described, and their detection in tissue could serve as a prognostic marker. NETs are delicate and filigree structures; hence good tissue preservation is essential for their detection. Indeed, analysis of paraffin-embedded tissue has proven superior to the study of cryo sections. Though, under favorable conditions, the presence of NETs can be detected in tissue sections stained with histological dyes, definitive identification of NETs needs the colocalization of immunofluorescent signals for both nuclear and granular (or cytoplasmic) NET components. We tested diverse antigen retrieval methods and various combinations of commercially available antibodies and present here staining protocols to detect NETs in human and murine tissue sections.

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INTRODUCTION

Neutrophil extracellular traps (NETs) probably evolved to counteract invading pathogens (1). When their production or degradation is not controlled, they have pathogenic potential and are involved in numerous diseases including autoimmunity, thrombosis, lung diseases, infertility, diabetes, cancer, and neurodegeneration, reviewed in (2). NETs are composed of chromatin, granular, and some cytoplasmic constituents (3). In naive neutrophils, granular and nuclear antigens are spatially separated and during NETosis, some granular proteins, like neutrophil elastase (NE) and myeloperoxidase (MPO) gradually migrate to the nucleus (4, 5). Also, histones are citrullinated during NETosis (6, 7). Thus, the localization of granular proteins in the nucleus and the citrullination of histones provide unique feature for neutrophils undergoing NETosis that can be exploited for identification of these cells in tissue sections.

In vitro, there are a variety of protocols to detect and quantify NETs (8–12). It is important to note that the widely spread out strands associated with NET images generated *in vitro* are probably an artifact of fixation. Indeed, in life cell imaging studies with isolated neutrophils, NETs appear as a diffuse cloud formed by the strands of NETs floating in the medium (13). It is not clear how NETs appear in tissues, where space is restricted, and the NETs are unlikely to appear as the large areas observed *in vitro*. Classical histological stains, like hematoxylin/eosin, may indicate the presence of NETs, but there are only few examples of NETs detection with histological stains (14, 15). Notably, there is no general protocol to identify NETs in tissues.

While they are well suited for staining with most antibodies, cryo sections from freshly frozen tissue have the disadvantage that due to ice crystal formation neutrophils in the tissue can be damaged, thus NET-like structures can be generated as a preparation artifact. In contrast, fixation with buffered paraformaldehyde solution, ideally by perfusion, preserves the tissue architecture including NETs. Here, we present methods for formalin-fixed and paraffin-embedded sections. These sections

are available from pathological studies and can be conserved indefinitely before analysis.

Most antibodies will not readily bind to their epitopes in formalin-fixed tissue. The reason for this is the formation of intra- and intermolecular cross-links by methylene bridges that mask most epitopes (16, 17). For successful immunohistological staining, antigen retrieval is required that normally involves heating of the rehydrated sections in a suitable heat-induced epitope retrieval (HIER) buffer (18, 19). This breaks the methylene bridges that prevent binding of the antibody and renders the epitopes accessible. In a study with histopathologically important antibodies, it was shown that most epitopes detected by clinically relevant antibodies are linear and can be reversibly blocked by binding to neighboring proteins during fixation (20).

We tested a series of antibodies against NET components for their ability to bind to their epitopes in formalin-fixed paraffin-embedded tissue. We selected nine antibodies with good staining properties and tested various antigen retrieval methods to find suitable combinations for double or triple immunofluorescence. In this paper, we present protocols that allow simultaneous staining for nuclear and granular or cytoplasmic NET components in paraffin-embedded tissue sections after antigen retrieval.

For the identification of NETs it is necessary to determine if nuclear antigens are colocalized with granular and/

or cytoplasmic components. Hence, micrographs have to be prepared at a primary magnification of at least 20×. The resulting images can be used to quantify the fluorescent signals as an unbiased means for the detection and measurement of NETs in tissue.

MATERIALS AND EQUIPMENT

Archived paraffin blocks of mouse lungs and of a human brain fungal abscess were used. Mouse breeding and experiments were approved by the Berlin state authority Landesamt für Gesundheit und Soziales, permit G0200/15. Pathology sample collection was approved by the ethical committee of Charité University Hospital, Berlin, Germany.

All antibodies were obtained from commercial suppliers (Table 1). The following antigen retrieval solutions were used: R-Universal Buffer pH7, 10× (Aptum APO 0530500), Target Retrieval Solution pH9 10mM Tris (TRS) 10× (Dako S236784), and Target Retrieval Solution pH6 10mM Citrate 10× (Dako S236984-3).

Fluorescence images were recorded using a Leica SP8 confocal or a Leica DMR widefield microscope (equipped with bandpass filter blocks and a Jenoptik ProgRes MF USB camera).

Complete tissue sections were digitized using a ZEISS Axioscan Z1 slide scanner.

TABLE 1 | Overview of the antibodies that allow immunostaining for NET components in paraffin-embedded tissue and the respective most effective antigen retrieval protocol.

Source	Clone	Specificity	Host dilution	37°C		50°C		50°C		60°C		96°C	
				Citrate pH6	TRS pH9	R-Univ pH7	Citrate pH6	TRS pH9	Citrate pH6	TRS pH9	Citrate pH6	TRS pH9	
Abcam ab134211	–	Histone H2B	ck 1:500	H2B	+++ ¹ N	+/-	+++ ¹ N	+++ ¹ N Q	+++ ¹ N	+++ N	+++ ¹ N	+++ N	+++ N
Antibodies-Online ABIN1735464	–	Histone H3	sh 1:100	H3	+/-	+/- N	+++ ¹ N (Q)	+++ ¹ N Q	+++ ¹ N Q	+++ ¹ N (Q)	+++ N	+++ N	+++ ¹ N
Abcam ab5103	–	Histone H3 citrulline (R2 + R8 + R17)	rb 1:50	H3cit	+++ ¹ (N)	+/-	+++ N Q	+++ N Q	+++ ¹ (N) Q	+/- N	+ (N)	+++ N	+++ N
Millipore 481001	–	Neutrophil elastase	rb 1:50	NE	++ (N)	+/-	++ N	++ N	++ (N)	+ (N)	–	–	–
LS-B4244	–	HN elane ^a	sh 1:200	NEsh	++	++	++	++ N	++ N	++	++	–	–
R&D Systems AF3667	–	Myeloperoxidase	gt 1:200	MPO	++	++	++	++	++	++	++	++	++
Biorbyt orb316605	–	CalgrA S100A8 MRP14	rb 1:200	CalA	+++ N	+++ N	+++ N	+++ N	+++ N	+++ N	+++ N	+++ N	+++ N
Biorbyt orb315186	–	CalgrB S100A9 MRP14	rb 1:200	CalB	+++ N	+++ N	+++ N	+++ N	+++ N	+++ N	+++ N	+++ N	+++ N
BDPharmingen 551459	1A8	Ly6G	rt 1:200	Ly6G	++	+++	+++	+++	+++	+++	+++	+++	+++

^aOnly for human tissue.

R-Universal Buffer pH7 (RUB) 10× Aptum APO 0530500.

Target Retrieval Solution pH9 10mM Tris (TRS) 10× Dako S236784.

Target Retrieval Solution pH6 10mM Citrate 10× Dako S236984-3.

N, NET visualizations.

+++¹, strong staining of expected epitopes.

++¹, satisfying specific positive result.

+¹, moderate specific positive result.

+/-¹, weak or partially positive.

–¹, completely negative result.

Q, suitable as NET marker for quantification under mentioned conditions.

¹, decondensed nuclei+++¹, nuclei +/-¹, or +¹.

(), limitations.

STEPWISE PROCEDURES

Immunofluorescence of Tissue Sections

The mouse tissue had been fixed *in situ* by transcardial perfusion with 2% paraformaldehyde solution in TRIS-buffered saline (TBS, pH 7.4). Following this, the lungs were carefully removed and post-fixed in 2% paraformaldehyde for 16–20 h at RT. The tissue was then dehydrated and paraffin-embedded (60°C) using a Leica TP 1020 tissue processor. Human brain fungal abscess tissue was from archived paraffin blocks; fixation conditions are not known.

Paraffin blocks were cut at 3 μm , sections were mounted and dried on Superfrost Plus slides (Thermo Scientific) avoiding temperatures above 37°C. After dewaxing and rehydration, sections were incubated in one of the HIER buffers at different temperatures [20 min at 96°C in a steam cooker (Braun) or 90 min at lower temperatures in a water bath, details in **Table 1**].

After antigen retrieval, sections were left in the respective HIER buffer at RT to cool below 30°C, rinsed with deionized water three times, TBS pH7.4 one time, and permeabilized for 5 min with 0.5% Triton X100 in TBS at RT, followed by three rinsing steps with TBS.

Sections were surrounded with PAP-pen and treated with blocking buffer for 30 min to prevent non-specific binding. Primary antibodies (**Table 1**) were diluted in blocking buffer and incubated on the sections over night at 37°C. At any one time, two or three primary antibodies requiring the same antigen retrieval protocol raised in different hosts were combined. We used secondary antibodies raised in donkey and pre-absorbed against serum proteins from multiple host species (Jackson ImmunoResearch). Dilution and blocking buffer was TBS supplemented with 1% BSA/2% donkey NS/5% cold water fish gelatin/0.05% Tween 20/0.05% Triton X100.

Hematoxylin/Eosin Histology

Consecutive sections were stained with hematoxylin/eosin using standard protocols.

Image Analysis

Image sets were analyzed using the Fiji-ImageJ software package (21) and a common spreadsheet application. The FigureJ plugin (22) was used to assemble **Figures 2** and **3**.

RESULTS

Suitable Combinations of Antibodies and Antigen Retrieval Methods

Table 1 summarizes the results of immunofluorescence staining for NET components and neutrophil marker proteins under different antigen retrieval conditions. **Figure 1** shows immunofluorescence images resulting from different antigen retrieval protocols. Immunodetection of NE is dependent on the incubation temperature, which may not exceed 60°C (**Figure 1C**). Staining is stronger at pH6 compared to pH9. The cytoplasmic NET component calprotectin (3), a heterodimer consisting of Calgranulin A and B, is readily detected at all temperatures

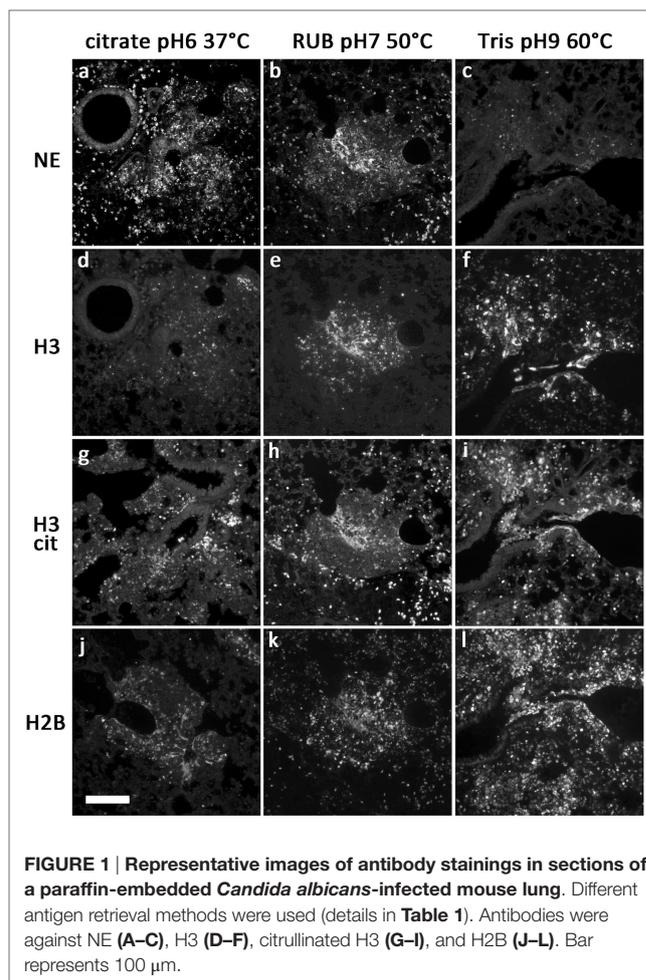


FIGURE 1 | Representative images of antibody stainings in sections of a paraffin-embedded *Candida albicans*-infected mouse lung. Different antigen retrieval methods were used (details in **Table 1**). Antibodies were against NE (**A–C**), H3 (**D–F**), citrullinated H3 (**G–I**), and H2B (**J–L**). Bar represents 100 μm .

tested. As expected, the staining patterns for both subunits did not differ.

We tested various antibodies against histones. Only the ones that gave reliable immunostaining with NETs are specified in **Table 1**. Interestingly, both antibodies against H3 and H2B produced different staining patterns depending on the antigen retrieval temperature. Incubation of the sections at temperatures above 55°C resulted in a strong staining of NETs and nuclei (**Figures 1F,I**). In contrast, at temperatures between 37 and 50°C, both antibodies reacted predominantly with relaxed chromatin in netting neutrophils and NETs, while normal neutrophils and other cells show a weak nuclear staining (**Figures 1D,E,J,K**; also **Figure 3A**). A similar staining pattern has been described for an antibody against a subnucleosomal complex in NETs derived *in vitro* from isolated neutrophils (13). Taken together, this difference in staining is probably due to the compaction of chromatin and the state of the antigen detected. Importantly, antibodies against citrullinated H3 (H3cit) reacted at all temperatures tested, and the staining pattern was nearly exclusively in areas with netting neutrophils and NETs (**Figures 1G–I**) (6, 7).

To clearly identify NETs in tissue, colocalization of granular and nuclear components has to be detected. We chose antibodies against NE and either H3 or H2B in combination with detection

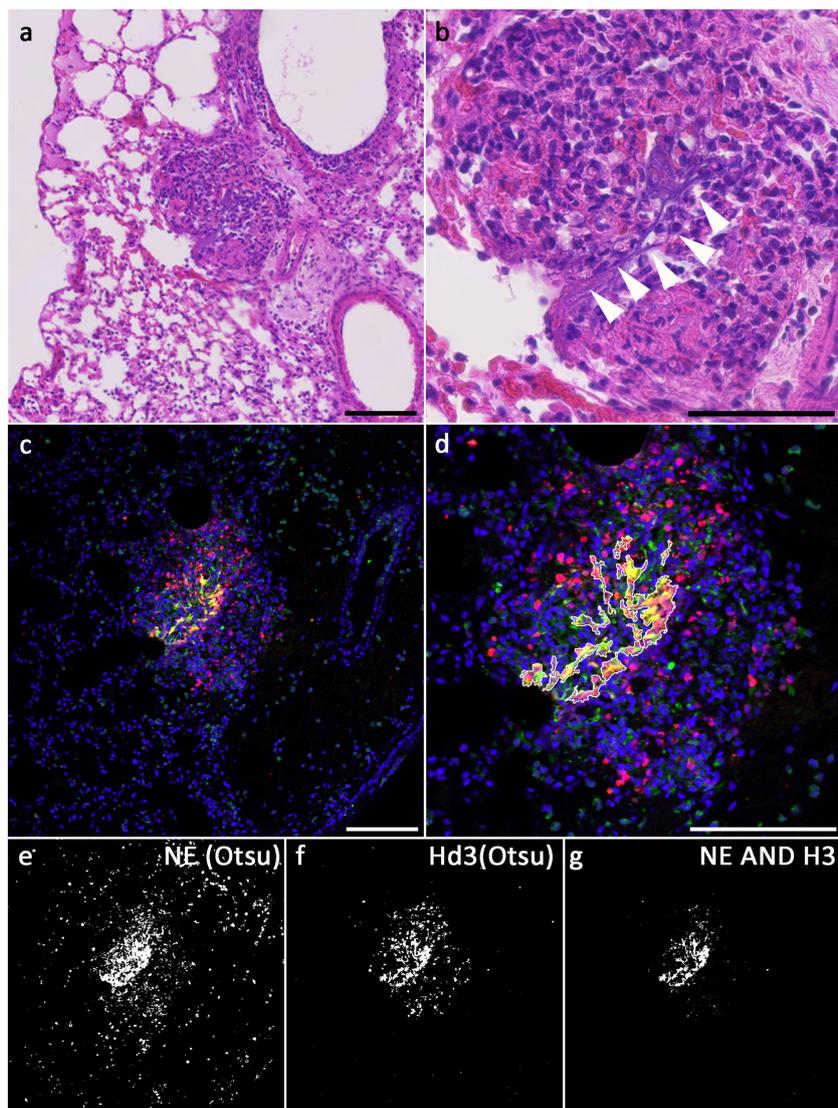


FIGURE 2 | NETs in a *Candida*-infected mouse lung; (A,B) hematoxylin/eosin staining of a *Candida albicans*-infected mouse lung, consecutive section to the one used in (C–G). Areas of extracellular DNA are present (arrowheads), which stain for NET markers (C). (C) Merged fluorescence images showing DNA: blue, NE immunolabel: green, H3 immunolabel: red; (E,F) binarized images after Otsu thresholding, (E) NE above threshold, (F) H3 above threshold, (G) NETs [intersection of both, $NE \wedge H3$; outline of this area is superimposed in (D)]; (D) magnified section of the merged fluorescence image with outline of NETs segmentation (excluding areas <30 pixels). Bars represent 100 μm .

of citrullinated H3. As a compromise for the different conditions of antigen retrieval, we chose Buffer R-Universal at neutral pH, which allows simultaneous immunodetection of histones and NE. At magnifications of 20 \times or higher, the resulting images can be used for automatic detection of NET-containing areas in tissue (Figure 2C).

Segmentation of Areas Positive for Nuclear and Granular NET Components Allow Quantification of NETs in Tissue

Figure 2C shows a confocal image of a neutrophil-rich area of a mouse lung infected with *Candida albicans* and stained for NE

(green, Millipore 48101) and H3 (red, ABIN 1735464) as well as for DNA (Hoechst 33342). Antigen retrieval was performed with R-Universal Buffer at 50°C. Using automatic Otsu thresholding, positive areas for both channels were depicted white, while areas below threshold were depicted black (Figures 2E,F). The overlap of both indicating the NET-positive area is shown in Figure 2G and superimposed on the tissue staining (Figure 2D).

Hematoxylin/eosin staining of the same area in a consecutive section is presented in Figures 2A,B. The overview shows infiltration of neutrophils (Figure 2B, center). At higher magnification, extracellular strands of DNA are visible (arrowheads in Figure 2B). Identification of these strands as NETs needs immunofluorescence.

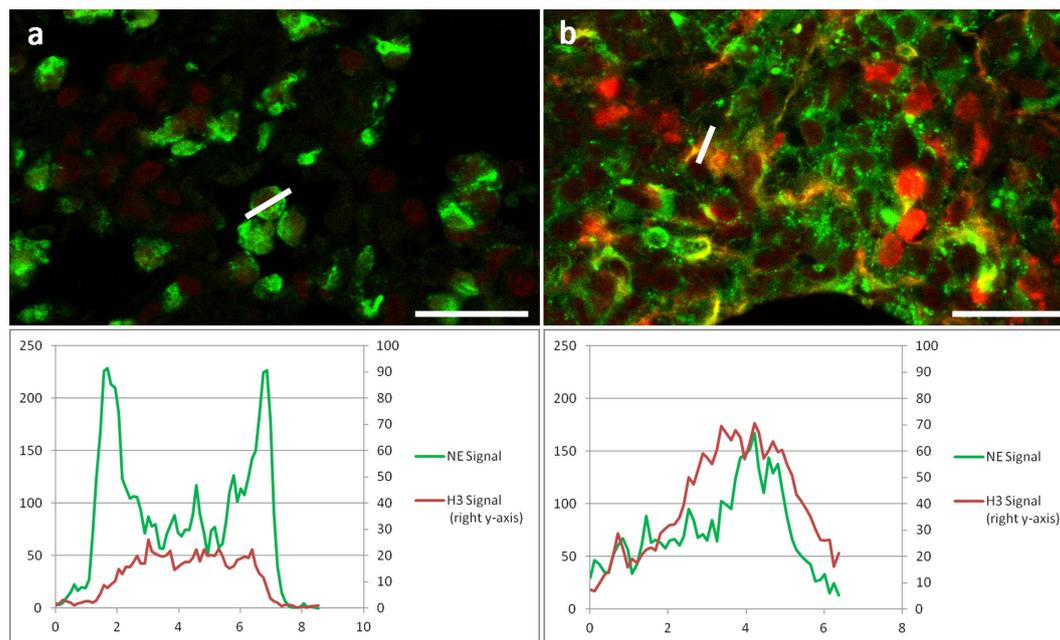


FIGURE 3 | Two areas of the same section of a *Candida albicans*-infected mouse lung at higher resolution showing NE immunolabel: Green, H3 immunolabel: red. (A) Area with non-NETotic Neutrophils with a line profile of a neutrophil granulocyte. (B) Area with NETs with line profile of a NET; both areas were imaged with identical settings. Below images are the respective fluorescence intensity plots of the line profiles for both channels. Bar represents 20 μ m.

Staining Pattern of Antibodies against H3 and H2B Is Dependent on the Antigen Retrieval Protocol

We further analyzed the staining pattern of anti-histone antibodies. When antigen retrieval was accomplished with incubations above 55°C, a strong staining of all nuclei and NETs was detected (Figures 1E,I). In contrast, only decondensed nuclei of neutrophils as well as NETs stained strongly with these antibodies if the antigen retrieval incubation did not exceed 50°C (Figure 3B). Images recorded under identical conditions of normal and netting neutrophils in the same tissue section revealed clear differences in fluorescence brightness. While H3 fluorescence in normal neutrophils rarely reached intensity values of 60 (red channel in Figure 3A), using identical settings, intensity in areas with NETosis often reached saturation (256, Figure 3B). Representative intensity line profiles are presented below the micrographs including the intensity values for NE (green). As expected, in normal neutrophils, highest NE intensity was found surrounding the nuclear area (in the cytoplasm), as opposed to netting neutrophils showing similar line plots for NE and H3 indicating colocalization of nuclear and granular NET components.

Immunodetection of NETs in Archived Human Tissue

The tissue section (archived human brain fungal abscess) depicted in Figure 4 was stained with antibodies against NE (LS-B4244, Figure 4A, and green in Figure 4D) and against Histone 2B (H2B, Abcam ab 134211, Figure 2B, and red in Figure 4D) after antigen retrieval using R-Universal Buffer at 50°C. DNA was

stained with Hoechst 33342 (Figure 4C and blue in Figure 4D). NE-staining shows a very strong granular signal (green arrows in Figures 4A,D) compared to the rather faint diffuse staining in NETs (green arrowheads in Figures 4A,D). Conversely, the signal for H2B is stronger in NETs (red arrowheads in Figures 4B,D) than in compact nuclei (red arrows in Figures 4B,D). This pattern of strong histone staining in NETs but weak staining in compact nuclei is similar to that of H3 described for staining in mouse tissue (Figure 3). DNA staining is strongest at areas with high DNA concentration (compact nuclei, blue arrows in Figures 3A,B), while relaxed chromatin in NETs shows a diffuse staining (blue arrowheads in Figures 3A,B).

DISCUSSION

Due to their pathogenic potential, identification of NETs in tissue samples both from patients and from laboratory animals is important and could be of diagnostic value. In contrast to staining of NETs derived from isolated neutrophils stimulated *in vitro*, NETs in tissue are not easily identified, and staining for just one component, e.g., DNA, is not sufficient.

In order to allow simultaneous immunodetection of two or three NET components, we tested a series of commercially available antibodies for their property to react with NET markers in paraffin-embedded tissue. Each antibody was tested using various antigen retrieval protocols (Table 1). We use unlabeled primary antibodies from different hosts and detect the bound antibodies with species-specific secondary antibodies, which are cross-absorbed against serum proteins of a number of hosts. This avoids false-positive staining due to unspecific cross-labeling and

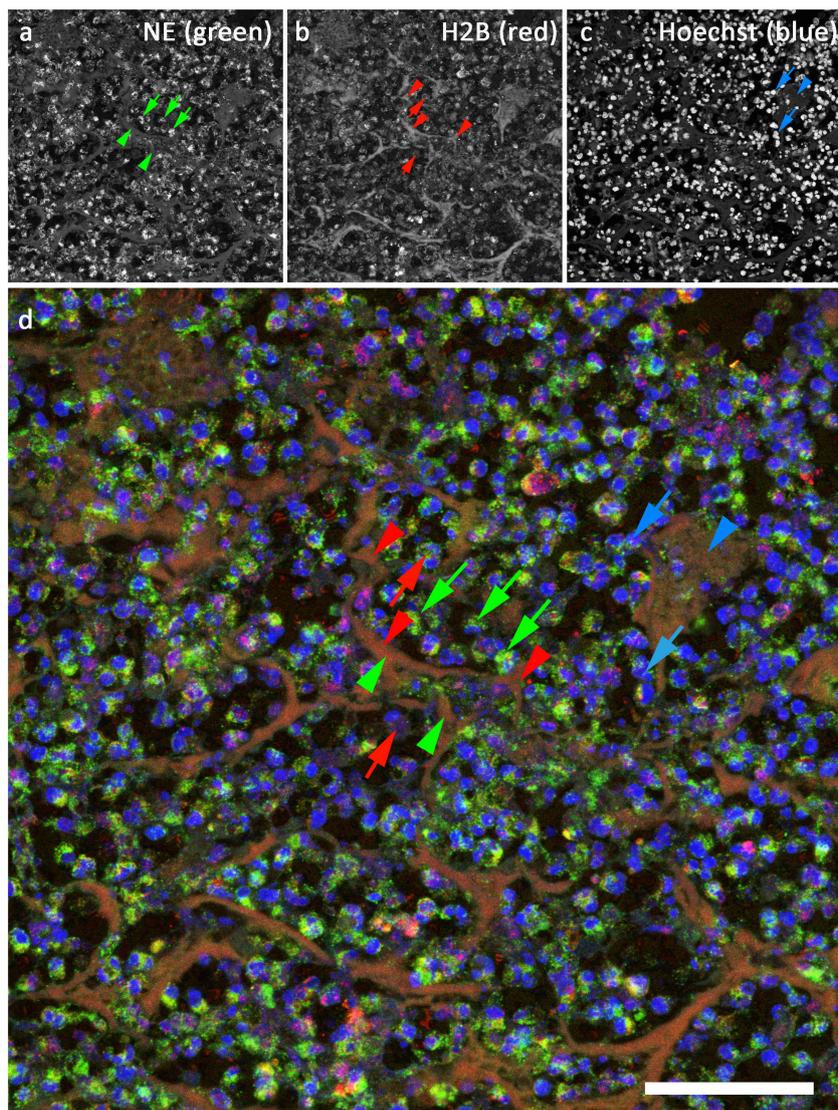


FIGURE 4 | Confocal image of NETs in an archived sample of human brain fungal abscess. (A) Staining for neutrophil elastase (LS-B4244). Green in (D). **(B)** Staining for histone 2B (Abcam ab134211), red in (D). **(C)** DNA stain (Hoechst 33342), blue in (D). Bar represents 50 μ m.

facilitates dye swaps using different combinations of secondary antibodies. We found that good results were obtained performing antigen retrieval at neutral pH and temperatures between 37 and 50°C, which allow combinations of various antibodies against nuclear, granular, and cytoplasmic NET components (Figure 1).

In resting neutrophils, these are clearly segregated, and immunofluorescence staining reveals no overlap of the signals if magnifications of 20 \times or higher are used given that the section thickness does not exceed 3–5 μ m or confocal microscopy is used. During NETosis, NET components gradually intermingle to a homogenous mixture in late phases of NETosis and in NETs. Accordingly, fluorescence signals for nuclear and granular or cytoplasmic NET components overlap increasingly. These fluorescence signals can be segmented automatically (Figures 2C,D), and the area of signal overlap defines NETs (Figures 2B,E). In

hematoxylin/eosin-stained tissue slices, NETs can appear as dark diffuse strands [Figures 2F,G and Ref. (14)], but the positive identification of these smears demands overlapping immunodetection of NET components.

We found that under mild antigen retrieval conditions, antibodies against H3 (Figure 3) and H2B (Figures 4B,D) stain relaxed chromatin as present in NETs and netting neutrophils stronger than the compact chromatin of normal nuclei. This property can be used to scan at low power magnifications for areas that may contain NETs for subsequent detailed analysis using a second NET marker.

Figure 4 depicts a section of an archived sample of human brain fungal abscess stained for NE (Figure 4A, green in Figure 4D), H2B (Figure 4B, red in Figure 4D), and DNA (Figure 4C, blue in Figure 4D). Interestingly, the staining

intensity for NE is very high in granules but rather low in NETs. This is probably due to differences in protein concentration. In contrast, staining for H2B is generally lower in condensed nuclei than in NETs. Presumably, the epitope of this antibody is better accessible in relaxed compared to compact chromatin. Notably, this preference for binding to decondensed chromatin depends on the temperature used for antigen retrieval: when HIER buffer is heated above 55°C, antibodies against H2B and H3 react strongly both with NETs and with compact nuclei. Apparently, antigen retrieval at higher temperatures exposes histone epitopes that are normally hidden.

Ly6G is a differentiation antigen, which is expressed in mature neutrophils. In areas of massive infiltration, neutrophils are densely packed leaving nearly no space between the cells. Under these conditions, GPI-anchored Ly6G delineating the cell membrane can come in close contact to extracellular NETs that may result in the interpretation of Ly6G-positive areas as NETs (23). It has been shown that NETs do not contain membrane proteins, and Ly6G was not found as a NET constituent (3). For proper

identification of NETs in tissue, immunostaining for generally accepted NET markers as presented in this protocol should be employed.

We have identified a set of antibodies, which can be used to detect NET components in paraffin-embedded tissue both of human and murine origin. Using mild antigen retrieval protocols, many of these antibodies can be combined to yield a satisfactory signal intensity. We hope that these protocols will be useful for a more reliable detection of NETs in tissue.

AUTHOR CONTRIBUTIONS

Designed study: VB, UA, CG, and AZ. Tissue immunostainings: UA. Microscopy: UA and VB. Image analysis: CG. Wrote the manuscript: VB, UA, CG, and AZ.

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Current Challenges and Limitations in Antibody-Based Detection of Citrullinated Histones

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Studies on NETosis demand reliable and convenient markers to monitor the progress of this form of cell death. Because a determining step in the release of nuclear chromatin NETs requires the conversion of arginine residues to citrulline residues in histones by peptidylarginine deiminase, citrullinated histones can provide such a marker. Here, we evaluate antibody reagents for the detection of citrulline residues in histones and observe alarming differences between commercial antisera and mouse and rabbit monoclonal antibodies in their ability to detect their nominal target residues. Differences between antibodies that are currently used to detect citrulline residues in histones could jeopardize efforts to reach a scientific consensus and instead lead to inconsistent and even conflicting conclusions regarding the regulation of histone deimination. Our results will assist others in planning their initial or ongoing studies on peptidylarginine deiminase activity with the use of currently available antibodies. Furthermore, we argue that, along with the careful attention to experimental conditions and calcium concentrations, validated antibody reagents are urgently needed to avoid possible setbacks in the research on NETosis.

Keywords: NETs, NETosis, deimination, peptidylarginine deiminase, antibodies, immunodetection, citrulline

The years since 2004 have seen an explosive rise in interest in neutrophil cell death mechanism. Much of the interest was sparked by the discovery of NETosis by Brinkmann et al. (1), which implicated this unique form of cell death in infectious and autoimmune diseases. It is difficult to adequately summarize all the remarkable discoveries since that landmark paper was published. Some examples include the relation between NETosis, autophagy (2), apoptosis (3), necroptosis (4), and granzyme-mediated cell death (5). Insights into the regulation of NETosis have defined the roles of cell surface receptors (6), protein kinases (7), elastase, and myeloperoxidase (8). The participation of NETs has been demonstrated in inflammatory diseases such as acute lung injury (9), thrombosis (10), cystic fibrosis (11), vasculitis (12), gout (13), diabetes (14), and even Alzheimer's (15). NETosis also directly contributes to the induction of autoantibodies in major autoimmune diseases such as rheumatoid arthritis (16) and systemic lupus erythematosus (17). The process of chromatin NET release may not be unique to vertebrates, as plants (18) and slime molds (19) have mechanisms to release nuclear chromatin under specific circumstances. Subtypes of NETs have been reported and an important form of NETosis has been identified in which neutrophils release nuclear DNA but continue certain functions such as chemotaxis despite the casting of NETs (20). Similarly, NETs consisting of mitochondrial DNA have been characterized that may be compatible with continued functions of neutrophils (21–23). NETs are also released by other granulocytes (24), macrophages (25), and mast cells (26).

NETosis is characterized by large-scale morphological transitions that can be seen as the swelling of the lobed nucleus, the rupture of the nuclear envelope, and the release of NETs that can stretch to many times the size of a single neutrophil (27). Thus, NETosis has been measured by automated image analysis (28), by quantitative fluorescence activated cytometry (29), and by immunofluorescence detection of colocalized DNA and neutrophil granule components (30). The detection of deiminated histones represents an important hallmark of NETosis because the enzyme responsible for histone modification, peptidylarginine deiminase IV (PAD4), is activated on a massive scale during the progress of NETosis (31). Indeed, the activation of PAD4 is intimately linked with the production of autoantibodies to citrullinated proteins. These antibodies are sensitive and predictive criteria in a number of autoimmune diseases (3, 32–34). Importantly, several groups of researchers have provided evidence that NET release is dramatically impaired by the genetic deficiency (35) or the pharmacological inhibition of PAD4 (36), and that PAD4 inhibitors offer promising starting points to develop autoimmune disease therapies (37, 38).

The detection of deiminated histones *in vivo* has been interpreted as evidence for NETs, as may occur in nephritis associated with vasculitis (39), thrombus formation (29), lung injury (40), and due to alum adjuvant stimulation (41). Detection of histone deimination has also been helpful in testing aspects of PAD4 regulation (42). However, inconsistencies between results reported by different labs have also appeared in the literature. For example, one widely used stimulus, PMA, has resulted in conflicting results in the literature. Thus, PMA was observed to induce deimination (35) or suppress deimination (7). Our result was surprising due to the frequent use of PMA to induce the release of NETs, and the common assumption that PAD activity is required for NET release to occur. Therefore, we carefully analyzed the phenomenon and observed that PMA also suppressed histone deimination in the presence of A23187 ionophore, a compound that by itself is a strong inducer of deimination (7). We further established that PMA inhibited PAD4 *via* activation of PKC α/β . Our results have been confirmed by Doua et al. who characterized two alternate forms of neutrophil cell death leading to NET release (42). In addition, apoptosis induction may block histone deimination (3) or promote it (5). Certainly, the conflicting results could be explained by various differences in the execution of these experiments, including details of buffers and media used during stimulation, yet one testable possibility was that the reagents for detecting deimination were inconsistent.

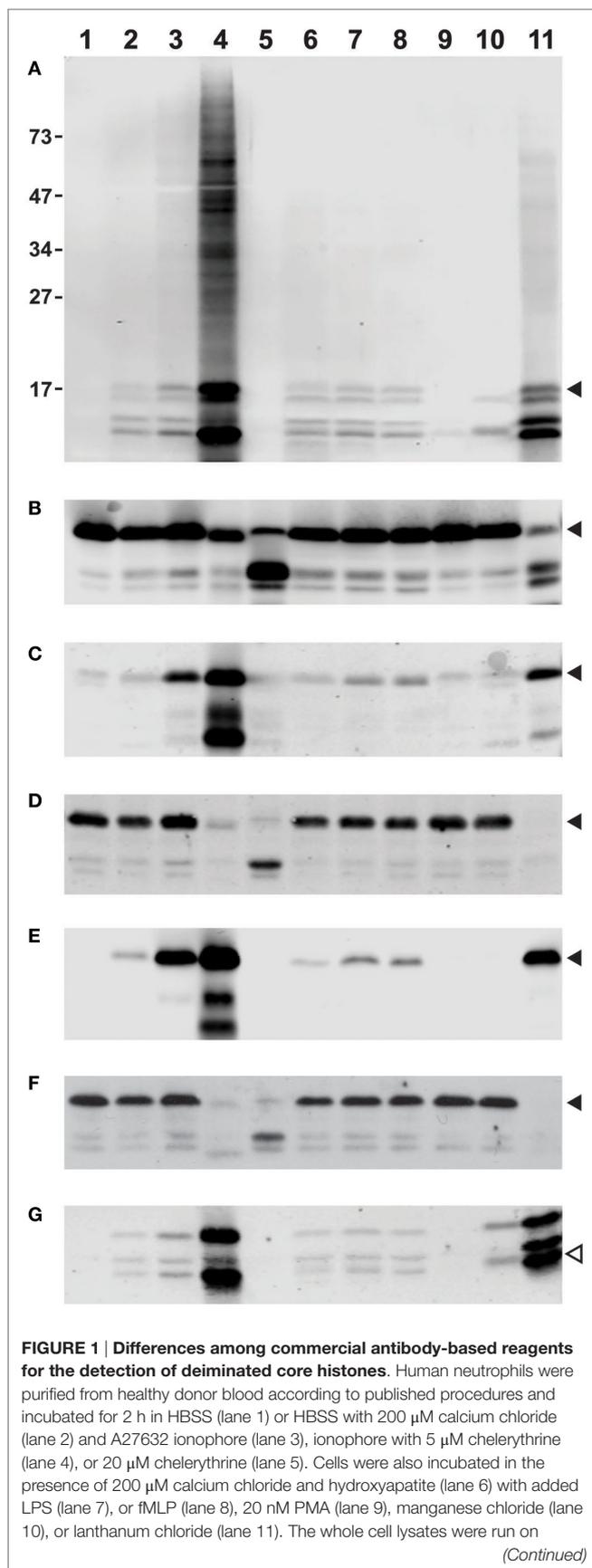
The most convenient way to measure histone deimination is with antibodies that recognize citrulline residues within their specific antigenic epitope. Various commercial antibodies based on polyclonal sera or monoclonal antibodies (Mab) are available for immunochemical detection of deiminated histones. Caution is advised, as polyclonal antisera may differ from animal to animal according to stochastic events that generate antibody specificity. Conversely, Mabs can be highly specific but may also be sensitive to subtle changes in the epitope due to contributions from flanking residues.

Thus, we set out to assess the reliability and consistency of different commercial antibodies against deiminated histones.

To provide samples for our analysis, we prepared whole cell lysates from freshly isolated human neutrophils that were treated with diverse stimuli to induce or suppress histone deimination. For a commonly accepted baseline, we analyzed the lysates with antibodies to diacetyl monoxime/antipyrine-modified citrullines (**Figure 1A**), using a detection kit from Millipore (43). To assess the quantity and integrity of the core histones, we used antibodies to total histone H3 (Cell Signaling Technologies, #4620S) to generate the blot shown in **Figure 1B**. All incubations, except for unstimulated neutrophils (lanes 1), contained 200 μ M calcium in addition to the diverse stimuli. In all cases, the yields of intact H3 were comparable, except in samples treated with 20 μ M chelerythrine along with ionophore (lanes 5) or lanthanum 200 μ M (lanes 11), which showed partial cleavage of H3 (**Figure 1B**). Most treatments induced moderate to high levels of histone deimination, except, as previously reported, 20 μ M chelerythrine and 20 nM PMA (lanes 5 and 9, **Figure 1A**), which showed little to no deimination (7). The most intense deimination was observed in cells that were incubated with 5 μ M chelerythrine and ionophore (lanes 4), and cells incubated with lanthanum (**Figure 1A**).

A widely used polyclonal antibody that recognizes histone H3 with citrulline residues at positions 2, 8, and 17 (Abcam #ab5103, Lot GR247556) could detect histone deimination (**Figure 1C**) in roughly similar measure as the modified citrulline antibody (**Figure 1A**), although the Abcam antibody also reacted weakly with unstimulated and 20 nM PMA-treated neutrophil lysates. We have compared different lots of this antibody and observed lot-to-lot variability (data not shown). This lot detected a small increase in deimination in the presence of extracellular calcium (lane 2), presumably reflecting the enzyme's requirement for calcium. However, a significant increase was noted, once ionophore opened access for calcium across the plasma membrane (lane 3). There was an even greater enhancement of deimination with 5 μ M chelerythrine (lane 4), a selective inhibitor of certain protein kinase C isozymes. Raising the concentration of chelerythrine to 20 μ M impaired histone deimination (lane 5). Mechanical damage to the plasma membrane, as may be induced by frustrated phagocytosis of hydroxyapatite crystals, also enhanced deimination (lane 6, **Figure 1C**), and this effect could be further stimulated by addition of LPS or fMLP (lanes 7 and 8). In contrast, incubation in calcium with 20 nM PMA did not stimulate deimination (lane 9), despite the fact that NETosis is greatly induced by this compound (data not shown). Manganese, and, more intensely, the combination of calcium and lanthanum (lanes 10 and 11), induced deimination.

To compare the rabbit antisera to MAbs, which represent more stable immunological reagents, we examined three commercially available MAbs that were promoted as detection reagents for citrulline residues. A mouse MAb to citrullinated histone H3 (Abcam, #ab80256, clone 7C10), which was listed as reacting against H3 with citrullines at positions 2, 8, and 17, showed a drastically different pattern of reactivity than the antisera used above (**Figure 1D**). In this instance, the antibody appeared insensitive to large increases in deimination, as the antibody did not distinguish between unstimulated lysates and lysates prepared following the incubation with ionophore. In fact, the signal was low to absent in the samples treated with 5 μ M chelerythrine or

**FIGURE 1 | Continued**

a denaturing 12% PAGE and blotted to nitrocellulose membrane prior to reaction with diacetyl monoxime/antipyrine and detection of modified citrullines with the antibody and following instructions from Millipore (A). Alternatively, blots were blocked for 1 h at RT with 5% bovine serum albumin (BSA) or 5% milk in TBST [Tris-buffered saline (TBS) and Tween 20, 25 mM Tris (pH 7.2), 150 mM NaCl, and 0.1% Tween 20] and rinsed before overnight incubation at 4°C with a dilution of primary Abs (as recommended by the supplier) in 2.5% BSA in TBST. Subsequently, membranes were washed and incubated for 1 h with donkey anti-rabbit secondary Ab IR800 (catalog #925-32213) or goat anti-mouse (catalog #926-32210) as secondary antibodies available from LI-COR, washed three times with TBST and twice with TBS alone and developed on an Odyssey imaging system. Blots were reacted with antibodies to total histone H3, obtained from Cell Signaling Technologies (B), rabbit antisera to citrullines at positions 2, 8, and 17 of histone H3, Abcam, catalog #ab5103 (C), mouse Mab to citrullines at the same positions, also from Abcam, clone 7C10 (D), a rabbit Mab to citrulline at position 2 of H3, Abcam, catalog #176843 (E), and a mouse IgM Mab to poly-citrulline, F95 from Millipore (F). For comparison, we used a rabbit antiserum to citrullines in the amino terminus of H4, supplied by Millipore under #07-596 (G). Filled arrowheads indicate position of H3 on the membrane, whereas the open symbol points to the position of H4. The membrane in (A) displays additional reactivity to proteins of slower mobility on the gel (lane 4). The distance to which marker proteins had migrated and their masses in kilodaltons are indicated on the margin of (A).

lanthanum, which gave the most intense signals with the two antisera used above (Figures 1A,C).

In contrast, a rabbit Mab from the same supplier (Abcam, #176843) that binds to citrulline at the second position of histone H3, could detect a strong increase of H3 deimination with ionophore treatment and a further enhancement with 5 μ M chelerythrine (Figure 1E). This antibody also gave a strong signal with the lysate treated with lanthanum, although it showed only a marginal signal increase with lysates from cells incubated with hydroxyapatite in combination with LPS or fMLP, and no reaction with manganese or PMA-treated cell lysates.

Results with F95 (Figure 1F), a mouse IgM reported to recognize citrullines in a context-independent manner (Millipore, MABN328), further emphasized the drastic differences between MABs to citrulline epitopes. The same neutrophil lysates as used in the examples above showed no increase in histone deimination with addition of ionophore or hydroxyapatite treatment in calcium buffer. Thus, the signal that was detected with histones from unstimulated cells showed little to no modulation following induction of deimination. In addition, enhanced deimination that was induced with 5 μ M chelerythrine or with lanthanum was nearly completely undetectable (Figure 1F). These results were inconsistent with the previous observations. On a longer exposure, F95 IgM could detect citrullination of other proteins, as seen by the increased reactivity with proteins of increased molecular weights (data not shown). Thus, there is a specific problem with the detection of citrullines in histones by F95.

To extend our analysis to antibodies to other deiminated core histones, we tested a rabbit antiserum to the citrullinated amino terminus of histone H4 (Millipore, #07-596). This polyclonal antibody detected the enhanced level of deimination in the presence of 5 μ M chelerythrine and lanthanum (Figure 1G), but it was somewhat less sensitive to calcium/ionophore or hydroxyapatite than the mouse antiserum to H3 (Figure 1C). This antibody

showed cross-reactivity with proteins of greater molecular weight than H4, as acknowledged by the supplier, which could be due to a shared amino acid motif between histone H4 and H2A. Nevertheless, the results with this antibody were comparable to those obtained with the tri-citrullinated H3 antibody (Figure 1C) and the rabbit MAb (Figure 1E).

In conclusion, we uncover a surprising level of inconsistency between commercially available antibodies to citrulline-containing epitopes. Others have recently pointed out the need for commercial suppliers of antibodies to more carefully assay and validate different lots of antibodies (44–48). This is especially relevant for antibodies to histone post-translational modifications, as histones incur numerous modifications that are relevant for the functional properties of chromatin. An International Working Group for Antibody Validation was recently convened and published a set of recommendations for antibody validation because of the enormous losses of research funds due to the inconsistent data that arise based on currently available antibody reagents (49). One useful resource is an online repository of antibody-binding specificities that currently lists over 100 antibodies to histone post-translational modifications (47). Our results argue for a cautious approach to interpretations of any single antibody for the determination of deiminase activity in neutrophils or other cell types of interest. Although the modified citrulline antibody is more complicated to use than the other antibodies, a prudent approach may include the use of

this antibody for comparison to the other reagents. Other ways of detecting citrulline have been reported, but they may require more sophisticated equipment or more complicated analyses. Generally, the current challenge in the field of NETosis research with regard to histone deimination is acute and requires reliable, accepted, and broadly available reagents. Increased efforts to isolate monoclonal mouse or rabbit Ab, including possibly by recombinant methods, should be promoted. Notably, advances in phage display technology have led to the discovery of antibodies that are specific for various post-translational protein modifications, including acetylation, phosphorylation, methylation, and citrullination (50) and that allow their efficient conversion into IgG molecules (51).

AUTHOR CONTRIBUTIONS

IN conducted the laboratory research and assisted in the drafting and revision of the manuscript. MR conceived the experimental approach, interpreted the data, and wrote the manuscript.

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Peptidylarginine Deiminase Inhibitor Suppresses Neutrophil Extracellular Trap Formation and MPO-ANCA Production

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Myeloperoxidase-antineutrophil cytoplasmic antibody (MPO-ANCA)-associated vasculitis is a systemic small-vessel vasculitis, wherein, MPO-ANCA plays a critical role in the pathogenesis. Neutrophil extracellular traps (NETs) released from activated neutrophils are composed of extracellular web-like DNA and antimicrobial proteins, including MPO. Diverse stimuli, such as phorbol myristate acetate (PMA) and ligands of toll-like receptors (TLR), induce NETs. Although TLR-mediated NET formation can occur with preservation of living neutrophilic functions (called vital NETosis), PMA-stimulated neutrophils undergo cell death with NET formation (called suicidal NETosis). In the process of suicidal NETosis, histones are citrullinated by peptidylarginine deiminase 4 (PAD4). Since this step is necessary for decondensation of DNA, PAD4 plays a pivotal role in suicidal NETosis. Although NETs are essential for elimination of microorganisms, excessive formation of NETs has been suggested to be implicated in MPO-ANCA production. This study aimed to determine if pan-PAD inhibitors could suppress MPO-ANCA production *in vivo*. At first, NETs were induced in peripheral blood neutrophils derived from healthy donors ($1 \times 10^6/\text{ml}$) by stimulation with 20 nM PMA with or without 20 μM propylthiouracil (PTU), an anti-thyroid drug. We then determined that the *in vitro* NET formation was inhibited completely by 200 μM Cl-amidine, a pan-PAD inhibitor. Next, we established mouse models with MPO-ANCA production. BALB/c mice were given intraperitoneal (i.p.) injection of PMA (50 ng at days 0 and 7) and oral PTU (2.5 mg/day) for 2 weeks. These mice were divided into two groups; the first group was given daily i.p. injection of PBS (200 $\mu\text{l}/\text{day}$) ($n = 13$) and the other group with daily i.p. injection of Cl-amidine (0.3 mg/200 μl PBS/day) ($n = 7$). Two weeks later, citrullination as an indicator of NET formation in the peritoneum and serum MPO-ANCA titer was compared between the two groups. Results demonstrated that citrullination in the peritoneum was significantly reduced in the Cl-amidine-treated mice compared with the vehicle-injected control mice (38% reduction). Additionally, the serum MPO-ANCA titer of the Cl-amidine-treated mice (32.3 ± 31.0 ng/ml) was significantly lower than that in the vehicle-injected mice (132.1 ± 41.6 ng/ml). The collective findings indicate that excessive formation of NETs may be implicated in MPO-ANCA production *in vivo*.

Keywords: MPO-ANCA-associated vasculitis, MPO-ANCA, neutrophil extracellular trap, peptidylarginine deiminase 4, peptidylarginine deiminase inhibitor

INTRODUCTION

Antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis is a systemic small-vessel vasculitis (1). The major target antigens of ANCA are myeloperoxidase (MPO) and proteinase 3 (PR3). Neutrophils primed by pro-inflammatory cytokines, such as TNF- α , express MPO and PR3 on the cell surface. ANCA bind to the antigens and then activate neutrophils directly and/or through binding to bystander Fc γ receptors. Consequently, the activated neutrophils induce vascular endothelial cell injury resulting in the development of small-vessel vasculitis (2, 3). ANCA, therefore, play a critical role in the pathogenesis of ANCA-associated vasculitis. Although the mechanism of MPO-ANCA production was unknown for a long time, recent studies have suggested the involvement of neutrophil extracellular traps (NETs) in the mechanism (4–6).

Neutrophil extracellular traps are firstly reported in 2004 as extracellular web-like DNA studded with antimicrobial proteins, including MPO, which are released from phorbol myristate acetate (PMA)-stimulated neutrophils (7). The PMA-stimulated neutrophils undergo cell death with the formation of NETs (8), though not all stimuli induce cell death in NET-forming neutrophil (9). It has been demonstrated that NET formation can occur with preservation of living neutrophilic functions, including phagocytosis and chemotaxis (10, 11). Currently, NET formation undergoing cell death is called suicidal NETosis, whereas that preserves living neutrophilic functions is called vital NETosis. NETs can trap microorganisms by the extracellular DNA and kill them using the antimicrobial proteins. Thus, NETosis is considered as an important event in innate immunity. However, excessive NETosis can result in vascular endothelial cell injury (12), thrombosis (13, 14), and impairment of diabetic wound healing (15, 16). In addition, disordered regulation of NETosis has been suggested to be involved in the pathogenesis of autoimmune diseases, including systemic lupus erythematosus (SLE) (17) and anti-thyroid drug propylthiouracil (PTU)-induced MPO-ANCA-associated vasculitis (5). In the last two studies, the possibility of extracellular components in persistent NETs recognized as autoantigens by the immune system was discussed.

Suicidal NETosis is induced in response to diverse stimuli, including PMA (7, 18). These stimuli activate the Raf–mitogen-activated protein kinase kinase–extracellular signal-regulated kinase pathway, NADPH oxidase-dependent production of reactive oxygen species, and receptor-interacting protein kinase/mixed lineage kinase domain-like-mediated signals (19, 20). In this pathway, peptidylarginine deiminase 4 (PAD4) yields citrullination of histones, around which DNA coils.

The PAD enzymes convert arginine residues to citrulline in a variety of protein substrates (21). Among the PAD family, which includes PAD 1–4 and 6, PAD4 is expressed mainly in hematopoietic cells, such as neutrophils (22). In the process of suicidal NETosis, PAD4-dependent citrullination of histones that yields decondensation of DNA is an essential step to mix DNA and intracytoplasmic proteins. This mixture is subsequently extruded from the ruptured plasma membrane. Accordingly, PAD4 plays a pivotal role in the process of suicidal NETosis (23).

In the present study, we aimed to determine if a pan-PAD inhibitor, Cl-amidine, could suppress MPO-ANCA production *in vivo*. For this purpose, we attempted to generate novel mouse models of MPO-ANCA-associated vasculitis according to our previous protocol utilized for establishment of a rat model of this disease (5). The mouse models would be more useful models that require lower doses of reagents than the rat model.

MATERIALS AND METHODS

Human Neutrophil Isolation

Human peripheral blood neutrophils were obtained from healthy volunteers by density centrifugation using Polymorphprep (Axis-Shield, Dundee, Scotland) according to the manufacturer's instructions.

NET Induction *In Vitro*

The *in vitro* NET induction was conducted similarly to our earlier study (5). In brief, human peripheral blood neutrophils were re-suspended in RPMI 1640 medium supplemented with 5% fetal bovine serum and then seeded in wells of 4-well chamber slides (Thermo Fisher Scientific, Yokohama, Japan) (1×10^6 /ml). After incubation for 30 min at 37°C, the cells were exposed to 0 or 20 nM PMA (Sigma-Aldrich, St. Louis, MO, USA) with or without 20 μ M PTU (Chugai Pharmaceutical, Tokyo, Japan) and incubated for another 2 h at 37°C.

PAD Inhibitor Administration *In Vitro*

Fifteen minutes prior to the PMA/PTU administration, 200 μ M Cl-amidine (Calbiochem, San Diego, CA, USA), a pan-PAD inhibitor, was added alternately into the wells. The concentration of Cl-amidine was adopted according to the previous report (24).

Quantification of NETs *In Vitro*

After 2 h of incubation, the medium containing the reagents was removed, and the remaining cells were washed with PBS followed by fixation with 4% paraformaldehyde for 15 min. Thereafter, the specimens were made to react with 5 μ g/ml of rabbit anti-human citrullinated histone 3 polyclonal antibody (Abcam, Cambridge, UK) for 60 min at room temperature. After removal of unbound antibody, the specimens were next allowed to react with 1:500 dilution of Alexa Fluor 594-conjugated goat anti-rabbit IgG antibody (Invitrogen, Tokyo, Japan) for 60 min at room temperature. After washing with PBS, the specimens were finally mounted with the 4', 6-diamidino-2-phenylindole (DAPI)-containing solution (Sigma-Aldrich). NET formation was observed under a fluorescent microscope and was quantified by counting the citrullinated histone 3-positive cells per $\times 100$ power field of view. Data from five random fields of view ($\times 100$) were subjected to the quantitative analysis.

Establishment of Mouse Models with MPO-ANCA Production

BALB/c, New Zealand White (NZW), C57BL/6N (B6/N), C57BL/6J (B6/J), and DBA mice (14-week-old female) were purchased from Clea Japan (Tokyo, Japan). The mice were given

intraperitoneal (i.p.) injection of PMA (50 ng at days 0 and 7) and oral administration of PTU dissolved in 5% glucose water for 4 weeks ($n = 5/\text{strain}$). Since preliminary experiments revealed that each mouse ingested at least 2.5 ml of the drug-containing glucose water, the concentration of PTU was set as 1 mg/ml in order to administer exactly 2.5 mg PTU per day. These mice were maintained under specific pathogen-free condition in accordance with the guidelines for the care and use of laboratory animals in Hokkaido University (Permission No. 12-0077).

Laboratory Data

Mouse urine was collected during the last 24 h using metabolic cages. Blood samples were obtained at days 14 and 28. Hematuria was assessed by dipsticks (Siemens Healthcare, Tokyo, Japan). Biochemical examinations for blood nitrogen urea (BUN) and creatinine (Cr) were performed at Daiichi Kishimoto Clinical Laboratory (Sapporo, Japan). Serum titer of MPO-ANCA was determined by enzyme-linked immunosorbent assay (ELISA) at A-CLIP Institute (Chiba, Japan).

Histological Evaluation

The lungs, kidneys, and peritoneal tissues were obtained at day 28 and then fixed in 10% formalin. The pulmonary and renal sections were subjected to hematoxylin and eosin staining. The sections of the peritoneal tissues were subjected to immunohistochemistry for citrullinated histone 3 as described previously (25, 26).

PAD Inhibitor Administration *In Vivo*

BALB/c mice (14-week-old female) were given i.p. injection of PMA (50 ng at days 0 and 7) and oral PTU (2.5 mg/day, aforementioned protocol) for 2 weeks. These mice were divided into two groups. The first group was given daily i.p. injection of PBS (200 $\mu\text{l}/\text{day}$) ($n = 13$). The second group was given daily i.p. injection of Cl-amidine (0.3 mg/200 μl PBS/day) ($n = 7$). The dose of Cl-amidine was adopted according to the previous report (27). Each mouse was administered at least 10 mg/kg of Cl-amidine per day. Mouse urine was collected during the last 24 h using metabolic cages. Blood and tissue samples were obtained at day 14.

Statistical Analysis

The *in vitro* data were presented as mean \pm standard deviation (SD) values obtained from experiments repeated for at least five times. The *in vivo* data were also presented as mean \pm SD values. Mann-Whitney *U*-tests were applied for statistical evaluation with StatPlus software. *P*-values of <0.05 were regarded as statistically significant.

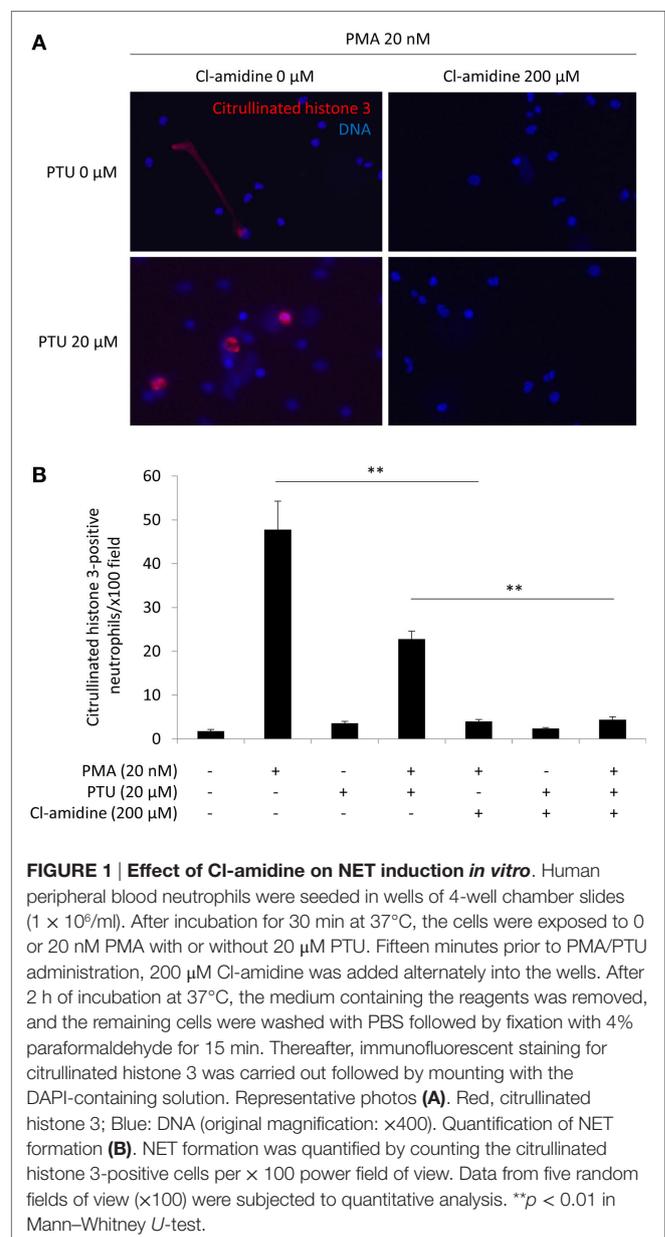
RESULTS

Effect of PAD Inhibitor on NET Induction *In Vitro*

Human peripheral blood neutrophils were exposed to 0 or 20 nM PMA with or without 20 μM PTU. Fifteen minutes prior to PMA/PTU exposure, the neutrophils were treated with or without 200 μM Cl-amidine. Stimulation without PMA did not induce

NETs (data not shown). It has been also determined that 200 μM of Cl-amidine did not affect the viability of neutrophils *in vitro* (Figure S1 in Supplementary Material).

The neutrophils stimulated by 20 nM PMA alone formed extended NETs, whereas neutrophils stimulated by 20 nM PMA plus 20 μM PTU formed non-extended round-shaped NETs (Figure 1A). These findings corresponded with our earlier observations (5) and suggested that PTU yielded abnormal conformation of NETs induced by PMA. Although the morphology of NETs was different, both stimuli (PMA alone and PMA plus PTU) were shown to induce citrullination of histone 3 (5). Therefore, PAD4 was likely involved in the NET induction by these stimuli. As expected, the pan-PAD inhibitor, Cl-amidine (200 μM) inhibited the NET formation induced by both PMA alone and PMA plus PTU *in vitro* (Figures 1A,B). Furthermore,



these findings suggest that Cl-amidine can inhibit not only the formation of PMA-induced conventional (extended) NETs but also PTU-mediated unconventional (non-extended round-shaped) NETs.

Establishment of Mouse Models with MPO-ANCA Production

In our earlier study, WKY rats were employed to establish an animal model of MPO-ANCA-associated vasculitis (5). In this study, we attempted to establish novel mouse models of MPO-ANCA-associated vasculitis according to the protocol for the rat model. For this purpose, BALB/c, NZW, B6/N, B6/J, and DBA mice ($n = 5$ /strain) were given i.p. injection of PMA (50 ng at days 0 and 7) and oral PTU (2.5 mg/day) for 4 weeks (Figure 2A). The serum titers of MPO-ANCA at day 28 were 100.4 ± 12.0 ng/ml in BALB/c, 96.1 ± 12.8 ng/ml in NZW, 41.3 ± 0.90 ng/ml in B6/N, 31.6 ± 5.79 ng/ml in B6/J, and 32.0 ± 4.06 ng/ml in

DBA mice (Figure 2B). Contrary to the rat model, no vasculitic lesion was observed in the lungs and kidneys of all mouse strains examined. Correspondingly, renal dysfunction was not detected in the urine and blood samples. Based on these findings, we employed BALB/c mice to construct the mouse model with MPO-ANCA production. The serum MPO-ANCA titer at day 14 was 79.0 ± 5.70 ng/ml in the BALB/c model. MPO-ANCA was not detected in the vehicle-injected BALB/c mice.

Effect of PAD Inhibitor on Citrullination *In Vivo*

BALB/c mice (14-week-old female) were given i.p. injection of PMA (50 ng at days 0 and 7) and oral PTU (2.5 mg/day) for 2 weeks. These mice were divided into two groups. The first group was given daily i.p. injection of PBS (200 μ l/day) ($n = 13$) and the other group with daily i.p. injection of Cl-amidine (0.3 mg/200 μ l PBS/day) ($n = 7$) (Figure 3A). At day 14, the peritoneal tissues were subjected to immunohistochemistry for citrullinated histone 3 for the evaluation of NET formation *in vivo*. As a result, the rate of citrullinated histone 3-positive cells in polymorphonuclear cells was significantly reduced in the Cl-amidine-treated mice in comparison with the vehicle-injected control mice (38% reduction) (Figure 3B).

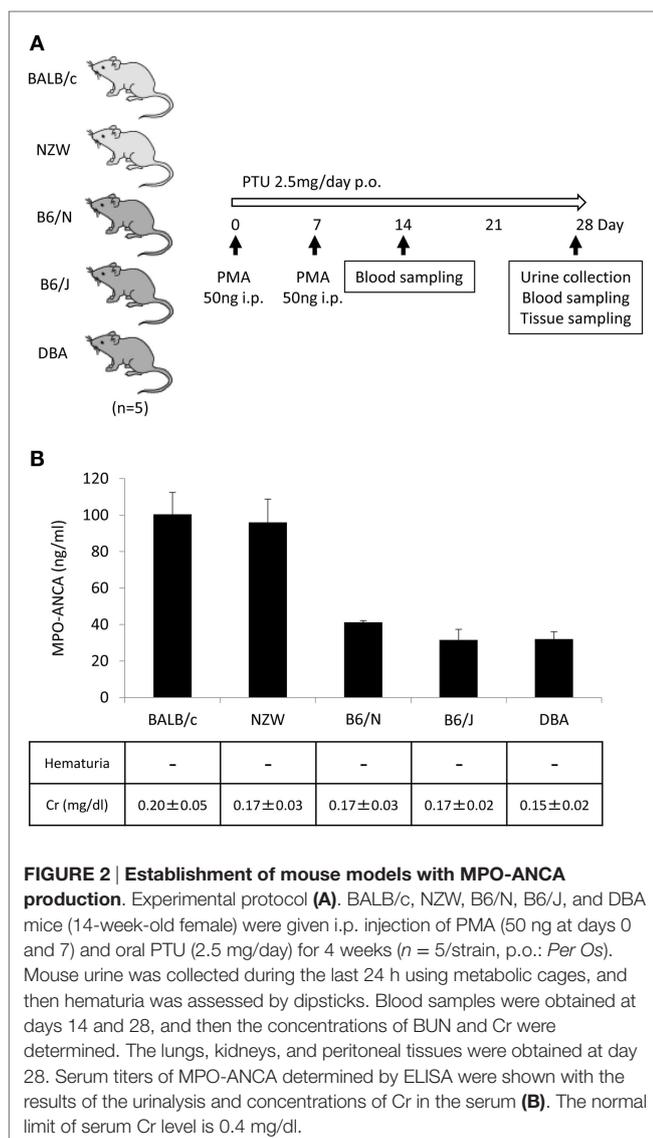
Effect of PAD Inhibitor on MPO-ANCA Production *In Vivo*

The serum titers of MPO-ANCA in the Cl-amidine-treated mice (32.3 ± 31.0 ng/ml) were significantly lower than the vehicle-injected control mice (132.1 ± 41.6 ng/ml) (Figure 3C). The collective findings clearly indicate that NET formation is inhibited by the pan-PAD inhibitor, Cl-amidine, both *in vitro* and *in vivo*, and that MPO-ANCA production is suppressed by Cl-amidine *in vivo*.

DISCUSSION

The PMA-stimulated neutrophils extrude decondensed DNA, which forms extracellular web-like structures decorated with bactericidal proteins (7). Since this substance, called NETs, can bind and kill bacteria, NET formation is regarded as an important event in innate immunity. Chronic granulomatous disease (CGD) patients who cannot generate NETs are susceptible to diverse bacteria and fungi indeed, and it was shown that restoration of NET formation in CGD resulted in resistance to such infections (28). Currently, it is considered that NET appears to be a form of innate response that binds microorganisms, prevents them from spreading, and ensures a high local concentration of antimicrobial agents derived from neutrophils (29).

The PMA-stimulated neutrophils undergo cell death with NET formation (8). Since the characteristics of cell death resembled neither typical necrosis nor apoptosis, Steinberg et al. coined NETosis for the neutrophil death with NET formation (30). However, Clark et al. have demonstrated that neutrophils do not necessarily undergo cell death after NET formation (31). It has been shown that NET formation can occur with preservation of neutrophilic functions, including phagocytosis and



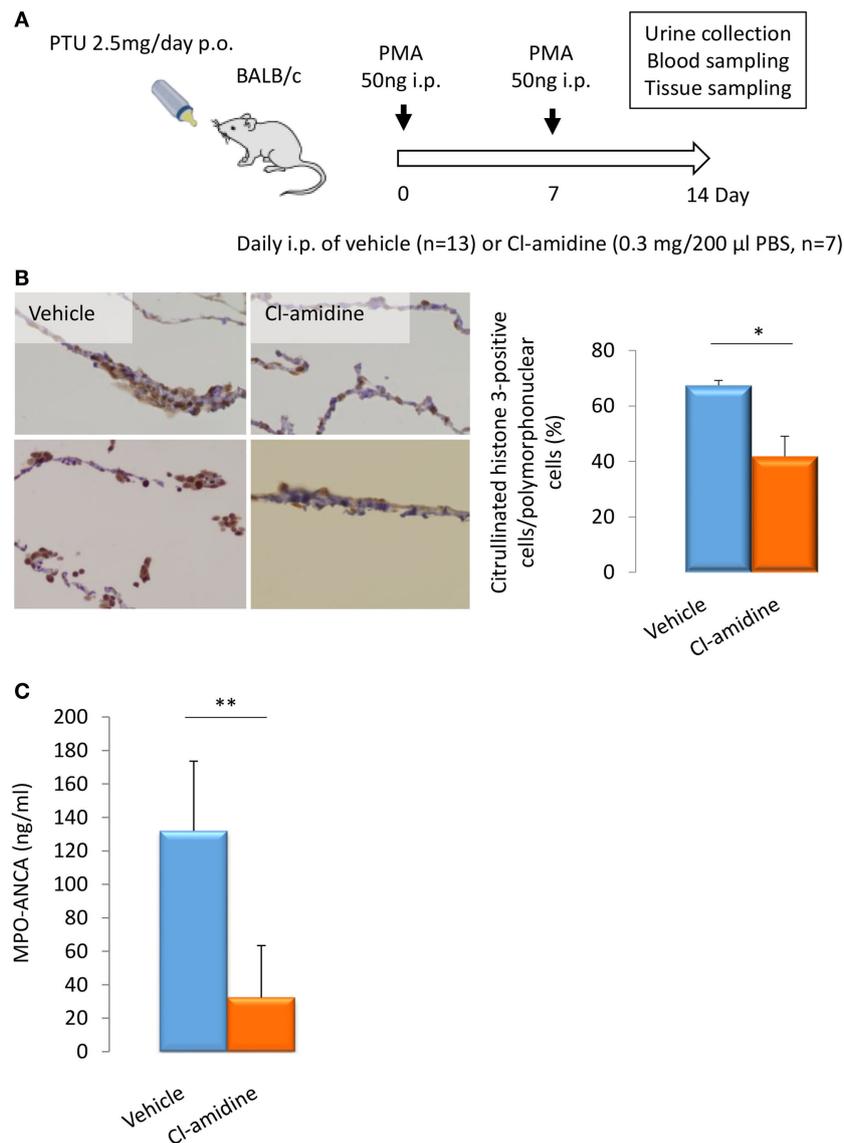


FIGURE 3 | Effect of Cl-amidine on citrullination and MPO-ANCA production *in vivo*. Experimental protocol (A). BALB/c mice (14-week-old female) were given i.p. injection of PMA (50 ng at days 0 and 7) and oral PTU (2.5 mg/day) for 2 weeks (p.o.: *Per Os*). These mice were divided into two groups. The first group of mice was given daily i.p. injection of PBS (200 µl/day) ($n = 13$). The other group was given daily i.p. injection of Cl-amidine (0.3 mg/200 µl PBS/day) ($n = 7$). Mouse urine was collected during the last 24 h using metabolic cages. Blood and tissue samples were obtained at day 14. NET formation in peritoneal tissues (B). The formalin-fixed paraffin-embedded sections of peritoneal tissues were subjected to immunohistochemistry for citrullinated histone 3. Representative photos among five random fields of view ($\times 100$) were shown. NET induction was quantified by calculating the rate of citrullinated histone 3-positive cells in polymorphonuclear cells in the five random fields of view. $*p < 0.05$ in Mann-Whitney *U*-test. Serum titers of MPO-ANCA determined by ELISA (C). $**p < 0.01$ in Mann-Whitney *U*-test.

chemotaxis (10, 11). This phenomenon termed vital NETosis (9). On the contrary, aforementioned dying NETosis has been called suicidal NETosis.

Although NETs play an essential role in the innate immunity, some harmful aspects of NETs have been demonstrated (32, 33). They not only have direct cytotoxic and thrombotic effects on vascular endothelium (12–14), but NETs are also associated with pathogenic autoantibody production (5, 17). For example, impairment of NET degradation potential (low activity of DNase I) in the serum is present in 30–40% of patients

with SLE and is suggested to lead the production of anti-DNA antibodies, which are related to disease severity (17). In another case, morphologically abnormal and DNase I-resistant NETs are generated by the anti-thyroid drug, PTU, and then MPO in the NETs seems to be recognized by the immune system resulting in the production of MPO-ANCA in rats administered with PTU (5). It has been shown that a part of PTU is metabolized by MPO. Simultaneously, conformational alteration of MPO could be induced by PTU (34), which could result in the tolerance break to MPO.

In the present study, we established mouse models with MPO-ANCA production. We treated five strains of mouse with PMA and PTU corresponding to the previously established protocol for the rat model of MPO-ANCA-associated vasculitis. Interestingly, there was a strain-dependency in the response to produce MPO-ANCA in mouse. Although the precise mechanism of the strain-dependency has to be determined in future studies, the Th2 phenotype of BALB/c (35) and autoimmune-prone genetic background of NZW (36) may be associated with the susceptibility to produce autoantibodies. In addition, no vasculitic lesion was observed in the mouse models with MPO-ANCA production, whereas WKY rats with MPO-ANCA developed pulmonary capillaritis and glomerulonephritis (5). Species-dependency may be present in the development of PMA plus PTU-induced MPO-ANCA-associated vasculitis. Nonetheless, we employed BALB/c mice to construct *in vivo* models of MPO-ANCA production.

Neeli et al. first demonstrated that PAD4-dependent histone deimination occurred in activated neutrophils under inflammatory conditions (37). Since PAD4-deficient neutrophils did not generate NETs in response to PMA, PAD4 plays a pivotal role in the NET formation (23). Correspondingly, inhibition of PAD4 using the pan-PAD inhibitor, Cl-amidine, prevented citrullination of histone 3 and significantly reduced NET release from HL60 cells, which were differentiated into mature neutrophils, in response to Ca²⁺ ionophore or *Shigella flexneri* exposure (24). In addition, Cl-amidine has been shown to suppress NET formation in lupus-prone mice (27). It has been shown that Cl-amidine can modify the cysteine of PAD and then inactivate it irreversibly (38). Based on these findings, we conducted *in vitro* and *in vivo* experiments to inhibit citrullination/NET formation using Cl-amidine as a pan-PAD inhibitor. The present study reproduced and extended the results of previous reports that investigated Cl-amidine both *in vitro* and *in vivo* and demonstrated that MPO-ANCA production was suppressed by Cl-amidine *in vivo*. These findings suggest that excessive formation of NETs may be implicated in MPO-ANCA production *in vivo*. In order to demonstrate the direct implication of PAD4-dependent NET formation in MPO-ANCA production, further studies using PAD4-deficient mice should be designed.

One limitation of this study is the lack of quantification of released NETs, which can be usually detected as MPO-DNA complexes in the serum. However, it has been shown that the PMA plus PTU-induced NETs hardly converted into soluble form (5). Thus, there is no better methodology to evaluate NETs in the

murine model than the immunohistochemistry for citrullinated histone 3.

No vasculitic phenotype was observed in the PMA plus PTU-induced mouse models with MPO-ANCA production. Therefore, we could not examine the effect of PAD inhibitors on MPO-ANCA-associated vasculitis in this model, which is another limitation of this study. It should be determined whether increased doses of PMA/PTU and/or longer duration could induce vasculitis in the mouse models in future studies.

It has been shown that MPO-ANCA is the major pathogenic factor in MPO-ANCA-associated vasculitis (2, 3). Thus, it is expected that PAD inhibitors, which can suppress the production of the pathogenic autoantibody, would be applied for the treatment of patients with MPO-ANCA-associated vasculitis. Preceding studies have demonstrated the protective effects of PAD inhibitors on the models of SLE (27) and other NET-related diseases, including multiple sclerosis (39), collagen-induced arthritis (40), and inflammatory bowel disease (41). Although further studies are needed to clarify their safety and effectiveness, PAD inhibitors are potential candidates as novel therapeutic agents for various NET-related diseases, including MPO-ANCA-associated vasculitis.

AUTHOR CONTRIBUTIONS

YK, HS, FH, and AM performed the experiments. YK, DN, SM, SN, UT, TA, and AI analyzed and discussed the data. YK, DN, UT, and AI designed the research. YK, UT, and AI wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at <http://journal.frontiersin.org/article/10.3389/fimmu.2016.00227>

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B Cell Tolerance to Deiminated Histones in BALB/c, C57BL/6, and Autoimmune-Prone Mouse Strains

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Deimination, a posttranslational modification of arginine to citrulline carried out by peptidylarginine deiminases, may compromise tolerance of self-antigens. Patients with connective tissue autoimmunity, particularly rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), or Felty's syndrome, present with autoantibodies to deiminated histones (dH), which thus form a category of antibodies to citrullinated protein antigens (ACPA). In general, ACPA are a sensitive diagnostic for RA and may form in response to the release of nuclear chromatin (DNA plus dH) from granulocytes, usually referred to as neutrophil extracellular traps. The aim of this study was to examine spontaneously autoimmune mice for autoantibodies and T cell responses to dH. We compared IgG binding to deiminated and non-deiminated histones (nH) by ELISA and Western blotting in spontaneously autoimmune strains of (NZB × NZW) F₁ and NZM2410 together with their derivative congenic strains, C57BL/6.*Slc1* and C57BL/6.*Slc1.Slc3*, which display profound autoreactivity against nuclear self-antigens. The splenocyte proliferation against the two antigens was determined in the spontaneously autoimmune (NZB × NZW) F₁ strain from which other autoimmune strains used in the study were derived. Immunizations with dH and nH were attempted in BALB/c mice to assess their splenocyte response. Splenocytes from BALB/c mice and from autoimmune mice at the time of conversion to autoimmunity proliferated strongly in response to dH, yet serum IgG from autoimmune (NZB × NZW) F₁, NZM2410, and C57BL/6.*Slc1.Slc3* mice displayed a remarkable bias against binding to dH. At the time of seroconversion, the antibodies already exhibited preference for nH, and only nH were recovered from circulating immune complexes. Analysis of histone deimination showed constitutive deimination in thymic extracts from C57BL/6 and C57BL/6.*Slc1.Slc2.Slc3* triply congenic mice and in spleens of autoimmune triply congenic mice. Our study demonstrates that tolerance mechanisms against dH are intact in BALB/c and C57BL/6 mice and continue to be effective in mice with overt autoimmunity to nH. We conclude that, in contrast to human RA and SLE patients, where we frequently observe autoantibodies against dH, autoimmune mice maintain strong tolerance mechanisms to prevent the development of autoantibodies to dH.

Keywords: autoimmunity, antibodies to citrullinated protein antigens, citrullines, B cells, lupus erythematosus, rheumatoid arthritis, autophagy, tolerance

INTRODUCTION

Antibodies to citrullinated protein antigens (ACPA) are diagnostic markers for rheumatoid arthritis (RA) (1) and also arise in other human autoimmune disorders such as systemic lupus erythematosus (SLE) and Felty's syndrome (2, 3). Citrullines are introduced into proteins by peptidylarginine deiminase (PAD) family of enzymes (4), and much effort has been devoted to learning the circumstances that activate PADs and lead to the PAD-mediated conversion of arginine residues into citrulline residues (5, 6). Several citrullinated antigens have been identified in RA, and a common mechanism has been proposed to account for the generation of citrullinated autoantigens (7–9). The proposed mechanism places particular importance on PAD2 and PAD4, enzymes that are expressed in cells of the innate and adaptive immune system (10, 11). These calcium-dependent enzymes are activated under inflammatory conditions (5). Direct stimuli of PADs include microbial pathogens and pro-inflammatory chemokines and cytokines (5). The enzymes are also activated by sterile inflammatory stimuli, such as cholesterol and urate crystals (8). In fact, it has been argued that any perforation to the plasma membrane could lead to the activation of PADs (12).

One particularly relevant event that is linked to PAD activation and may contribute to the induction of ACPA is a form of granulocyte cell death, which is induced by microbes and inflammatory stimuli and results in the release of nuclear chromatin (5, 13). Such neutrophil extracellular traps (NETs) are considered an innate antimicrobial response because the externalized chromatin is associated with neutrophil granule components such as myeloperoxidase and elastase, which, together with histones themselves, assist in bacterial killing and microbial entrapment (14). In the process of NET release, termed NETosis, PADs gain access to multiple intracellular and extracellular substrates such as histones, filaggrin, fibrinogen, and collagen, which are frequently targeted by ACPA (15). So, it is a prevalent hypothesis that NETosis provides conditions that lead to the production of deiminated (citrullinated) autoantigens that may stimulate cells of the adaptive immune system in the context of an inflammatory response. Moreover, the structural components of NETs, DNA, and histones, also become externalized during NETosis and, in an infection, may become entangled with bacteria and activate the immune system. Interestingly, dendritic cells respond to NETs with the production of interferons and other pro-inflammatory cytokines (16, 17). Other forms of cell death may also have consequences for the induction of autoantibodies, as autoantibodies bind to acetylated histones, a modification of apoptotic chromatin that may elicit autoantibody responses in mice and humans (18, 19).

To provide a mouse model for the study of ACPA, we sought to identify spontaneous mouse models of systemic autoimmunity that would produce autoantibodies to citrullinated histones. Although PAD4 expression parallels the severity of the inflammatory process in mouse models of RA (20), PAD4's contribution to the production of ACPA has been more difficult to ascertain (21, 22). Because ACPA are difficult to induce in most strains of mice (23), questions have been raised whether mouse ACPA participate in RA pathogenesis at all (22). Human ACPA often

react with deiminated histones (dH), and antibody binding to citrullinated histone peptides is a sensitive diagnostic test for RA (2, 24). Because histones are the major substrates of PADs in neutrophils, and dH are built into NETs, we expected that spontaneous anti-histone autoantibodies would preferentially bind to PAD-modified histones. However, we observed that mouse autoantibodies from (NZB × NZW) F₁ (NZB/W) mice and their recombinant derivative strains, including NZM2410 and C57BL/6J.*Sle1* (B6.*Sle1*) or C57BL/6J.*Sle1.Sle3* (B6.*Sle1.Sle3*) congenics, showed strong preference for non-deiminated histones (nH) over dH by ELISA and Western blot. Thus, even after tolerance to histones was broken and autoantibodies to nH were expressed, autoimmune-prone congenic strains retained B cell tolerance toward dH. B cell binding to dH was repressed, whereas autoantibody binding focused instead on PAD4 substrate arginines. The B cell bias against dH argues that dH remain effective tolerogens in autoimmune mice. In support of this possibility, we observed elevated levels of dH in thymus extracts from B6 and B6.*Sle1.Sle2.Sle3* (B6.TC mice) and spleens of autoimmune B6.TC mice. Our observations suggest that, even in overtly autoimmune lupus mice, central (thymic) tolerance inhibits B cells that react with a deiminated variant of an important nuclear autoantigen. These results point to unexpected intricacies in the murine immune response to deiminated autoantigens. We interpret these results as possible outcomes of PAD expression in antigen-presenting cells.

MATERIALS AND METHODS

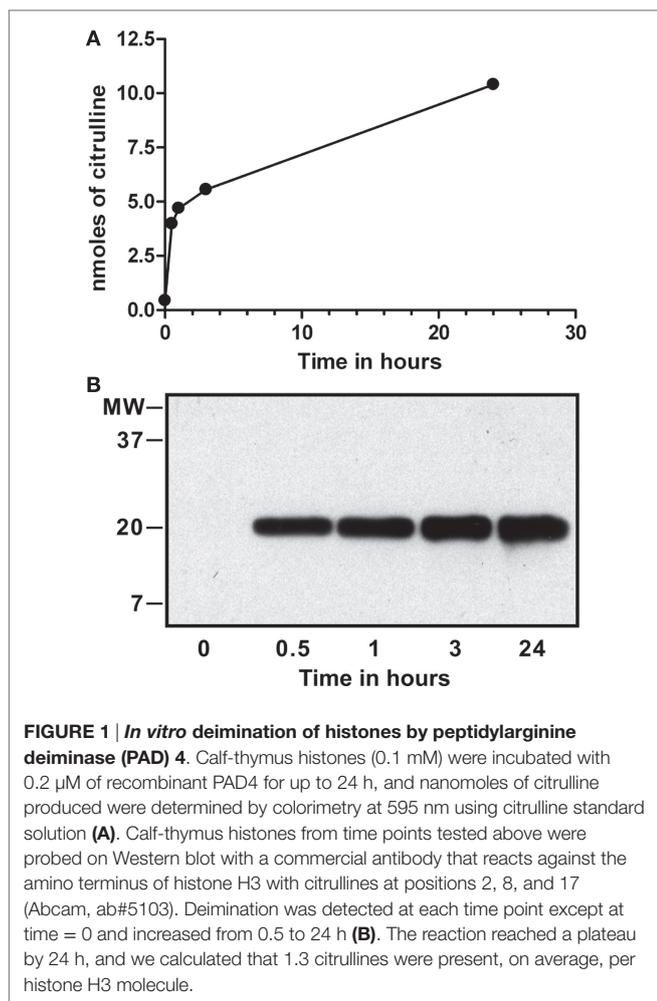
Mice

Sera were obtained from B6 mice, as well as from NZB/W, NZM2410, B6.*Sle1*, and B6.*Sle1.Sle3* mice at 6–8 months of age. Tissues were prepared from groups of matched B6 and B6.TC mice of 4–6 months of age. Splenocytes were isolated from 6 BALB/c mice of 4 months of age and 13 NZB/W F₁ female mice that were divided into 3 age groups: 6–10 weeks of age, 20–21 weeks of age, and 25–30 weeks of age. The treatment and care of animals were in accordance with the guidelines of the Office of Research, UTHSC, the University of Florida and the Norwegian Ethical and Welfare Board, and the study overall was approved by UTHSC Institutional Animal Care and Use Committee under the protocol #11-164.

ELISA

For binding assays, we treated purified calf-thymus histones with recombinant PAD4 *in vitro*, as described previously (2, 3). To assess the extent of deimination, we analyzed the progress of the reaction by colorimetry of citrullines and testing the resulting dH by Western blot with an antibody to citrullinated histone H3 (Abcam, ab#5103). The results of this analysis are shown in **Figure 1**.

Flat bottom, 96 well microtiter plates (Immulon 4HBX; Thermo Electron Corp.) were coated overnight with 5 μg/ml of nH, poly L-lusine, bovine serum albumin (BSA) (Sigma), ovalbumin (OVA) (Sigma), protamine sulfate (Sigma), or dH, as previously described (25). Plates were washed three times with



0.05% Tween-20 in PBS and blocked with 2.5% BSA in 0.02% NaN_3 and PBS for 2 h. A 1:100 initial dilution of primary sera along with threefold serial dilutions in 1.6% Tween-20 and 1% BSA in PBS were incubated for 1 h in the plates. Then, serum dilutions were removed, and wells were washed with 0.1% Tween-20 in PBS. Alkaline phosphate-conjugated goat anti-mouse kappa (Southern Biotech) was added at 1:1,000 dilution in 1% BSA with 0.05% Tween-20 in PBS for 1 h. Phosphatase substrate (Sigma) was used to develop the ELISA, and OD values were read at 405 nm on a Multiscan Plus plate reader (Labsystems).

Serum antibodies against dsDNA were detected by ELISA exactly as described (26, 27). In short, calf-thymus dsDNA (10 μ g/ml in PBS) was coated on microtiter plates (MaxiSorb; Nunc, Copenhagen, Denmark). Sera from mice were diluted twofold from 1:100 to 1:3,200 in PBS containing 0.02% Tween-20 and incubated in wells. ELISA readings were obtained with peroxidase-conjugated rabbit anti-mouse $\text{Fc}\gamma$ antibodies at 405 nm.

Ex Vivo Tissue Lysate Preparation

Seven-month-old B6.TC autoimmune female mice and age-matched control B6 IgH^a were dissected to recover a portion of spleen, bone marrow, kidney, and liver. Thymi from

4- to 6-month-old mice were similarly obtained. Tissue was cut, minced with scissors, and crushed between two sterile frosted glass slides. Dissociated tissues were washed in PBS (without Ca^{++}) and centrifuged at $5,000 \times g$ for 5 min to pellet cells. Cell pellets were mixed with lysis buffer (65mM Tris pH 7.2, 2%SDS, 10% glycerol), containing protease inhibitors. To test for dH in tissue lysates, equal amounts of total protein were analyzed by Western blotting, as described below.

Western Blot

For Western blot analysis, proteins were resolved on 15% SDS-PAGE and transferred to nitrocellulose. Membranes were blocked in 5% BSA in 0.1% Tween-20 in TBS (TBST) overnight at 4°C. Subsequently, the membranes were incubated with sera at 1:500 dilution in TBS containing 2.5% BSA, 1% NP-40, and 0.1% SDS. After 2 h of incubation, membranes were washed with 1% NP-40 in TBS. Anti-mouse IgG-HRP was used for detection at 1:20,000 dilution in TBST for 1 h, and blots were developed using chemiluminescence (PerkinElmer).

Peptide inhibition assays included a preceding step, in which 3 μ g of 20-mer peptides, both matching the amino terminus of H3 but either containing arginines or citrullines at positions 2, 8, and 17, were incubated with 1:300 dilutions of mouse sera for 1 h prior to use in binding to dH and nH on the membrane. Results of these Western blots were quantitated by infrared emission of secondary anti-mouse IgG antibodies (Odyssey). Separately, autoimmune sera were treated with DNase1 prior to Western blotting. Briefly, 400 μ l of a 1:200 dilution of sera were incubated with 20 units of DNase1 (New England Biolabs) for 1 h at room temperature to limit the possibility that DNA-anti-DNA complexes present in sera contribute to the observed histone binding. Following this incubation, the sera were diluted 1:500 in Western blot binding buffer.

To probe for deiminated histone H3 (dH3) in B6.TC and B6 mice, equal amounts of tissue lysates were resolved on 12% SDS-PAGE and transferred to nitrocellulose. Membranes were blocked with 5% BSA in TBST for 30 min and incubated with anti-dH3 anti-citrullinated histone H3 rabbit antibodies (Abcam, ab#5103) overnight at 4°C. Membranes were washed and incubated with HRP-conjugated goat-anti-rabbit secondary IgG antibody for 1 h at room temperature, washed three times in TBST, and twice in TBS alone. The HRP activity was detected as above.

Splenocyte Proliferation Assay

BALB/c mice were boosted twice with 100 μ g of total histones (dH or nH) or 100 μ g OVA, 14 and 2 days prior to the splenocyte proliferation assay. Ninety-six well tissue culture plates (Corning Incorporated) were filled with 100 μ l aliquots of 100 μ g/ml (or threefold serial dilutions) of dH, nH, or OVA in RPMI 1640 (Mediatech Inc.) supplemented with 10% FBS. Splenocytes were isolated and resuspended in RPMI with 10% FBS at 1×10^6 cells/ml. One hundred microliters of cell suspension was added to each well, and plates were incubated at 37°C in 5% CO_2 for 72 to 96 h. Tritiated thymidine (1 μ Ci/well) was added for the last 17 h of incubation. Plates were harvested onto glass fiber filters, and thymidine incorporation was assessed by scintillation counting. Splenocyte proliferation assays were also performed using

female NZB/W mice purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Splenocytes were collected from NZB/W mice of different ages. Following red blood cell lysis, the splenocytes were resuspended in DMEM-10 media with 10% FCS and 10,000 U/ml penicillin and 10 mg/ml streptomycin. One hundred thousand cells were incubated with dH or nH (20 µg/ml of protein) in triplicate wells. Tritiated thymidine incorporation (1 µCi/well) was measured after 20 h, 3 or 6 days by liquid scintillation, as described (28, 29).

RESULTS

Spontaneously Arising Anti-Histone Autoantibodies

To assess the production of anti-nH/dH autoantibodies in mice that spontaneously develop antinuclear autoantibodies, we tested sera from NZB/W and their recombinant inbred derivative strain NZM2410 for binding to nH and dH. In addition, we tested the contribution of lupus-predisposing genetic intervals *Sle1* and *Sle3* that were back-crossed from the NZM2410 onto the B6 background (B6.*Sle1* and B6.*Sle1.Sle3*). *Sle1* is a locus that breaks tolerance to chromatin, whereas *Sle3* affects functions of myeloid cells (30). The parental strains, NZB and NZW, have distinct MHC, H-2^d and H-2^z, respectively. The lupus-predisposing H-2^z was maintained in the NZM2410 congenics, whereas the *Sle1* and *Sle3* congenics have the H-2^b from B6. Antibody binding to dH and nH was assessed by ELISA (Figure 2) and Western blot (Figure 3). As controls, sera from age- and sex-matched B6 mice were used.

By ELISA, NZB/W (Figure 2A), B6.*Sle1* (Figure 2B), B6.*Sle1.Sle3* (Figure 2C), and NZM2410 (Figure 2D) sera showed preference for nH over dH. This preferential binding was statistically significant for NZB/W, NZM2410, and B6.*Sle1.Sle3*, as assessed by paired, one-tailed *T*-test. Binding differed for different mice and dilutions but, in general, binding to nH was stronger than the binding to dH. We also confirmed the additive effect of *Sle1* and *Sle3* loci, as the B6.*Sle1.Sle3* combination resulted in greater absorbance values relative to the B6.*Sle1* mice. In parallel assays, sera from B6 mice (Figure 2E) showed no reactivity to histones. Both dH and nH were present in equivalent concentrations on the plates, as shown by the nearly identical binding of the LG2.2 monoclonal antibody (Figure 2F) whose epitope, the first 13 amino acid residues of histone H2B, is identical between nH and dH (31).

To examine the possibility that antibodies to dH arise first but are replaced by antibodies to nH, we collected mouse sera over time to identify mice during the conversion to anti-histone autoimmunity. In Figure 2G, we show that binding preference to nH over dH was present at an early time when anti-histone reactivity first appeared. This result indicates the two reactivities arise jointly, rather than in succession, as might be predicted by epitope spreading.

To dissect the preferential binding to nH, we used Western blotting. The stringency of binding was increased by including both SDS (0.05%) and NP-40 (0.5%) in the binding buffer.

Indeed, under these conditions, the binding of serum antibodies from NZB/W, NZM2410 B6.*Sle1*, and B6.*Sle1.Sle3* mice to dH was weaker relative to the binding to nH, such that many of the sera bound exclusively to nH (Figure 3A). A variety of binding patterns were observed, including exclusive binding to one or two core histones. Binding to dH3 was observed most often, whereas binding to deiminated H4 or H2A was rare. In addition, binding to a band with the mobility of the deiminated linker histone H1 was observed in several blots. Overall, binding was more biased toward nH over dH, although some IgG dH was also observed in individual NZM2410 and B6.*Sle1.Sle3* mice. In no instance did binding to nH exceed binding to dH.

To examine the possibility that the binding to dH represented a truly separate population of antibodies, we conducted inhibition experiments using 20-mer peptides that matched the amino terminus of histone H3 and either contained arginines or citrullines at positions 2, 8, and 17 (Figure 3B). We observed that the arginine-containing peptide (orange bars) was a more effective inhibitor of binding to both nH and dH, relative to the citrulline-containing peptide (blue bars), suggesting that the antibody binding to either antigen reflects antibody specificity for nH and that the binding to dH likely represents cross-reactivity due to shared epitope structure.

Splenocyte Proliferation

We asked whether T cells from autoimmune mice also recognize dH by using NZB/W mice that spontaneously develop an autoimmune response against nuclear Ags, including DNA and histones (32). Splenocytes from NZB/W mice of different ages were tested for proliferation in the presence of dH or nH. At 6–10 weeks of age, prior to any measurable anti-DNA reactivity, the splenocytes did not proliferate in response to either form of histone (Figures 4A,B). At 20–21 weeks of age, anti-DNA autoantibodies could be detected in the sera of some NZB/W mice (indicated at the top of each panel), and splenocytes from these mice showed low levels of proliferation in response to nH and dH (Figures 4C,D). Thus, splenocyte responses to histones arose in parallel with, or slightly prior to, humoral responses to DNA.

Twenty-five-week-old NZB/W mice with established autoimmunity showed splenocyte proliferation in response to dH and nH (Figures 4E,F), suggesting the presence of histone-reactive T cells in the spleens of autoimmune mice. Although some mice showed a tendency to preferentially respond to dH, others preferred nH, as shown by data from two of the analyzed mice. Notably, preference could switch, depending on the length of stimulation (Figures 4E,F), suggesting the presence of a limited number of T cell clones with distinct specificities and growth characteristics. Because proliferation generally showed a bias for dH or nH rather than being equal, we infer that epitopes containing arginines or citrullines were both presented by the MHC and recognized by T cells in splenocytes.

To examine the ability of dH to drive a T cell response, we examined T cell proliferation *in vitro*. Splenocytes from BALB/c mice immunized with dH proliferated during incubation with dH (Figure 4G) to comparable extent as splenocytes from mice

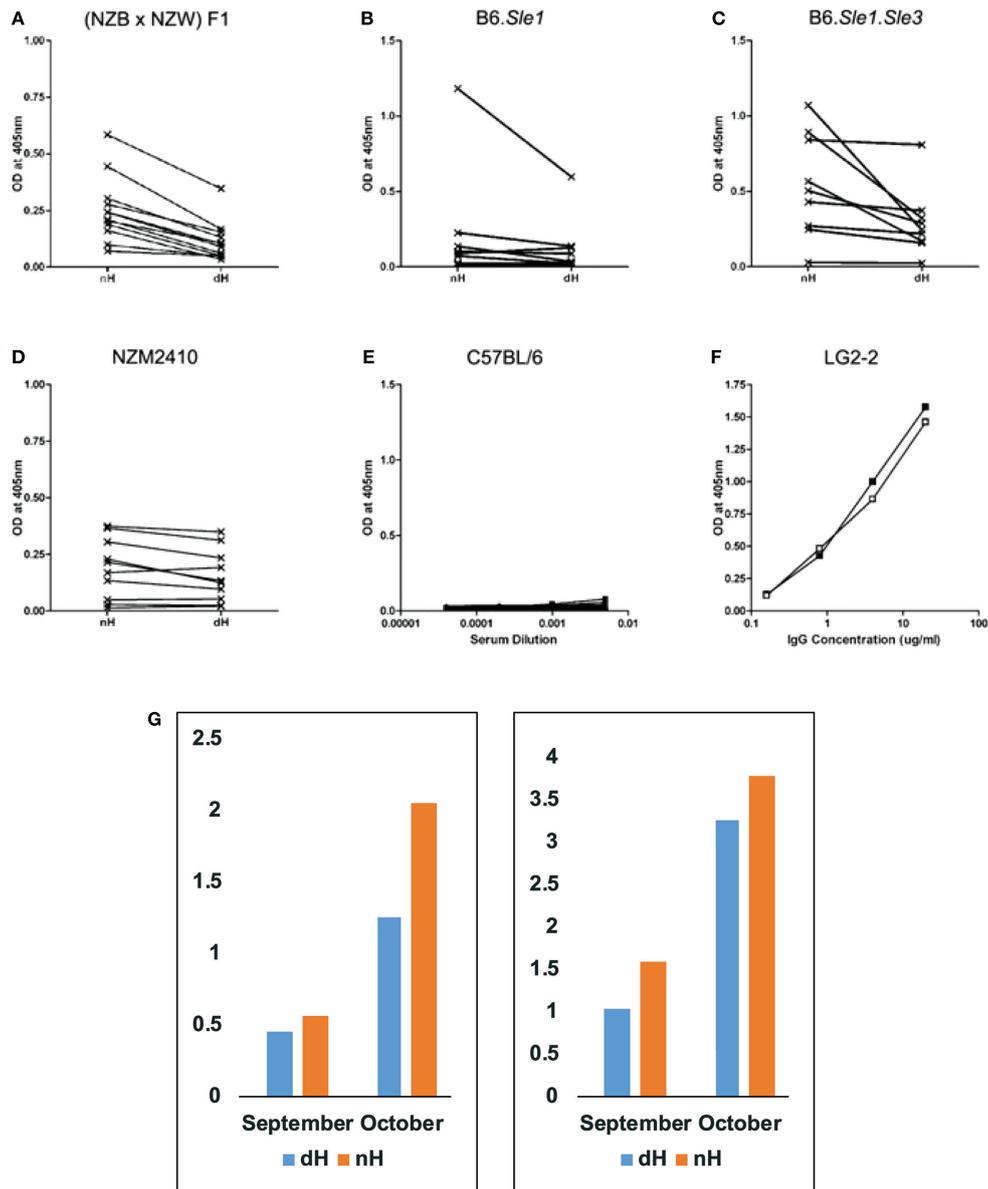


FIGURE 2 | Binding of IgG from autoimmune-prone and control mice to non-deiminated histones (nH) and deiminated histones (dH). Sera from NZB/W (A), B6.Sle1 (B), B6.Sle1.Sle3 (C), NZM2410 (D), and control B6 (E) mice were tested for IgG binding to nH and dH. Complete binding curves were obtained, and OD values for a single dilution were plotted in panels (A–D). We plotted data from 1:1,000 dilutions in panels (A–C) and from the 1:200 dilution in panel (D). Absorbance values for each serum corresponding to IgG binding to dH and nH are shown and are connected by a line indicating the pairs of data for the binding of each serum to the two antigens. Significance of the readings was tested by paired, one-tailed *T*-test. Binding to dH was significantly less than to nH for NZB/W ($p < 0.0001$), NZM2410 ($p < 0.016$), and B6.Sle1.Sle3 ($p < 0.016$). The binding of B6.Sle1 IgG tended to be lower to dH ($p < 0.10$). IgG from mice with systemic lupus erythematosus susceptibility genes showed preferential binding to nH. In comparison, control B6 mice showed negligible binding to either form of histones (E). As control for equal coating of Ags, we used LG2-2, a mouse anti-histone H2B mAb (F), whose epitope does not include any residues that are substrates for deimination (31). Thus, the binding curves for dH (filled symbols) and nH (open symbols) are nearly superimposable. Individual mice were followed over time (G), to observe the initial development of anti-histone autoreactivity. Two NZB/W mice that first showed anti-histone reactivity at 4 months of age (September) reacted more strongly to nH than to dH, and the preferential antibody binding was maintained at 5 months of age (October). The sera were diluted 1:300, and the measurements were performed three times with consistent results. The Y-axis displays values of optical density measured by ELISA.

immunized with OVA and incubated with OVA (Figure 4I). By contrast, splenocytes from BALB/c mice immunized with nH showed no enhanced proliferation regardless of whether they were incubated with dH, nH, or media alone (Figure 4H).

Spleens of Autoimmune Mice Have Increased Levels of dH

To test for the presence of dH *in vivo*, we prepared tissue lysates of bone marrow, spleen, liver, kidney, and thymus from B6.TC

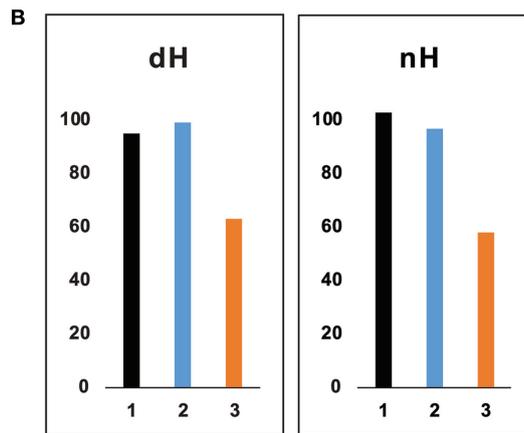
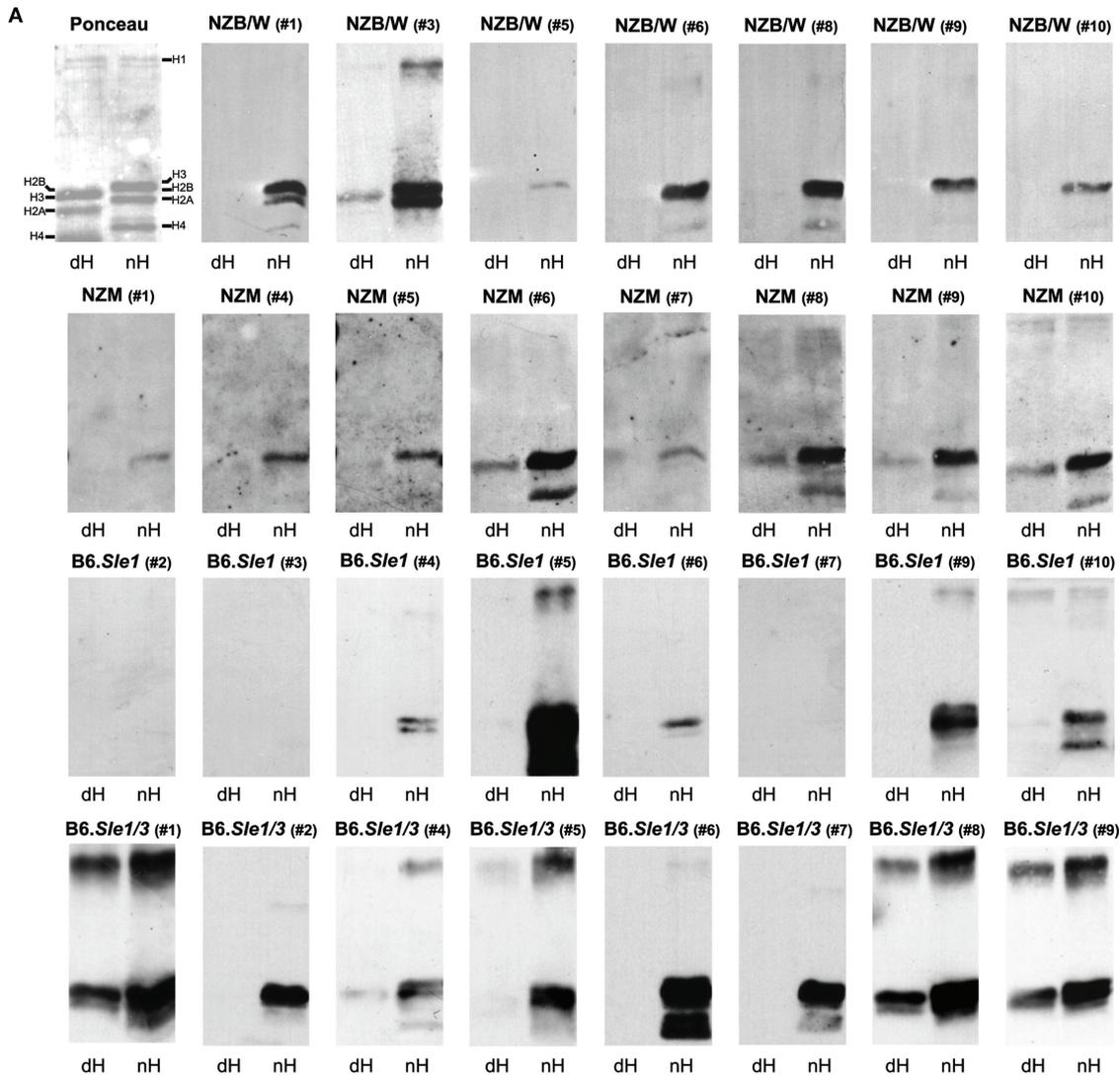
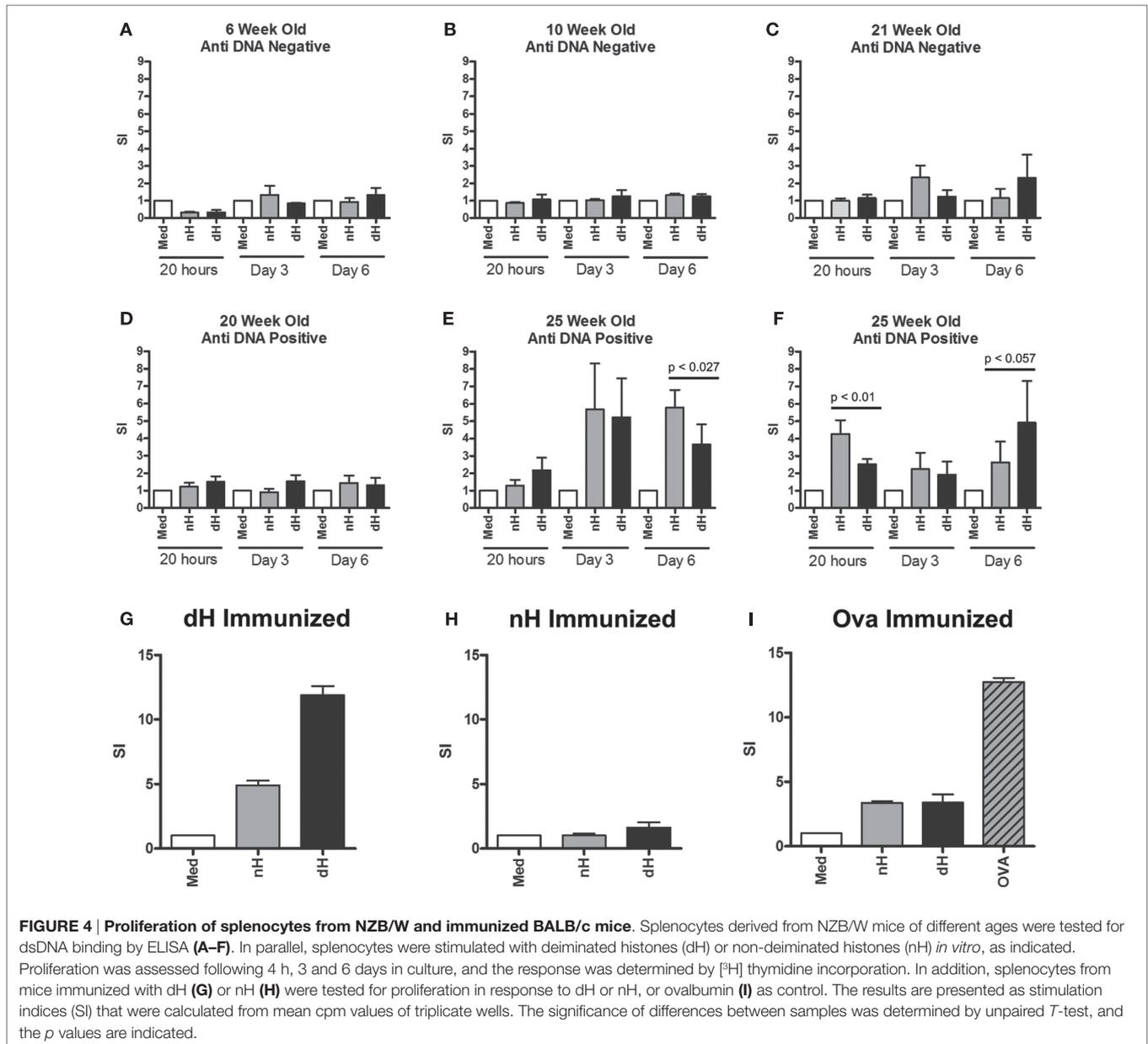


FIGURE 3 | Continued

FIGURE 3 | Continued

Western blot of IgG to deiminated histones (dH) and non-deiminated histones (nH). Equal amounts of dH and nH were resolved on SDS-PAGE, transferred to nitrocellulose, and stained with Ponceau red (upper left panel). The stained bands migrating with the mobilities of core and linker histones are indicated along the margins. Note that due to the replacement of the positively charged arginine by the neutral citrulline, the electrophoretic mobility of certain core histones is increased in the dH sample, indicating nearly complete modification by peptidylarginine deiminase 4. **(A)** Strips of membrane containing nH or dH were probed with NZB/W, NZM2410, B6.*Sle1*, and B6.*Sle1.Sle3* sera at 1:500 dilution and developed with anti-mouse IgG-horseradish peroxidase. Autoimmune-prone mouse sera bound nH in preference to dH. The experiments were performed at least three times with consistent results. **(B)** To explore whether the binding to histones on the membranes is equally sensitive to inhibition by 20-mer peptides matching the H3 amino terminus and containing arginine residues (nH peptide; orange bars) versus citrulline residues (dH peptide; blue bars) at positions 2, 8, and 17, we preincubated an NZB/W serum that showed binding to both nH and dH (black bars) with either peptide, as described in Section "Materials and Methods," and carried out the Western blots. The nH peptide was a more effective inhibitor of binding to both histones than the dH peptide. The Y-axis indicates binding intensities in units of infrared emission (IE).



mice and probed them with anti-dH by Western blot. The bone marrow lysates of autoimmune B6.TC mice and control mice at 7 months of age did not appreciably react with antibodies to

dH3 (Figure 5A). By contrast, spleens of B6.TC mice had clearly increased levels of dH3 as compared to B6 controls or lysates of the Jurkat lymphoma cells (Figure 5B). These results provide a

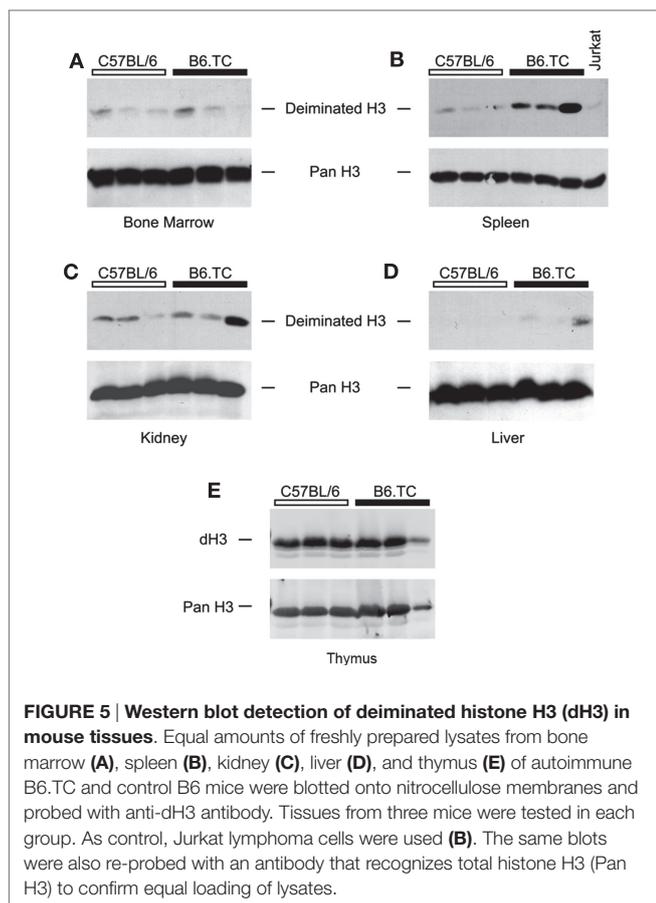


FIGURE 5 | Western blot detection of deiminated histone H3 (dH3) in mouse tissues. Equal amounts of freshly prepared lysates from bone marrow (A), spleen (B), kidney (C), liver (D), and thymus (E) of autoimmune B6.TC and control B6 mice were blotted onto nitrocellulose membranes and probed with anti-dH3 antibody. Tissues from three mice were tested in each group. As control, Jurkat lymphoma cells were used (B). The same blots were also re-probed with an antibody that recognizes total histone H3 (Pan H3) to confirm equal loading of lysates.

qualitative estimate rather than a precise measure of deimination. This is by necessity, as the cellular composition and disease process may affect histone deimination in a complex manner in a tissue such as the spleen. The overall amounts of histone H3 were similar in all samples, as indicated by the equivalent immunoreactivity of an anti-H3 antibody. Therefore, the spleens of autoimmune B6.TC mice contained increased quantities of dH3. In addition, the kidneys and the liver from individual autoimmune mice exhibited increased levels of dH3 (Figures 5C,D). Importantly, thymic extracts from 4- to 6-month-old B6 and B6.TC mice clearly showed constitutively elevated levels of dH (Figure 5E).

DISCUSSION

In this study, we observed that autoimmune mice exhibit a striking B cell bias toward binding nH over dH, a bias that is present at the earliest stages of anti-histone autoimmunity (Figure 2G). In 28 of 28 autoimmune NZB/W, NZM2410, B6.*Sle1*, and B6.*Sle1.Sle3* mice that made IgG anti-histone antibodies, preference was invariably in favor of nH (Figure 3A). Even more strikingly, in 18 animals that produced antibodies to nH, immunoblotting could not detect antibodies to dH. To exclude the possibility that anti-dH antibodies are only transiently expressed, or ensconced in immune complexes, we carried out longitudinal antibody-binding

assays that consistently revealed preferential or exclusive binding to nH (Figure 2G). These results indicate that, even in mouse strains that spontaneously convert to autoimmunity, deimination reduces IgG binding to histones, and autoantibody binding is focused on arginine-containing epitopes that are absent from dH. It follows that dH remain effective tolerogens even after development of autoimmunity in the tested mouse strains. We conclude this is an important characteristic of autoimmune mice, and we propose that a more detailed comparison with autoimmune disease patients may shed light on the induction of autoimmunity. Moreover, we argue that the specific regulation of PAD4 underlies these results.

Peptidylarginine deiminase 4 is most abundant in granulocytes and other cells of the innate immune system. However, PAD4 is also expressed in another immunologically relevant context. In elegant studies, Ireland and Unanue described the fact that antigen-presenting cells in mice express PAD4 and PAD2 in a compartment that is regulated by proteins in the autophagy pathway (33). The deiminase activity is constitutively expressed in dendritic cells and macrophages, whereas it is inducible in B cells by stress or stimulation through the Ig antigen receptor (33). The authors reported that arginine residues in antigenic peptides are converted to citrullines, and that T cells respond to target antigenic epitopes containing citrulline. This mechanism was demonstrated by using foreign antigens, in their case, hen egg lysozyme that was administered in a conventional immunization. The resulting T cell clones bound preferentially, or even exclusively citrullinated lysozyme peptides. Our results support this mechanism because splenocytes from BALB/c mice proliferated as vigorously against dH as against OVA (Figure 4G versus Figure 4I). By contrast, splenocytes from NZB/W mice, after these mice had converted to autoimmunity, showed comparable proliferation to either antigen (Figures 4E,F). This fundamental difference in outcome points to differences in immunized versus spontaneous autoimmune responses to nH.

As further shown by Ireland and Unanue, autophagy induction in B cells is necessary for their inducible expression of PAD4 activity associated with antigen processing (33). Conversely, a transient or sustained impairment of autophagy in B cells could provide conditions that would support the presentation of histone epitopes lacking citrullines. Under these conditions, B cells would express peptide epitopes that could act as neo-antigens for T cells and solicit T cell help. Autophagy has been linked by genetics to autoimmunity (27). A contribution of the autophagic processes to autoimmunity is consistent with the deficient or impaired functions of ATG5 (and other components of non-canonical autophagy) in SLE, but the mechanism for this relationship is unclear (27, 28). Our data suggest that effective autophagy may be required to maintain certain aspects of immune tolerance in mice.

However, an unanswered question is whether deiminated peptide presentation also occurs during thymic development, and whether tolerizing peptides expressed by thymic antigen-presenting cells are also deiminated. In support of this possibility, we found that thymus lysates from B6 and B6.TC mice show abundant histone deimination (Figure 5E), a result that suggests antigen presentation in the mouse thymus is tightly linked to

deimination. If so, citrulline-containing epitopes of autoantigens such as histones may induce powerful tolerance in mice. In that scenario, only B cells that bound to nH and presented non-deiminated epitopes would break tolerance and receive T cell help. Consequently, autoimmunity might initially be directed against non-deiminated peptides, provided that B cells, at an early stage of autoimmune pathogenesis, suspend or shut off the deimination of processed epitopes. Support for this alternative comes from the consistent anti-nH response that we observed in numerous autoimmune mice from different autoimmune strains (Figures 1 and 2). Thus, only B cells that no longer expressed PAD4 activity in their antigen processing compartment may escape tolerance. Our hypothesis of the key dependence of self-tolerance on the adequate function of autophagy for the presentation of dH peptides is in line with the remarkable preference of autoimmune mouse antibodies for nH. As corollary, a steady-state balance between nH and dH may be maintained under pre-autoimmune conditions, but an imbalance between the supply, processing, or recognition of nH, likely coincident with a disturbance of autophagic antigen processing in B cells, may result in an antigen-specific response to nH that may break immune tolerance and result in a sustained autoimmune response to nH.

To conclude, we briefly address the difference between mice and humans in their ability to express antibodies to dH. As we and others have shown, autoantibodies in various human autoimmune conditions preferentially bind dH (3, 24, 34), in striking contrast with the opposite bias in mice. Again, the key may be in the expression of PAD4 activity in B cells. Our tissue expression results indicate the increased presence of dH in the spleens of autoimmune mice (Figure 5B). By contrast, healthy human B cells appear incapable of expressing PAD4, as indicated by data

in the Human Protein Atlas (35). There, evidence suggests that human B cells, even after B cell antigen engagement in the white pulp, fail to express detectable PAD4. Thus, expression of dH epitopes on human B cells may not be intrinsic to the B cells, and presentation of dH epitopes may not engender tolerance that it is as effective as it is in mice. Consequently, B cell presentation of deiminated peptides in humans may be more likely to break tolerance and lead to ACPA generation.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and approved the final version to be published. MR had full access to all the data in the study and took responsibility for the integrity of the data and the accuracy of data analysis. Study conception and design: ND, MS, LM, OR, and MR. Acquisition of data: ND, AH, YZ, IN, and MR. Analysis and interpretation of data: ND, AH, YZ, IN, MS, LM, OR, and MR.

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Neutrophil Extracellular Traps Enhance Early Inflammatory Response in Sendai Virus-Induced Asthma Phenotype

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Paramyxoviral infection in childhood has been linked to a significant increased rate of asthma development. In mice, paramyxoviral infection with the mouse parainfluenza virus type I, Sendai virus (Sev), causes a limited bronchiolitis followed by persistent asthma traits. We have previously shown that the absence of cysteine protease dipeptidyl peptidase I (DPPI) dampened the acute lung inflammatory response and the subsequent asthma phenotype induced by Sev. Adoptive transfer of wild-type neutrophils into DPPI-deficient mice restored leukocyte influx, the acute cytokine response, and the subsequent mucous cell metaplasia that accompanied Sev-induced asthma phenotype. However, the exact mechanism by which DPPI-sufficient neutrophils promote asthma development following Sev infection is still unknown. We hypothesize that neutrophils recruited to the alveolar space following Sev infection elaborate neutrophil extracellular traps (NETs) that propagate the inflammatory cascade, culminating in the eventual asthma phenotype. Indeed, we found that Sev infection was associated with NET formation in the lung and release of cell-free DNA complexed to myeloperoxidase in the alveolar space and plasma that peaked on day 2 post infection. Absence of DPPI significantly attenuated Sev-induced NET formation *in vivo* and *in vitro*. Furthermore, concomitant administration of DNase 1, which dismantled NETs, or inhibition of peptidylarginine deiminase 4 (PAD4), an essential mediator of NET formation, suppressed the early inflammatory responses to Sev infection. Lastly, NETs primed bone marrow-derived cells to release cytokines that can amplify the inflammatory cascade.

Keywords: neutrophils, NETs, cytokines, dipeptidyl peptidase I, Sendai virus-induced asthma

INTRODUCTION

Respiratory viral illnesses are common in early life. A majority of children with an initial episode of viral-induced bronchiolitis will have recurrent wheezing, prompting the association between viral infection, and subsequent development of asthma (1–3). In the mouse, infection with the paramyxovirus Sendai virus (Sev) causes a limited bronchiolitis followed by chronic persistent asthma traits characterized by mucous cell metaplasia and airway hyperreactivity (AHR) (4). We have previously shown that the asthma phenotype was attenuated in the absence of dipeptidyl peptidase I (DPPI), a cysteine protease with many immunomodulatory activities (5). This attenuation was accompanied by significant reduction in the number of alveolar neutrophils

and local production of inflammatory cytokines in the acute phase of infection. Adoptive transfer of WT neutrophils into DPPI-deficient mice led to enhanced accumulation of DPPI-deficient neutrophils and inflammatory cytokine production in the alveolar space on day 4 post-infection (PI) and subsequent asthma phenotype development. The fact that DPPI-deficient neutrophils exhibited normal chemotaxis in response to various stimuli (5) but failed to accumulate at the site of inflammation (alveolar space) suggests a yet-to-be defined mechanism by which DPPI and DPPI-sufficient neutrophils modulate the early inflammatory responses to Sev.

Neutrophil extracellular traps (NETs) were initially described as a neutrophil defense mechanism to trap and kill bacteria (6). NETs have since been implicated in the pathogenesis of several inflammatory diseases, including bronchial asthma (7). Herein, we showed that Sev infection led to NET formation in the lung of WT mice. We also established that DPPI-deficient neutrophils exhibited a defect in NET formation *in vitro* in response to multiple stimuli. We hypothesized that the absence of DPPI attenuated NET formation in response to Sev infection, thus interrupting the inflammatory cascade and suppressing the ongoing leukocyte influx. Indeed, administration of DNase 1, which dismantled NETs, reduced free DNA–myeloperoxidase (MPO) complexes in the alveolar space and plasma, as well as attenuating the early inflammatory responses to Sev infection. Inhibition of peptidylarginine deiminase 4 (PAD4), an essential mediator of NET formation also suppressed alveolar leukocyte accumulation and cytokine production in the acute phase of infection, confirming the contribution of NETs to Sev-induced phenotype. Moreover, NETs from Sev-infected bronchoalveolar lavage fluid (BALF) stimulated bone marrow-derived cells (BMDCs) [dendritic cells (DCs) and macrophages] to release inflammatory cytokines.

MATERIALS AND METHODS

Animals

Dipeptidyl peptidase I^{-/-} mice were generated in 129/SvJ as previously described (8) and backcrossed to C57BL/6J for >10 generations. Microsatellite genotyping showed that DPPI^{-/-} mice were 99.2% congenic with C57BL/6J mice (The Jackson Laboratory). WT C57BL/6J mice were obtained from The Jackson Laboratory. WT and DPPI^{-/-} mice were kept in pathogen-free environment until the time of Sev infection. All animal experiments were performed in compliance with federal laws and in strict accordance with the guidelines established by the Division of Comparative Medicine at Washington University in St. Louis.

Viral Infection

Mice of 6–8 weeks of age were anesthetized with isoflurane and inoculated intranasally (i.n.) with 2,500 50% egg infectious dose of Sev (Fushimi strain) as previously described (5). Experimental infection with Sev was performed in biohazard containment facility. Some animals received DNase 1 (0.5 mg i.n./mouse) on days 0–2. Cl-amidine, a pan-PAD inhibitor (cat#506282, Calbiochem) was dissolved in DMSO/PBS (5% v/v) and administered i.p. at 10 mg/kg daily on days –2 and –1 prior to infection then twice a

day on days 0–2 PI. Controls received the same volume of DMSO/PBS (5% v/v). At different time points, mice were sacrificed and their blood, BALF, and lung harvested for cell count, cytokine, MPO–DNA, and histologic analysis.

Lung and BALF Analysis

After sacrifice, BALF was obtained as previously described (5). The cells were pelleted and analyzed by flow cytometry using: FITC anti-CD69 (cat# 561929; BD Pharmingen), PerCP anti-CD8a (cat# 45-0081-82; eBioscience), APC anti-CD4 (cat# 100516; BioLegend), PE anti-CD11c (cat#553802, BD Pharmingen), anti-Siglec-H (cat# MCA4647GA, AbD Serotec, Raleigh, NC, USA), APC anti-CD317 (cat# 127015, BioLegend), PerCP anti-CD11b (cat# 101229, BioLegend), FITC anti-rat IgG (cat# 712-095-150, Jackson ImmunoResearch Laboratories). In general, 10⁶ cells were blocked with the anti-FcR mAb 2.4G2, stained with the indicated antibodies for 20 min at 4°C and then washed and resuspended in FACS buffer for analysis. Flow cytometry was performed on a BD FACSCalibur™. Data analysis was performed using BD CellQuest™ Pro software. Cell-free BALF was subjected to cytokine analysis by cytometric bead arrays (CBA) or MPO–DNA complex analysis. The lung was snap frozen in OCT compound and examined for *in vivo* NET formation.

In Vivo NET Detection

Cross sections (9 μm) of OCT-embedded frozen lung tissues were fixed in 4% paraformaldehyde, blocked in 8% BSA in PBS and incubated with the primary antibodies: anti-Histone H2B (1:100 dilution; Cat # SC-8651; Santa Cruz Biotechnology), anti-mouse MPO (1:100 dilution; Cat # HM1051BT; Hycult Biotech) followed by the appropriate rhodamine red- or FITC-conjugated secondary antibody (1:100–1:200; Jackson ImmunoResearch Laboratories). DNA was stained with DAPI. All images were acquired with QCapture software on a Nikon Eclipse microscope.

In Vitro NET Induction

Neutrophils were isolated from bone marrow as previously described (9). Isolated neutrophils were seeded on Thermanox plastic coverslips (Cat # 174950, Thermo Fisher Scientific Inc.) or 5-mm round glass coverslips (Cat # 101413-528, VWR), placed in 24-well plates (75,000 cells/well) and incubated for 1 h at 37°C to allow adherence to coverslips. The following activating agents were used: LPS (10 μg/ml, Cat # L2762, Sigma-Aldrich), PMA (10 nM, cat# P8137, Sigma-Aldrich) or Sev (5,000), and rmTNF-α (10 μg/ml, cat# 410-MT, R&D Systems). After 30 min of stimulation, cells were fixed with 4% paraformaldehyde in PBS overnight and the DNA was stained with Sytox green (Cat # S7020, Invitrogen). NETs were visualized on a Nikon Eclipse fluorescence microscope and low magnification images (40×) were acquired with QCapture software on non-overlapping random images (7–11 separate fields/coverslips, derived from 3 wells/condition or genotype). NETs were manually identified on acquired images as Sytox-positive structures emanating from neutrophils with an overall length at least twice as long as the cell diameter (10) and expressed as percentage of neutrophils with released DNA. Each experiment was repeated at least three times.

MPO–DNA ELISA

Anti-MPO antibody (5 µg/ml, cat# HM 1051BT, Hycult Biotech Inc) was used to coat 96-well plate overnight at 4°C. After three washes with washing buffer (PBS with 0.05% Tween 20), 20 µl of BALF or plasma samples were added to the wells with 80-µl incubation buffer containing a peroxidase-labeled anti-DNA monoclonal antibody (dilution 1:25, cat# 11544675001, Cell Death Detection ELISA, Roche). The plate was incubated for 2 h at room temperature. After three washes, 100 µl of peroxidase substrate (cat# DY999, R&D Systems) was added. The reaction was stopped with 1M H₂SO₄ and OD of samples measured at 450 nm (Molecular Devices SpectraMax Plus 384). Specific OD was obtained by subtracting total OD from background OD generated without the addition of peroxidase-labeled anti-DNA antibody (less than 10%).

Cytokine Analysis

Cytokine concentrations in BALF samples and co-cultures were measured using the CBA for Mouse Inflammation Kit (cat# 552364, BD Biosciences), according to the manufacturer's protocols.

In Vitro Co-cultures

Murine bone marrow cells were cultured *in vitro* with GM-CSF (10 µg/ml, cat# PMC2015, Thermo Fisher Scientific) and IL-4 (5 µg/ml, cat# 404-ML/CF, R&D Systems) for 7 days. Cultured cells were plated in 24-well plates at 0.5×10^6 cells/well. BALF collected on day 3 from Sev-infected mice were added at a final dilution of 44%. Supernatants were collected 2 days later and cytokine concentrations were analyzed by CBA. Baseline cytokine levels from BMDCs alone are subtracted from co-culture cytokine levels and values are presented as % of mean WT level, which is set at 100%.

Statistical Analysis

Comparisons between two groups were performed by student's *t*-test and comparisons between multiple groups (≥ 3) were performed by one-way ANOVA and Bonferroni's correction for multiple comparisons was performed. Numerical data were expressed as mean \pm SEM. *P* values <0.05 were considered significant.

RESULTS

Sev Infection Induces NET Formation *In Vivo* and *In Vitro*

WT C57BL/6J and DPPI-deficient (DPPI^{-/-}) mice were infected *i.n.* with 2,500 50% egg infectious dose of Sev as previously described (5). On day 3 PI, mice were sacrificed and their lung examined for NET formation by immunostaining with anti-histone and anti-MPO antibodies; DAPI stained DNA. NETs were easily detected in WT lung but greatly decreased in DPPI^{-/-} mice (Figure 1A). Concomitant administration of DNase 1, which dismantled NETs (11, 12), also suppressed Sev-induced NET formation in Sev-infected WT mice (Figure 1A).

To further quantify the NET content in Sev-infected mice we measured concentration of MPO–DNA complexes in the BALF

and in plasma. The concentration of MPO–DNA complexes in BALF and plasma was increased in Sev-infected WT mice (Figures 1B,C). By contrast, we detected significantly lower levels of MPO–DNA complexes in the lung and plasma of Sev-infected DPPI^{-/-} mice on days 2–4 PI (Figures 1B,C). DNase 1 treatment also lowered the concentration of MPO–DNA complexes in BALF and plasma, confirming that the release of these complexes was partly NET dependent (Figure 1D).

In vitro, Sev enhanced NET release from TNF-primed, bone marrow-derived WT neutrophils (Figure 2). TNF-primed DPPI^{-/-} neutrophils generated lower levels of NETs in response to Sev (Figure 2) although the overall rate of Sev-induced NET formation was lower compared to PMA or LPS stimulation (Figure 2). Regardless of the stimulus, DPPI^{-/-} neutrophils consistently generated lower level of NETs, consistent with the *in vivo* results and previous studies (13, 14). Taken together these results suggest that Sev can directly induce NETs; however, in the lung milieu following Sev infection, several inflammatory cytokines likely contribute to or enhance Sev-induced NET formation.

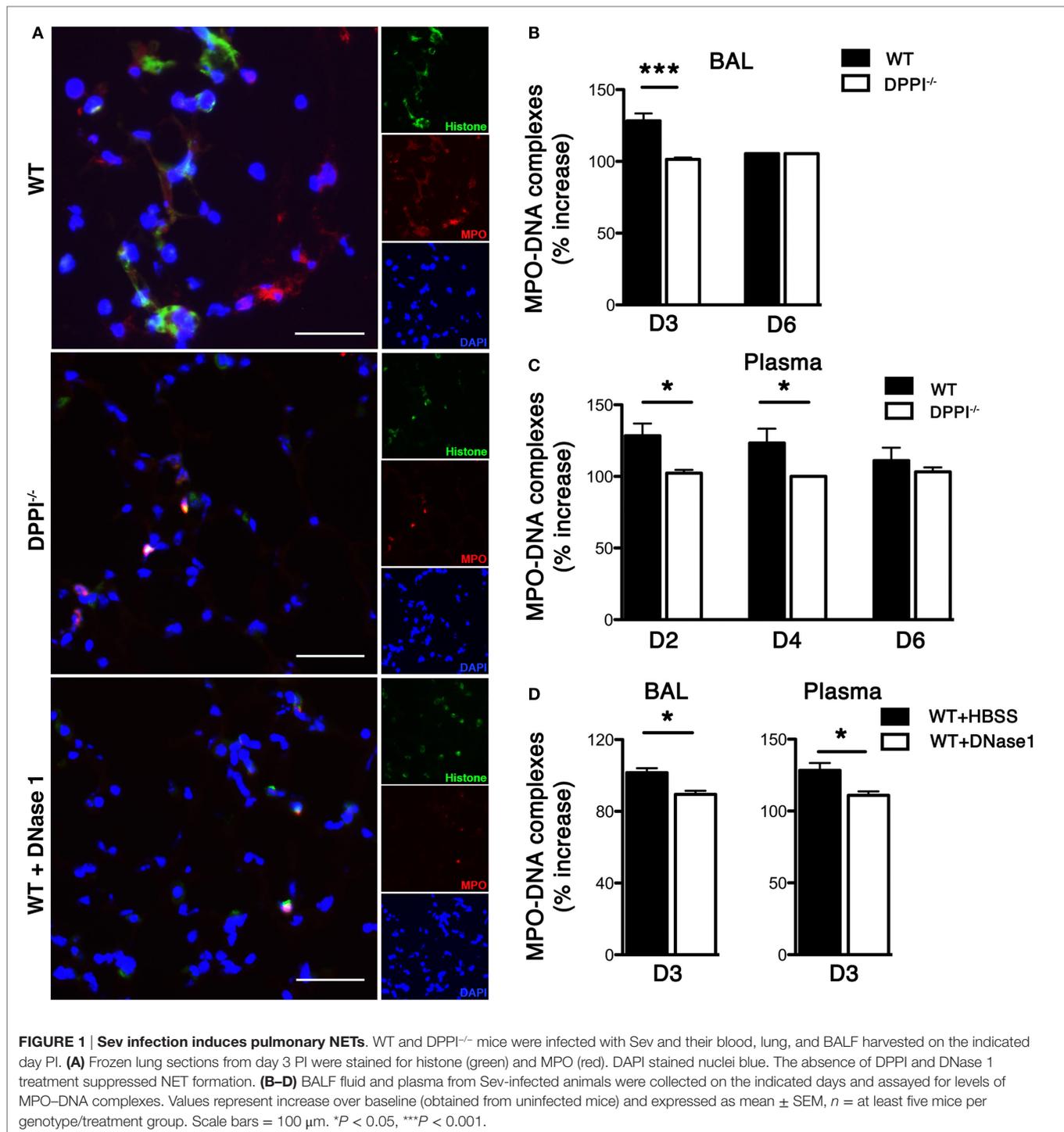
DNase 1 Attenuates the Early Sev-Induced Alveolar Inflammation

We have previously shown that Sev infection induced the expression of several cytokines/chemokines and absence of DPPI dampened this acute inflammatory response (5). To determine whether NETs modulated the acute response following Sev infection, we examined alveolar leukocyte accumulation and cytokine expression in WT mice treated with DNase 1. Administration of DNase 1 (*i.n.*) significantly reduced the total number of leukocytes recruited to the alveolar space (Figure 3). In addition, the levels of several BALF inflammatory cytokines, including TNF- α and IL-6 were suppressed with DNase 1 treatment (Figure 3). The extent of suppression with DNase1 treatment was equivalent to that observed with DPPI^{-/-} mice (Figure 3). DNase 1 treatment not only suppressed the acute cytokine response but also dampened the recruitment and activation of CD4⁺ and CD8⁺ T cells, as well as plasmacytoid DCs (CD317⁺ CD11c⁺ CD11b⁻ Siglec-H⁺) (Figure 4).

To further confirm that NET plays a role in the early inflammatory responses to Sev infection, we administered Cl-amidine, a pan PAD inhibitor with preferential irreversible inactivation of the calcium bound form of PAD4 (15, 16) to Sev-infected mice. PAD4 catalyzes the conversion of specific arginine residues to citrulline (17) and is essential for the formation of NETs via PAD4-mediated citrullination of histones (18). Cl-amidine suppressed neutrophil accumulation and inflammatory cytokine release in the alveolar space (Figure 5). Combined with the DNase 1 treatment results, these findings further confirmed the involvement of NETs in early host responses to Sev.

NET Containing BALF Stimulates Dendritic Cells/Macrophages to Release Inflammatory Cytokines

Previous studies suggest that the early phase antiviral cytokine response correlates with chronic lung immunopathology, including the airway remodeling and AHR that accompany the asthma



phenotype (19). In addition to Th2-types cytokines, Th1 cytokines, such as TNF- α , have also been shown to play an important role in the pathogenesis of asthma. TNF- α is increased in the sputum of patients with asthma (20, 21), aggravates AHR (22) as well as recruit inflammatory cells in animals (23). Blockade of TNF- α significantly inhibits AHR and reduces airway inflammation (24). To this end, we examined whether BALF-derived NETs from

Sev-infected mice modulated TNF- α (and other inflammatory cytokine) release from *in vitro* GM-CSF-induced BMDCs that comprise conventional DCs and macrophages (25). We observed that NET-containing BALF from WT Sev-infected mice directly stimulated BMDCs to release substantial amount of TNF- α (as well as IL-6 and MCP-1) while BALF from DPPI^{-/-} mice and animals treated with DNase 1 was less efficient (Figure 6A). Cl-amidine

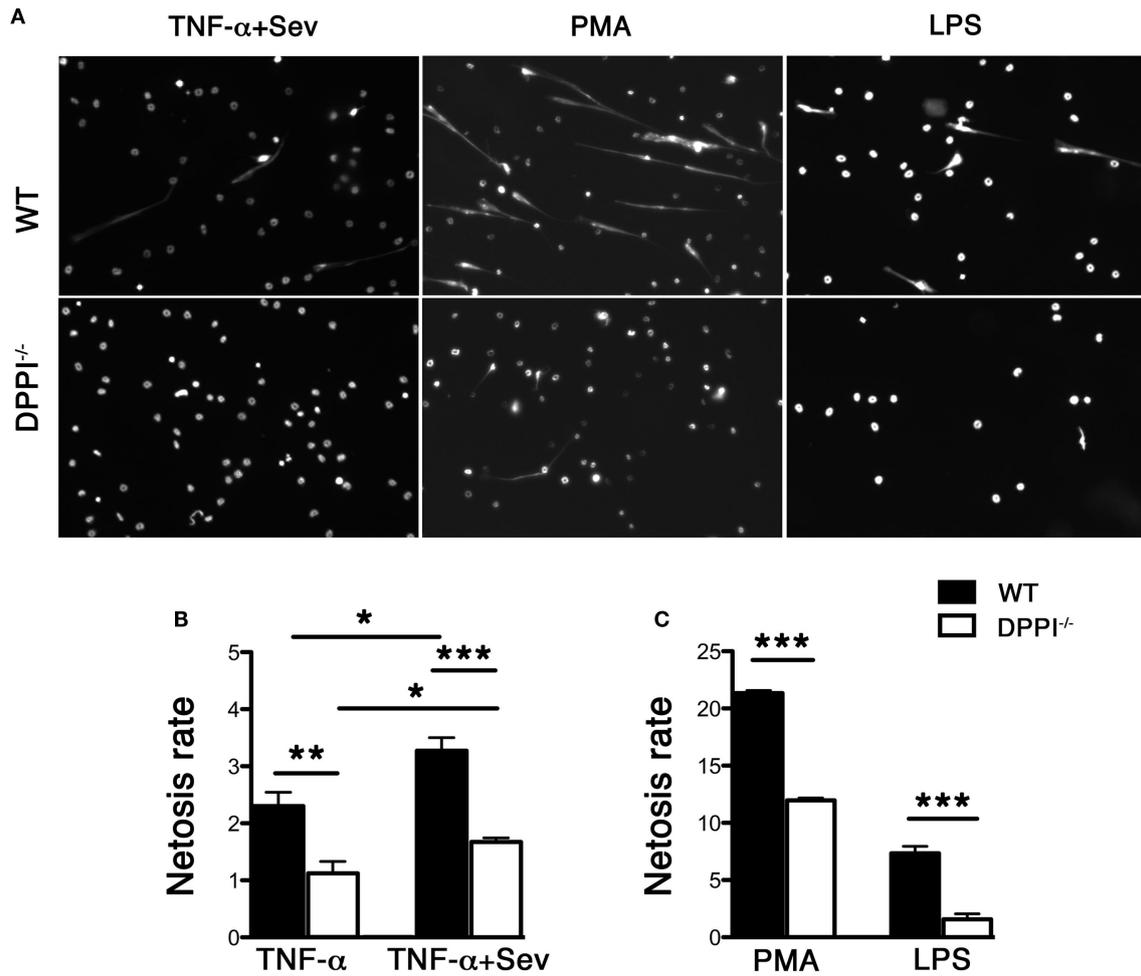


FIGURE 2 | Absence of DPPI attenuates NET formation *in vitro*. (A) Bone marrow-derived neutrophils from WT and DPPI^{-/-} mice were stimulated with TNF-α + Sev, PMA, or LPS for 30 min and stained for DNA with Sytox green. (B,C) Quantification of NETs with different stimuli (mean ± SEM derived from three separate experiments). **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

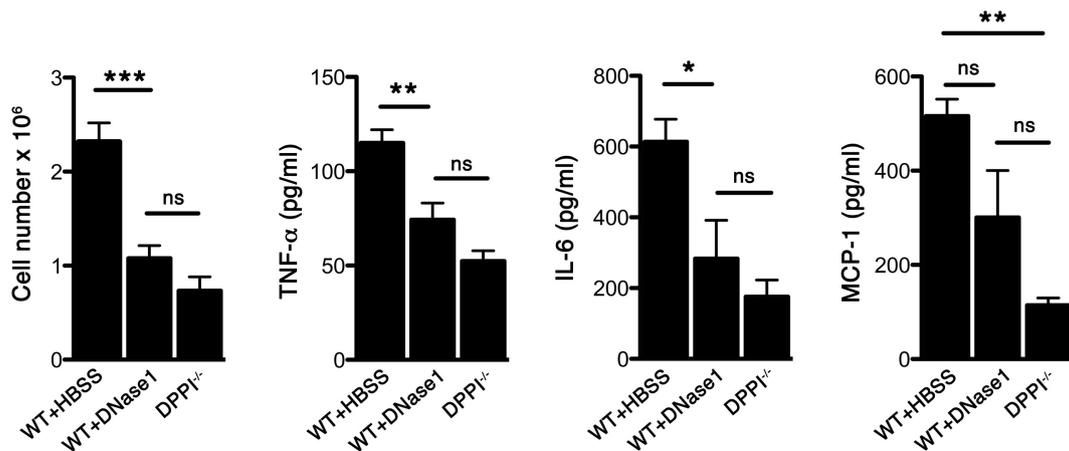


FIGURE 3 | DNase 1 treatment suppresses the acute Sev-induced inflammatory responses. WT and DPPI^{-/-} mice were infected with Sev and their BALF harvested on day 3 PI. Some WT mice received DNase 1 i.n. on days 0–2 PI. The cells were enumerated and cell-free BALF assayed for cytokines by CBA. Values represent mean ± SEM, *n* = 4–5 mice per genotype/treatment group. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, ns = not significant.

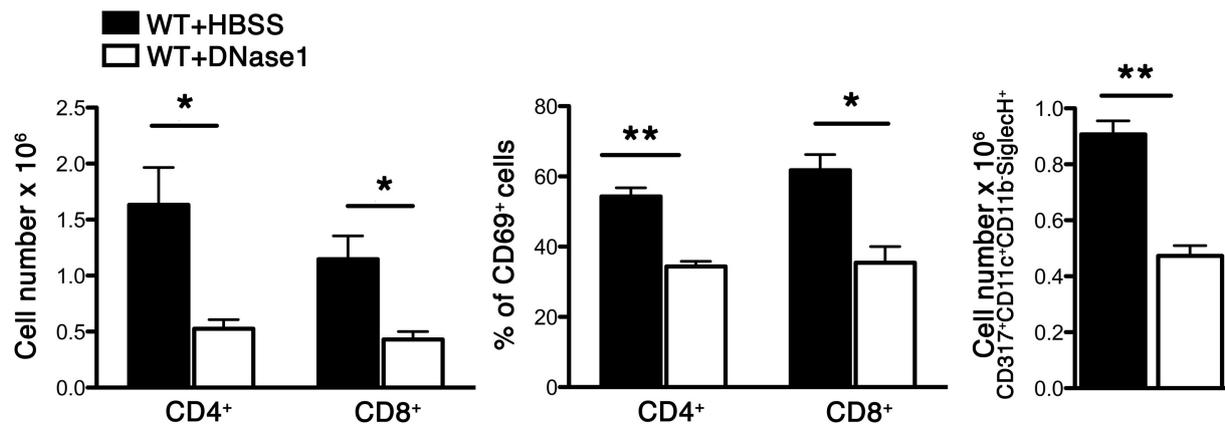


FIGURE 4 | DNase 1 treatment suppresses immune cell recruitment and activation. WT mice were infected with Sev and treated i.n. with HBSS or DNase 1. Cells were harvested from BAL on day 3 PI and analyzed for CD4, CD8, CD69 (activation marker), and CD317+CD11c+CD11b-SiglecH+ (plasmacytoid dendritic cell markers) by multicolor FACS analysis. Values represent mean \pm SEM, $n = 4-5$ mice/treatment group. * $P < 0.05$, ** $P < 0.01$.

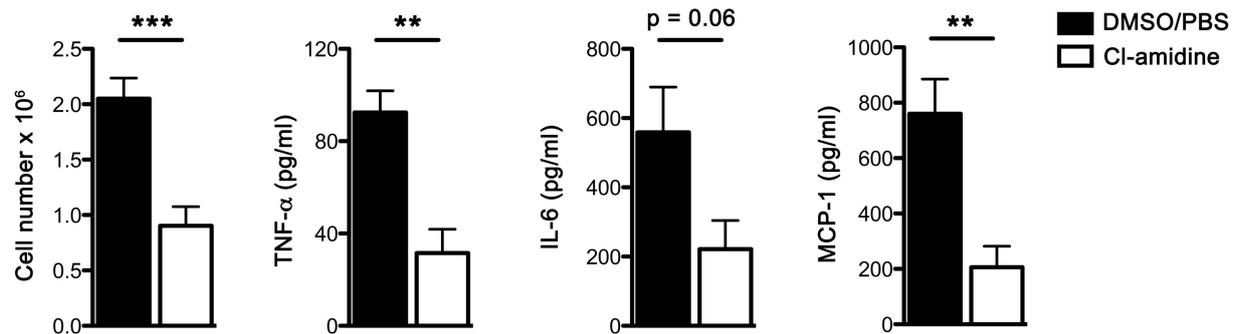


FIGURE 5 | Cl-amidine administration attenuates acute Sev-induced inflammatory responses. WT mice were administered Cl-amidine i.p. daily 2 days prior to infection and treatment was continued (twice a day) until sacrifice as detailed in Section "Materials and Methods." Controls received the same i.p. regimen of DMSO/PBS (5% v/v). On day 3 BALF was collected; cells were enumerated and cell-free BALF assayed for cytokines by CBA. Values represent mean \pm SEM, $n = 5$ mice/treatment group. ** $P < 0.01$; *** $P = 0.001$; note that IL-6 level in Cl-amidine treatment group approached statistical significance ($P = 0.06$).

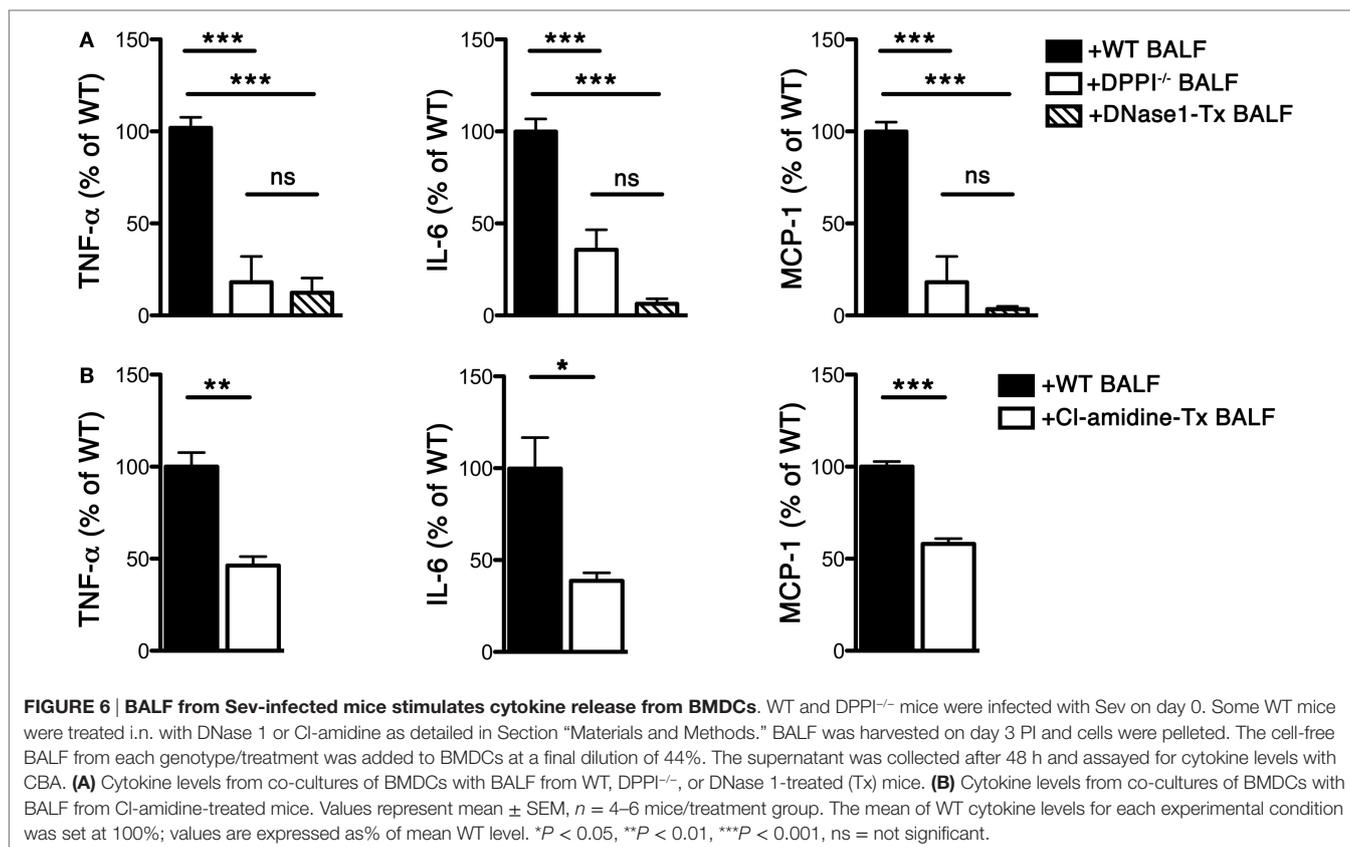
treatment also significantly attenuated the release of inflammatory cytokines by BALF-stimulated BMDCs (Figure 6B). Taken together, these above findings suggest that NETs released by recruited neutrophils play an important role in shaping the early immune response that likely impacts the Sev-induced chronic lung immunopathology.

DISCUSSION

Neutrophil extracellular traps can have anti- and pro-inflammatory effects on disease pathogenesis. On the one hand, NETs have been shown to promote the resolution of neutrophilic inflammation in monosodium urate crystal-induced arthritis (gout) (26). On the other hand, NETs are increasingly implicated in driving the pathogenesis of various diseases (11, 27–31). Their role in asthma development, however, remains largely unknown. Asthma is considered an inflammatory disease involving different mediators and cell types, with eosinophil predominance. Non-eosinophilic

asthma, however, comprises 50% of asthma and is often associated with accumulation of neutrophils in the airways, possibly due to bacterial endotoxin exposure as well as viral infections (32). Neutrophils are often present in acute asthma exacerbations (33) and noted in specific circumstances, such as status asthmaticus (34) but their role in asthma pathogenesis has not been extensively explored. A more recent study suggests that NETs are generated in human atopic asthmatic airways (35); however, their pathophysiologic role in allergic asthma also remains undefined. Using a physiologic murine model of Sev-induced asthma phenotype, we previously established a critical role for neutrophils and neutrophil-associated DPPI in the disease pathogenesis (5). In the studies herein, we further extend the investigation to show that neutrophil-derived DPPI is an important mediator of NET formation and NETs mediate the early inflammatory responses in Sev infection.

In addition to Sev-induced asthma phenotype, the absence of DPPI protects against various inflammatory conditions,



including preclinical models of rheumatoid arthritis (36), sepsis (37), abdominal aortic aneurysm (38), and ANCA-associated glomerulonephritis (39). DPPI deficiency also attenuates early atherosclerotic lesion in LDL-receptor-deficient mice (40). A recent report established that the absence of DPPI abrogates *in vitro* NET formation (13). DPPI is a ubiquitously expressed cysteine protease that is required for the expression of neutrophil serine proteases (NSPs), neutrophil elastase (NE), cathepsin G (CG) and proteinase 3 (PR3) in mature neutrophils (13, 36, 41). The role of NSPs in NET formation is well accepted (42). It is suggested that NE, but not CG or PR3, translocates to the nucleus where it contributes to chromatin decondensation by cleaving histones, an essential step in NET formation (43, 44). However, Martinod et al. recently showed that NE is dispensable for *in vitro* and *in vivo* NETs (45). Consistent with these results, we found that murine NE-deficient neutrophils release normal levels of NETs *in vitro* in response to various stimuli while the absence of NE and PR3 results in a NET defect similar to that observed with DPPI-deficient neutrophils (14). These discrepancies may reflect species-dependent requirements for NET formation, as previous studies used human neutrophils and NSP inhibitors.

Airway remodeling is an important histologic and structural change seen in asthma. It is a complex and incompletely understood process, involving several cell types and mediators. Neutrophils have been linked to airway remodeling presumably through their ability to release proteases, including metalloproteases and NE that induce degradation of extracellular matrix and

modulate inflammatory cell trafficking (46–48). Herein, we show that DNase 1 treatment significantly reduced the recruitment and activation of leukocytes, including CD4⁺ and CD8⁺ T cells, in the acute phase PI and suppressed inflammatory cytokine release. BALF from DNase 1- and Cl-amidine-treated animals was also less efficient at stimulating bone marrow-derived macrophages and DCs from releasing inflammatory cytokines. Taken together these results suggest that NETs released during the acute phase of Sev infection induce airway inflammation, leading to the elaboration of inflammatory cytokines that further recruit and activate immune cells, thus promoting the eventual chronic asthma phenotype.

In summary, these findings suggest that controlling neutrophil activation and NET formation in non-eosinophilic asthma may attenuate acute exacerbations and potentially prevent further airway remodeling.

AUTHOR CONTRIBUTIONS

AA and LS designed and performed the experiments, and analyzed the data; AA, LS, and CP wrote the manuscript. All authors approved the final version of the manuscript.

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Oxidative Burst-Dependent NETosis Is Implicated in the Resolution of Necrosis-Associated Sterile Inflammation

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Necrosis is associated with a profound inflammatory response. The regulation of necrosis-associated inflammation, particularly the mechanisms responsible for resolution of inflammation is incompletely characterized. Nanoparticles are known to induce plasma membrane damage and necrosis followed by sterile inflammation. We observed that injection of metabolically inert nanodiamonds resulted in paw edema in WT and *Ncf1*^{**} mice. However, while inflammation quickly resolved in WT mice, it persisted over several weeks in *Ncf1*^{**} mice indicating failure of resolution of inflammation. Mechanistically, NOX2-dependent reactive oxygen species (ROS) production and formation of neutrophil extracellular traps were essential for the resolution of necrosis-induced inflammation: hence, by evaluating the fate of the particles at the site of inflammation, we observed that *Ncf1*^{**} mice deficient in NADPH-dependent ROS failed to generate granulation tissue therefore being unable to trap the nanodiamonds. These data suggest that NOX2-dependent NETosis is crucial for preventing the chronification of the inflammatory response to tissue necrosis by forming NETosis-dependent barriers between the necrotic and healthy surrounding tissue.

Keywords: necrosis, inflammation, nanodiamonds, NETosis, resolution, reactive oxygen species

INTRODUCTION

Sterile inflammation usually occurs as a reaction to tissue injury and cell death (1). Endogenous molecules released from necrotic cells (damage-associated molecular patterns, DAMPs) usually trigger and augment the inflammatory response to the insult and cell necrosis (2). For instance, the high-mobility group box 1 (HMGB1), a *bona fide* DAMP is released from necrotic cells (3) and triggers a neutrophil-mediated injury amplification loop that involves the receptor for advanced glycation end products (4). Neutrophils, the most abundant leukocytes in blood, are considered the initial line of defense, as they are the first cells recruited to sites of injury. Upon recruitment, neutrophils produce ROS, degranulate, and release pro-inflammatory cytokines to ensure the inactivation of the putative aggressor (5).

Importantly, neutrophils also release neutrophil extracellular traps (NETs) to entrap and kill microorganisms (6). This suicidal process is referred to as NET formation or NETosis (7). NETs are released in a coordinated series of events that involve chromatin decondensation and translocation of granular proteins. Upon neutrophil activation, the integrity of the granular membranes is lost. In consequence, neutrophil elastase (NE) and myeloperoxidase (MPO) translocate to the nucleus where they contribute to histone degradation and chromatin decondensation, respectively (8, 9). PAD4-mediated citrullination of histone H3 (citH3) has been shown to foster chromatin decondensation (10, 11). Reactive oxygen species (ROS) contribute to the release of NE and MPO from the granules and their translocation to the nucleus (8, 12) and probably at later stages to the disruption of the plasma membrane. Released DNA gets then decorated with NE, MPO, and citH3. Besides pathogens, other stimuli, such as cytokines (13), phorbol myristate acetate (PMA) (6), ionomycin (14), or monosodium urate (MSU) crystals (15), reportedly induce NETosis.

Currently, the functions of neutrophils are considered a double-edged sword: on one side, they exert pro-inflammatory actions during infections that contribute to the development of both innate and adaptive immunity (16, 17); on the other side, they are involved in tissue damage and in the initiation and perpetuation of immune dysregulation in chronic autoimmune diseases such as RA (18) and SLE (19). Nevertheless, neutrophils have also been implicated in the resolution of acute inflammation. When the initial wave of neutrophils is missing or when neutrophils are impaired in generating NETs, the inflammatory responses to zymosan or MSU tend to chronify (20, 21). In this case, an aggregate of NETs confines the inflammatory stimulus and degrades inflammatory cytokines and chemokines limiting further neutrophil recruitment and orchestrating the resolution of inflammation (20, 21).

We have recently shown that diamond nanoparticles induce membrane damage in a broad spectrum of cells *in vitro* and *in vivo* (22). The ability to penetrate through plasma membranes was also demonstrated for C60 fullerenes (23) and for single- and multi-walled carbon nanotubes (24). The exposure to carbon nanotubes of mice resulted in the formation of granulomata in skin, lungs, and peritoneum (25, 26). Taking together, metabolically inert nanoparticles induce sterile tissue damage upon injection. Since small nanodiamonds also cause NETosis in neutrophils (22), we hypothesized that such a necrosis-inducing trigger does not only mount sterile inflammation but also a robust resolution response. Thus, we aimed to create an *in vivo* model of permanent sterile inflammation to reveal the role of oxidative

burst-dependent NETosis in the context of tissue damage in the absence of pathogens.

Here, we describe that nanodiamonds induced necrosis and self-limited sterile inflammation when injected into wild-type mice. Nanodiamonds also emerged as potent triggers for NETosis in cultured neutrophils. When nanodiamonds were injected into ROS-deficient mice lacking functional NETosis; however, the resolution of inflammation was severely impaired. NETs efficiently entrap nanodiamonds and shield healthy from necrotic tissue.

RESULTS

Characterization of Diamond Particles

The structural and surface properties of the nanodiamonds, sized 10 nm, were verified by high-resolution transmission microscopy (HRTEM), X-ray photoelectron spectroscopy (XPS), and Raman spectroscopy. The Raman images of the diamonds are shown in Figure S1A in Supplementary Material. The spectra reveal the characteristic feature of diamonds with a peak at 1336/cm. The presence of graphitic carbon with bands at 1350 and 1580/cm is virtually absent. The XPS survey spectra of the diamonds showed signals of carbon (10 nm: 90.4%; 1000 nm: 90.1%) as dominant element together with the presence of oxygen (10 nm: 4.2%; 1000 nm: 9.6%) and nitrogen (10 nm: 1.8%; 1000 nm: 0.3%). Figure S1B in Supplementary Material shows (high resolution) transmission microscopy pictures of 10 nm nanodiamonds with a diameter of about 7 ± 4 nm. The lattice fringes with a spacing of 2.06 Å are assigned to the diamond (111) plane. The physicochemical properties of the diamonds are listed in Table 1.

Induction of Plasma Membrane Damage in Human Leukocytes

Freshly isolated human polymorphonuclear leukocytes (PMN) and peripheral blood mononuclear cells (PBMC) showed rapidly increasing membrane permeability, represented by an increase of the SYTOX Green signal due to its increased accessibility to DNA (Figure 1A) when exposed to 10 nm diamonds (nanodiamonds). SYTOX Green is a cell membrane-impermeable dye that specifically intercalates into accessible DNA, thereby increasing its fluorescence up to 500 times. Interestingly, the increase of the SYTOX signal in PBMC was less pronounced than the one observed in PMN. Next, we compared the SYTOX signal induced by nanodiamonds with the one induced by PMA as a classical stimulus for DNA externalization in conjunction

TABLE 1 | Physicochemical characteristics of diamond particles.

Particle size (nm)	Diameter/nm ^a	Hydrodynamic diameter/nm ^b (pH = 7.4)	ζ-potential/mV (pH = 7.4)	Hydrophobicity
10	7 ± 4	618 ± 25	+34 ± 1	High
1000	1010 ± 300	970 ± 50	-38 ± 1	High

^aDetermined by TEM.

^bDetermined by DLS.

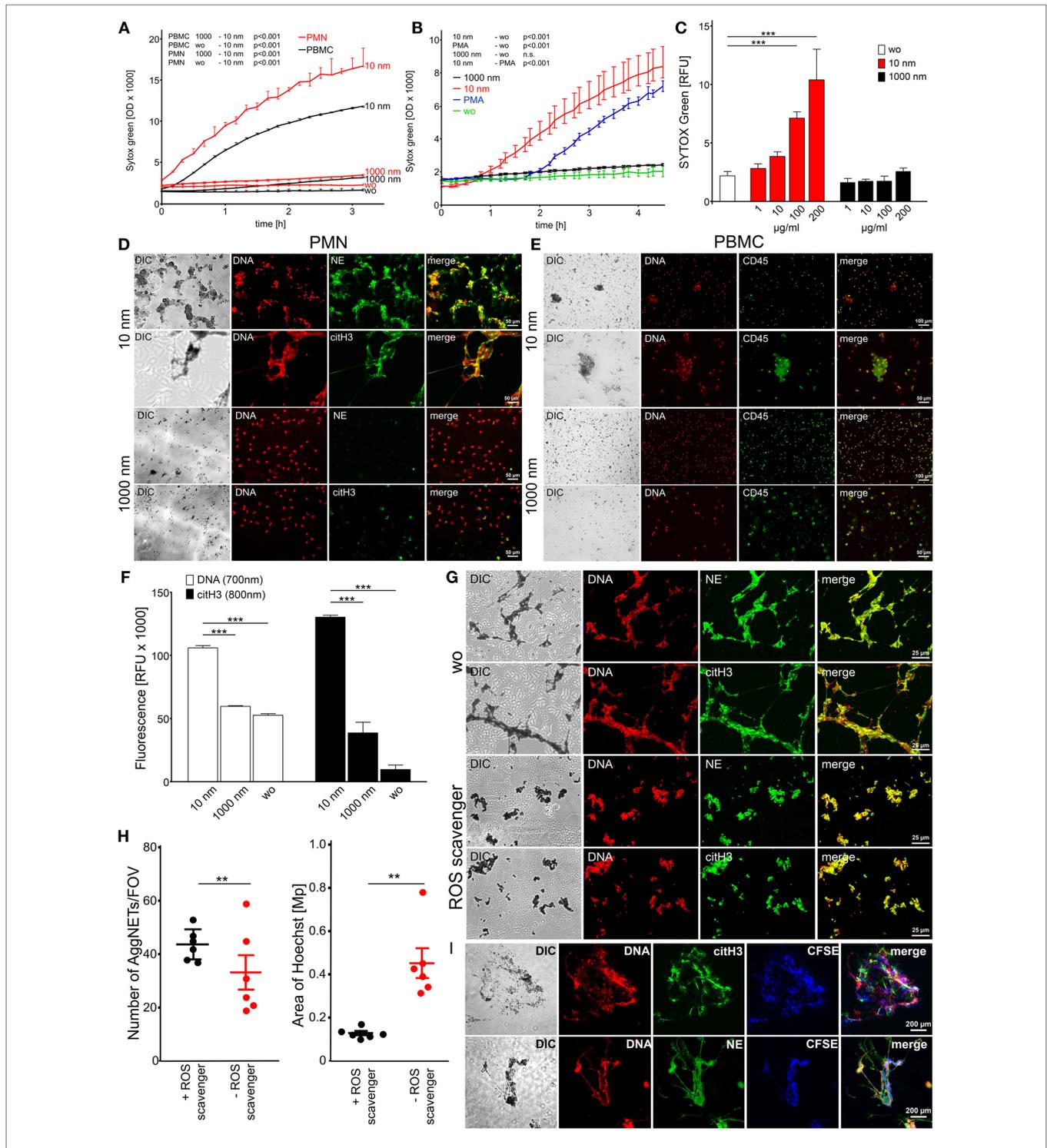


FIGURE 1 | Diamond nanoparticles induce plasma membrane damage and NET formation in human leukocytes. (A) Assessment of DNA exposure in neutrophils (PMN) and peripheral blood mononuclear cells (PBMC). Cells without stimulus (wo) or incubated with nanodiamonds (10 nm) or microdiamonds (1000 nm) were measured using SYTOX Green. **(B)** Exposure of DNA by neutrophils was measured in response to phorbol 12-myristate 13-acetate (PMA) to nanodiamonds (10 nm) or microdiamonds (1000 nm) and without stimulus (wo) using SYTOX Green. **(C)** Dose-dependent increase of DNA exposure in PMN without stimulus (wo) or incubated with nanodiamonds (10 nm) or microdiamonds (1000 nm) by SYTOX Green after 150 min. **(D)** Microscopic analysis of PMN incubated with nanodiamonds (10 nm) or microdiamonds (1000 nm) and stained for DNA (Hoechst33342), neutrophil elastase (NE), or citrullinated histone H3 (citH3). Diamonds are visible in differential interference contrast (DIC) images. Cy5 fluorescence was artificially colored green. **(E)** Microscopic analysis of PBMC incubated (Continued)

FIGURE 1 | Continued

with nanodiamonds (10 nm) or microdiamonds (1000 nm) and stained for DNA (Hoechst33342) and CD45-FITC. Diamonds are visible in DIC and provide strong background fluorescence in FITC. **(F)** Quantification of extracellular DNA and citH3 in human PMN without stimulus (wo) or incubated with nanodiamonds (10 nm) or microdiamonds (1000 nm) after 240 min. Significances below 0.001 are depicted. **(G)** Microscopic analysis of PMN incubated with nanodiamonds and treated with the ROS scavenger *N*-acetyl cysteine stained for DNA (Hoechst33342), NE, or citH3. Diamonds are visible in DIC. **(H)** *In silico* quantification of microscopic pictures regarding the number and area of DNA (Hoechst33342)-stained NET structures (AggNET) formed by PMN incubated with nanodiamonds in the absence or presence of the ROS scavenger *N*-acetyl cysteine. Each dot represents one analyzed field of view (FOV). **(I)** Microscopic analysis of NET formation in response to nanodiamonds induced necrosis of CFSE-labeled PBMC stained for DNA (Hoechst33342), NE, or citH3. Diamonds are visible in DIC. Data of one representative experiment reflecting the result of three independent experiments are shown as medians with interquartile ranges of triplicates. Two-way ANOVA **(A–C)**, one-way ANOVA **(F)**, Kruskal–Wallis one-way analysis of variance **(H)**, and Mann–Whitney *U* test **(I)** were used to evaluate differences among means; ***p* < 0.05, ****p* < 0.001, and relative fluorescence units (RFU) field of view (FOV).

with NETosis. In response to nanodiamonds, the SYTOX signal was enhanced earlier (60 min) and more pronounced than with PMA (**Figure 1B**). In contrast, upon incubation with the control 1000 nm diamonds (microdiamonds), the SYTOX signal was comparable to unstimulated cells, showing no membrane damage and no accessibility of the DNA in PBMC or PMN (**Figure 1A**). The membrane damage in response to nanodiamonds was dose dependent as represented by the increase of the SYTOX signal in PMN incubated with increasing amounts of particles (**Figure 1C**). We conclude that nanodiamonds induce rapid cell membrane rupture rendering DNA accessible in white blood cells.

In order to characterize the nuclear appearance of leukocytes upon contact with diamonds, we microscopically analyzed PMN and PBMC after co-incubation with nanodiamonds or microdiamonds. DNA was stained by propidium iodide or Hoechst 33342 and the DNA-associated proteins citH3 and NE by immunofluorescence. PMN incubated with nanodiamonds exhibited large spread aggregates composed of DNA and nanodiamonds co-localizing with citH3 and NE (**Figure 1D**). In contrast, employing microdiamonds no such structures were observed. The nuclei displayed a lobular shape, characteristic of neutrophils, and the signals of NE and citH3 were localized intracellularly. The nuclear appearance of PBMC incubated with nanodiamonds differed strongly from that of similarly treated PMN (**Figure 1E**). Only sporadic diamond and necrotic cell aggregates with normal nuclear morphology were observed in association with nanodiamonds. NET-like structures trapping nanodiamonds were not observed in PBMC samples. Incubation with microdiamonds induced nuclear modifications neither in PBMC nor in PMN (**Figures 1D,E**).

Live cell imaging confirmed the fast and uncontrolled rupture of the plasma membrane of PMN (Video S1 in Supplementary Material) and PBMC (Video S2 in Supplementary Material) in response to nanodiamonds. This process is represented by the conversion of the blue Hoechst3342 signal, being cell membrane permeable, to the red PI signal, intercalating into accessible DNA. The nuclear appearance of PMN markedly differed from that of PBMC. The lobulated nucleus of viable neutrophils, stained by Hoechst3342, became PI-positive, and displayed a decondensed morphology as soon as 30 min after the stimulus (Video S1 in Supplementary Material). This process was followed by externalization and spreading of the DNA. In contrast, nuclei of PBMC quickly became PI-positive, indicative for plasma membrane damage, but the DNA was not externalized and spread (Video

S2 in Supplementary Material). Microdiamonds associated with both, PMN (Video S3 in Supplementary Material) and PBMC (Video S4 in Supplementary Material), but did not affect the integrity of their cellular membranes. However, some spontaneous cell death was observed most likely due to phototoxicity in time-lapse fluorescence microscopy (27).

Quantification of the signal for extracellular DNA and citH3 revealed a significant increase when PMN were incubated with nanodiamonds in comparison to microdiamonds or unstimulated cells (**Figure 1F**). Externalized chromatin of neutrophils co-localizing with citH3 is indicative for NETosis (11). In order to further evaluate NETosis, we added nanodiamonds to PMN pre-incubated with the ROS scavenger *N*-acetyl L-cysteine (NAC). Although we still observed NET formation in the presence of NAC, the aggregation of the nanodiamonds by NETs was reduced when compared to that resulting from the incubation of PMN with nanodiamonds alone (**Figure 1G**). This was confirmed by *in silico* morphometric quantification of NET aggregation concerning the number and area of NET structures (**Figure 1H**). In the presence of the ROS scavenger, the number of NET aggregates (AggNET) was significantly higher, but the area was significantly decreased. This reflects a reduced aggregation of NETs in the absence of ROS. Next, we employed CFSE-labeled PBMC, which had previously been incubated with nanodiamonds to stimulate freshly isolated viable PMN. Interestingly, we observed that necrotic PBMCs and nanodiamonds were entrapped in the NET aggregates characterized by large DNA filaments decorated with NE and citH3 (**Figure 1I**).

We conclude that nanodiamonds induced fast rupture of the plasma membrane when encountering leukocytes (PMN and PBMC). The mononuclear cells rapidly died by membrane rupture and became necrotic. Contrarily, PMN formed NETs, which tended to aggregate in the presence of ROS to confine nanodiamonds as well as necrotic mononuclear cells.

Induction of Cellular Damage in Murine Bone Marrow-Derived Immune Cells

In order to determine the effect of nano- and microdiamonds on murine leukocytes, we quantified the accessibility of DNA for SYTOX Green in isolated bone marrow cells. To further analyze the role of the oxidative burst on nanoparticle-induced NETosis, experiments were conducted with bone marrow cells of both, WT and *Ncf1*^{**} mice. Latter harbor a single-nucleotide polymorphism in the gene for the regulatory p47^{phox} subunit (*Ncf1*) of the NADPH oxidase NOX2. This mutation leads to

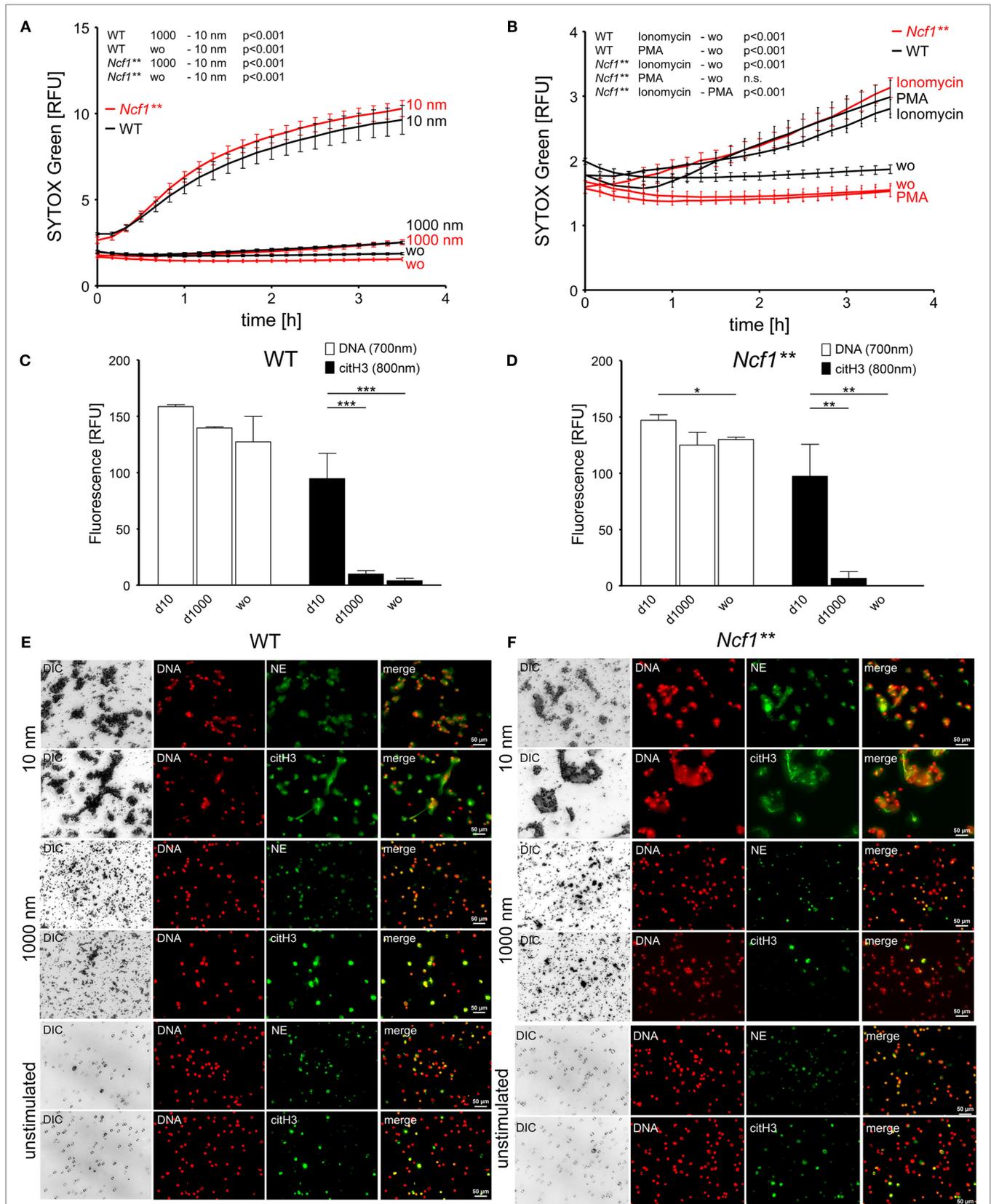


FIGURE 2 | Diamond nanoparticles induce plasma membrane damage and NET formation in murine bone marrow cells.

(Continued)

FIGURE 2 | Continued

(A) Analysis of DNA exposure by bone marrow cells of WT and *Ncf1*^{**} mice in response to nanodiamonds (10 nm) or microdiamonds (1000 nm) or without stimulus (wo) using SYTOX Green. **(B)** Analysis of DNA exposure by bone marrow cells of WT and *Ncf1*^{**} mice in response to NET-inducing stimuli (PMA and ionomycin) or without stimulus (wo) using SYTOX Green. **(C)** Quantification of extracellular DNA and citrullinated histone H3 (citH3) in bone marrow cells of WT mice without stimulus (wo) or incubated with nanodiamonds (10 nm) or microdiamonds (1000 nm) after 240 min. **(D)** Quantification of extracellular DNA and citH3 in bone marrow cells of *Ncf1*^{**} mice without stimulus (wo) or incubated with nanodiamonds (10 nm) or microdiamonds (1000 nm) after 240 min. **(E)** Microscopic analysis of bone marrow cells of WT mice incubated with nanodiamonds (10 nm) or microdiamonds (1000 nm) and unstimulated cells stained for DNA (Hoechst33342), neutrophil elastase (NE), or citH3. Diamonds are visible in differential interference contrast (DIC). Cy5 fluorescence was artificially colored green. **(F)** Microscopic analysis of bone marrow cells of *Ncf1*^{**} mice incubated with nanodiamonds (10 nm) or microdiamonds (1000 nm) and unstimulated cells stained for DNA (Hoechst33342), NE, or citH3. Diamonds are visible in DIC. Cy5 fluorescence was artificially colored green. Data of one representative experiment reflecting the result of three independent experiments are shown as medians with interquartile ranges of triplicates. Two-way ANOVA **(A,B)** and one-way ANOVA **(C,D)** were used to evaluate differences among means; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and relative fluorescence units (RFU).

strongly diminished NOX2-dependent ROS production resulting in a deficiency of neutrophils to undergo NOX2-dependent NETosis.

Isolated bone marrow cells contained about 80% CD45⁺ leukocytes. More than 50% of these were identified as granulocytes (Figures S1C,D in Supplementary Material). Consistent with previous experiments employing human leukocytes, we observed a fast permeabilization of the plasma membrane in response to nanodiamonds in bone marrow cells of both, WT and *Ncf1*^{**} mice (Figure 2A), while the microdiamonds were inert. Stimulation with ionomycin of bone marrow cells triggered DNA release in both, WT and *Ncf1*^{**} mice (Figure 2B). This stimulus reportedly induces NETosis independent of NOX2-dependent ROS production (28). Importantly, only leukocytes from WT mice underwent DNA externalization in response to PMA, a NOX2-dependent ROS inducing stimulus (29), whereas *Ncf1*^{**}-derived cells did not. Similar to human cells, the quantification of extracellular citH3 revealed significantly elevated signals after exposure to nanodiamonds in comparison to microdiamonds or unstimulated cells (Figures 2C,D). Analyses by microscopy of WT or *Ncf1*^{**} bone marrow cells incubated with nanodiamonds revealed a similar appearance to that of human PMN (Figures 2E,F). The nanodiamonds induced NET structures of DNA co-localizing with citH3 or NE and sequestering the particles in both, WT- and *Ncf1*^{**}-derived cells (Figures 2E,F). The microdiamonds did not induce the formation of such structures (Figures 2E,F). Lower magnification pictures showing NET structures or nuclear morphology in a larger area are depicted in Figures S1E,F in Supplementary Material. From these observations, we can conclude that the direct effects of nanodiamonds on both human and murine leukocytes are ROS independent.

Tissue Damage-Induced Inflammation Does Not Resolve in *Ncf1*^{**} Mice

Nanodiamonds induced rapid and substantial cellular damage *in vitro* independently of the leukocyte type in both human and mice. Since nanodiamonds cannot be digested enzymatically, we hypothesized that their persistent presence might result in a continuous induction of inflammation *in vivo*. To investigate the role of NOX2-dependent NETosis in nanoparticle-induced inflammation, we injected 1 mg of nanodiamonds or microdiamonds into the metatarsal region of the hind paws of WT or *Ncf1*^{**} mice. Paw edema in the particle-injected foot was recorded as a specific sign of local inflammation over 28 days and compared

to the sham-treated control foot. Already 24 h after injection, we observed the development of significant paw edema in both mouse strains injected with nanodiamonds (Figure 3A). In WT mice, the inflammation resolved within 3 days. *Ncf1*^{**} mice developed a sustained inflammation that did not resolve until the end of the experiment at day 28. In contrast, microdiamonds neither induced paw swelling in WT nor in *Ncf1*^{**} mice (Figure 3B). In an additional experiment, we injected nanodiamonds in the presence of DNase I into WT mice (Figure 3C). To avoid further injection-induced tissue damage, DNase I was applied only once together with the nanodiamonds as well as i.v. 24 and 48 h after injection of nanodiamonds. Paw edema was recorded for 28 days. Since no differences between the groups at later time points were observed, values until day 10 are shown. DNase I-treated mice showed prolonged inflammation until day 5, while in the untreated group, the inflammation resolved already at day 4.

Dissection of the WT hind paws disclosed that nanodiamonds were wrapped in membrane-like structures resembling granulation tissue (Figure 3D). In contrast, *Ncf1*^{**} hind paws showed bare nanodiamonds without visible association to connective tissue. The microdiamonds injected into hind paws showed no signs of clumping or granulation (Figure 3D). Moreover, nanodiamonds were tightly attached to the overlaying skin in WT paws, while they appeared more loose and spread in the surrounding tissues in the paws of *Ncf1*^{**} mice (Figure 3E). Histological analysis of the skins revealed packing of nanodiamonds associated with DNA and NE in WT mice (Figure 3F). In contrast, nanodiamonds were dispersed in the skin section and not associated with extracellular neutrophil markers in *Ncf1*^{**} mice.

In summary, nanodiamonds trigger a strong local inflammatory response when injected into tissues by inducing membrane rupture and necrosis. In WT mice, cell death in response to nanodiamonds induced ROS-dependent NETosis, organization of nanodiamonds in the tissue, and resolution of the initial inflammation. In the absence of NOX2-dependent NETosis; however, inflammation does not resolve, nanodiamonds are dispersed in the tissue, and this may trigger chronic inflammation.

DISCUSSION

Consistent with previous findings employing epithelial and red blood cells (RBC) (22), we report that nanodiamonds induce cellular damage in human and murine leukocytes *in vitro*. In contrast, microdiamonds did not affect the membrane

integrity of these cells. In the case of PMN, rupture of the plasma membrane in response to nanodiamonds was accompanied by DNA decondensation and release of DNA decorated with citH3 and NE. These NETs sequestered and entrapped the nanodiamonds. NETosis induced by particulate matter has already been reported for MSU (30), calcium carbonate (31), calcium oxalate (32), and diamonds (22). The mechanism of crystal-induced NETosis is associated with the necroptosis pathway (33). Several other studies support a role for the size of nanoparticles and their effects on cells and tissues (34–36). Most of these studies are limited to macrophage-mediated particle engulfment (37, 38) and clearance (39). In the case of neutrophils exposed to nanomaterials, investigations focused on cytotoxicity (40–42), degranulation (43), or phagocytic uptake (44). Recently, MPO-mediated degradation of single-walled carbon nanotubes has been shown to abrogate carbon nanotube-induced pulmonary inflammation (45). Further reports on neutrophils reacting to tissue damage induced by nanoparticles are scarce. We have recently demonstrated that the size of particles critically determines NETosis, and this is associated with the resolution of an initial neutrophil-driven

inflammation in air pouches (22). Microdiamonds of 1 μm were not taken up by granulocytes *in vitro*, whereas small nanodiamonds induced NETosis due to their size and hydrophobicity. This result seems to contradict other findings showing that neutrophils undergo NETosis selectively in response to large pathogens, but not in response to small yeast or single bacteria (46). However, it is likely that neutrophils respond differently to pathogens than nanodiamonds, which differ greatly in size and properties from pathogens like bacteria or yeast.

The role of NETosis in spatially and temporally restricted sterile inflammation following necrosis has not been investigated. Since nanodiamonds induced substantial cellular damage *in vitro* and persist at the site of injection, we hypothesized that they provoke chronic inflammation after *in vivo* injection. Indeed, we observed that nanodiamonds triggered inflammation within 24 h after injection. However, inflammation resolved within 3 days in WT mice despite continuous persistence of the nanodiamonds *in situ*. Conversely, mice deficient in NOX2 developed a sustained inflammatory response indicating a role for ROS-dependent NETosis in the resolution of necrosis-associated sterile inflammation. This observation is also supported by the fact that the

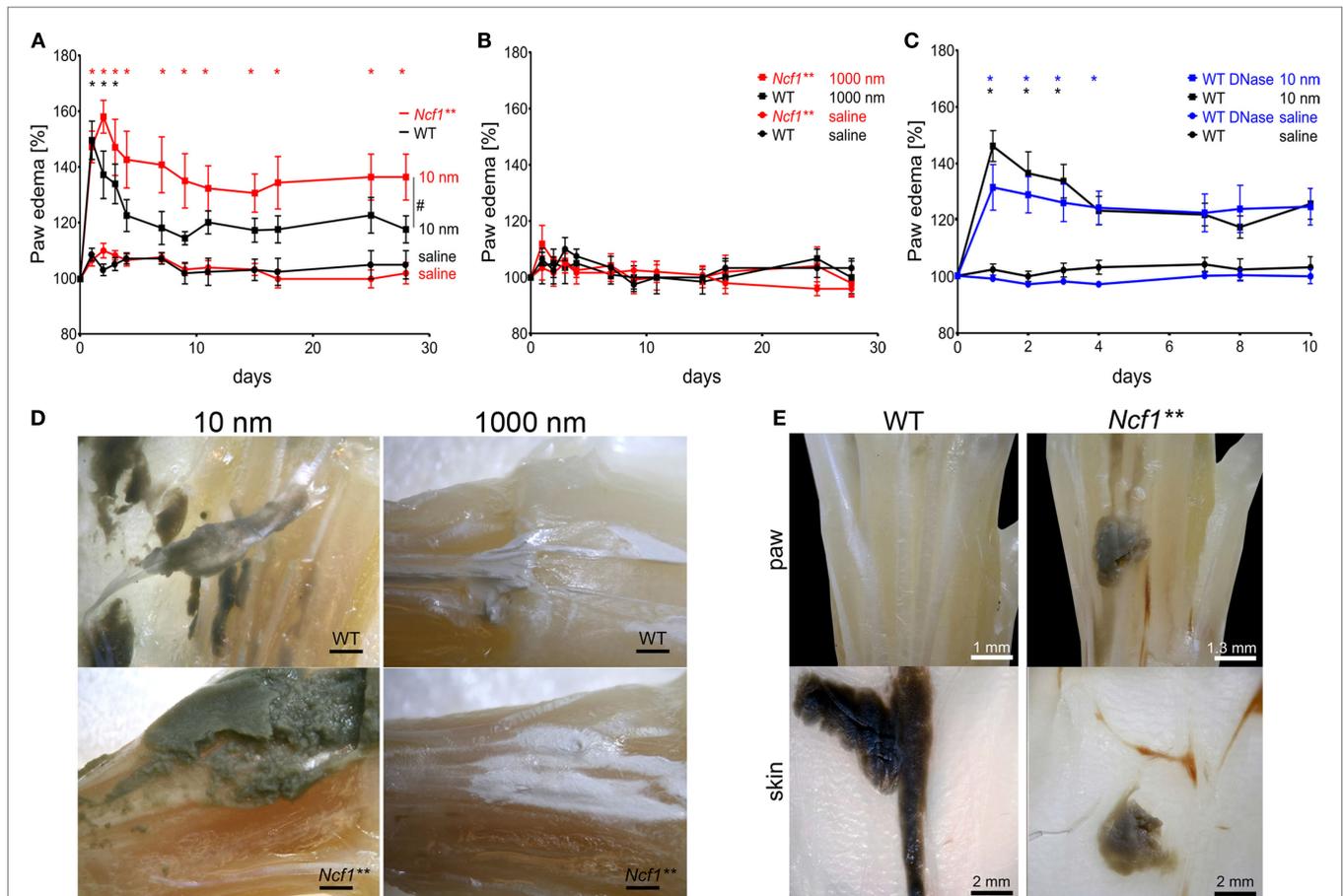
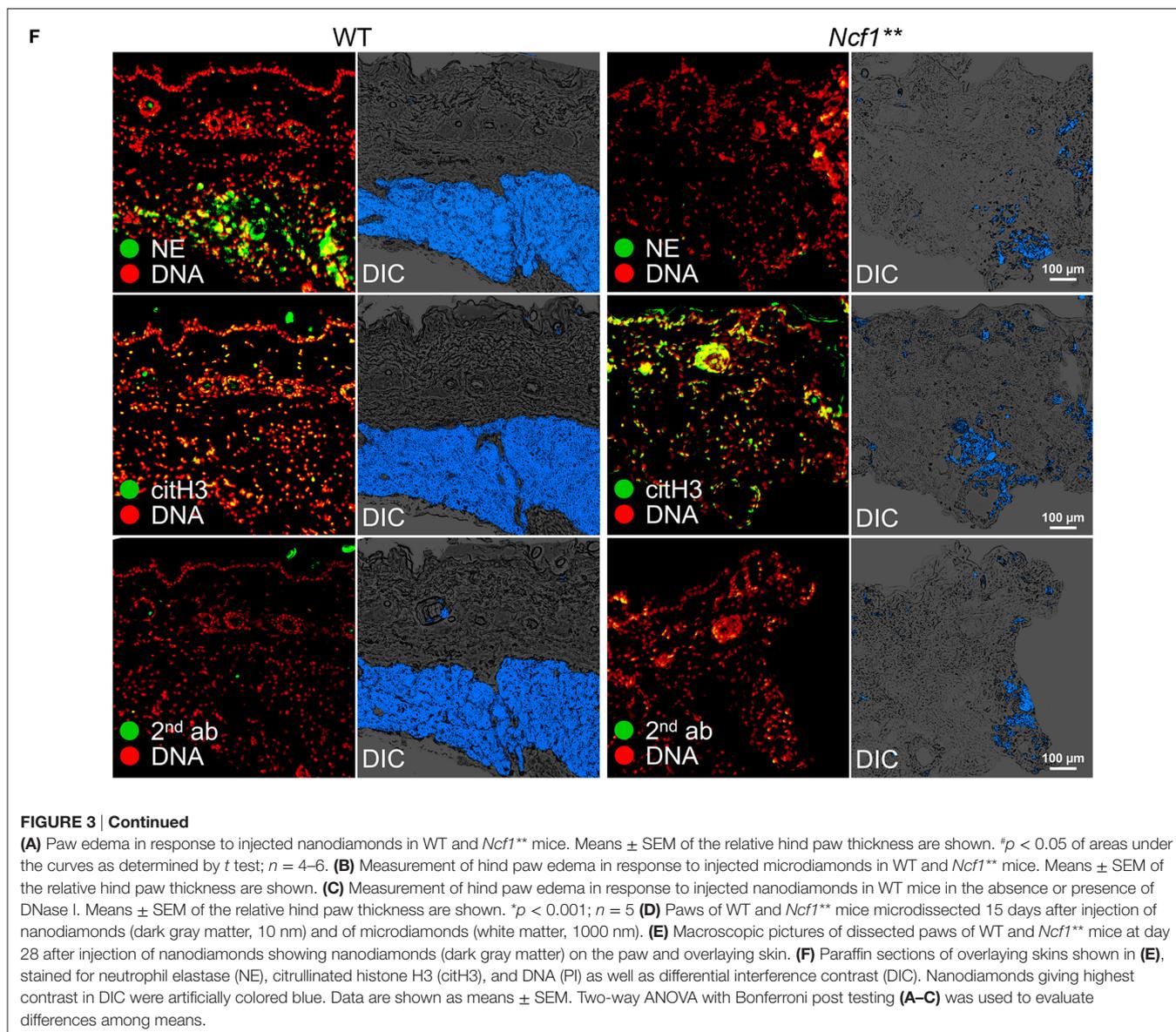


FIGURE 3 | Chronification of necrosis-associated sterile inflammation in NOX2-deficient mice.

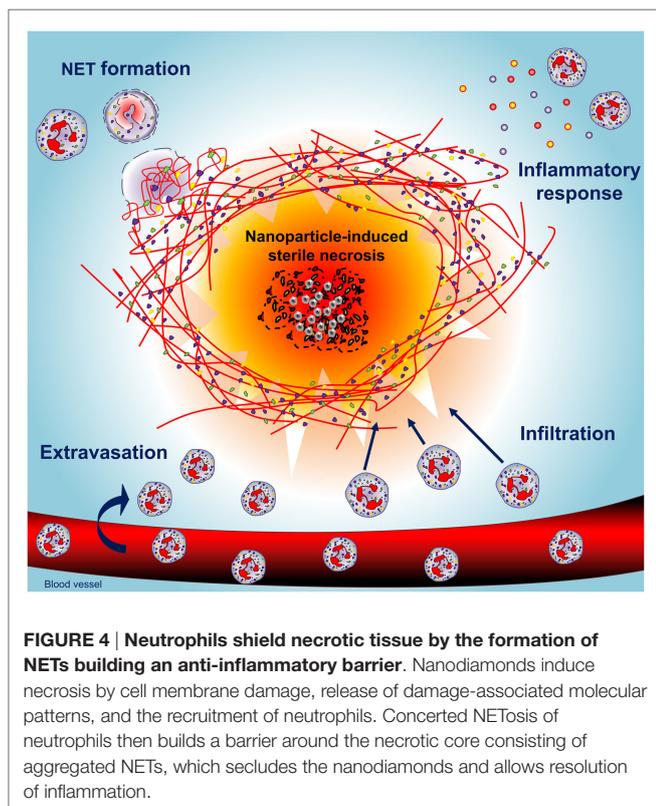
(Continued)



formation of large NET aggregates is notably reduced in the presence of the ROS scavenger *N*-acetyl L-cysteine. Consistently, DNase I-treated mice showed prolonged paw edema due to NET degradation. However, the effect of DNase I was not sufficient to induce chronification of nanodiamond-induced inflammation. Macroscopical analyses revealed that the diamonds were not enzymatically degraded, as expected, nor cleared from the tissue and remained at the site of injection. In contrast, we observed granulation-like tissue surrounding nanodiamonds exclusively in WT animals but not in ROS-deficient *Ncf1*^{**} mice. Unfortunately, immunohistochemical evaluation of the whole paw could not be done, since diamonds precluded sectioning of the tissue samples. However, histological analyses of adjacent skin tissue collected at day 28 revealed tight packing of nanodiamonds in WT mice and anatomical association of nanodiamonds with neutrophil markers. Contrarily, *Ncf1*^{**} mice lacking oxidative burst-dependent

NETosis were not able to encapsulate nanodiamonds and therefore the inflammatory trigger remained bare. These observations support the hypothesis that neutrophils contribute to the entrapping and isolation of particulate matter by NETosis.

Recently, we reported that the formation of aggregated NETs is implicated in the resolution of inflammation in patients with gout (20). NETs enclose MSU crystals and form tophi. This amorphous material can be clinically silent for a long time. Thus, the acute inflammation caused by MSU crystals is ameliorated if they are entrapped in large NET aggregates. This reaction represents the basis of granuloma formation in patients with gout (15). Similar mechanisms may operate during the infection with *Mycobacterium tuberculosis*, a condition characterized by massive granuloma formation (47). Thus, the formation of granuloma during an inflammatory process can be considered a mechanism to terminate excessive inflammation.



Necrotic injury is usually accompanied by massive neutrophil infiltration. Depending on the affected organ, it may result in acute life-threatening conditions (4). Neutrophil infiltration is the key initial process of the inflammatory response to sterile necrosis (48). We observed that the concurrence of necrotic cells and NETosis leads to the entrapment of the dead cells. A recent report on NETosis induced by apoptotic cells *in vitro* also supports the link between cell death and NETosis (49).

In summary, we conclude that aggregated NETs contribute to the resolution of sterile inflammation induced by nanoparticle-mediated cell necrosis. Neutrophils, recruited to sites of nanoparticle-induced cell death, undergo NETosis and form NET aggregates segregating the damaged area from the surrounding viable tissue (Figure 4). The initial inflammatory response may involve several cytotoxic mechanisms including ROS-independent NETosis. However, in the course of inflammation, the absence of ROS-dependent NETosis leads to a chronic inflammatory response. NET aggregates therefore essentially contribute to the termination of the inflammatory response.

MATERIALS AND METHODS

Preparation of Human Material

All analyses of human material were performed in full agreement with institutional guidelines and with the approval of the Ethical committee of the University Hospital Erlangen (permit # 193 13B). Human peripheral PMN and PBMC were isolated from heparinized (20 U/ml) venous blood of normal healthy donors (NHD)

by Lymphoflot (Bio-Rad, Hercules, CA, USA) density gradient centrifugation as described elsewhere (50). Briefly, whole blood was carefully pipetted on Lymphoflot solution and centrifuged for 30 min at 1400 rpm. Then, the plasma was carefully removed and the PBMC layer was collected. The PMN-rich layer on top of RBC was taken and subjected to hypotonic lysis of RBC. Cell viability was assessed by trypan blue exclusion.

Mice

Ncf1^{**} mice, harboring a single-nucleotide polymorphism in the gene for the regulatory p47phox subunit (*Ncf1*) of the NADPH oxidase NOX2 (51, 52), originate from The Jackson Laboratories and were backcrossed over more than 10 generations to the BALB/c background and maintained at the animal facilities of the University of Erlangen. The animal studies were approved by the Veterinary Office of the Government of lower Franconia (permit # 55.2 DMS-2532-2-103) and conducted according to the guidelines of the Federation of European Laboratory Animal Science Associations (FELASA). Genotyping of *Ncf1*^{**} and WT littermates was done by pyrosequencing, as described (53).

Characterization and Preparation of Diamond Particles

Transmission electron microscopy (TEM) images were recorded on a JEOL JEM-2011 electron microscope operated at an accelerating voltage of 200 kV. XPS measurements were performed with an ESCALAB 220 XL spectrometer from Vacuum Generators featuring a monochromatic Al K α X-ray source (1486.6 eV) and a spherical energy analyzer operated in the CAE (constant analyzer energy) mode (CAE = 100 eV for survey spectra and CAE = 40 eV for high-resolution spectra), using the electromagnetic lens mode. No flood gun source was needed due to the conducting character of the substrates. The angle between the incident X-rays and the analyzer is 58°. The detection angle of the photoelectrons is 30°. Zeta (ζ) potential measurements were performed with a Zetasizer Nano ZS (Malvern Instruments S.A., Worcestershire, UK). The pH of all the samples was maintained at ~7.4. Micro-Raman spectroscopy measurements were performed on a Horiba Jobin Yvon LabRam HR micro-Raman system combined with a 473 nm (1 mW) laser diode as excitation source. Visible light is focused by a 100 \times objective. The scattered light is collected by the same objective in backscattering configuration, dispersed by a 1800-mm focal length monochromator and detected by a CCD camera. In order to exclude endotoxin contamination, diamonds were treated with NaOH, dried out in ethanol, and treated with 300°C heat before used in cultures and animal experiments.

Nanoparticle-Induced Paw Swelling

We injected 1 mg of 10 or 1000 nm diamonds in 70 μ l 0.9% sterile NaCl solution into the metatarsal region of the hind paws of *Ncf1*^{**} and WT mice. The contralateral paw was injected with 70 μ l 0.9% NaCl solution serving as control treatment. In order to assess the role of NETs in the resolution of nanoparticle-induced inflammation, we injected 1 mg of 10 nm diamonds in 70 μ l 0.9% sterile NaCl solution containing 200 μ g DNase I (Sigma-Aldrich)

into the metatarsal region of the hind paws of WT mice. The contralateral paw was injected with 70 μ l 0.9% NaCl solution containing 200 μ g DNase I serving as control treatment. Additionally, 500 μ g DNase I was injected intravenously 24 and 48 h after particle injection. To monitor paw edema as a sign of inflammation, foot pads were measured with an electronic caliper at the indicated time points. On day 28, mice were sacrificed by CO₂ aspiration. Some mice were sacrificed at day 15 and hind paws were fixed in 4% paraformaldehyde then transferred to 70% ethanol for dissection.

Isolation of Bone Marrow Cells

Bone marrows of femurs and tibiae of BALB/c WT and *Ncf1*^{**} mice were flushed out with PBS and RBC were lysed using Tris-buffered (0.15 M) ammonium chloride (0.16 M) adjusted to pH 1.65.

Plate Reader-Based Quantification of DNA Accessibility

Freshly isolated human PMN and PBMCs or murine bone marrow cells were adjusted to a concentration of 2×10^6 cells/ml in HBSS containing 5.55 mM glucose, 1.2 mM calcium, and 0.5 mM magnesium (Thermo Fisher Scientific) and plated in 96-well plates (Greiner Bio-One, Frickenhausen, Germany) in a final cell density of 1×10^6 cells/ml. The DNA dye SYTOX Green (Thermo Fisher Scientific) was added in a final concentration of 2.5 μ M. Diamonds of 10 and 1000 nm were added in a final concentration of 200 μ g/ml or as indicated. The traditional NETosis-inducing stimuli ionomycin (InvivoGen, San Diego, CA, USA) or PMA (Sigma, Darmstadt, Germany) were added in final concentrations of 1 μ g/ml or 100 ng/ml, respectively. Plates containing PMA, ionomycin, 10 or 1000 nm diamonds in 100 μ l of HBSS, respectively, were incubated at 37°C and 5% CO₂ prior to addition of cells to equilibrate pH. Living cells were added and plates were covered. DNA externalization was analyzed for 210 min. on an Infinite[®] 200 PRO plate reader (TECAN, Crailshaim, Germany) under controlled temperature. Excitation was performed at 485 nm and emission was detected at 535 nm. Values displayed in the graphs were normalized according to absorption or addition of fluorescence by 10 or 1000 nm diamonds, respectively.

NETosis Assay for Subsequent Immunofluorescence Staining

For microscopic analysis, 200,000 per well freshly isolated PMN or PBMC or 600,000 murine bone marrow cells per well were seeded in RPMI (Thermo Fisher Scientific, Waltham, MA, USA) in LabTek2 Chamber Slides (Thermo Fisher Scientific, Waltham, MA, USA). After addition of 200 μ g/ml, 10 or 1000 nm diamonds (Sigma-Aldrich, St. Louis, MO, USA), 10 μ g/ml PMA (Sigma-Aldrich, St. Louis, MO, USA), or 1 μ g/ml ionomycin (Sigma-Aldrich, St. Louis, MO, USA) cells were incubated for 4 h at 37°C and 5% CO₂. For experiments using the ROS scavenger *N*-acetyl L-cysteine (NAC) (Sigma-Aldrich, St. Louis, MO, USA), PMN were pre-incubated for 30 min at 37°C and 5% CO₂ with 5 mM NAC for 30 min before addition of 200 μ g/ml 10 nm nanodiamonds and incubation for 4 h at 37°C and 5%

CO₂. For experiments with CFSE-labeled cells, freshly isolated PBMC were labeled with CellTrace™ CFSE (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Then, cells were fixed with 0.1% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) for 15 min and stained, as described below.

Histology and Immunofluorescence Staining

For analysis of murine tissue samples, histology was performed using paraffin embedding. Tissue samples were fixed overnight in 4% formalin, dehydrated with ethanol, and subsequently embedded in paraffin. This was followed by immunofluorescence staining. Chamber slides and deparaffinized sections were blocked for 18 h at 4°C with PBS containing 10% FBS (Merck Millipore, Billerica, Waltham, MA, USA). Primary antibodies detecting NE (ab21595, Abcam, Cambridge, UK) or citH3 (ab5103, Abcam, Cambridge, UK) were added to the slides in a 1:200 dilution and incubated for 1.5 h at room temperature. This was followed by incubation with the Cy5-conjugated secondary detection antibody AffiniPure Goat Anti-Rabbit IgG (H + L) (Jackson Immuno Research Labs, West Grove, PA, USA) in a dilution 1:400 for 1 h at room temperature in the dark together with Hoechst 33342 (Thermo Fisher Scientific, Waltham, MA, USA). Slides were washed with PBS and H₂O and samples were embedded in DAKO fluorescent mounting medium (Agilent Technologies, Santa Clara, CA, USA). Slides were analyzed either using the Eclipse Ni-U (Nikon Corporation, Tokyo, Japan) or the BZ-X700 microscope (Keyence Corporation, Osaka, Japan). Z-stacks were performed to increase depth of field. Post-processing of pictures was performed using Photoshop CS5 (Adobe, München, Germany).

Immunofluorescence Quantification of Samples Containing Diamond Particles

The 200,000 PMN or PBMC or murine bone marrow cells resuspended in RPMI were seeded in poly L-lysine (Sigma-Aldrich, St. Louis, MO, USA)-coated 96-well flat bottom cell culture plates. Cells were then incubated with 200 μ g/ml 10 or 1000 nm diamonds, 10 μ g/ml PMA (Sigma-Aldrich, St. Louis, MO, USA), or 1 μ g/ml ionomycin (Sigma-Aldrich, St. Louis, MO, USA) for 4 h at 37°C, and 5% CO₂. After incubation, DNA was stained with the membrane-impermeable DNA dye DRAQ7 (BioLegend, San Diego, CA, USA) at room temperature for 15 min. After centrifugation for 5 min at 1800 rpm, samples were measured using the near-infrared fluorescence imaging system Odyssey[®] CLx Imaging System (LI-COR, Lincoln, NE, USA) at 700 nm. After DNA measurement, cells were fixed with 0.1% paraformaldehyde for 15 min and blocked for 18 h at 4°C in PBS containing 10% FBS. Samples were incubated with the primary antibody recognizing citH3 for 1.5 h at room temperature, followed by incubation with the secondary goat anti-rabbit detection antibody IRDye[®] 800 CW (LI-COR, Lincoln, NE, USA) for 1 h at room temperature. After centrifugation for 5 min at 1800 rpm, samples were measured with the Odyssey[®] CLx Imaging System at 800 nm. Post-processing of pictures and quantification of the signals was performed using Photoshop CS5 (Adobe, München, Germany).

Live Cell Imaging

Isolated PMN and PBMC were adjusted to a concentration of 1×10^6 cells/ml in RPMI. Cell suspensions were added to an 8-well Nunc chamber slide (VWR, Darmstadt, Germany). Chamber slides were pre-incubated at 37°C at least 30 min prior to addition of 200 μ l of 10 or 1000 nm diamonds with a concentration of 200 μ g/ml each. Staining solution containing 0.1 μ g/ml Hoechst 33342 and 1 μ g/ml PI in PBS was added shortly before addition of the diamonds. Slides were analyzed on a BZ-X710 microscope (Keyence, Neu-Isenburg, Germany). Z-stacks were performed to increase depth of field. Post-processing of pictures was performed with Photoshop CS5 (Adobe, München, Germany).

Statistics

Results are represented as the mean \pm SEM of the indicated number of replicates/experiments. We performed computations and created charts using the GraphPad Prism 5.03 software. For calculation of statistical differences among the groups, we used ANOVA test with Bonferroni *post hoc* correction, where applicable. Adjusted *p*-values <0.05 were considered to be statistically significant.

AUTHOR CONTRIBUTIONS

MB, MP, and JK planned and performed most of the *in vitro* and *in vivo* experiments, conducted data analysis, and wrote the manuscript. CR, DK, DW, CM, JH, and RoBi performed *in vivo* and *in vitro* experiments and conducted data analyses. AB, RaBo, and SS performed micro-Raman spectroscopy, X-ray photoelectron spectroscopy, and transmission electron microscopy analyses. GS and MaHo provided scientific input and wrote the manuscript. LM and MaHe supervised the project, planned and conducted experiments, data analysis, and wrote the manuscript. All the authors read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The supplementary material for this article can be found online at <http://journal.frontiersin.org/article/10.3389/fimmu.2016.00557/full#supplementary-material>.

FIGURE S1 | (A) Raman spectra of used nanodiamonds. The spectra representing the features of 10 nm nanodiamonds (red) and 1000 nm microdiamonds (black) showing a characteristic peak at 1336/cm; **(B)** nanodiamonds are visualized using transmission electron microscopy (TEM) and high-resolution TEM (HRTEM). Lattice fringe spacing is depicted in angstrom **(A)** in the HRTEM picture. **(C)** Frequency of CD45⁺ cells in bone marrow preparations of WT (*n* = 2) and *Ncf1*^{**} (*n* = 3) mice. Data are shown as means \pm SEM. **(D)** Frequency of CD11b⁺ Ly6Cint cells in the population of CD45⁺ bone marrow cells. **(E)** Microscopic analysis of bone marrow cells of WT mice incubated with nanodiamonds (10 nm) or microdiamonds (1000 nm) stained for DNA (Hoechst33342), neutrophil elastase (NE), or citH3 at a low magnification. Diamonds are visible in differential interference contrast (DIC). Cy5 fluorescence was artificially colored green **(F)** Microscopic analysis of bone marrow cells of *Ncf1*^{**} mice incubated with of nanodiamonds (10 nm) or microdiamonds (1000 nm) stained for DNA (Hoechst33342), NE, or citH3 at a low magnification. Diamonds are visible in DIC. Cy5 fluorescence was artificially colored green.

VIDEO S1 | Time-lapsed fluorescence microscopy of polymorphonuclear leukocytes (PMN) and nanodiamonds. Freshly isolated PMN (blue intracellular nuclei, Hoechst 33342) incubated with nanodiamonds undergo rapid rupture of the plasma membrane and subsequent release of DNA (red extracellular DNA, propidium iodide).

VIDEO S2 | Time-lapsed fluorescence microscopy of peripheral blood mononuclear cells (PBMC) and nanodiamonds. Freshly isolated PBMC incubated with nanodiamonds retain nuclear morphology (blue intracellular nuclei, Hoechst 33342) and undergo rapid rupture of the plasma membrane exposing DNA to the propidium iodide dye (red intracellular).

VIDEO S3 | Time-lapsed fluorescence microscopy of polymorphonuclear leukocytes (PMN) and microdiamonds. Freshly isolated PMN incubated with microdiamonds retain nuclear morphology (blue intracellular nuclei, Hoechst 33342) and do not release DNA to the extracellular space (propidium iodide).

VIDEO S4 | Time-lapsed fluorescence microscopy of peripheral blood mononuclear cells (PBMC) and microdiamonds. Freshly isolated PBMC incubated with microdiamonds retain nuclear morphology (blue intracellular nuclei, Hoechst 33342) and do not release DNA to the extracellular space (propidium iodide).

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Neutrophil Extracellular Traps Form a Barrier between Necrotic and Viable Areas in Acute Abdominal Inflammation

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Neutrophils form neutrophil extracellular traps (NETs) of decondensed DNA and histones that trap and immobilize particulate matter and microbial pathogens like bacteria. NET aggregates reportedly surround and isolate large objects like monosodium urate crystals, which cannot be sufficiently cleared from tissues. In the setting of acute necrotizing pancreatitis, massive tissue necrosis occurs, which is organized as pancreatic pseudocysts (1). In contrast to regular cysts, these pseudocysts are not surrounded by epithelial layers. We hypothesize that, instead, the necrotic areas observed in necrotizing pancreatitis are isolated from the surrounding healthy tissues by aggregated NETs. These may form an alternative, putatively transient barrier, separating necrotic areas from viable tissue. To test this hypothesis, we investigated histological samples from the necropsy material of internal organs of two patients with necrotizing pancreatitis and peritonitis accompanied by multiple organ failure. Tissues including the inflammatory zone were stained with hematoxylin and eosin and evaluated for signs of inflammation. Infiltrating neutrophils and NETs were detected by immunohistochemistry for DNA, neutrophil elastase (NE), and citrullinated histone H3. Interestingly, in severely affected areas of pancreatic necrosis or peritonitis, chromatin stained positive for NE and citrullinated histone H3, and may, therefore, be considered NET-derived. These NET structures formed a layer, which separated the necrotic core from the areas of viable tissue remains. A condensed layer of aggregated NETs, thus, spatially shields and isolates the site of necrosis, thereby limiting the spread of necrosis-associated proinflammatory mediators. We propose that necrotic debris may initiate and/or facilitate the formation of the NET-based surrogate barrier.

Keywords: neutrophils, sepsis, inflammation, neutrophil extracellular traps, neutrophil elastase

INTRODUCTION

Neutrophils are known to produce neutrophil extracellular traps (NETs) of decondensed DNA and histones that trap and immobilize microbial pathogens, e.g., bacteria/fungi (2) or small inert nanoparticles (3). Recent reports on NETosis demonstrate its role in immobilization and sequestering (isolating) foreign objects, which can otherwise not be cleared from the body [e.g., monosodium

urate (MSU) crystals during gout (4) or hydrophobic nanoparticles]. Whereas neutrophil extravasation plays an important role in mediating immune response (5) and was reportedly related to inflammatory insults of the invaded tissues (6), it has recently been reported that neutrophils patrol several body tissues that are not inflamed (7), protecting internal organs, exocrine glands, and their ducts from invading intestinal bacteria (8).

In acute necrotizing pancreatitis, massive tissue necrosis occurs in the abdominal cavity. The massive release of intracellular mediators of pancreatic acinar cells determines the degree of systemic toxicity and consequent multi-organ failure and lethality (9). Therefore, immunological mechanisms need to prevent the further spread of necrosis-derived mediators. Formation of NETs was reported in the pancreata and in blood of mice with acute pancreatitis (10). Pancreatic necrosis present early as acute necrotic collections and are consequently organized and separated from adjacent healthy tissues by granulation tissue ("walled-off necrosis") (11). Even in less severe cases of acute edematous pancreatitis, inflammatory barrier formation may occur and may lead to the formation of pancreatic pseudocysts. In contrast to regular cysts, pseudocysts are not surrounded by an epithelial layer. Thus, we hypothesize that the acute necrotic areas often observed in massive abdominal inflammation are isolated from surrounding healthy tissues by aggregated NETs. These may form a provisional tissue barrier separating necrotic areas from remains of viable tissue. To check this possible involvement, we analyzed a series of necropsy samples of patients of Lviv Regional Clinical Hospital, who have died from multi-organ failure and systemic toxicity of massive inflammation in the abdominal region.

CASE PRESENTATIONS

Patient I. A 40-year-old man with a history of 5 days alcohol abuse and severe epigastric abdominal pain radiating into the back, with suspect of pancreatitis was hospitalized in December 2014. Upon hospitalization, blood pressure of 110/80 mmHg, heart rate of 152 beats/min (90, hereinafter normal values are represented in square brackets), body temperature of 38.7°C, and respiration rate of 24/min (<20) were recorded. The clinical picture was compatible with systemic inflammatory response syndrome (SIRS). Physical examination revealed that the abdomen was mildly distended with tenderness over the epigastric area. Routine laboratory investigations revealed increased white blood cell count of $14.0 \times 10^9/L$ (< $9.0 \times 10^9/L$), as well as levels of pancreatic lipase & amylase, total bilirubin, aspartate transaminase, alanine transaminase, and creatinine, indicating a biliary origin of the pancreatitis and impaired renal function. Levels of procalcitonin and C-reactive protein were increased [1.56 ng/mL (<0.5 ng/mL) and 306.8 mg/L (10 g/L), correspondingly]. A CT scan of the abdomen was performed at day 2 to evaluate the morphological extent of acute pancreatitis (**Figure 1**). It revealed destructive severe hemorrhagic pancreatitis with signs of necrosis at the body of the pancreas, a necrotic-hemorrhagic mass in the abdomen, ascites, and reactive retroperitoneal lymphadenopathy. Head (49 mm) and tail (39 mm) of the pancreas were clearly differentiated, with no visible pathological inclusions. The patient was admitted to the intensive care unit and managed with aggressive

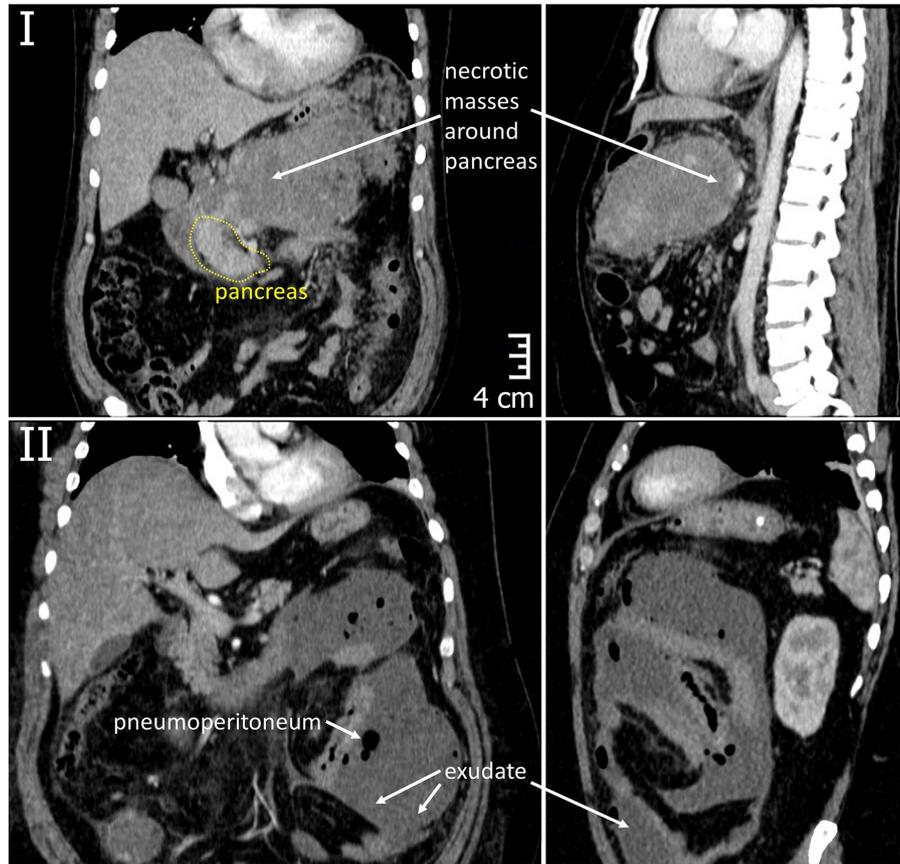
fluid resuscitation and broad-spectrum antibiotics. The patient displayed daily fever. Seven days after hospitalization, the patient underwent laparotomy, necrosectomy, and peripancreatic drainage. Seven hours after surgery, the patient died despite all measures of intensive care due to multiple organ failure.

Patient II. A 50-year-old man was hospitalized in March 2015 with acute pancreatitis. The patient had suffered from pain and dyspepsia for 10 days before hospitalization. Upon hospitalization, blood pressure of 90/60 mmHg, heart rate of 130 beats/min (<90 beats/min), body temperature of 39.1°C, and respiration rate of 22/min (<20) were recorded. Patient displayed nausea and appetite loss, impaired renal function with creatinine of 192 mM/L (<106 mM/L), WBC count of $14.3 \times 10^9/L$ (< $9.0 \times 10^9/L$), and highly elevated procalcitonin level of 16.24 ng/mL (<0.5 ng/mL). Ultrasonic examination and blood test for lipase and amylase activity confirmed the diagnosis of acute pancreatitis. The patient also suffered from obesity, diabetes mellitus (glucose level of 17 mM/L on the hospitalization decreasing to 7.7 mM/L upon treatments) and fatty liver disease, with aspartate transaminase level of 68.8 U/L (<31 U/L) and alanine transaminase level of 46.5 U/L (<41 U/L). Thirteen days after hospitalization, the peritoneal cavity contained a leukocytic exudate. A CT scan was made at that time (**Figure 1**) indicating pneumoperitoneum and inflammatory masses in the pancreatic region. Laparotomy was performed to achieve necrosectomy and drainage, in 1 day (day 21 after hospitalization), intestinal content was recovered from abdominal drainages, which called for repeated surgical interventions (detailed at **Figure 1**) and massive peritoneal adhesion. Pneumonia have developed at 6th week after hospitalization, causing respiratory failure (day 48). Despite intensive care therapy, multiple organ failure progressed and resulted in the patient's death at day 51.

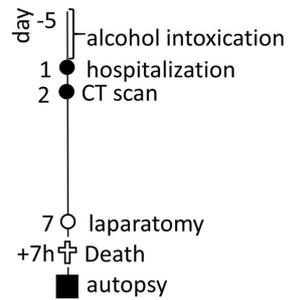
RESULTS

Necropsy samples of internal organs (19 samples of Patient I and 24 samples of Patient II) were analyzed by hematoxylin and eosin (H&E) staining to reveal areas of the interface between intact and necrotizing tissue. Seven of them were selected and additionally stained for neutrophil elastase (NE) expression. From these samples, we have selected for further detailed analysis the sample of tissue of Patient I being the part on the pancreas in the interface between normal tissue and necrosis area (**Figure 2**) and of Patient II being the part of peritoneum at the interface of healthy and necrotic areas (**Figure 3**). Both samples were subjected to immunohistochemistry with antibodies against NE and citrullinated histone H3 (citH3) to reveal molecular markers attributable to neutrophils and NETs.

Hematoxylin and eosin staining of the pancreas of Patient I demonstrated that a part of the organ still displayed a regular morphology, while other areas were completely destroyed by necrosis. At the interface between viable and necrotic tissue, the typical histological structure of granulation tissue was to be observed (**Figures 2A,B**). The superposition of immune histochemistry images for neutrophil elastase staining with H&E staining revealed that the surface of granulation tissues [often referred to as surface leukocyte necrotic layer (12)] is highly positive



Patient I



Patient II

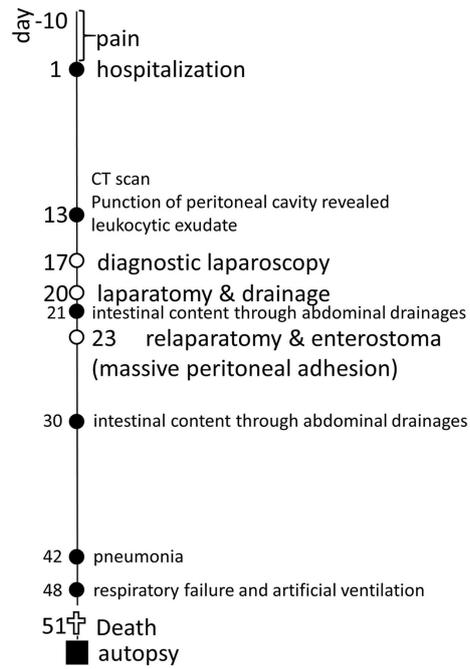


FIGURE 1 | Top – CT images of the abdominal region of Patients I and II demonstrating the areas of inflammation around pancreas (I) and in peritoneum (II). Bottom – graphical representation of disease courses of Patients I & II. Important events are indicated with filled circles, operations – open circles, time of sample collection – filled squares.

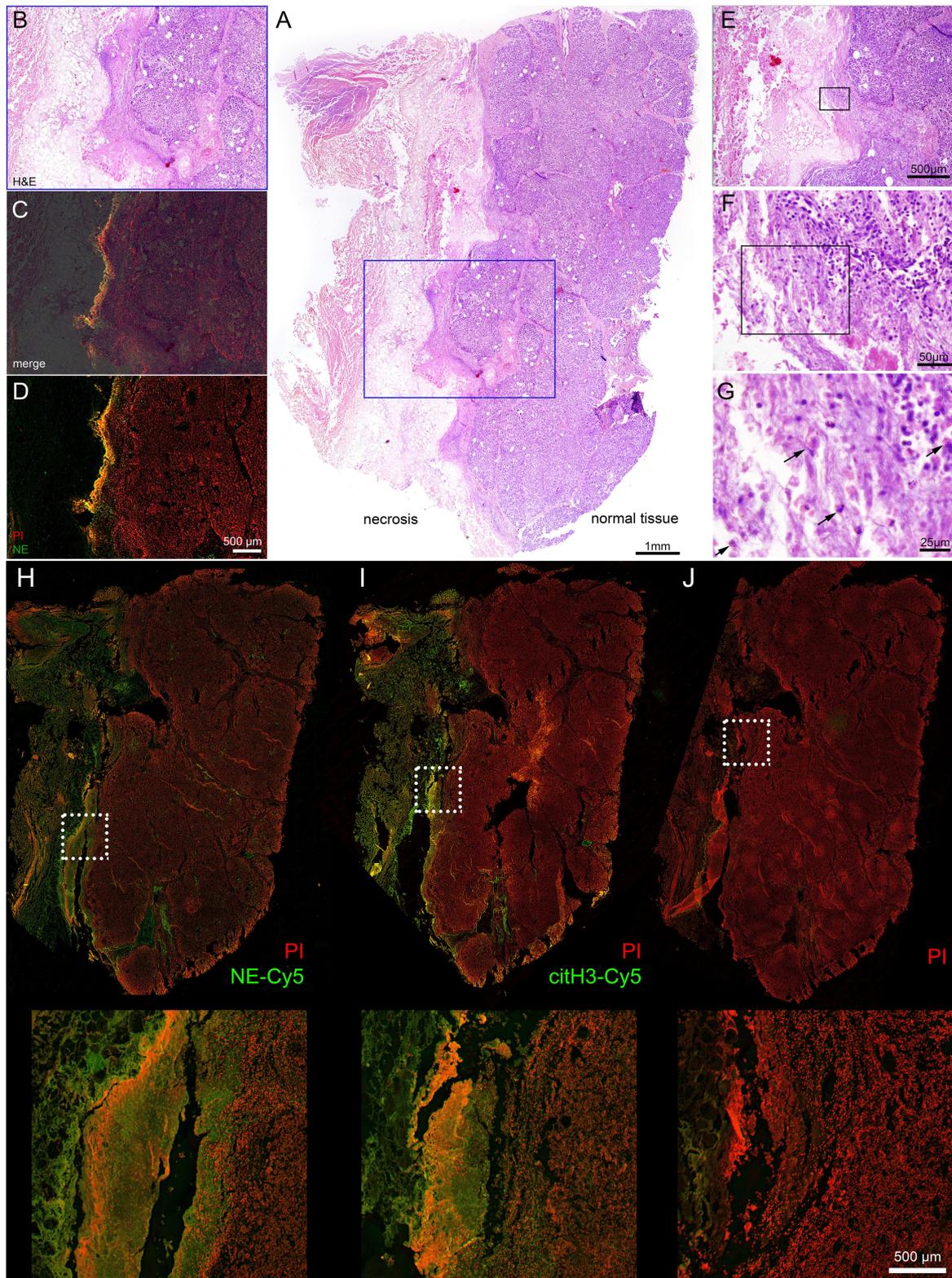


FIGURE 2 | Interface between normal tissue of the pancreatic gland and necrotic tissue in the area of inflammation, acute pancreatitis. Patient 1. (A) – overview (H&E) staining, (B–D) – details of region highlighted blue, (B,E,C) – merged NE-Cy5 (pseudo colored green) & PI with subsequent slide image stained with H&E, (D) – NE-Cy5 and PI, (E–G) – sequential details of the indicated areas. (H) – immunohistochemistry of histological slide close to (A), stained with NE-Cy5, (I) – immunohistochemistry with citH3-Cy5 and PI, (J) – secondary Ab and PI staining. The arrows in (G) point to cells with swollen nuclei.

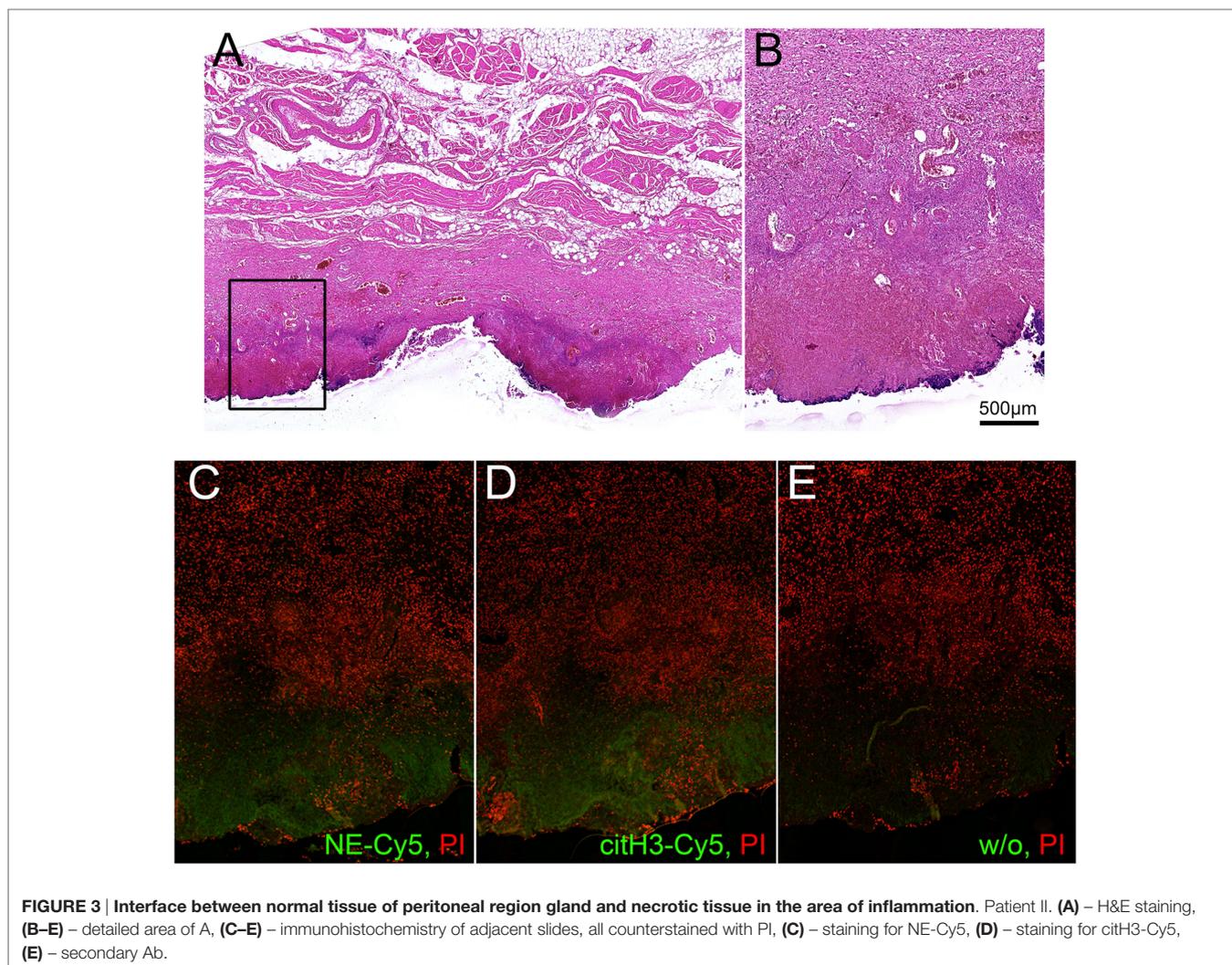


FIGURE 3 | Interface between normal tissue of peritoneal region gland and necrotic tissue in the area of inflammation. Patient II. (A) – H&E staining, (B–E) – detailed area of A, (C–E) – immunohistochemistry of adjacent slides, all counterstained with PI, (C) – staining for NE-Cy5, (D) – staining for citH3-Cy5, (E) – secondary Ab.

for NE deposits and DNA (detected by PI staining, detailed at **Figure 2J**); no regular cellular components were to be detected at this region (**Figures 2C,D**). Between the marginal zone and viable tissue, high resolution microscopy revealed numerous cellular infiltrates, with nucleus morphology being typical for those of neutrophils. Swelling and release on neutrophil content was also observed (**Figures 2E–G**). Immunohistochemistry revealed that the mentioned marginal zone was positive for both NE and citH3 signals, while high resolution microscopy demonstrated that areas covering the granulation tissue and displaying neutrophil components were also devoid of cellular material but with abundant amounts of extracellular DNA, judged by the PI signal (**Figures 2H–J**). High magnification images of this areas are shown on Figure S1 in Supplementary Material, demonstrating different stages of NETosis progression. Thus, these barrier-forming structures possessed typical features of NETs.

Histological analysis of the selected tissue from Patient II also demonstrated the formation of a clear separation zone between the viable tissue of the peritoneal region, mainly composed of muscular fibers and connective tissue, and the zone of necrosis (**Figures 3A,B**). Deposition of NE and citH3 were clearly seen in

this marginal zone followed by the adjacent zone infiltrated with leukocytes that preserved their viable morphology (**Figures 2C–E**).

DISCUSSION AND CONCLUDING REMARKS

Evaluating our data, we propose that the formation of neutrophils extracellular traps serves to form a transient barrier that isolates necrotic tissues during acute inflammatory processes. NETs form in response to necrotic debris and provide a biological sink for damage-associated molecular patterns (DAMPs) by binding to the NET. Potentially harmful DAMPs are then degraded in the NET as previously shown for inflammatory cytokines *via* proteolytic digestion (4). Extracellular DNA and chromatin-associated proteins then contribute to coagulation and reinforce fibrin clots formed at the NET surface (13). This barrier may finally be transformed into fibrotic tissue by slowly migrating and proliferating fibroblasts (14). We have observed abundant NETosis surrounding necrotic areas in two clinical cases of pancreatitis and peritonitis, the abundance of NETosis in other clinical cases, and at different diseases still need further investigations.

The long-term consequences of abundant NETosis surrounding inflammation areas should be taken into account, as products of proteolytic digestion were abundant in pancreatic pseudocysts (15), and the ability of dying cells to modulate immune response was demonstrated (16). As except from cytokine-degrading ability, neutrophil-released enzymes can also possess an ability to modify glycan on the surrounding tissues (17). At the same time, the connection between modification of glycans of IgG molecules and disease activity of lupus was shown (18). Inappropriate clearance of DNA-released material is leading to autoimmune responses, thus connecting NETosis and autoimmunity (19). Abundant production of ROS during neutrophils-induced inflammation is important for the destruction of pathogens, but high levels of ROS in case of massive neutrophil involvement can lead to oxidative stress with ensuing cell death and necrosis (20). Recently, the molecular mechanism of NETosis in response to action to crystals was revealed and shown to include RIPK1-RIPK3-MLKL signaling (21), the involvement of Raf-MEK-ERK pathway was also demonstrated (22); however, it is difficult to check the specific mechanism in the described cases due to massive necrosis and associated release of cellular factors.

Different stages of NETosis, such as early stage of NETosis, progression of NETosis, and aggregated NETs were revealed at histological sections of interfaces between viable and necrotic tissues, as depicted on Figure S2 in Supplementary Material. Staining for DNA (with PI) and for NE allowed the discrimination of those stages. To better understand the different types of cells death in relation to NETosis detection, we summarized the current knowledge of different types of cells death stained for DNA, NE and citH3 in **Table 1**.

Thus, the NETosis role in limiting the spread of necrotic tissues was demonstrated; however, the possible long-term consequences of such process are to be determined and can be connected with frequent complications in patients who suffered from acute inflammation of internal organs.

MATERIALS AND METHODS

Human Samples

Human tissue samples in the form of formalin-fixed paraffin-embedded blocks, obtained as a post mortem biopsies of patients with acute systemic inflammation (sepsis), was obtained from Main Pathoanatomical Laboratory and PathoAnatomical Archive of Danylo Halytsky Lviv National Medical University

upon approval by the Ethics Council of the University. Tissue samples were fixed in formalin and then processed according to the routine H&E staining protocol.

Computed Tomography

The computed tomography was performed with a multidetector spiral computer tomograph “Aquilion” (Toshiba medical systems, Japan). Exposure doze was 6.0 mSv for Patient I and 5.0 mSv for Patient I. Contrast enhancement with iodine-containing compounds was applied in both cases.

Histology and Immunohistochemistry

Tissue samples in the form of formalin-fixed paraffin-embedded blocks were used. For general morphology studies, 5–7 μ m thick sections were stained with H&E. For immune histochemical analysis, paraffin sections were deparaffinized in toluene-isopropanol-water and incubated for 20 min at 90°C in citrate-containing antigen-retrieval solution. Slides were cooled to room temperature, rinsed in water (2 \times) and PBS and blocked with 20% of normal goat serum and 2% BSA solution in PBS for 1 h at RT, samples were washed with PBS, and incubated with primary antibodies dissolved in 10% normal goat serum and 2% BSA solution in PBS and incubated overnight at 4°C. The following antibodies were used: rabbit polyclonal antibody to neutrophil elastase (ab68672, Abcam), dilute 1:200 and rabbit polyclonal to histone H3 (citrulline R2 + R8 + R17) – ChIP Grade (ab5103, Abcam). Slides were washed 3 \times with PBS and incubated with Cy5 AffiniPure Goat Anti-Rabbit IgG (H + L), diluted 1:400 in 10% normal goat serum and 2% BSA solution in PBS and incubated for 1.5 h at 4°C. Slides were washed twice with PBS, counterstained with propidium iodine (2 μ g/mL), 10 min at RT, and covered with water soluble fluorescence mounting medium (DAKO).

Fluorescent Microscopy

Fluorescence microscopy was performed using Keyence bz-x700 robotized microscope using objective 20 \times NA0.75. Z-stitching was performed for the whole depth of the slide with combining images of maximal sharpness (usually 10 images with 1.0 μ m depth), XY-stitching was done for whole slide areas, generating composite image picture. Imaging of PI and Cy5 (pseudo colored green on the presented figures) was done using appropriate filter sets: for PI – OP-87764 with ex. 545/25, em. 605/70, dichroic 565 nm; for Cy5 OP-87766 with ex. 620/60, em. 700/75, dichroic

TABLE 1 | NE and DNA localization during different stages of cell death.

Type of cell death	DNA (PI)	NE	PI-NE colocalization	CitH3
Viable neutrophil	Nuclear	Granular kept inactive by SERPINA1 (23)	No	Few if any
Early NETosis	Cytoplasmic	Cytoplasmic	Yes	Enriched in decondensed chromatin
NETs	Externalized	Externalized bound to DNA in an active form	Yes	Decorating NETs; partially released from chromatin into vicinity of the NETs
Apoptotic cell	Fragmented nuclei	Granular	No	Few if any
Apoptotic body	Apoptotic body	Inside the body	No	Few if any
Secondary necrotic	Fragmented nuclei	Partially released (24)	No	Few if any
Cell remnant				
Primary necrotic	Nuclear	Released as granula with unknown activity	No	Few if any

660 nm. The use of abovementioned filter set allowed simultaneous staining with PI and Cy5 labels.

AUTHOR CONTRIBUTIONS

RB, VF, VV, ML, TD, and MH planned experiments and performed most of *in vivo* experiments, conducted data analysis, and wrote the manuscript. VF performed patient's treatment, operations, and sample collection, arranged, and conducted the CT analyses. VC and GS provided scientific input and wrote the manuscript. RB and MH supervised the project, planned and conducted experiments, data analysis, and wrote the manuscript. All authors read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at <http://journal.frontiersin.org/article/10.3389/fimmu.2016.00424>

FIGURE S1 | Interface between normal tissue of the pancreatic gland and necrotic tissue in the area of acute pancreatitis of Patient 1 demonstrating different stages of NETosis. Immunohistochemistry with staining for PI and NE-Cy5.

FIGURE S2 | Details from the interface area between viable and necrotic tissue of Patient 1, stained with NE & PI, demonstrating colocalization of NE and DNA during the progression of NETosis. 1–2, 3–4, 5–7, and 8 represent viable neutrophils, early stage of NETosis, progression of NETosis, and aggregated NETs, respectively.

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Conflict of Interest Statement: The 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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Deficient Neutrophil Extracellular Trap Formation in Patients Undergoing Bone Marrow Transplantation

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Overwhelming infection causes significant morbidity and mortality among patients treated with bone marrow transplantation (BMT) for primary immune deficiencies, syndromes of bone marrow failure, or cancer. The polymorphonuclear leukocyte (PMN; neutrophil) is the first responder to microbial invasion and acts within the innate immune system to contain and clear infections. PMNs contain, and possibly clear, infections in part by forming neutrophil extracellular traps (NETs). NETs are extensive lattices of extracellular DNA and decondensed chromatin decorated with antimicrobial proteins and degradative enzymes, such as histones, myeloperoxidase, and neutrophil elastase. They trap and contain microbes, including bacteria and fungi, and may directly affect extracellular microbial killing. Whether or not deficient NET formation contributes to the increased risk for overwhelming infection in patients undergoing BMT remains incompletely characterized, especially in the pediatric population. We examined NET formation *in vitro* in PMNs isolated from 24 patients who had undergone BMT for 13 different clinical indications. For these 24 study participants, the median age was 7 years. For 6 of the 24 patients, we examined NET formation by PMNs isolated from serial, peripheral blood samples drawn at three different clinical time points: pre-BMT, pre-engraftment, and post-engraftment. We found decreased NET formation by PMNs isolated from patients prior to BMT and during the pre-engraftment and post-engraftment phases, with decreased NET formation compared with healthy control PMNs detected even out to 199 days after their BMT. This decrease in NET formation after BMT did not result from neutrophil developmental immaturity as we demonstrated that >80% of the PMNs tested using flow cytometry expressed both CD10 and CD16 as markers of terminal differentiation along the neutrophilic lineage. These pilot study results mandate further exploration regarding the mechanisms or factors regulating NET formation by PMNs in patients at risk for overwhelming infection following BMT.

Keywords: bone marrow transplantation, neutrophil, neutrophil extracellular traps, infection, innate immune response

INTRODUCTION

The polymorphonuclear leukocyte (PMN; neutrophil) is the first circulating leukocyte to respond to tissue damage or infection and the primary effector cell of innate immunity and acute inflammation (1, 2). PMNs rapidly infiltrate areas of injury or infection where they participate in wound healing, infection containment, and microbial killing. Disorders associated with a deficiency or impairment of neutrophil number or function, such as neutropenia, chronic granulomatous disease (CGD), or leukocyte adhesion deficiency syndrome, predispose to severe bacterial and fungal infections (3) and are associated with significant morbidity and mortality (1). Novel neutrophil activities continue to be elucidated suggesting that PMNs influence all aspects of immunity (2).

Recently, neutrophils isolated from healthy adult donors were shown to undergo programmed cell death distinct from apoptosis and necrosis to form neutrophil extracellular traps (NETs) (4, 5). NETs are extensive lattices of extracellular DNA and decondensed chromatin decorated with antimicrobial proteins and degradative enzymes, such as myeloperoxidase and neutrophil elastase (NE). NETs affect extracellular killing of bacteria and fungi (6–9). We recently identified impaired NET formation as a novel innate immune deficiency in human newborn infants (10). PMNs isolated from the umbilical cord blood of newborn infants, whether born at term or prematurely, demonstrate impaired NET formation and extracellular bacterial killing in the first week of life as compared with PMNs isolated from healthy adults (10, 11). Thus, severe, early onset, neonatal infections may be associated with deficient PMN NET formation leading to impaired extracellular microbial containment and killing.

Pediatric and adult patients undergoing bone marrow transplant (BMT) also exhibit an increased risk for severe infection. A 2006 study found that 85% of cord blood transplant recipients and 69% of hematopoietic stem cell transplant recipients developed at least one severe infection within a 3-year median follow-up time (12). Such infections accounted for 59 and 61% of infection-related mortality for cord blood and peripheral blood stem cell transplant recipients, respectively (12). Posttransplant immune reconstitution is thought to mirror neonatal immune development, and the complicated process of immune system reconstitution continues over 1–2 years (13). However, the risk for microbial infection is greatest in the pre-engraftment period when patients are neutropenic (14). A previous study concluded that an unknown element of neutrophil dysfunction exists in patients after BMT which contributes to the high risk for infection (15). In addition, a recent report looked at NET formation following BMT in 12 adult patients and demonstrated a significant decrease in NET formation compared with healthy adult controls (16). We hypothesized that this increased risk for infection results, in part, from deficient NET formation by neutrophils produced by the nascent, peri-engraftment immune system and tested this hypothesis in a primarily pediatric population. We now show that NET formation by PMNs isolated from pediatric and

adult patients before and after BMT exhibit diminished NET formation *in vitro* and that this failure of NET formation is not simply the result of neutrophil immaturity.

MATERIALS AND METHODS

PMN Isolation

Polymorphonuclear leukocytes were isolated from EDTA anti-coagulated venous blood of 24 participants either undergoing BMT or after completion of BMT using a research protocol approved by the IRB at the University of Utah. Fourteen of the participants were <18 years old at the time of BMT (**Table 1**), and the median age for our study participants was 7 years. Eighteen of our study participants only had NET formation assessed after engraftment following BMT. Four of these participants had only qualitative assessment of NET formation using live cell imaging, while the additional 14 of these participants had NET formation assessed both qualitatively and quantitatively. For 6 of the 24 participants, we obtained serial, peripheral blood samples at three different time points: pre-BMT ($n = 3$), pre-engraftment ($n = 6$), and post-engraftment ($n = 6$) (**Table 1**). For all experiments, we performed assays of NET formation and granulocytic differentiation markers in parallel using PMNs isolated from the peripheral blood of healthy adults as positive controls for NET formation and PMN differentiation (10, 11, 17). In all, we collected peripheral blood from 24 adult donors as controls for the experiments outlined. These adult donors are described as healthy, fasting, medication free, and non-hospitalized, consenting control subjects aged ≥ 21 years who donate peripheral blood routinely under a University of Utah IRB approved protocol. No attempt was made to match control donors to study participants with regard to age, sex, or ethnicity. PMN suspensions (>96% pure) were prepared by positive immunoselection using anti-CD15-coated microbeads and an auto-MACS cell sorter (Miltenyi Biotec, Inc.) and were resuspended at 2×10^6 cells/mL concentration in serum-free M-199 media warmed at 37°C.

Live Cell Imaging of NET Formation

Qualitative assessment of NET formation was performed, as previously referenced (10, 17). Briefly, participant primary PMNs were incubated with control buffer or stimulated with LPS (100 ng/mL) for 2 h at 37°C in 5% CO₂/95% air on glass coverslips coated with poly-L-lysine. After stimulation, PMNs were gently washed with PBS and incubated with a mixture of cell permeable (Syto Green, Molecular Probes) and impermeable (Sytox Orange, Molecular Probes) DNA fluorescent dyes. Confocal microscopy was accomplished using a FV300 1X81 Microscope and the FluoView software (Olympus). Both 20 \times and 60 \times objectives were used. Z-series images were obtained at a step size of 1 μ m over a range of 20 μ m for each field. The Olympus FluoView software and the Adobe Photoshop CS2 software were used for image processing. Semiquantitative analysis of NET formation was accomplished using the ImageJ analysis software (NIH) and a standardized grid system with rigorous NET quantitation.

TABLE 1 | Patient characteristics according to age group.

Characteristics, n(%)	Age group (years)			
	Combined	0–10	11–30	≥31
n	24	15 (62.5%)	6 (25%)	3 (12.5%)
Gender				
Female	12 (50%)	9 (60%)	1 (17%)	2 (67%)
Male	12 (50%)	6 (40%)	5 (83%)	1 (33%)
Primary disease				
SCID	2 (8%)	2 (13%)	0 (0%)	0 (0%)
Relapsed ALL	6 (25%)	4 (27%)	2 (33%)	0 (0%)
Bilineal leukemia	2 (8%)	2 (13%)	0 (0%)	0 (0%)
Relapsed AML	1 (4%)	1 (7%)	0 (0%)	0 (0%)
Neuroblastoma	3 (13%)	3 (20%)	0 (0%)	0 (0%)
AML	3 (13%)	0 (0%)	2 (33%)	1 (33%)
MDS	1 (4%)	0 (0%)	0 (0%)	1 (33%)
AML/MDS	1 (4%)	1 (7%)	0 (0%)	0 (0%)
ALL	1 (4%)	0 (0%)	1 (17%)	0 (0%)
CLL	1 (4%)	0 (0%)	0 (0%)	1 (33%)
Hodgkins lymphoma	1 (4%)	0 (0%)	1 (17%)	0 (0%)
Anaplastic ependymoma	1 (4%)	1 (7%)	0 (0%)	0 (0%)
Aplastic anemia	1 (4%)	1 (7%)	0 (0%)	0 (0%)
HLA				
Matched, unrelated	10 (42%)	4 (27%)	4 (67%)	2 (67%)
PTD of PMN engraftment, mean	20.4	25.3	17.5	16.5
Matched, related	4 (17%)	3 (20%)	0 (0%)	1 (33%)
PTD of PMN engraftment, mean	23	25	–	17
Cord blood	5 (21%)	4 (27%)	1 (17%)	0 (0%)
PTD of PMN engraftment, mean	20.2	16.8	34	–
Autologous	5 (21%)	4 (27%)	1 (17%)	0 (0%)
PTD of PMN engraftment, mean	11	10.8	12	–
Survival (as of October 2014)				
Alive	16 (67%)	10 (67%)	3 (50%)	3 (100%)
Dead	8 (33%)	5 (33%)	3 (50%)	0 (0%)

PTD, posttransplant day.

Statistical comparisons were made *via* one way ANOVA with Tukey's multiple comparisons *post hoc* testing.

Quantitation of NET Formation – Supernatant Histone H3 Content

We determined supernatant total histone H3 content as a surrogate for NET formation, as previously referenced (17). After live cell imaging of control and stimulated primary PMNs (2×10^6 cells/mL; LPS 100 ng/mL), the cells were incubated with new media containing DNase (40 U/mL) for 15 min at room temperature to break down and release NETs formed in response to stimulation. The supernatant was gently removed and centrifuged at $420 \times g$ for 5 min. The cell-free supernatant was then mixed 3:1 with $4 \times$ Laemmli buffer prior to Western blotting. We used a polyclonal primary antibody against human histone H3 protein (Cell Signaling Technology) and infrared secondary antibodies (Li-Cor Biosciences). Imaging and densitometry were performed on the Odyssey™ infrared imaging system (Li-Cor Biosciences). Statistical comparisons were made *via* one way ANOVA with *post hoc* testing.

Assessment of PMN Differentiation

We assessed PMN differentiation through analysis of CD16 and CD10 protein surface expression on PMNs isolated from BMT patients using flow cytometry, as previously described (18). PMNs were isolated by positive immunoselection using anti-CD15-coated microbeads and an auto-MACS cell sorter (Miltenyi Biotec, Inc.) as described (10, 17, 18) and prepared for flow cytometry using FACS lysis buffer (Becton-Dickinson). PMN CD16 and CD10 cell surface protein expression was determined by incubating directly conjugated antibodies against human CD 16 (PE; 10 μ L/test) and human CD10- (FITC; 10 μ L/test) or isotype-matched control antibodies in the dark at 4°C for 30 min (All antibodies from Becton-Dickinson). FACS analysis was accomplished in the University of Utah Flow Cytometry Core using a Becton-Dickinson FACS Scan Analyzer and Flow-Jo analysis software (Version 9.7.6).

RESULTS

In this study, we assessed *in vitro* NET formation in response to LPS stimulation by PMNs isolated from 24 BMT patients with varying ages at and indications for BMT (Table 1). In 18 of these BMT patients, we were only able to assess post-engraftment NET formation (posttransplant day range: 14–199). For six BMT patients, however, we assessed peri-engraftment NET formation at the time of BMT, collecting pre-BMT, pre-engraftment, and post-engraftment PMN samples for analysis of NET formation. All six of these patients had received chemotherapy for malignancies prior to pre-BMT conditioning. Chemotherapeutic agents used for these patients included busulfan, carboplatin, etoposide, cyclophosphamide, daunorubicin, vincristine, cytarabine, and mitoxantrone. We did not obtain pre-BMT neutrophil samples from the two patients in this cohort undergoing BMT for severe combined immunodeficiency syndrome and, therefore, did not have pre-BMT chemotherapy. Consistent with a previous report (16), we demonstrated a significant decrease in NET formation by PMNs isolated both pre- and post-engraftment following BMT in response to LPS stimulation as compared with healthy adult control PMNs (Figures 1B–D). Furthermore, using semiquantitative image analysis, we demonstrated a statistically significant decrease in NET formation by LPS-stimulated PMNs isolated from study participants prior to BMT (Figure 1B). While our histone H3 release assay results did not show a correlative statistically significant decrease in NET formation by LPS-stimulated pre-BMT PMNs compared with healthy control PMNs (Figure 1C), an apparent trend toward decreased NET formation was detected (Figure 1C); this correlates with the qualitative and semiquantitative image analysis results (Figures 1A,B).

Of the PMNs isolated post-engraftment from 18 BMT patients, NET formation following LPS stimulation, while detectable, was decreased compared with healthy control PMNs (Figures 1A–C). Furthermore, a subset of patients analyzed showed decreased NET formation despite being 60–199 days removed from their BMT (not shown). This suggests that decreased NET formation may be an unrecognized aspect of PMN dysfunction after BMT that contributes to the prolonged risk for severe infection seen in these patients, which may last up to 3 years (13).

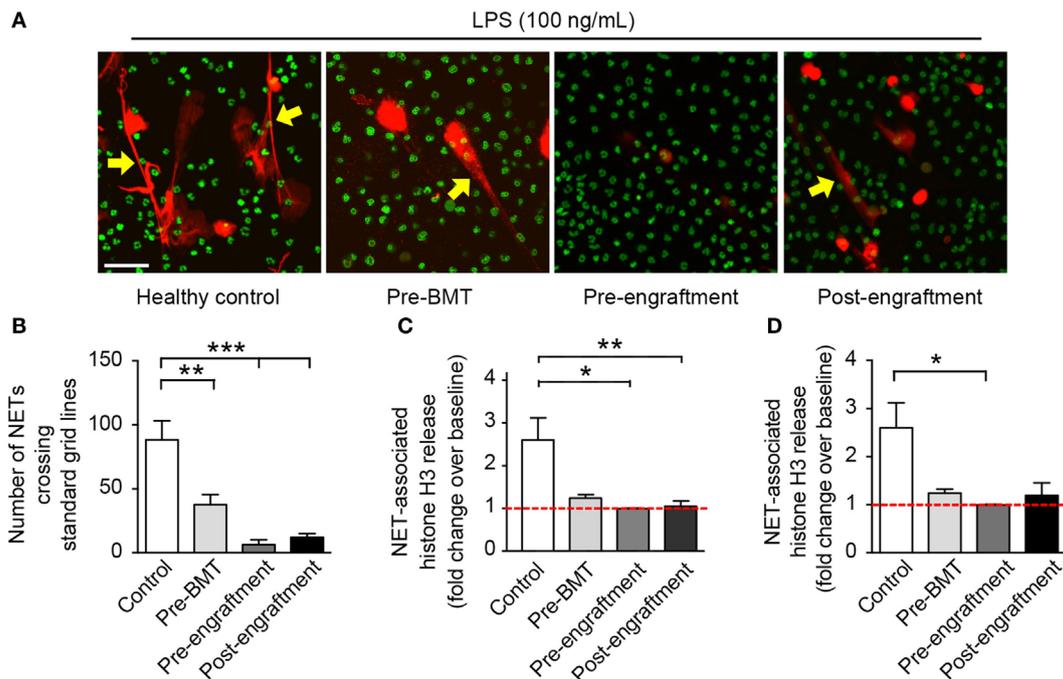


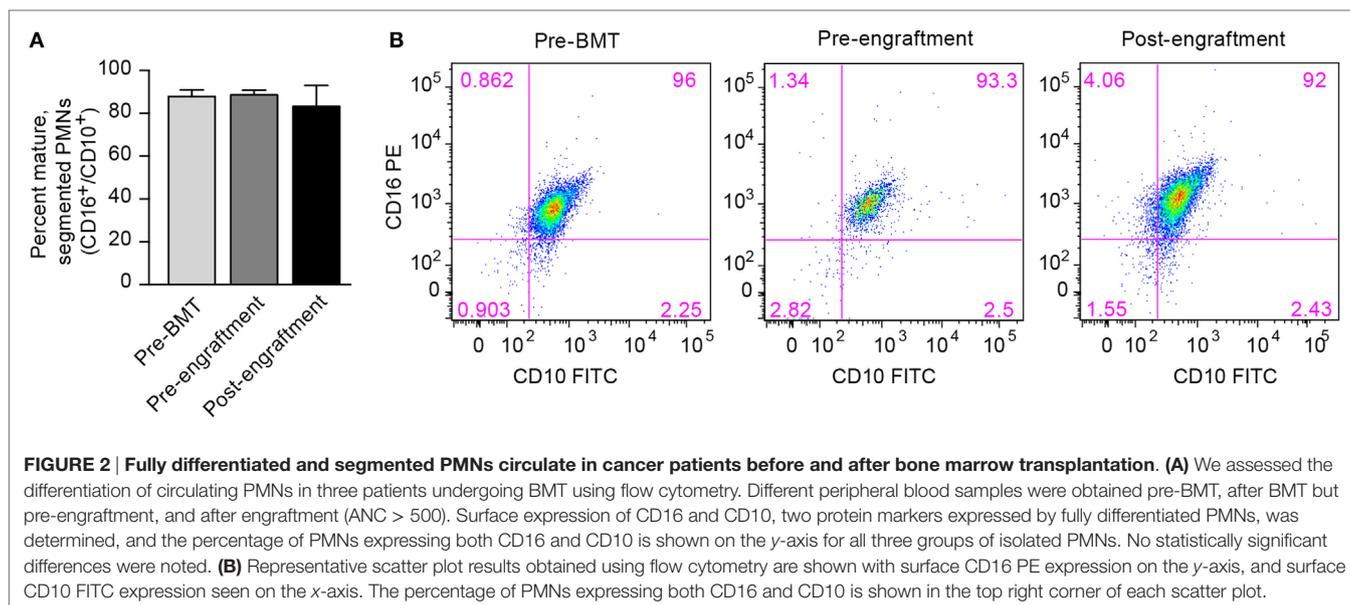
FIGURE 1 | PMNs isolated from patients at BMT demonstrate decreased NET formation following *in vitro* stimulation with LPS. We assessed NET formation by LPS-stimulated PMNs isolated from patients undergoing bone marrow transplant ($n = 3$ pre-BMT, $n = 6$ pre-engraftment, $n = 19$ post-engraftment) compared with control LPS-stimulated PMNs isolated from healthy adult donors. PMNs were stimulated with LPS (100 ng/mL; 1 h) and NET formation was determined qualitatively and quantitatively using (A) live cell imaging (60 \times magnification), (B) semiquantitative image analysis, and (C,D) release of histone H3 (fold change over baseline; mean \pm SEM). (A) NET-associated, extracellular DNA is shown in red fluorescence (yellow arrows). Nuclear DNA is shown in green fluorescence. (B) We analyzed NET formation in a semiquantitative manner using ImageJ analysis software and a standardized grid system for all captured live cell imaging results (20 \times magnification, $n > 6$ images analyzed per patient group). The y -axis depicts the number of times that NETs crossed the standardized grid lines (** $p < 0.01$ and *** $p < 0.001$). We employed a one way ANOVA statistical tool with Tukey's *post hoc* testing. (C) NET-associated histone H3 release is shown as fold change over baseline on the y -axis (* $p < 0.05$ and ** $p < 0.01$) compared with baseline (red dashed line), arbitrarily set at 1. We employed a one way ANOVA statistical tool with Tukey's *post hoc* testing. (D) Here, we reanalyze the NET-associated histone H3 release data for only these study participants from whom serial PMN samples were obtained ($n = 3$ pre-BMT, $n = 6$ pre-engraftment, $n = 6$ post-engraftment, and $n = 11$ controls). NET-associated histone H3 release is again shown as for (C) * $p < 0.05$ compared with baseline (red dashed line), arbitrarily set at 1. We employed a one way ANOVA statistical tool with Dunn's *post hoc* testing.

One possible cause for this prolonged NET deficiency following BMT is that a large proportion of neutrophils isolated pre-engraftment and right after engraftment may be developmental precursors of fully differentiated PMNs. Therefore, we assessed surface expression of the protein markers of PMN differentiation CD16 and CD10 by PMNs isolated from three BMT patients at the pre-BMT, pre-engraftment, and post-engraftment stages. Surface expression of CD16 and CD10 denotes differentiation of granulocytic precursors into fully differentiated, segmented PMNs (19). We found no differences in PMN differentiation between pre-BMT, pre-engraftment, and post-engraftment PMNs, and the PMN preparations from all three groups showed that >80% of the isolated PMNs expressed both CD16 and CD10 (Figure 2).

DISCUSSION

This brief report demonstrates that NET formation by PMNs isolated from both pediatric and adult patients following BMT is impaired in response to LPS stimulation and that this does not

result from incomplete differentiation of the PMNs circulating in these patients. These findings extend the literature as a confirmatory report of decreased NET formation in adult patients following BMT and are the first to show this potential immune deficiency following BMT in pediatric patients. A recent report by Domingo-Gonzalez et al. demonstrated a similar decrease in NET formation by PMNs isolated from BMT patients (16). Their study characterized phorbol-12-myristate (PMA)-induced NET formation by PMNs isolated from 12 patients undergoing BMT for indications, including various cancers and myelodysplastic disorders. These patients were all adults and the median participant age in their study was 59 years. In contrast, while we did include five adult patients in our study cohort, the median age for our study participants was 7 years. Another difference between our studies was the use of different agonists to induce NET formation. While PMA is a known, non-physiologic PMN stimulatory agonist and does induce NET formation (5), our use of LPS for NET induction more closely approximates the *in vivo* triggering by infectious agents.



Our results are particularly pertinent given the pronounced predisposition of BMT patients, both adult and pediatric, to severe infections, especially in the pre-engraftment period (13, 20). We acknowledge the low sample size for these investigations as a weakness of this pilot study, but note that this study is the largest to date to specifically examine NET formation after BMT in humans and the only one to examine NET formation before human BMT. An additional, potential weakness of this study is the failure to control for age by using age-matched controls in our assays of NET formation. While this represents a potential confounder in this study, we note that age-specific normal values for NET formation in both pediatric and adult populations have not been determined. To date, non-disease specific abnormalities in NET formation have only been characterized in the very young (10, 11) and very old (21). Another study weakness is our inability to examine the effects of autologous versus allogenic bone marrow reconstitution on NET formation following BMT. This very important question will be addressed by planned future studies where a larger number of autologous and allogenic BMT patients can be enrolled. In addition, we also point to the diversity in indications for and times after BMT as strengths of this report suggestive that deficiencies of NET formation in these patients may contribute to their increased risk for severe infection (20). Bacterial and fungal infections continue to cause significant morbidity and mortality in the pretransplantation, pre-engraftment, and post-engraftment periods, which together last through 100 days after BMT (13). While controversy exists regarding the extent of their direct antimicrobial activity, NETs clearly trap bacteria and fungi (22). This effect alone can lead to containment and clearance of an infectious agent, a finding confirmed in a murine model of necrotizing fasciitis with NET inhibition in PAD4 knockout mice (23). Correlative data in the human system also suggest a role for NET formation in clearance of microbes during severe infection. Bianchi et al. reported the use of gene therapy to

restore NET formation and to control refractory pulmonary aspergillosis in a patient with CGD (8, 9). These studies and our results suggest a need to examine NET formation prospectively in a larger cohort of BMT patients where the number, types (bacterial, fungal, and parasitic), and severity of infections may be correlated with indications for BMT, pre-BMT chemotherapeutic regimens, and aspects of neutrophil function in addition to NET formation.

Clearly, further studies are warranted to determine the mechanisms or factors leading to this deficit in NET formation after BMT. We have previously shown NET formation to be deficient in undifferentiated HL-60 leukocytes, a cancer cell line arrested in the pro-myelocytic stage of PMN differentiation (17). However, retinoic acid-induced differentiation of HL-60 leukocytes leads to robust NET formation in these leukocytes in response to LPS stimulation (17). These findings led to our initial hypothesis regarding the mechanism for decreased NET formation following BMT. With this study, we have disproven our initial hypothesis that decreased NET formation by PMNs isolated after BMT results from neutrophil developmental immaturity, a finding that might also have been expected given our results in PMNs isolated from newborn infant cord blood (10). In their recently published paper, Domingo-Gonzalez et al. also studied PMNs isolated from a murine model of BMT and demonstrated that increased levels of prostaglandin E₂ (PGE₂) in mice following BMT inhibited PMA-induced NET formation (16). This finding is also consistent with the results of Shishikura et al. who showed that PGE₂ inhibits NET formation by PMA-induced murine and human PMNs through increased production of cyclic AMP (24). Together, these reports elucidate yet another regulatory pathway governing NET formation in human and murine PMNs. Our report, in contrast, focused on PMNs isolated from human BMT patients or healthy controls and examined only one possible reason for decreased NET formation – that of decreased myeloid cell maturity after BMT. Still, future studies in PMNs isolated from

BMT patients, both before and after BMT, will need to include interrogation of the many known molecular pathways governing NETosis. Examples of such studies would include an examination of the toll-like receptor signaling pathways; an investigation of BMT effects on intracellular reactive oxygen species generation (5) and autophagy (25), with the BMT effects on the role of NE/myeloperoxidase in triggering NETosis in these patients as well (26); and finally an assessment of PAD4 activity as an enzyme leading to nuclear decondensation, a precursor step toward NETosis (23, 27).

Finally, our findings suggest that PMNs isolated from BMT patients prior to BMT may exhibit a deficit in NET formation and mandate that NET formation be studied in PMNs isolated from other immunodeficiency patients as well as cancer patients in general. If confirmed, NET deficiency by pre-BMT PMNs may be a result of the specific disorders leading up to BMT or from chemotherapy prior to BMT. To date, the effects of cancer chemotherapeutic agents on NET formation by neutrophils have not been extensively studied. Our study examining the role of the mammalian target of rapamycin (mTOR) signaling pathway in the regulation of NET formation did investigate rapamycin, an immunosuppressant and chemotherapeutic agent used in solid organ transplantation and some cancer treatment regimens, as an inhibitor of NET formation. We found that rapamycin inhibited NET formation in an mTOR and hypoxia-inducible factor 1-dependent manner. None of the participants in this report were exposed to rapamycin, and none of the chemotherapeutic agents to which they were exposed have been studied with regard to their effects on NET formation. Clearly, with these findings and the findings of Domingo-Gonzalez et al., further investigation is warranted (16). Alternatively, the question of a circulating inhibitor of NET formation should be considered, with PGE₂ being a leading candidate. Identification of an endogenous inhibitor of NET formation might prove important given the newly described pathogenic role of dysregulated NET formation in syndromes of pathologic inflammation (28, 29).

ETHICS STATEMENT

Institutional Review Board – University of Utah School of Medicine: IRB Protocol #056286. Pediatric participants were recruited by Meghann P. McManus, D.O., Michael A. Pulsipher, M.D., and Michael Boyer, M.D. (Co-Investigators and Pediatric Bone Marrow Transplant Physicians) as they met with patients to coordinate their bone marrow transplant procedure. For adult study participants, patients were recruited by Michael A. Pulsipher, M.D., Michael Boyer, M.D., Thai Cao, M.D., and Schickwann Tsai, M.D. (Co-Investigators and Adult Bone Marrow Transplant Physicians). Informed consent was sought at

the time when the patients were consented for their bone marrow transplant procedure. The study information and procedures were discussed in detail with each patient and their family as applicable (pediatric patients). A copy of the study associated with Consent and Authorization Document (Adult) or Parental Permission and Authorization Document (Pediatric) was given to the individual patient or family to read. Time was provided for the individual and family to consider the issues and discuss their response privately. No attempts at coercion or use of undue influence were made. The patients and families were clearly told that refusal of consent for this study would not in any way change the attitude or care of the clinical staff to the patient.

AUTHOR CONTRIBUTIONS

JG performed, directed, and interpreted experiments, and wrote significant portions of the manuscript. MC performed experiments, provided key experimental approaches, and interpreted results. MM recruited study participants, collected participant blood samples, and assisted with collection and characterization of participant clinical demographic and treatment data. MP recruited study participants, interpreted experimental results, provided key clinical expertise in the area of BMT, and edited portions of the manuscript. JS participated in study conceptualization, recruitment of study participants, edited portions of the manuscript, and provided key clinical insight into human cancer diagnosis and treatment. CY provided overall direction and conceptualization to the project, provided expertise regarding neutrophil biology and NET formation, reviewed and analyzed all experiments, wrote sections of the manuscript, and edited all portions of the manuscript.

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NETosing Neutrophils Activate Complement Both on Their Own NETs and Bacteria *via* Alternative and Non-alternative Pathways

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Neutrophils deposit antimicrobial proteins, such as myeloperoxidase and proteases on chromatin, which they release as neutrophil extracellular traps (NETs). Neutrophils also carry key components of the complement alternative pathway (AP) such as properdin or complement factor P (CFP), complement factor B (CFB), and C3. However, the contribution of these complement components and complement activation during NET formation in the presence and absence of bacteria is poorly understood. We studied complement activation on NETs and a Gram-negative opportunistic bacterial pathogen *Pseudomonas aeruginosa* (PA01, PAKwt, and PAKgfp). Here, we show that anaphylatoxin C5a, formyl-methionyl-leucyl-phenylalanine (fMLP) and phorbol myristate acetate (PMA), which activates NADPH oxidase, induce the release of CFP, CFB, and C3 from neutrophils. In response to PMA or *P. aeruginosa*, neutrophils secrete CFP, deposit it on NETs and bacteria, and induce the formation of terminal complement complexes (C5b–9). A blocking anti-CFP antibody inhibited AP-mediated but not non-AP-mediated complement activation on NETs and *P. aeruginosa*. Therefore, NET-mediated complement activation occurs *via* both AP- and non AP-based mechanisms, and AP-mediated complement activation during NETosis is dependent on CFP. These findings suggest that neutrophils could use their “AP tool kit” to readily activate complement on NETs and Gram-negative bacteria, such as *P. aeruginosa*, whereas additional components present in the serum help to fix non-AP-mediated complement both on NETs and bacteria. This unique mechanism may play important roles in host defense and help to explain specific roles of complement activation in NET-related diseases.

Keywords: neutrophil extracellular traps, NETosis, complement system, alternative pathway, properdin, *Pseudomonas aeruginosa*

INTRODUCTION

Neutrophils play a central role in the innate immune system and function in inflammation and immune surveillance. At sites of inflammation, neutrophils kill pathogens *via* phagocytosis and release of proteolytic enzymes (1, 2). Recently, the ability of neutrophils to form web-like neutrophil extracellular traps (NETs) has been identified as an additional strategy for antimicrobial defense. The process of NET formation (i.e., NETosis) is a specific form of cell death, in which nuclear DNA undergoes decondensation with subsequent expulsion of chromatin that is coated with cytotoxic granular proteins, such as myeloperoxidase (MPO), elastase, and other proteases (3). NETs are released in response to a variety of stimuli, including NADPH oxidase (Nox) agonist, such as phorbol-12-myristate-13-acetate (PMA), inflammatory stimuli, and bacteria (4, 5). Two major types of NETosis have been reported to date: Nox-dependent NETosis and Nox-independent NETosis, in which reactive oxygen species (ROS) are generated by Nox and mitochondrial complexes, respectively (6–9). In both of these types of NETosis, neutrophil release chromatin coated with granular proteins as NETs. In the presence of C5a, GM-CSF-primed neutrophils undergo a vital NETosis, in which cells do not die, but release mitochondrial DNA. This type of NETosis is regulated by mitochondrial ROS production (10).

Once formed, NETs ensnare pathogens and expose them to high localized concentrations of antimicrobial proteins (11). NETs can also be cytotoxic and have been shown to contribute to thrombosis, sepsis, cystic fibrosis, asthma, systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) (12–24). Complement and infections have been implicated in the pathogenesis and exacerbation of many of these diseases. Although it has recently been described that NETs can activate and deposit complement alternative pathway (AP) components (25), the involvement of the different complement pathways and their components in the context of NETosis and bacterial infection has not been fully understood. This fundamental knowledge is essential for understanding molecular mechanisms involved in NET-related pathobiology.

The complement system consists of more than 30 proteins distributed in the circulation and on endothelial cells, and functions primarily in microbial defense and clearance of immune complexes and injured cells (26). Complement can be constantly active (*via* the complement AP) or become activated by immune complexes and dying cells [*via* the C1q-mediated classical pathway (CP)] or carbohydrate ligands on microorganisms [*via* the lectin pathway (LP)] (26). Complement factor P (CFP), the only positive complement regulator, acts as stabilizer of the AP convertase (C3bBbP) and selective pattern recognition molecule of certain microorganisms and host cells (i.e., apoptotic/necrotic cells) by serving as a platform for the assembly of the AP C3 convertase (27). Complement progression includes the activation of complement proteins C3 and C5 (to form the potent anaphylatoxins C3a and C5a and the opsonins C3b and C5b) and the subsequent activation of the terminal pathway with the formation of the potentially lytic membrane attack complex (MAC), C5b–9. AP activation is critically enhanced by the C3 convertase C3bBbP,

and requires tight regulation to maintain the balance between necessary activation and harmful over-activation (26). Bacteria are capable of inducing Nox-dependent NETosis (9, 28–30), and we aimed to identify possible links between NETosis, bacteria, and the complement system, in particular, the possibility that neutrophils mount a targeted complement response to infectious agents *via* the formation of NETs and deposition of complement components on NETs and microbial pathogens.

MATERIALS AND METHODS

Ethics

Informed written consent was obtained from all donors. The study protocol was approved by the Research Ethics Board at The Hospital for Sick Children, Toronto, ON, Canada.

Reagents

All buffer salts and reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise.

Bacterial Culture

Pseudomonas aeruginosa (mPA01, PAKwt, and PAKgfp) cultures were grown overnight in LB-broth. PAKgfp was maintained in 30 µg/ml gentamicin. The concentration of bacteria was calculated using $[CFU] \times 10^8 = (OD_{600}) 30.88 - 99,607$. For NETosis assays, *P. aeruginosa* sub-cultured for 3 h was used at a multiplicity of infection (MOI) of 10 or 100.

Neutrophil Isolation and Preparation of Neutrophil Lysates

Human peripheral neutrophils were purified from whole blood (20 ml) collected in BD EDTA-vacutainers from healthy donors using Polymorphprep™ (Axis-shield, Oslo, Norway). After lysing erythrocytes with hypotonic buffer, neutrophils were resuspended in RPMI 1640 (Wisent Bioproducts, Montreal, QC, Canada) (31). To obtain neutrophil lysate, cell pellets were resuspended in a lysis buffer [1% (v/v) Triton X-100, 50 mM Tris, pH 7.4, 10 mM KCl containing 2× complete, mini protease inhibitor cocktail (Roche Diagnostics, Laval, QC, Canada) supplemented with 0.5 mM EDTA, 25 µM leupeptin, 25 µM pepstatin, 25 µM aprotinin, 1 mM levamisole, 1 mM Na₃VO₄, 25 mM NaF, 1 mM PMSF], sonicated (VWR Sonics model 50D), and incubated for 15 min at 4°C. Neutrophil lysates were centrifuged at 25,000× g for 30 min at 4°C and stored at –80°C for future analysis.

Neutrophil Activation, Oxidative Burst, and Secretion

Neutrophils (2×10^7 cells/ml) were resuspended in RPMI 1640 with 10 mM Hepes, pH 7.4, and activated with C5a (CompTech, Tyler, TX, USA) (1 µM), formyl-methionyl-leucyl-phenylalanine (fMLP) (1 µM) or PMA (20 nM), and incubated [37°C, 5% (v/v) CO₂] for 30 min. Stimulation was terminated by incubating these cells at 4°C for 5 min. Neutrophils were pelleted (1000× g for 10 min) and the supernatant was collected and further

centrifuged at 25,000× *g* for 10 min at 4°C, immediately placed in 2× neutrophil Laemmli sample buffer, heated at 95°C for 5 min and stored at –80°C for future analysis. Neutrophil lysates were prepared from the remaining cell pellet as described in the Section “Neutrophil Isolation and Preparation of Neutrophil Lysates.” To determine respiratory burst, neutrophils (1 × 10⁶ cells/ml) were pre-loaded with dihydrorhodamine (DHR) 123 (10 μM), treated with the agonists as above and analyzed by flow cytometry (Gallios, Beckman Coulter, Mississauga, ON, Canada). Cells were first gated with forward and side scatters, and further gated for Hoechst (1 μg/ml) using 405/450 BP 40 filter channel. ROS was detected using 488/429 BP 28.25 filter channel.

Sytox Green Plate Reader Assay

Neutrophils (3 × 10⁴ cells) were seeded onto 96-well plates in the presence of cell-impermeable Sytox Green DNA-binding dye (5 μM) and were activated with agonists or three stains of *P. aeruginosa* (mPAO1, PAKwt, and PAKgfp) at MOIs of 10 and 100. For inhibition studies, neutrophils were preincubated with 2 μM of Nox inhibitor, diphenyleneiodonium (DPI) for 1 h before activation. Fluorescence intensity was measured by the POLARstar Omega microplate reader (BMG Labtech, Ortenberg, Germany) with excitation/emission (485/520), every 30 min. NET formation was normalized to total neutrophils DNA content determined by permeabilizing the cells with 0.5% (w/v) Triton X-100.

Western Blot

Neutrophil lysates (50 μg) were size-fractionated in 10% (w/v) SDS-polyacrylamide gels, transferred to nitrocellulose membranes, blocked with 5% (w/v) skim-milk + 0.05% (v/v) Tween-20 (TBST), probed with goat polyclonal antibody to complement proteins (1:1000 dilution; Complement Technology, Tyler, TX, USA) or mouse monoclonal antibody to β-actin (BA3R, 1:10,000 dilution; Thermo Fisher Scientific, Rockford, IL, USA) in 5% (w/v) skim-milk in TBST, washed, and incubated with secondary antibody in 5% (w/v) skim-milk in TBST. Proteins were detected using Western Lighting™ Plus-ECL, Enhanced (PerkinElmer, Waltham, MA, USA) and developed on radiographic film on a Kodak X-Omat 2000a processor.

Detection of Complement Proteins in NETs

Phorbol-12-myristate-13-acetate (20 nM) activated neutrophils at 240 min were fixed with 4% (w/v) paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA, USA), blocked with 3% (v/v) cold water fish skin gelatin and incubated with anti-complement antibodies: rabbit polyclonal antibody to CFP (SC-68366, 1:50 dilution; Santa Cruz Biotechnology, Dallas, TX, USA), rabbit polyclonal antibody to complement factor B (CFB) (SC-67151, 1:50 dilution; Santa Cruz Biotechnology, Dallas, TX, USA), and rabbit polyclonal antibody to complement C3 (ab97462, 1:100 dilution; Abcam, Cambridge, MA, USA). Neutrophils activated with PMA were co-stained with mouse monoclonal antibody to MPO (ab25989, 1:500 dilution; Abcam, Cambridge, MA, USA). Complexes were detected with donkey anti-primary antibodies conjugated with Alexa Fluor® 555 (Invitrogen, Eugene, OR, USA). Specimens were mounted with Dako Fluorescence Mounting

Media (Dako Canada, Burlington, ON, Canada) for analysis with spinning-disk confocal microscopy.

C5b–9 Formation on NETs

After 240 min of neutrophil activation, culture plates were centrifuged at 200× *g* for 5 min at 4°C. The media was replaced with 500 μl of 20% (v/v) Recludan® (Bayer Healthcare, Wayne, NJ, USA) fresh frozen PPP prepared in RPMI 1640 media or AP buffer (20 mM Hepes, pH 7.4, 144 mM NaCl, 7 mM MgCl₂, and 10 mM EGTA). For experiments with AP buffer, a buffer exchange was performed prior to the addition of plasma (Bayer Healthcare, Wayne, NJ, USA). After 15 min [37°C, 5% (v/v) CO₂], specimens were fixed with 4% (w/v) paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA, USA). In some experiments, DNase I (50 μg/ml) was added to digest DNA. After blocking with 3% (v/v) gelatin, mouse monoclonal antibody to C5b–9 (DIA 011-01, 1:200 dilution; Antibody Shop, Gentofte, Denmark) was incubated, washed, and further incubated with donkey anti-mouse secondary antibody conjugated with Alexa Fluor® 555 (Invitrogen, Eugene, OR, USA). PAKgfp signal was enhanced with Alexa Fluor® 488 conjugated rabbit polyclonal antibody to GFP (A21311, 1:400 dilution; Invitrogen, Eugene, OR, USA). For CFP inhibitor assays, optimal concentration of a mouse monoclonal anti-CFP antibody (Anti Factor P#1, A233; Quidel Corporation, San Diego, CA, USA) was determined by rabbit red blood cell lysis assay (32). An antibody concentration of 4 μg/ml was used in the final assays. Presence of C5b–9 was detected as described above. Wheat germ agglutinin was used for labeling neutrophil membrane. Specimens were mounted and analyzed with spinning-disk confocal microscopy.

Spinning-Disk Confocal Microscopy and Colocalization Analysis

Images were taken on an Olympus IX81 inverted fluorescence microscope using a 60×/1.35 oil immersion objective equipped with a Hamamatsu C9100-13 back-thinned EM-CCD camera and Yokogawa CSU X1 spinning-disk confocal scan head (with Spectral Aurora Borealis upgrade). The unit is equipped with four separate diode-pumped solid state laser lines (Spectral Applied Research, 405, 491, 561, and 642 nm) with emission filters: 447 ± 60, 525 ± 50, 593 ± 40, 620 ± 60, 676 ± 29, and 700 nm ± 75, and 1.5× magnification lens (Spectral Applied Research). Confocal images were taken with an Improvion Piezo Focus Drive. Z-stacks were taken at 0.25 μm. Images taken using the spinning-disk confocal microscope were deconvolved by iterative restoration using Volocity Software (PerkinElmer, Waltham, MA, USA) with confidence limit set to 95% and iteration limit set to 20.

Statistical Analysis

Student’s *t*-test, or one-way or two-way ANOVA with Tukey’s multiple comparison test was used for statistical comparison as needed. A *p*-value was set at 0.05, 0.01, or 0.001 for statistical significance. All statistical analyses were performed using GraphPad Prism (GraphPad Software, La Jolla, CA, USA) statistical analysis software (Version 6.0).

RESULTS

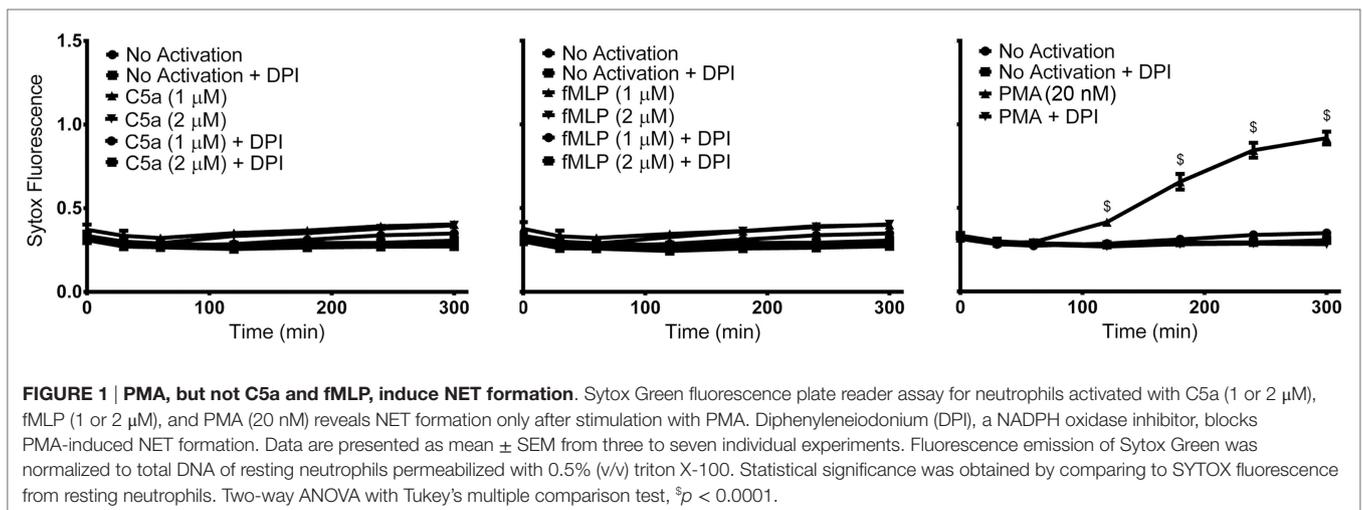
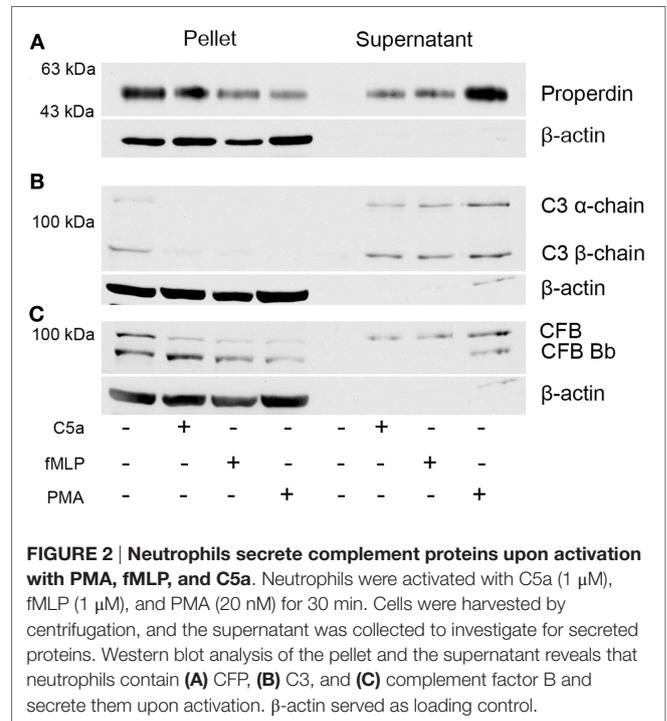
PMA, but not fMLP and C5a, Induces NETosis

Reactive oxygen species is considered to be important for NETosis. However, different agonists induce ROS to different degrees. Therefore, to determine whether ROS was sufficient to induce NETosis, ROS production was measured 30 min post neutrophil stimulation using DHR 123 and flow cytometry. C5a (1–2 μ M) did not generate ROS above baseline levels (Figure S1A in Supplementary Material); however, similar concentrations of fMLP led to a 1.5-fold increase in ROS compared to baseline values ($p < 0.05$; Figure S1B in Supplementary Material). The use of 20 nM PMA produced a ninefold increase of ROS in comparison to non-treated control neutrophils ($p < 0.05$; Figure S1C in Supplementary Material). The overall ability of these agonists to induce ROS production was PMA \gg fMLP $>$ C5a.

Although both PMA and fMLP induced ROS production, whether fMLP can induce NETosis is uncertain. Therefore, to identify the ability of PMA, fMLP, and C5a to independently elicit NETosis, we treated neutrophils with varying concentrations of these reagents. NETosis was monitored using a plate reader assay by measuring the production of extracellular DNA. This assay monitors the fluorescence generated by the binding of cell-impermeable DNA-binding Sytox Green fluorescent dye to NET DNA. Neither C5a nor fMLP (up to concentration of 2 μ M) induced NET formation within the observed 300-min time period (Figure 1). However, stimulation of neutrophils with PMA resulted in NET generation after approximately 120 min, as determined by Sytox Green plate reader assay (Figure 1) and nuclear morphology changes (Figure S2 in Supplementary Material). As expected, the use of the NADPH inhibitor DPI abrogated PMA-induced NET formation with levels remaining near baseline. This confirms that PMA induces NETosis *via* the Nox-dependent pathway, and that C5a and fMLP on their own do not induce NETosis.

Induction of NETosis Causes Release of Complement Factors

To identify whether stimulation of neutrophils results in the release of complement factors, Western blot analysis was performed on the cell pellets and cell-free supernatant (Figure 2). Protein levels of the complement proteins CFP, C3, and CFB were compared for neutrophils induced for 30 min either with C5a, fMLP, and PMA or with buffer. Complement proteins were not detected in the supernatant of non-activated neutrophils, with proteins being identified exclusively in cell pellet samples. The release of all three complement proteins was observed for all induction



methods; however, the use of PMA elicited the greatest release of both CFP and CFB within the supernatant (**Figures 2A–C**). Pelleted samples for activated cells also contained a large amount of complement proteins: primarily CFP and CFB (and its activation product Bb). Furthermore, CFB-Bb was only identified in PMA-induced neutrophils (**Figure 2C**). These results indicate the capability of PMA to not only induce NETosis but also cause the greatest release of complement proteins in comparison to C5a and fMLP.

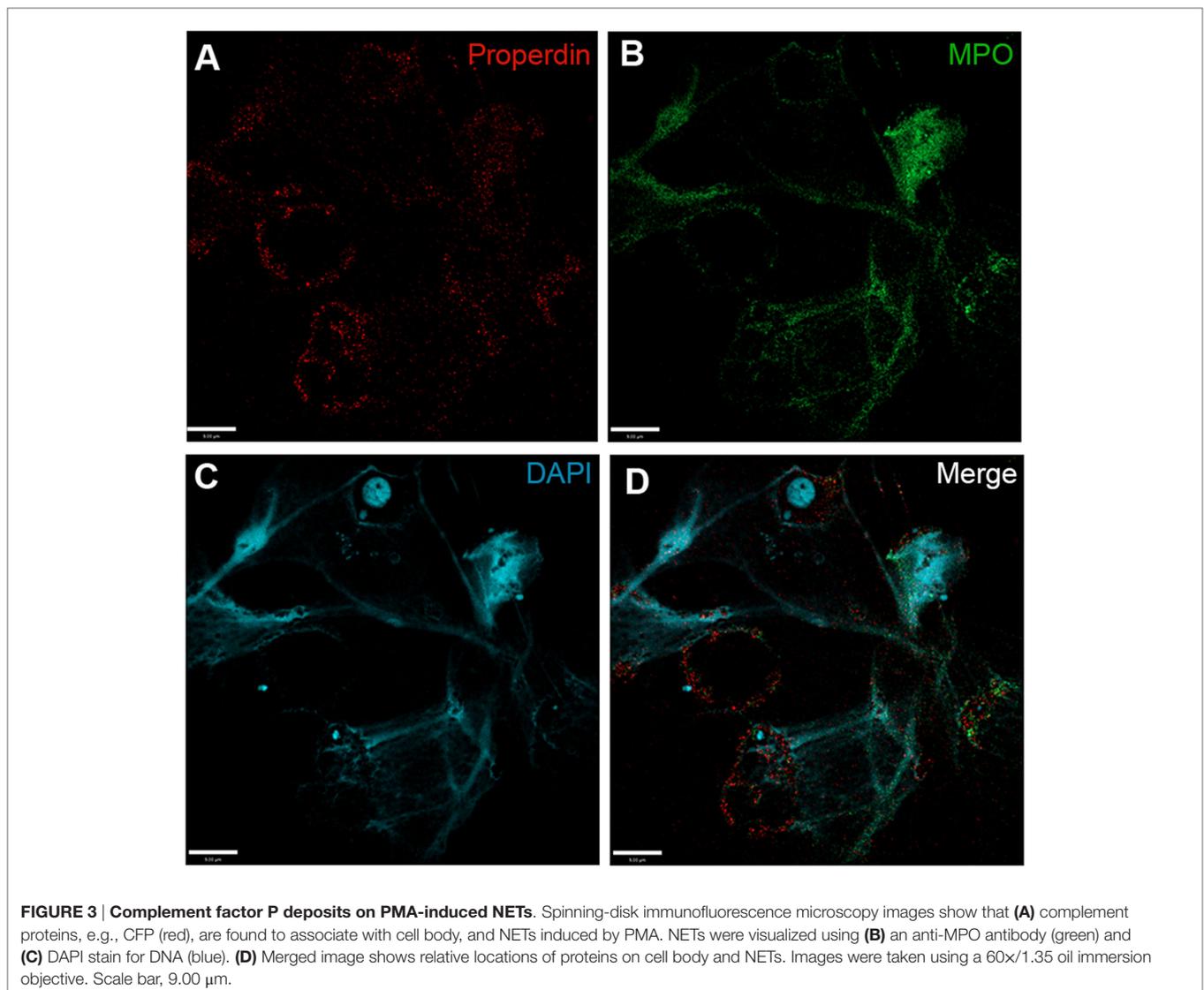
PMA-Induced NETosis Deposits CFP on NETs

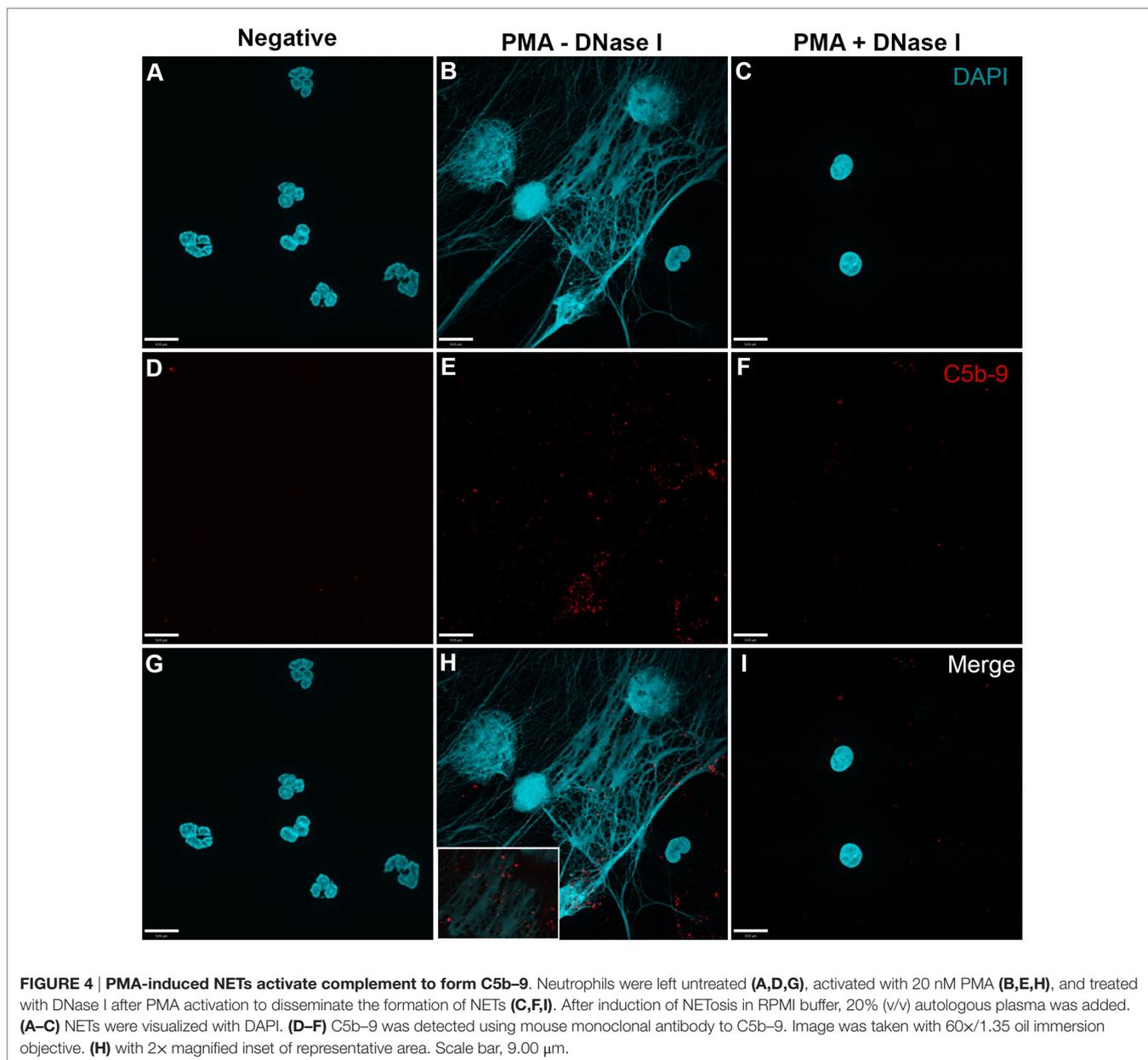
As the next step, we sought to identify whether CFP was capable of adhering to NETosing neutrophils and NETs. Immunofluorescence analysis performed on PMA-induced NETs showed the deposition of both CFP and MPO (another known NET-associated protein) on the surface of extracellular NETs (**Figure 3**). CFP could also be detected on the neutrophil membranes after NET formation.

Deposition pattern of both MPO and CFP shows substantial overlap throughout the extracellular DNA lattice structure. Therefore, neutrophils deposit CFP on NETs.

PMA-Induced NETs Activate Complement

In order to analyze whether complement activation occurs on NETs, neutrophils were stimulated with PMA, washed and incubated with 20% complement active plasma for 15 min in complement competent buffers. Under these conditions, both AP- and non-AP-mediated complement fixation can occur. Immunofluorescence microscopy was performed on these NETs to identify the deposition of terminal complement complex C5b–9. Images show that C5b–9 deposits on NETs (**Figures 4 and 5 – middle column; Figures 4H and 5H** with 2× magnified insets of representative areas). The use of DNase I, which removes pre-formed NETs, causes a large decrease in the detection of C5b–9 deposition. These data suggest that the presence of NETs is necessary to activate complement cascade and deposit terminal complement complex.





PMA-Induced NETosis Activates Complement Also *via* Alternative Pathway

To further identify the contribution of AP to NETosis, this experiment was repeated except that the plasma incubation step was performed in the presence of AP buffer allowing for AP activation only. Immunofluorescence microscopy images show that C5b-9 could be deposited *via* AP (Figure 6; Figures 6I–K with 2x magnified insets of representative areas). To determine whether blocking CFP is sufficient to prevent complement activation, 4 $\mu\text{g}/\text{ml}$ anti-CFP antibody was added during complement activation. This antibody concentration was chosen because AP-dependent rabbit erythrocyte hemolysis was inhibited in the presence of >4 $\mu\text{g}/\text{ml}$ anti-CFP antibody (Figure S3 in Supplementary Material). Immunofluorescence microscopy reveals that blocking

CFP fully prevents C5b-9 deposition in AP buffer conditions, but reduced C5b-9 deposition only slightly in complete buffer conditions (Figure 6). Therefore, both AP- and non-AP-mediated complement depositions occur on NETs induced by PMA.

P. aeruginosa-Induced NETosis Deposits CFP on NETs and Bacteria

To test the ability of complement fixation on pathogens during NETosis, neutrophils were exposed to various strains of *P. aeruginosa* (mPA01, PAKwt, and PAKgfp). Similar to the PMA experiments described above, NETosis was monitored using Sytox Green plate reader assays. All three strains of these bacteria induced NETosis, and followed similar kinetics in terms of post-infection time response (Figure 7). NETosis began at

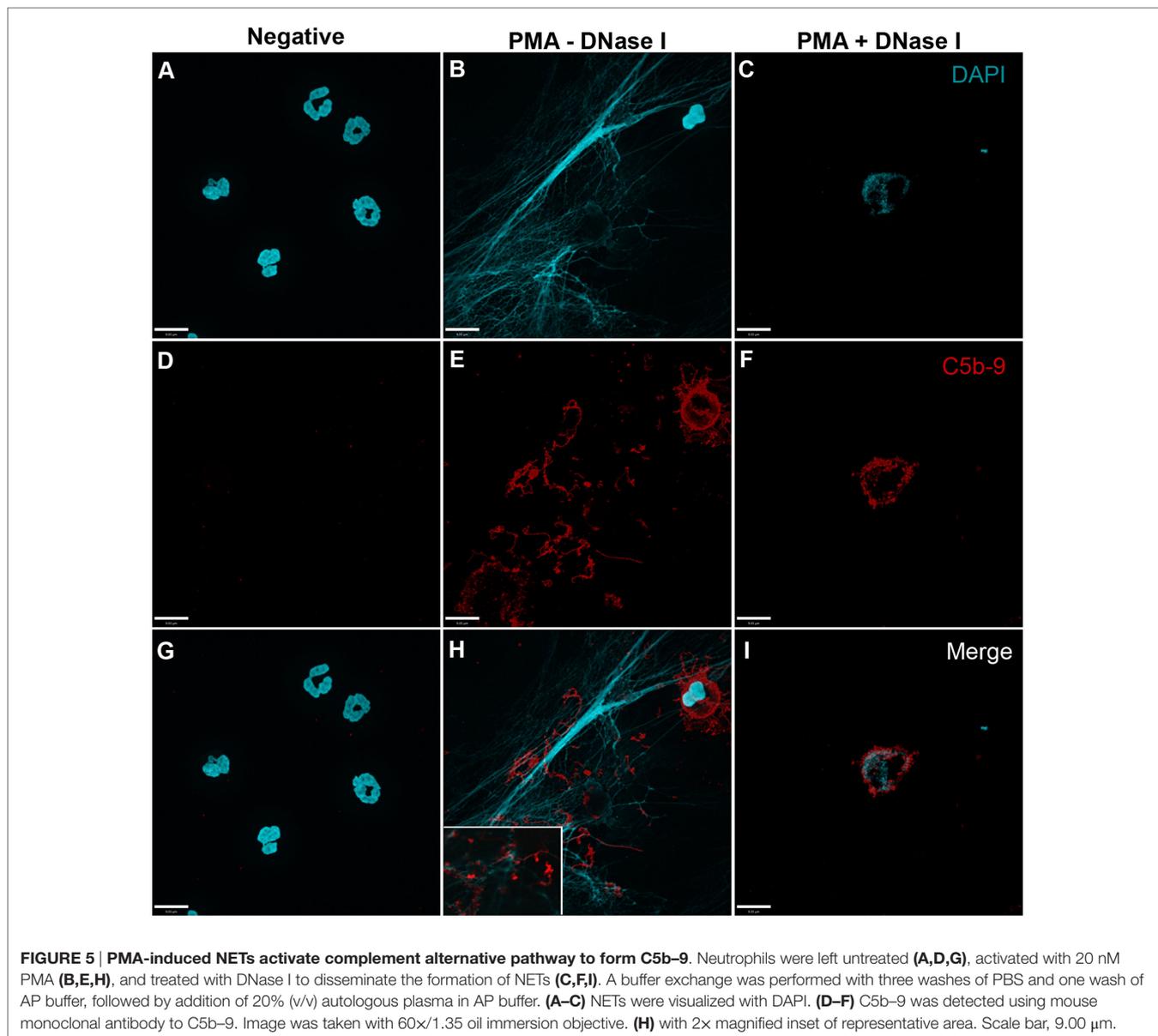


FIGURE 5 | PMA-induced NETs activate complement alternative pathway to form C5b-9. Neutrophils were left untreated (**A,D,G**), activated with 20 nM PMA (**B,E,H**), and treated with DNase I to disseminate the formation of NETs (**C,F,I**). A buffer exchange was performed with three washes of PBS and one wash of AP buffer, followed by addition of 20% (v/v) autologous plasma in AP buffer. (**A-C**) NETs were visualized with DAPI. (**D-F**) C5b-9 was detected using mouse monoclonal antibody to C5b-9. Image was taken with 60x/1.35 oil immersion objective. (**H**) with 2x magnified inset of representative area. Scale bar, 9.00 μm .

approximately 120 min and continued to increase throughout the 300-min experimental time period. Furthermore, this induction is bacterial load dependent with both a faster response and a larger response observed when the MOI was increased from 10 to 100. To determine whether this NETosis induction was dependent on Nox, DPI was included in the media. Nox inhibitor DPI fully abrogated *P. aeruginosa*-induced NETosis for all three stains at both MOIs ($p < 0.05$). This finding indicates that similar to PMA, bacteria-induced NETosis occurs in a Nox-dependent manner.

To determine CFP deposition during NETosis, specimens were immunostained. In the absence of serum, CFP was detected on both bacteria and NETs, and DNase I treatment abolished CFP deposition (**Figure 8; Figure 8K** with 2x magnified inset of a representative area). These results indicate that during *P. aeruginosa*-induced NETosis CFP is released from the neutrophils and deposits on NETs and bacteria.

***P. aeruginosa*-Induced NETosis Activates Complement on NETs**

To determine whether NET induction mediated by *P. aeruginosa* results in complement activation, C5b-9 deposition was determined by immunofluorescence microscopy. Images show that C5b-9 was deposited on NETs (**Figure 9; Figure 9K** with a 2x magnified inset of a representative area). This effect was abolished when the NET DNA lattice was removed by DNase treatment. Thus, complement deposits on NETs during NETosis.

***P. aeruginosa*-Induced NETosis Also Activates Complement via Alternative Pathway**

To determine whether the AP is involved in *P. aeruginosa*-induced NETosis and C5b-9 formation, we first examined C5b-9

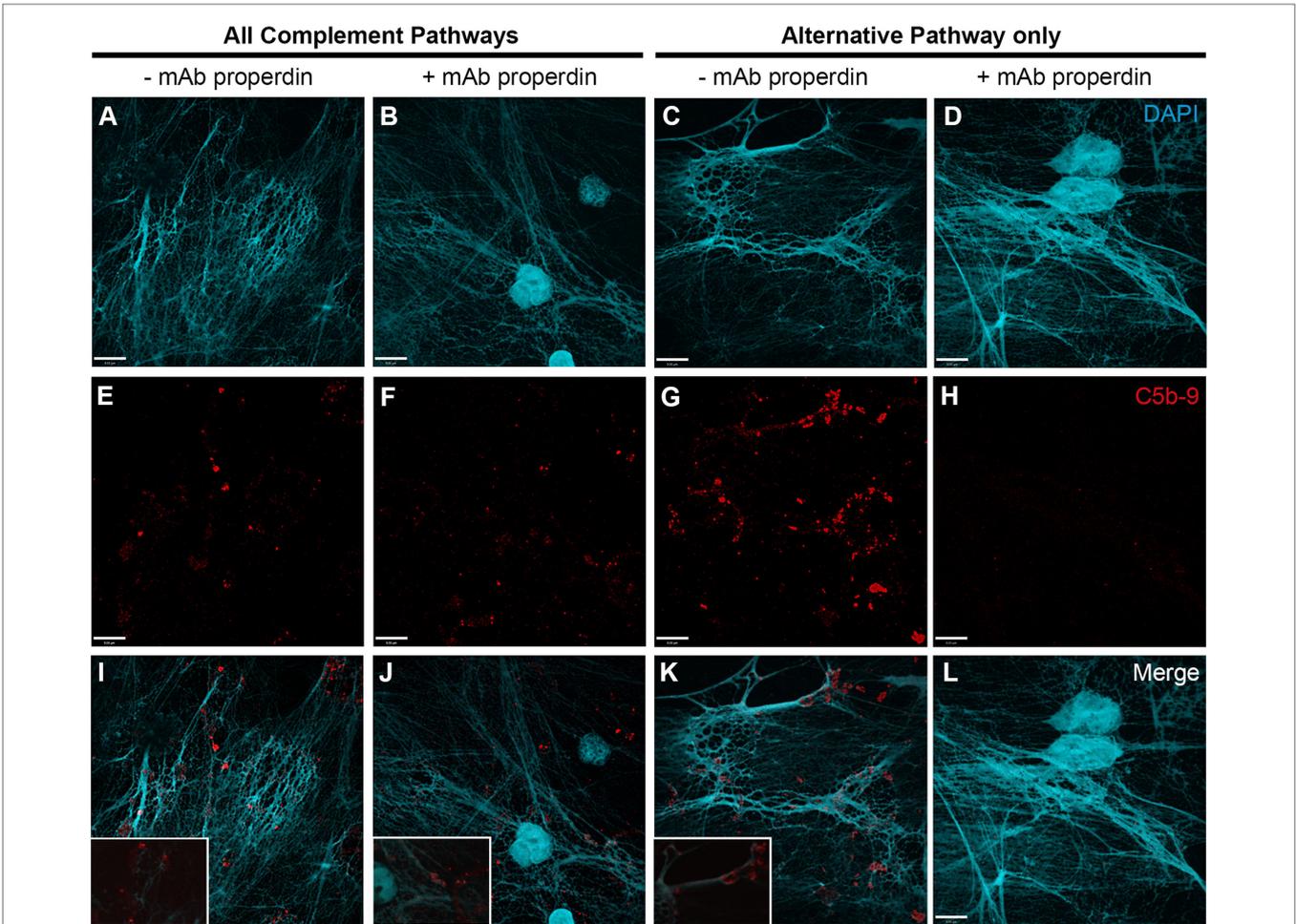


FIGURE 6 | Anti-CFP antibody blocks activation of complement AP to form C5b-9 on PMA-induced NETs. Neutrophils were activated with PMA (20 nM) to induce NET formation. Plasma with RPMI buffer was used to activate all complement pathways, AP buffer to only activate the alternative pathway. A monoclonal anti-properdin antibody was used to inhibit AP activation. A buffer exchange was performed followed by addition of 20% (v/v) plasma:RPMI1640 + 10 mM HEPES [far left column (A,E,I)], 20% (v/v) plasma:RPMI1640 + 10 mM HEPES + mAb properdin [left column (B,F,J)], 20% (v/v) plasma:AP buffer [right column (C,G,K)], 20% (v/v) plasma:AP buffer + mAb properdin [far right column (D,H,L)]. (A–D) show DAPI, (E–H) C5b-9 deposition, and (I–L) merged images. Images were taken with 60x/1.35 oil immersion objective. (I–K) with 2x magnified insets of representative areas. Scale bar, 9.00 μm.

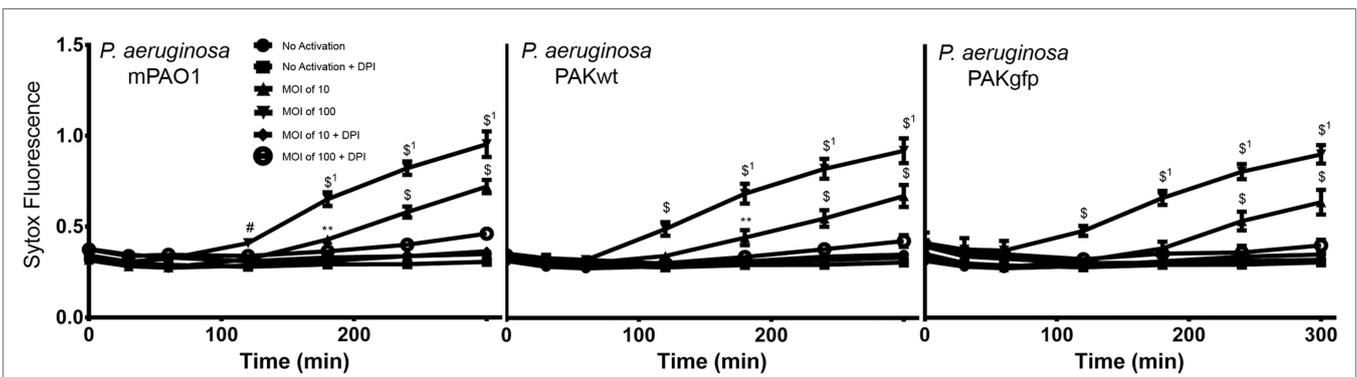
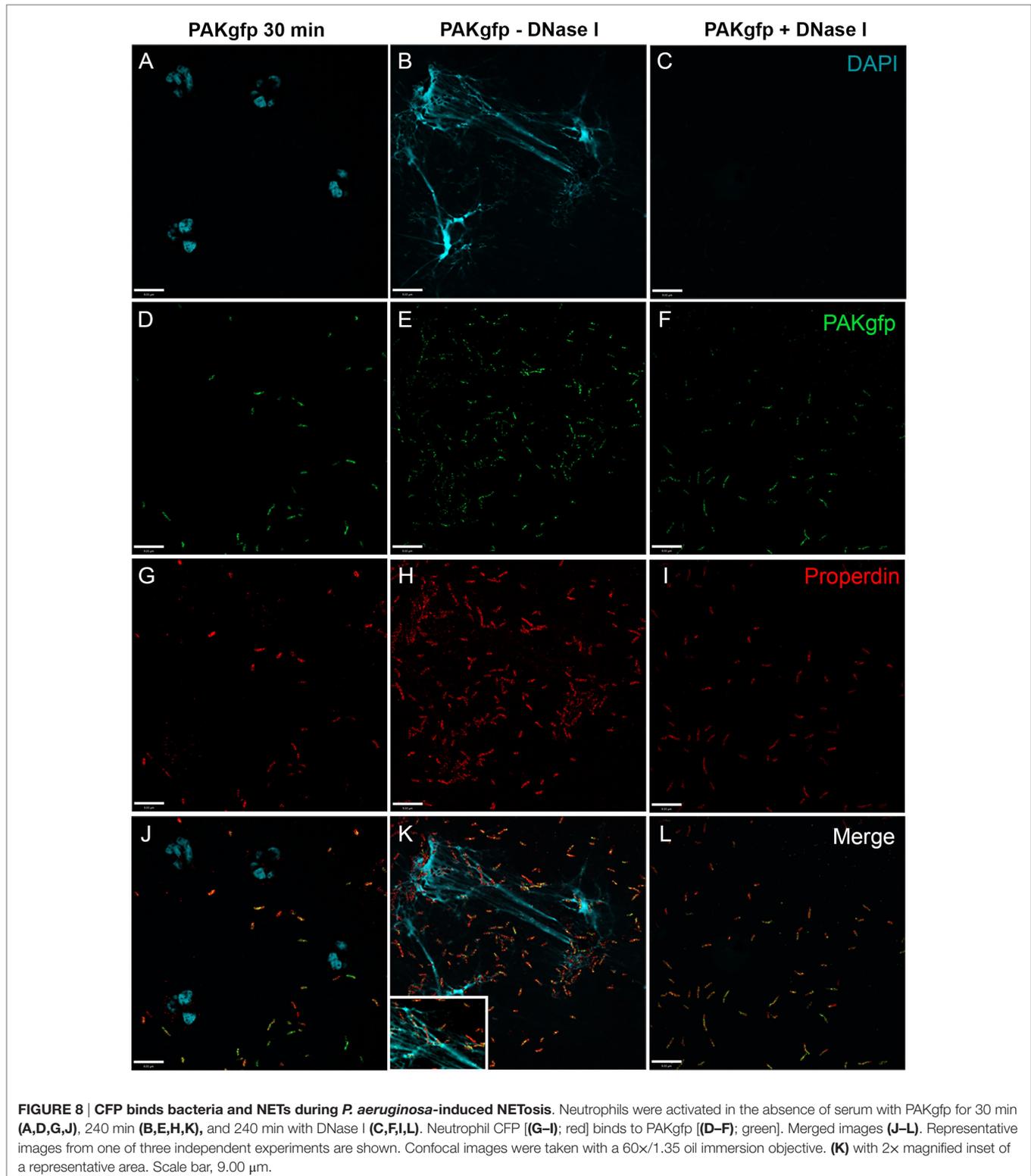
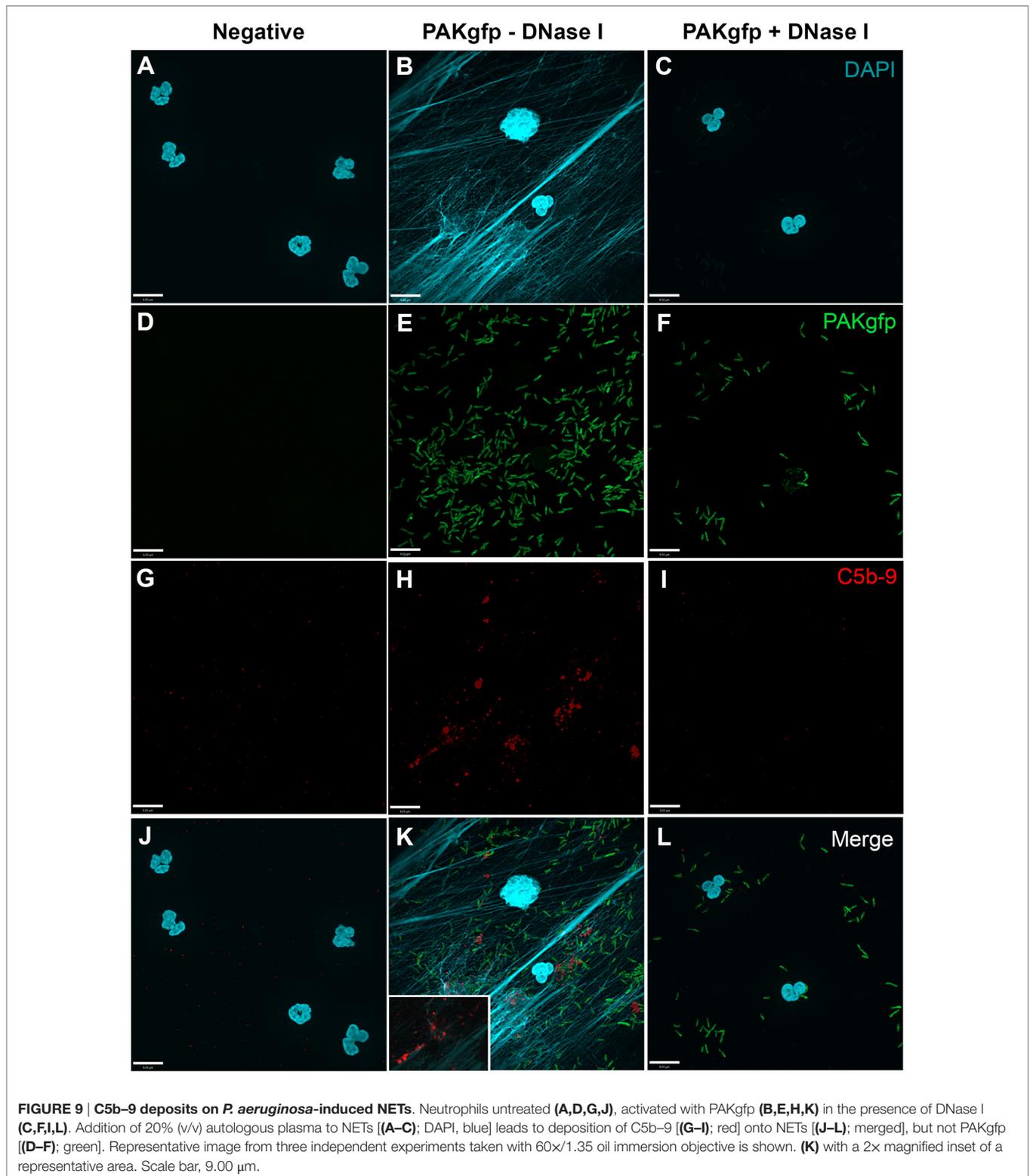


FIGURE 7 | *P. aeruginosa* induces Nox-dependent NETosis. Sytox Green plate reader assay showing the NETosis ability of different strains of *P. aeruginosa* (mPAO1, PAKwt, and PAKgfp). Fluorescence emission of Sytox green was normalized to total DNA of resting neutrophils permeabilized with 0.5% triton X-100. Nox inhibitor DPI suppresses the NETosis of all three strains. Data are presented as mean ± SEM from four to seven individual experiments. Statistical significance compared to Sytox fluorescence from resting neutrophils. ¹ denotes statistical significance between different multiplicities of infection. Two-way ANOVA with Tukey's multiple comparison test, **p* < 0.05, ***p* < 0.01, **p* < 0.001, ²*p* < 0.0001.



deposition under AP activation conditions. Immunofluorescence microscopy analysis shows that C5b–9 is deposited on NETs (**Figure 10; Figure 10K** with a 2× magnified inset of a representative area).

Next, we used anti-CFP antibody to test the importance of CFP under AP activation conditions (Figure S3 in Supplementary Material). Induction of NETosis was performed using PAKgfp at an MOI of 100. After NET induction with *PAKgfp*, samples



were maintained in complement competent RPMI media with the addition of 20% (v/v) autologous plasma (Figure 11 – first column; Figure 11M with a 2× magnified inset of a representative area). The addition of anti-CFP antibody to these samples did not change the formation and deposition of C5b-9

(Figure 11 – second column; Figure 11N with a 2× magnified inset of a representative area). Next, we incubated neutrophils with 20% (v/v) plasma in AP buffer. C5b-9 deposition was detected on NETs (Figure 11 – third column; Figure 11O with a 2× magnified inset of a representative area). Adding 4 μg/ml anti-CFP antibody

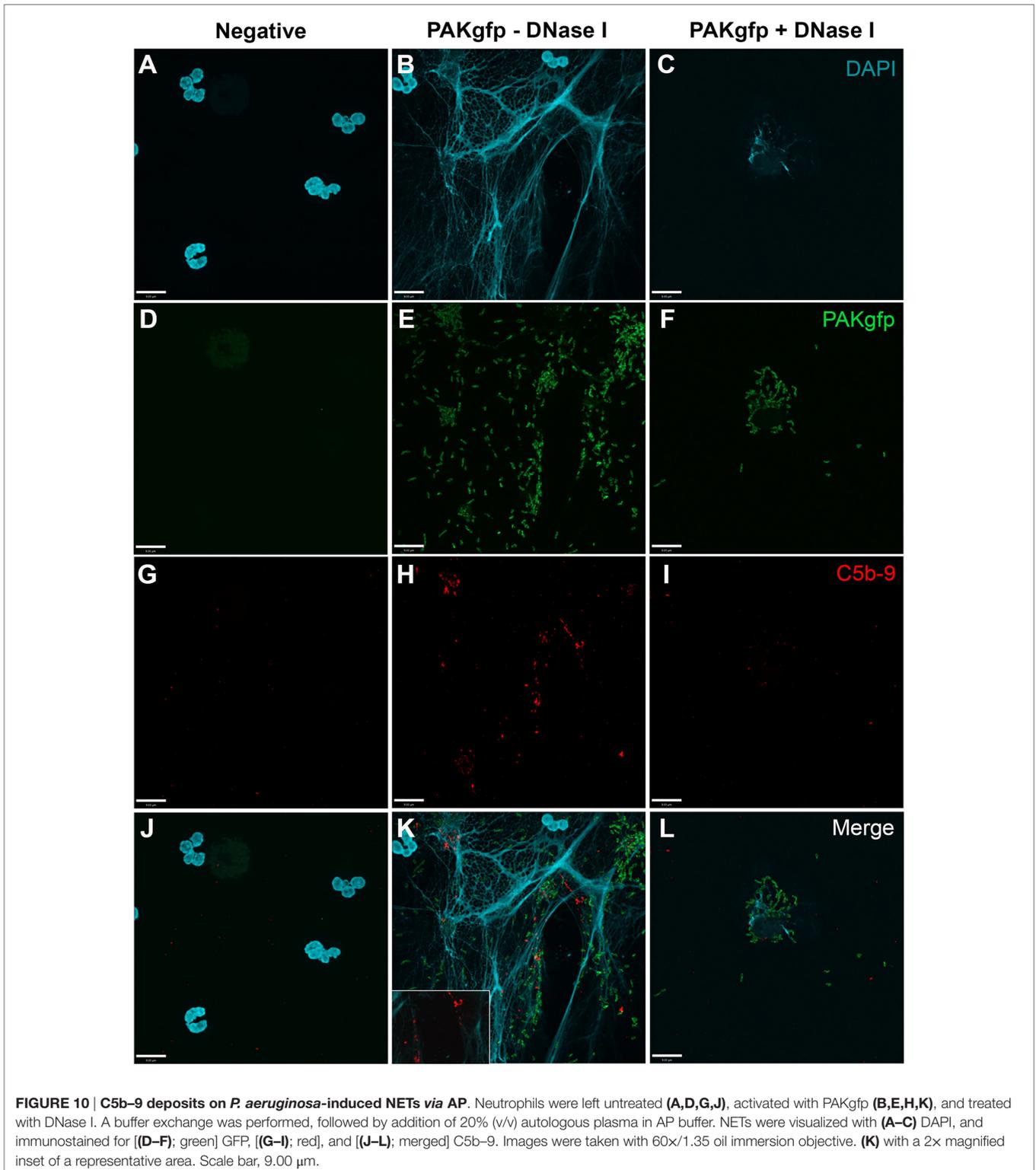


FIGURE 10 | C5b-9 deposits on *P. aeruginosa*-induced NETs via AP. Neutrophils were left untreated (A,D,G,J), activated with PAKgfp (B,E,H,K), and treated with DNase I. A buffer exchange was performed, followed by addition of 20% (v/v) autologous plasma in AP buffer. NETs were visualized with (A–C) DAPI, and immunostained for [(D–F); green] GFP, [(G–I); red], and [(J–L); merged] C5b-9. Images were taken with 60x/1.35 oil immersion objective. (K) with a 2x magnified inset of a representative area. Scale bar, 9.00 μm.

before incubating bacteria-induced NETs with plasma inhibited C5b-9 deposition on NETs (Figure 11 – fourth column). Taken together, these data show that complement activation and progression to C5b-9 formation on NETs occurs *via* AP and non-AP pathways, and that AP activation depends on CFP.

DISCUSSION

Over the past decade, the ability of neutrophils to generate NETs has led to studies attempting to determine their function and involvement in disease. Although extensive studies have been

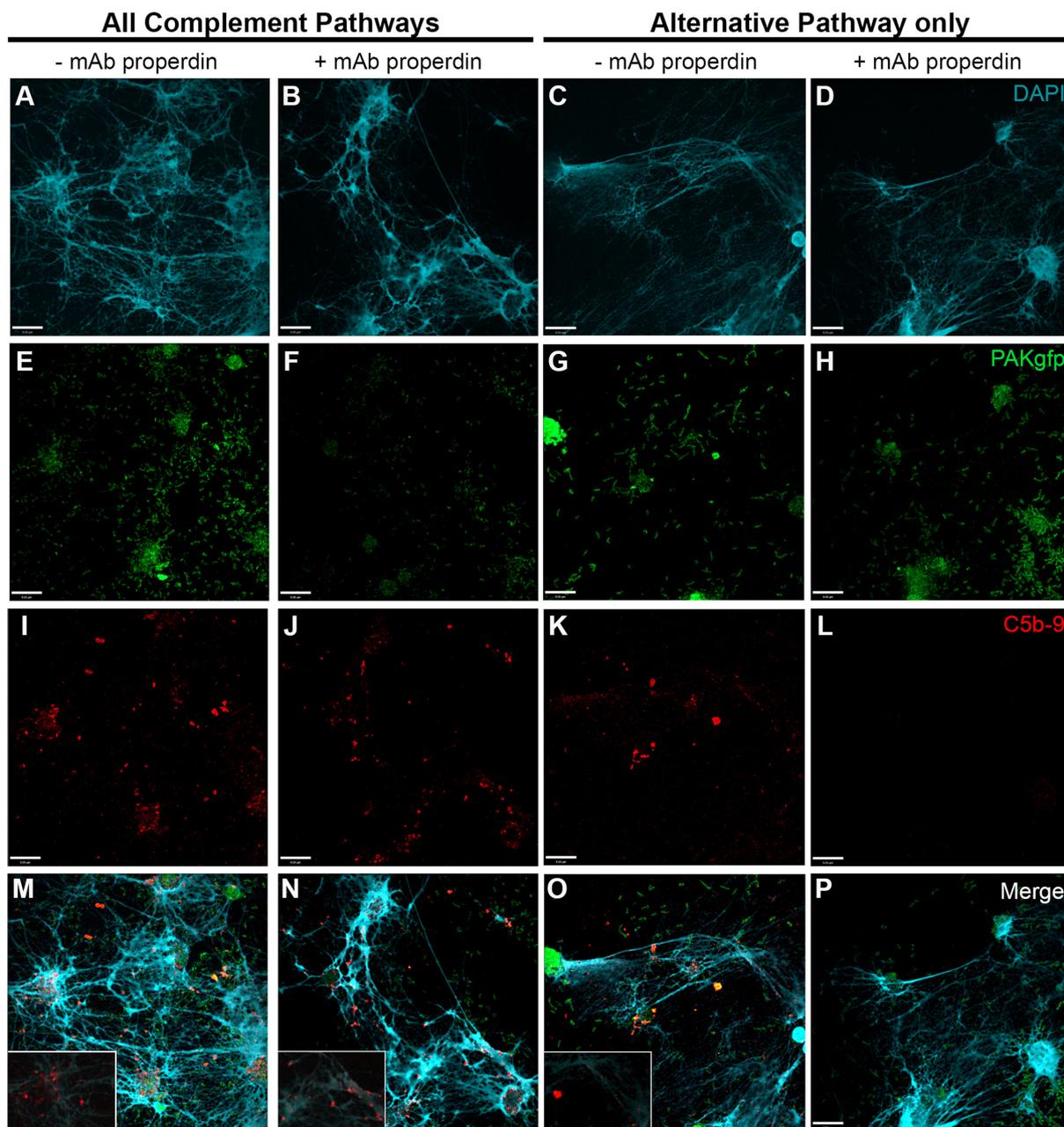


FIGURE 11 | *P. aeruginosa*-induced NETosis results in complement activation on NETs via CFP-dependent and -independent mechanisms. Neutrophils were activated with *P. aeruginosa* PAKgfp MOI 100 [(E–H); green] to induce the formation of NETs [(A–D); blue]. Samples were left in RPMI buffer (left two columns) followed by addition of 20% (v/v) autologous plasma in RPMI buffer, or in 20% plasma in AP buffer (AP activation only, right two columns). Samples were immunolabeled for C5b–9 [(I–L); red]. Merged images are shown in [(M–P)]. Addition of anti-properdin antibody has no effect on C5b–9 formation in RPMI buffer [(J,N)]. Incubation of samples in 20% plasma in AP buffer (AP activation only) with a anti-properdin antibody abolished C5b–9 deposition on NETs [(L,P)]. Images were taken with 60x/1.35 oil immersion objective. [(M–O)] with a 2x magnified insets of a representative area. Scale bar, 9.00 μm .

performed, the exact functions of NETs, and their mechanism of action, remain to be completely elicited. NETs have been identified in several diseases that are associated with complement activation (1, 13, 14, 17, 33). In this study, we sought to understand the involvement of the complement system in the context of bacteria and NETs.

The use of varying reagents to induce NETosis has been established in many studies (4, 5). Applying these conditions, we found that PMA, but not C5a and fMLP, induces NETosis *via* the production of ROS. In order to obtain a more physiological impression of the mechanism of NETosis and the interplay with components of the complement system, we incorporated the use of *P. aeruginosa*

in further studies. As previously seen (28–30), bacteria are capable of inducing Nox-dependent NETosis in a load-dependent manner.

Neutrophils recruited to sites of inflammation are a major determinant of AP activation (1). Neutrophil stimulation with PMA, C5a, and fMLP resulted in a quick release of the complement proteins CFP, C3, and CFB. These proteins are critical for the assembly of the AP convertase C3bBb, where CFP functions as a stabilizer (26). After demonstrating neutrophil release of complement proteins during NETosis, we confirmed that complement proteins became entangled within the NET structures. C5b–9 was deposited on the NETs, independent of the stimulus (PMA and PAKgfp). Deposition was abrogated by the use of DNase, signifying the requirement of the NET lattices for the generation of C5b–9. This finding supports a role for NETs in inducing and/or enhancing complement activation, which is in keeping with the recently published observation that NETs can activate and deposit complement AP components (25). CFP also binds to DNA exposed by necrotic or apoptotic cells, and neutrophil-secreted CFP has been linked to a positive feedback loop between neutrophil and complement activation (1, 34–37). We also found CFP deposition on NETs, and the use of an anti-CFP antibody blocking the AP allowed us to identify the complement pathways involved in NET-mediated C5b–9 activation (38).

Complement factor P blockade was efficient in preventing terminal pathway activation when experimental conditions limited complement activation (i.e., C5b–9) on NETs to the AP. When the classical and lectin pathways were also allowed to be activated, this AP-specific blocking effect was lost. Fluorescence microscopy data suggest the possibility of CFP-mediated C5b–9 formation on NETs – data consistent with recent studies, indicating that both AP- and non-AP-mediated complement activation can occur on NETs (1, 5, 25, 37, 39, 40). CFP binding to targets *via* C3 fragments (alone or in context of the C3-/C5-convertases) is also possible.

Taken together, our results demonstrate that the “AP tool kit” present in the neutrophils are released upon stimulus, and deposits on targets, such as NETs. In the presence of plasma, NET formation results in terminal pathway activation *via* both CFP-dependent and -independent mechanisms. In NET-mediated diseases, the formation of NETs might trigger complement activation and exert secondary effects, such as cell injury and death. This is of great importance, as therapeutic complement inhibitors (e.g., eculizumab) are now available for clinical use (41, 42).

AUTHOR CONTRIBUTIONS

JY designed and carried out the experiments, interpreted data, and wrote the first draft of the manuscript; AC, MR, and FP

contributed to designing and carrying out experiments, interpreting data, and writing the manuscript; DD provided technical assistance in designing and carrying out some of the experiments; MU provided the PAKgfp strain and contributed interpreting data; WK contributed designing experiments and interpreting data; NP and CL designed experiments, supervised the study, interpreted data, and wrote the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at <http://journal.frontiersin.org/article/10.3389/fimmu.2016.00137>

FIGURE S1 | PMA and fMLP, but not C5a, induce ROS production in neutrophils. Neutrophils were activated with (A) C5a (1 or 2 μ M), (B) fMLP (1 or 2 μ M), and (C) PMA (20 nM) and analyzed by flow cytometry for oxidative burst using dihydrorhodamine (DHR) 123. A significant ROS production was only observed for fMLP (1 μ M) and PMA (20 nM). Results are given as median fluorescence intensity (MFI) from three independent experiments. Student's *t*-test, **p* < 0.01.

FIGURE S2 | Confocal images showing PMA-mediated kinetics of NETosis. Neutrophils were activated with PMA (20 nM) to induce NET formation. Samples were fixed with 4% (w/v) paraformaldehyde and stained with DAPI for microscopy. (A) Four distinct nuclear morphologies (lobulated, delobulated, decondensed nuclei; NETs) can be identified during NETosis. (B) Percentage difference for nuclear morphologies was identified through manual counting of at least 118–220 cells from five different focal planes at 40 \times magnification. Data are presented as mean \pm SEM from three independent experiments. Statistical significance is shown only if percentage of nuclear morphology is significantly different compared to all other morphologies at the same time point. Two-way ANOVA with Tukey's multiple comparison test, **p* < 0.05, #*p* < 0.001, §*p* < 0.0001.

FIGURE S3 | Anti-CFP antibody concentrations of >4 μ g/ml block AP-mediated complement activation. Serial dilutions of a mouse monoclonal anti-properdin antibody were performed to determine the antibody concentration required to completely inhibit complement AP as determined by rabbit erythrocyte lysis. This was achieved using concentrations >4 μ g/ml. Data are presented as mean \pm SEM from three independent experiments. Student's *t*-test, **p* < 0.05, ***p* < 0.01.

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Effect of High-Fat Diet on the Formation of Pulmonary Neutrophil Extracellular Traps during Influenza Pneumonia in BALB/c Mice

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Obesity is an independent risk factor for severe outcome of influenza infection. Higher dietary fat consumption has been linked to greater morbidity and severe influenza in mouse models. However, the extent of generation of neutrophil extracellular traps (NETs or NETosis) in obese individuals during influenza pneumonia is hitherto unknown. This study investigated pulmonary NETs generation in BALB/c mice fed with high-fat diet (HFD) and low-fat diet (LFD), during the course of influenza pneumonia. Clinical disease progression, histopathology, lung reactive oxygen species, and myeloperoxidase activity were also compared. Consumption of HFD over 18 weeks led to significantly higher body weight, body mass index, and adiposity in BALB/c mice compared with LFD. Lethal challenge of mice (on HFD and LFD) with influenza A/PR/8/34 (H1N1) virus led to similar body weight loss and histopathologic severity. However, NETs were formed at relatively higher levels in mice fed with HFD, despite the absence of significant difference in disease progression between HFD- and LFD-fed mice.

Keywords: neutrophils, NETs, high-fat diet, influenza, pneumonia

INTRODUCTION

Neutrophils release neutrophil extracellular traps (NETs) to ensnare and kill pathogens, such as bacteria and fungi (1, 2), but limited studies have addressed their role in viral infections (3–5). NETs can entrap and neutralize HIV-1 in a TLR7- and TLR8-dependent manner (4). On the other hand, when NETs are present in large numbers, they can cause tissue damage by releasing cytotoxic proteins into the extracellular space. We previously revealed exuberant infiltration of activated neutrophils and extensive generation of pulmonary NETs in lethal challenge of mice with influenza virus that contribute to lung injury (6). We also demonstrated the association between pulmonary NETosis with histopathologic severity in mice during lethal pneumococcal superinfection following influenza lung infection (7, 8). These studies underscore the pathological role of pulmonary NETs during influenza pneumonia.

Obesity has been associated with chronic low-grade inflammation (9). Nutrient excess restricts blood supply to adipocytes leading to hypoxia that can incite necrosis and macrophage infiltration of adipose tissue, which in turn leads to oversecretion of pro-inflammatory cytokines (10, 11).

The adipocytes themselves secrete adipokines, such as TNF- α and IL-6, that are pro-inflammatory and associated with various metabolic conditions (12, 13). Augmented peripheral neutrophil activity, such as superoxide generation, is observed in healthy obese subjects and may be attributed to cytokines, such as IL-8 secreted from the adipocytes (14, 15). Even though pro-inflammatory cytokines, such as TNF- α and IL-8, can induce NETosis (1, 16), the effects of adiposity and its associated inflammation upon NETosis are still unclear.

Obesity was documented as an independent risk factor for complications arising from severe influenza during the 2009 H1N1 pandemic (17, 18). Earlier mouse models of diet-induced obesity (DIO) have also linked the degree of obesity with severe influenza outcome and the ensuing pulmonary pathology and immune dysfunction (19–21). The association between pulmonary NETosis and the outcome of influenza pneumonia in obese subjects is hitherto unknown. Given that adipose tissues favor a pro-inflammatory environment that may potentially activate neutrophils, we hypothesize that higher adiposity can contribute to greater pulmonary NETs formation that aggravates the pathologic outcome of influenza pneumonia. The objective of this study was to investigate and compare the effects of high-fat diet (HFD) versus low-fat diet (LFD) on the extent of NETosis in the lungs of BALB/c mice during lethal influenza challenge.

MATERIALS AND METHODS

Animals, Ethics, and Diet Regimen

All animal experiments were performed according to the regulations of the Institutional Animal Care and Use Committee, National University of Singapore (protocol number 050/11). Four-week-old male BALB/c mice were acclimatized for 1 week with standard chow before beginning a scheduled defined diet (Research Diets). Mice were randomly divided into two groups, i.e., each group was fed with either LFD (10% kcal from dietary fat) or HFD (45% kcal from dietary fat) for 18 weeks. Fresh feed was provided every week. Body weight, BMI [weight (g)/nose-to-anus length (mm²)], and food intake were measured weekly. The calories consumed were calculated based on food consumed (i.e., 1 g LFD = 3.85 kcal; 1 g HFD = 4.73 kcal). Blood glucose levels were measured using an Accu-Check Performa glucometer (Roche) at the start and end of the 18-week dietary schedule. At the end of the 18 weeks, organs such as lungs, brain, kidneys, heart, liver, spleen, white adipose tissue (WAT) of gonadal, perirenal regions, and interscapular brown adipose tissue (IBAT) from both groups of mice were harvested, weighed, and sectioned for hematoxylin and eosin (H&E) staining.

Infection of Mice with Influenza Virus

Influenza virus A/Puerto Rico/8/34 (H1N1) strain (PR8) from the American Type Culture Collection was propagated in embryonated eggs, as described previously (7). After the 18-week dietary schedule, mice from both diet groups were intratracheally challenged with a lethal dose of PR8 virus, i.e., 50 plaque-forming units (PFU). Anesthesia was performed using 75 mg/kg ketamine

and 1 mg/kg medetomidine, and reversed using atipamezole hydrochloride (5 mg/ml). Control mice received phosphate-buffered saline (PBS) alone. The mice were euthanized on 6 and 10 days post-infection (DPI), their lungs were excised, with one lobe fixed in 4% paraformaldehyde, while the other lobe was snap frozen for subsequent assays.

Histopathologic Scoring of Lung Tissue

Formalin-fixed lungs were dehydrated and embedded in paraffin. Lung sections (5 μ m) stained with H&E were subjected to histopathologic scoring in a blinded manner based on modified criteria (22). The final injury score was calculated according to the formula: 1 \times (alveolar hemorrhage, 0–3) + 2 \times (alveolar infiltrate, 0–3) + 2 \times (bronchiolar infiltrate, 0–3) + 2 \times (fibrin, 0–3) + 1 \times (alveolar septal congestion, 0–3), where, 0–3 refer to 0 = absent, 1 = mild, 2 = moderate, and 3 = severe.

NETs Staining and Quantification in Lung Tissue

Neutrophil extracellular traps in the lung sections were quantified by triple immunolabelling, as described previously (7). Briefly, lung sections (5 μ m) were stained with antibodies against histone H2B (Abcam) and myeloperoxidase (MPO, Abcam), and DAPI (Life Technologies). NETs were identified as single strands or clusters, and scored according to pre-determined criteria (0–10). Twenty fields were analyzed, and the sum was calculated for the final NETs score.

Determination of Viral Titers

Frozen lung tissues were homogenized using the gentleMACS tissue dissociator (Miltenyi Biotech). The viral titers were determined by plaque assay, as described previously (23).

Hydrogen Peroxide and Myeloperoxidase Assays

Hydrogen peroxide (H₂O₂) concentrations in lungs were measured by the Amplex red hydrogen peroxide/peroxidase assay kit (Invitrogen). MPO activity was determined, as described previously (8). Briefly, 10 μ l of lung homogenate was mixed with 190 μ l of freshly prepared assay solution (26.9 ml H₂O, 2.0 ml 0.1M sodium phosphate buffer pH 7.0, 0.1 ml 0.1M H₂O₂, and 0.048 ml guaiacol), and the absorbance was read immediately at 470 nm for 1 min. The MPO activity was calculated as units/ml = (Δ O.D. \times V_t \times 4)/(E \times Δ t \times V_s) \times 2, where Δ O.D. = optical density change, V_t = total volume (milliliters), E = 26.6 mM⁻¹ cm⁻¹ (extinction coefficient of tetraguaiacol product), Δ t = time of measurement (minutes), V_s = sample volume (milliliters), and 2 is the conversion factor to 1 cm path-length. All values were normalized to lung protein content as measured by the Bradford method (Bio-Rad).

Statistical Analyses

Statistical analyses were performed using the SPSS (version 22). Student's *t*-test was used for analyzing parametric data, whereas Mann–Whitney *U* test was used for non-parametric data analysis. ANOVA with Tukey *post hoc* correction was used for comparison

of more than two groups. *P*-values less than 0.05, 0.01, and 0.001 were considered significant to varying extents.

RESULTS

HFD Mice Gain Higher Body Weight and Body Fat Compared with LFD Mice

Mice on HFD showed significantly higher body weights ($P < 0.001$ on week 18) and BMI ($P < 0.01$ on week 18) compared with LFD-fed mice from 15 weeks onward, despite their weights being comparable at the start of the diet (Figures 1A,B). Although the amount of food consumed was generally similar between the groups, the HFD mice consumed more calories per mouse than the LFD mice due to higher fat percentage in HFD ($P < 0.05$ until week 15) (Figures 1C,D). However, their blood glucose levels did not reveal any significant difference ($P = 0.239$) (data not shown).

The HFD mice also displayed relatively higher accumulation of WAT in the gonadal ($P = 0.1$) and perirenal fat pads ($P = 0.2$), IBAT ($P = 0.1$) (Figure 2A). Organs, such as heart ($P = 0.7$) and kidneys ($P = 0.4$), along with fat pads exhibited relatively increased weights compared with LFD mice, albeit not statistically significant (Figure 2B). The weights of spleen ($P = 0.2$), lungs ($P = 1.0$), and liver ($P = 1.0$) were slightly decreased in HFD mice, whereas the weights of brain ($P = 1.0$) were comparable between the two groups. No significant difference was observed in the histology of adipose tissue between the two groups (data not shown).

Infected HFD Mice Have Relatively Elevated Pulmonary Viral Load but Exhibit Similar Lung Pathology as LFD Mice

Upon lethal challenge with influenza A virus, both LFD and HFD mice showed similar body weight loss ($P < 0.01$ of both infected groups versus controls) (Figure 3A). The lung viral titers were almost threefold higher in the HFD group than LFD group, albeit not statistically significant ($P = 0.401$) (Figure 3B). A previous DIO study also observed somewhat elevated influenza viral titers in HFD mice, indicating that obesity exerts only marginal influence on viral replication within the host (19). Although histopathologic analyses demonstrated a relative reduction of overall severity score in the infected HFD group at 6 DPI ($P = 0.108$), the score became comparable with the infected LFD group by 10 DPI ($P = 0.725$) (Figures 3C,D). Both groups of mice showed thickening of alveolar septae, enhanced inflammatory cellular infiltration in the alveolar and bronchiolar spaces, and by 10 DPI, increased alveolar fibrin deposition.

Relatively Enhanced Formation of Lung ROS and NETs in Infected HFD Mice

The H_2O_2 concentration in lungs was relatively higher in infected HFD mice at 6 DPI ($P = 0.08$), indicating heightened oxidative stress in the lungs compared with LFD mice (Figure 4A). However, MPO activity was relatively lower ($P = 0.151$ and 0.128 ; 6 and 10 DPI) in infected HFD mice compared with LFD mice (Figure 4B). The infected HFD mice showed a

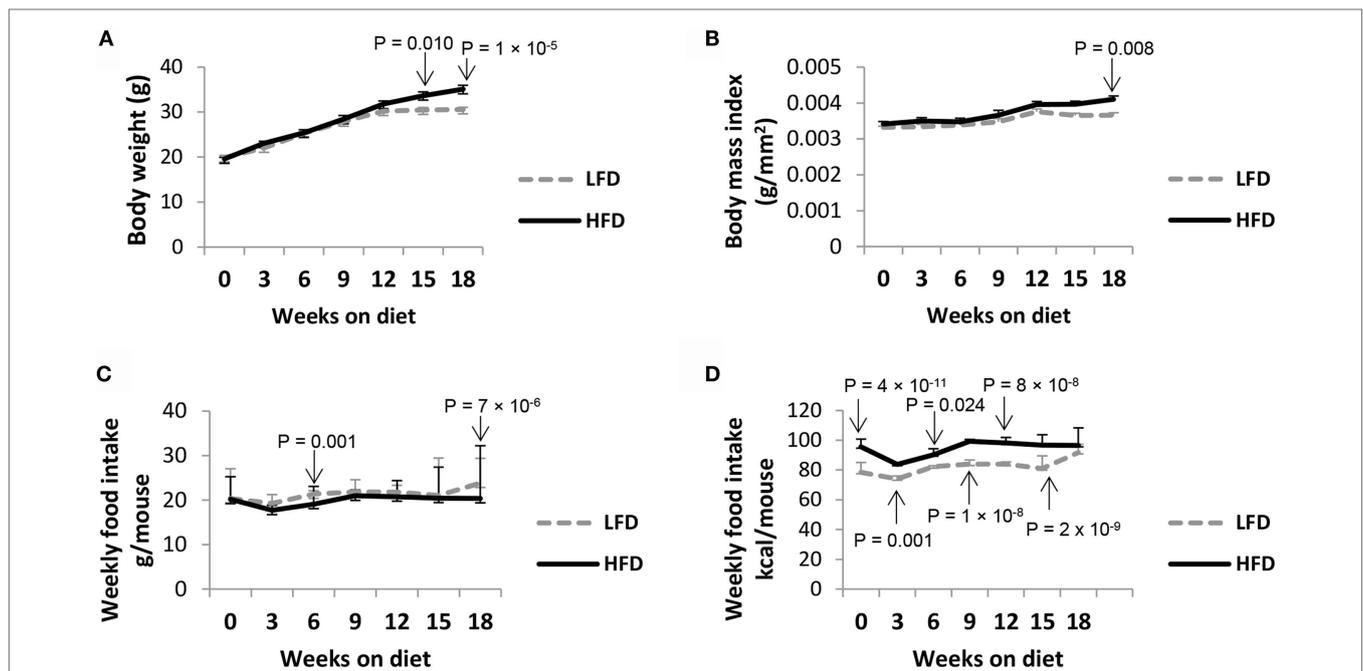
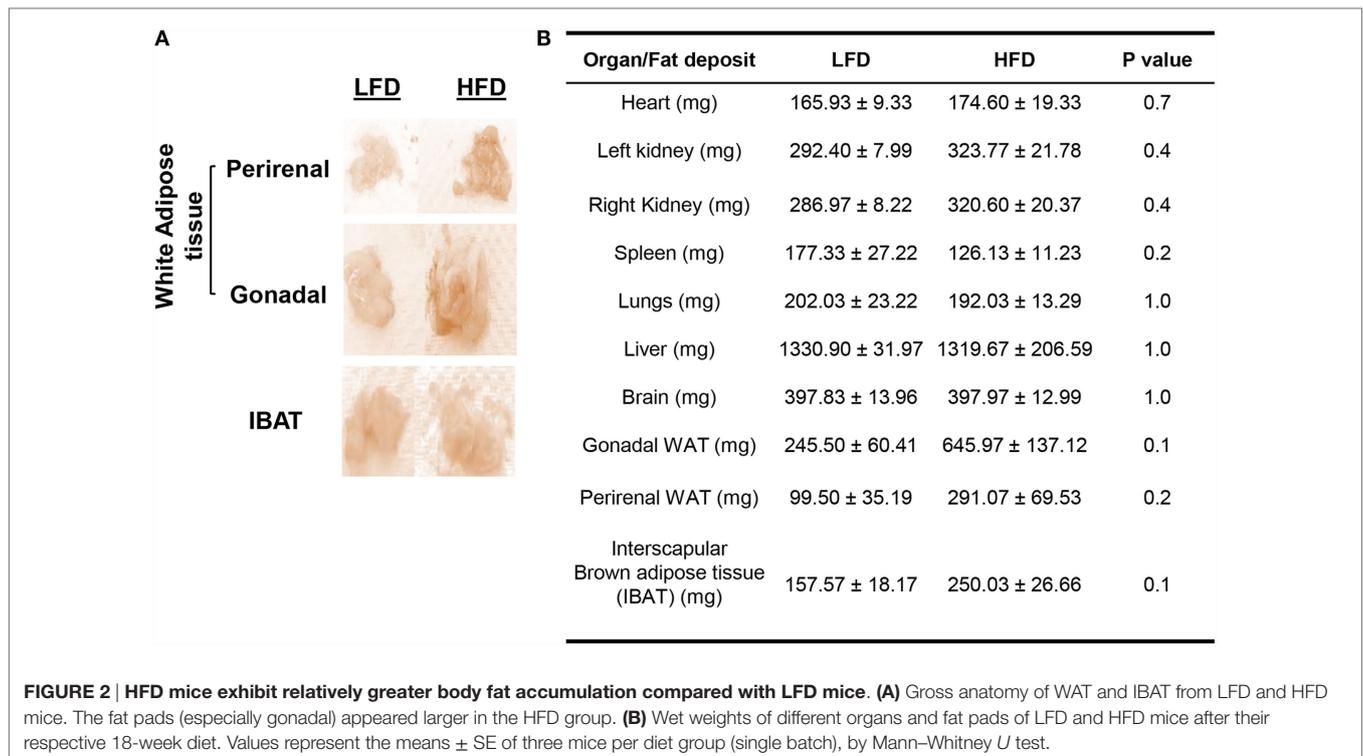


FIGURE 1 | HFD mice show higher body weight parameters compared with LFD mice. BALB/c mice (5-week-old) were fed with either HFD or LFD for 18 weeks. Upon completion of these diets, HFD mice showed significantly higher (A) weight gain and (B) BMI. Although (C) the weekly intake of food per mouse was comparable between the two groups, (D) the actual calories consumed varied due to higher dietary fat content in HFD. Values represent the means \pm SE of 15 mice per diet group (3 independent batches), by ANOVA with Tukey *post hoc* correction.



trend of relatively higher generation of NETs in their lungs at 6 DPI ($P = 0.104$) and 10 DPI ($P = 0.111$) (Figures 4C,D). Our findings are congruent with previous reports documenting augmented neutrophil activity in obese individuals and murine DIO models (14, 15, 24).

DISCUSSION

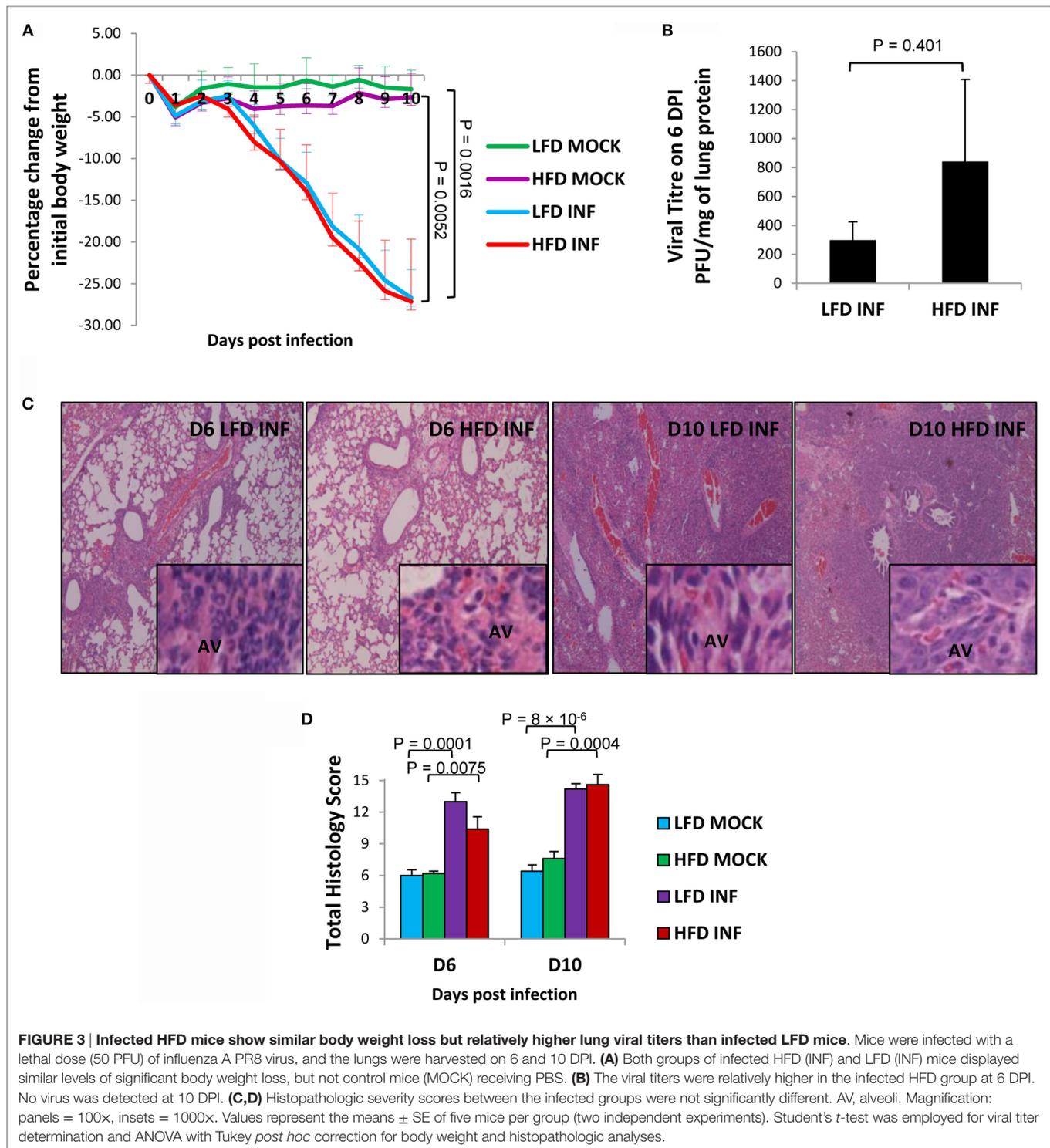
The widespread prevalence of obesity is a cause of concern for health authorities worldwide. Besides leading to a number of metabolic disorders, such as type 2 diabetes and atherosclerosis, obesity can impact respiratory health as well (25). Obesity escalates susceptibility to influenza-related complications and community-acquired pneumonia (17, 26). While earlier studies on DIO and influenza concentrated mostly on the functions of immune cells, such as T-cells, macrophages, and neutrophils, there are hitherto no reports on the extent of NETs generated in obese individuals. Since NETs are implicated in many pathologic conditions, their importance in obesity, especially during influenza pneumonia warrants closer attention (6, 7, 27, 28).

Although our study revealed that both groups of BALB/c mice on HFD and LFD suffered similar clinical progression during influenza pneumonia, there may be subtle differences in their lung pathophysiology and immune response. We assessed and compared viral titer, H_2O_2 concentration, MPO activity, histopathologic severity, and formation of pulmonary NETs between the two dietary groups. If HFD consumption alters the immune response in mice, it may influence viral replication. Higher pulmonary viral load may culminate in enhanced

NETs stimulation due to elevated levels of pro-inflammatory cytokine signaling and oxidative stress, given that NETosis is an oxidative process.

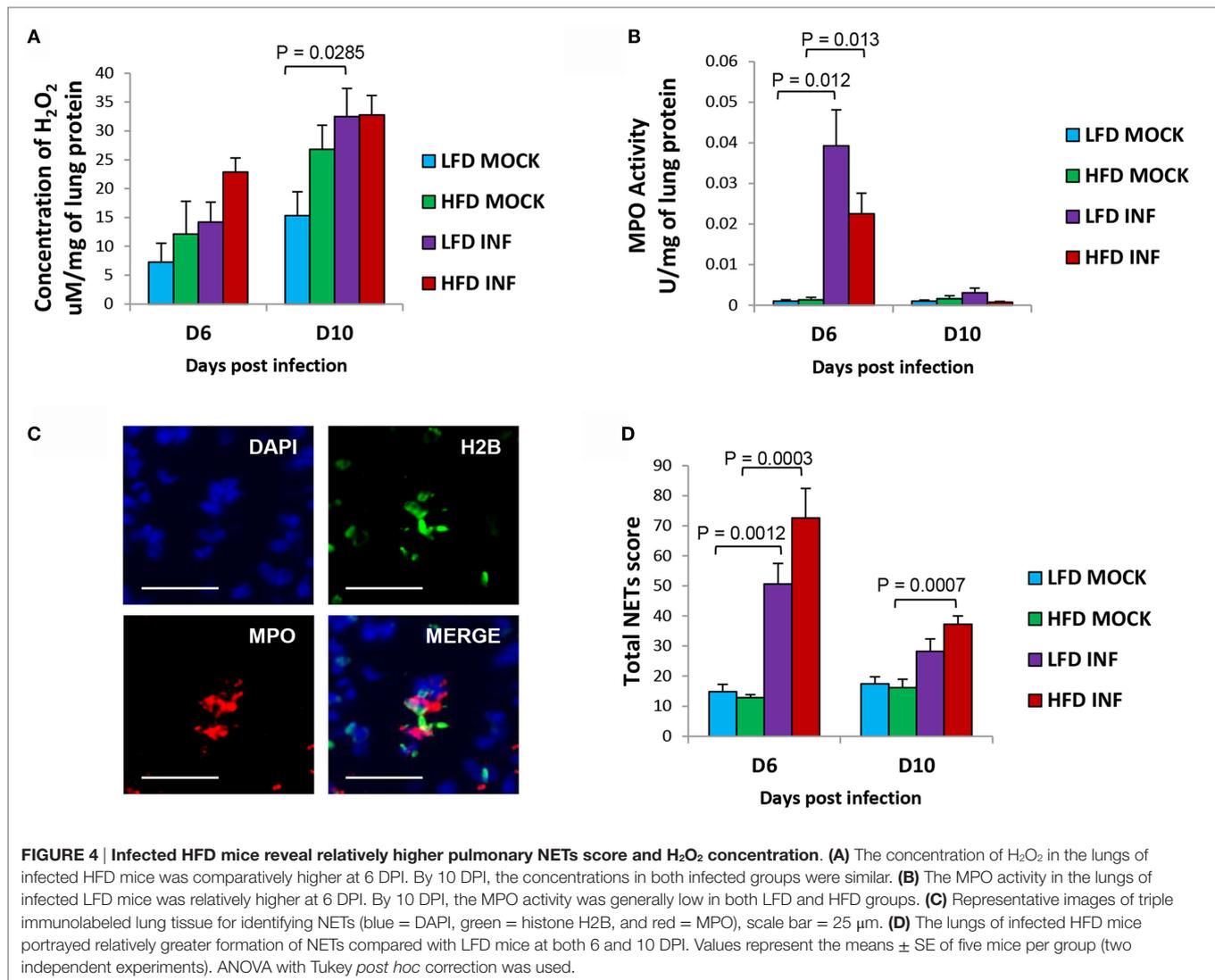
Generally, pulmonary viral burden, ROS concentration, and NETs formation were somewhat elevated in infected HFD mice compared with their LFD counterparts. This alludes to the subtle influences exerted by higher adiposity on these pathologic parameters. However, MPO activity was found to be slightly lower in infected HFD mice than LFD mice at 6 DPI. Similarly, the lung pathology scores were also relatively lower in HFD mice at 6 DPI. The relatively lower reduced lung viral load in infected leaner LFD mice implies a more functionally robust neutrophil response and inflammation to control the viral infection. However, we previously demonstrated that infection of neutrophils by influenza virus alone does not support active viral replication *in vitro* (29). Additional studies are thus justified to determine, if obesity and NETs release modulate virus replication and/clearance over the course of influenza pneumonia. Despite lower MPO activity, we also observed relatively greater formation of NETs in the lungs of infected HFD mice compared with infected LFD mice. Further detailed research is necessary given the involvement of MPO during NETosis, although it is still debatable whether MPO needs to be enzymatically active in this process. Contradictory reports suggest that the activity of MPO may either be non-essential during NETs stimulation (30) or is required along with its substrate H_2O_2 for antimicrobial activity of NETs (31).

In this study, we used BALB/c mice for consistency and comparison with our previous models on NETs during influenza pneumonia. Although the BALB/c mice on HFD were



not considered strictly “obese,” they displayed sufficiently greater amount of adipose tissue to result in significant difference in BMI and body weight, which are also parameters employed in human studies (17, 18). However, other mouse strains (e.g., C57/BL6, Swiss albino mice) on HFD should

also be tested for their degree of NETs generation following influenza challenge. Our study revealed a generally higher trend of pulmonary NETosis associated with adiposity. This suggests that in morbidly obese individuals, pulmonary NETs may form at significant levels in response to influenza infection



that may exacerbate lung injury and complications of influenza pneumonia. In conclusion, we demonstrate that increased adiposity due to prolonged consumption of HFD may lead to relatively greater formation of NETs in murine lungs during severe influenza pneumonia.

ETHICS STATEMENT

Experiments were performed after obtaining approval for all procedures to be performed on the animals, from the Institutional Animal Care and Use Committee, National University of Singapore (protocol number 050/11).

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AUTHOR CONTRIBUTIONS

AM designed and performed the experiments, analyzed the data, and wrote the manuscript. SW and KT contributed to histopathologic scoring. TN contributed to data analyses. VC conceived and supervised the experiments, and wrote the manuscript.

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Conflict of Interest Statement: The 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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Neutrophils Discriminate between Lipopolysaccharides of Different Bacterial Sources and Selectively Release Neutrophil Extracellular Traps

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The release of neutrophil extracellular traps (NETs), either during “suicidal” or “vital” NETosis, represents an important strategy of neutrophils to combat Gram-negative bacteria. Lipopolysaccharide (LPS), a major component of the outer membrane of Gram-negative bacteria, is a reported stimulus for NET formation. Although it is widely acknowledged that the structural diversity in LPS structures can elicit heterogeneous immune responses, species- and serotype-specific differences in the capacity of LPS to trigger NET formation have not yet been investigated. In the present study, we compared the NET-inducing potential of LPS derived from *Escherichia coli* (serotypes O55:B5, O127:B8, O128:B12, O111:B4, and O26:B6), *Salmonella enterica* (serotype enteritidis), and *Pseudomonas aeruginosa* (serotype 10), under platelet-free and platelet-rich conditions *in vitro*, and in whole blood *ex vivo*. Here, we demonstrate that under serum- and platelet-free conditions, mimicking tissue circumstances, neutrophils discriminate between LPS of different bacterial sources and selectively release NETs only in response to LPS derived from *E. coli* O128:B12 and *P. aeruginosa* 10, which both induced “suicidal” NETosis in an autophagy- and reactive oxygen species (ROS)-dependent, but TLR4-independent manner. Intriguingly, in whole blood cultures *ex vivo*, or *in vitro* in the presence of platelets, all LPS serotypes induced “vital” NET formation. This platelet-dependent release of NETs occurred rapidly without neutrophil cell death and was independent from ROS formation and autophagy but required platelet TLR4 and CD62P-dependent platelet–neutrophil interactions. Taken together, our data reveal a complex interplay between neutrophils and LPS, which can induce both “suicidal” and “vital” NETosis, depending on the bacterial origin of LPS and the presence or absence of platelets. Our findings suggest that LPS sensing by neutrophils may be a critical determinant for restricting NET release to certain Gram-negative bacteria only, which in turn may be crucial for minimizing unnecessary NET-associated immunopathology.

Keywords: NETosis, neutrophil extracellular traps, lipopolysaccharides, platelets, cell death

INTRODUCTION

Neutrophils are the most abundant terminally differentiated leukocytes circulating in the blood. Attracted by a chemotactic gradient of chemokines, neutrophils can rapidly traffic to inflammatory sites, where they utilize their antimicrobial arsenal of effector mechanisms to eradicate pathogens. In addition to phagocytosis and degranulation, the release of neutrophil extracellular traps (NETs)

represents a key antimicrobial strategy of neutrophils (1). NETs are released during a highly complex cell death pathway known as “suicidal” NETosis and comprise an expelled web of chromatin fibers that can bind pathogens, thereby inhibiting their spreading and facilitating their elimination (2, 3). Recent evidence indicates that NETs can also be released from viable neutrophils during an alternative pathway called “vital” NETosis, which requires activated platelets and is therefore thought to occur predominantly during sepsis (4, 5).

The release of NETs has been observed in response to many different bacteria, viruses, fungi, and parasites (6). Nevertheless, it is still incompletely understood how pathogens induce signaling events that result in NETosis. For Gram-negative bacteria, lipopolysaccharide (LPS) has been reported as important stimulus for NETosis (1). However, seemingly contradicting data in literature question whether the interaction between LPS and neutrophils can indeed trigger NET formation. Some reports describe that LPS often protects neutrophils against apoptosis but fails to induce NETosis (7, 8), whereas other reports claim that LPS-induced NETosis is only observed when additional factors are present, such as the immunomodulatory GM-CSF (9), apoptotic microparticles (10), or platelets (11).

Lipopolysaccharide has three main structural components: lipid A, a core domain containing an oligosaccharide component and a repetitive glycan polymer referred to as the O-antigen (12). Whereas the lipid A structure is relatively conserved, there is great variability in the composition of the O-antigen between bacterial strains, which provides the major basis for bacterial serotyping. The structural diversity in LPS has been associated with heterogeneous immune responses (13–16). However, species- and serotype-specific differences in the capacity of LPS to trigger NET formation have not yet been investigated. This study was undertaken to investigate the hypothesis that neutrophils are able to discriminate between LPS structures and thereby selectively release NETs in response to certain structures, which could partly explain the seemingly contradicting data concerning LPS-induced NETosis. Here, we compared the NET-inducing potential of commercially available LPS derived from seven different bacterial sources, i.e., *Escherichia coli* (serotypes O55:B5, O127:B8, O128:B12, O111:B4, and O26:B6), *Salmonella enterica* (serotype enteritidis), and *Pseudomonas aeruginosa* (serotype 10), under serum- and platelet-free or platelet-rich conditions, mimicking tissue and blood circumstances, respectively.

MATERIALS AND METHODS

Antibodies, Proteins, and Chemicals

Reagents were obtained from the following manufacturers: Sytox Orange (ThermoFisher Scientific, Cat. No. S11368, Duisburg, Germany), *N*-Methoxysuccinyl-Ala-Ala-Pro-Val p-nitroanilide (Sigma-Aldrich, Cat. No. M4765, Schnellendorf, Germany), phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich, Cat. No. P8139, Schnellendorf, Germany), micrococcal nuclease (MNase; Worthington Biochemical Corporation, Cat. No. LS004798, Lakewood, USA), TNF- α (eBioscience, Cat. No.

14-8329, Frankfurt, Germany), IL-6 (Prospec, Cat. No. cyt-213, Rehovot, Israel), IFN- α (Prospec, Cat. No. cyt-520, Rehovot, Israel), wortmannin (Enzo Life Sciences, Cat. No. BML-ST415, Raamsdonksveer, The Netherlands), diphenyleneiodonium chloride (DPI; Enzo Life Sciences, Cat. No. BML-CN240, Raamsdonksveer, The Netherlands), PKH26 Red Fluorescent Cell Linker Kit (Sigma-Aldrich, Cat. No. PKH26GL, Schnellendorf, Germany), polyclonal anti-TLR4 (InvivoGen, Cat. No. pab-hstlr4, Toulouse, France), anti-myeloperoxidase (BioLegend, Cat. No. 812801, Uithoorn, The Netherlands), anti-neutrophil elastase (Abcam, Cat. No. ab21595, Cambridge, UK), and anti-CD62P (Santa Cruz, Cat. No. sc-8419, Heidelberg, Germany). All LPS structures used in this study were purchased from Sigma-Aldrich (Schnellendorf, Germany) and are listed in **Table 1**.

Isolation of Neutrophils

Neutrophils were isolated as described earlier (17). Briefly, neutrophils were isolated at room temperature from EDTA-anticoagulated whole blood by Ficoll density gradient centrifugation using Lymphoprep™ (Stemcell Technologies, Cat. No. 07851). After centrifugation for 20 min at 800 \times g, the lower cellular fraction with neutrophils was collected, and residual erythrocytes were lysed in a hypotonic buffer. Neutrophils were counted with CASY cell counting technology (Scharfe System, Reutlingen, Germany) and adjusted to 1 million cells per milliliter in serum-free DMEM/F12 medium containing no phenol red (Life Technologies, Cat. No. 11039-021, Bleiswijk, The Netherlands).

Isolation of Platelets

After Ficoll density gradient centrifugation, platelet-rich plasma was collected and diluted 10 times in a buffer of PBS, 1% FCS, and 1 mM EDTA. Remaining leukocytes were pelleted at 190 \times g for 15 min at room temperature, after which the remaining supernatant with platelets was pelleted at 2500 \times g for 5 min at room temperature. The platelet pellet was immediately and carefully resuspended in DMEM/F12 medium to an equivalent volume as they were in the blood, yielding a solution of 100% platelets (v/v).

Induction and Quantification of NETosis

Purified neutrophils (3×10^5 cells per cm²) were seeded in well plates and stimulated with LPS from different bacterial sources, at the indicated concentrations and conditions, for 3–5 h at 37°C. Where indicated, stimulation of neutrophils with 100 nM PMA served as a positive control. After stimulation, neutrophils and adherent NETs were carefully washed twice with pre-warmed PBS (37°C) and isolated by partial NET digestion in DMEM/

TABLE 1 | LPS structures used in this study.

Species	Serotype	Abbreviation	Cat. No.
<i>Escherichia coli</i>	O55:B5	LPS-O55	L6529
	O127:B8	LPS-O127	L4516
	O128:B12	LPS-O128	L2755
	O111:B4	LPS-O111	L4391
	O26:B6	LPS-O26	L2654
<i>Salmonella enterica</i>	Enteritidis	LPS-SE	L7770
<i>Pseudomonas aeruginosa</i>	10	LPS-PA	L9143

F12 medium supplemented with 5 U/ml MNase (20 min at 37°C). Extracellular DNA in NET-containing supernatants was stained with 100 nM Sytox Orange and quantified by fluorometry (excitation/emission 530/640 nm). The activity of NET-associated neutrophil elastase (NE) and myeloperoxidase (MPO) was determined colorimetrically, using 100 μ M *N*-methoxysuccinyl-Ala-Ala-Pro-Val 4-nitroanilide (at 405 nm) or 1 mM 3,3',5,5'-tetramethylbenzidine (at 605 nm) as substrates for NE and MPO, respectively.

Immunofluorescence Imaging

Purified neutrophils (3×10^5 cells per cm^2) were seeded in slide-flask chambers (Thermo Scientific, Cat. No. 170920, Duisburg, Germany) and stimulated with LPS from different bacterial sources, at the indicated concentrations and conditions, for 3–5 h at 37°C. Where indicated, stimulation of neutrophils with 100 nM PMA served as a positive control. After stimulation, cells and NETs were fixed in 4% paraformaldehyde (30 min, room temperature), and slides were stained for DNA (Sytox Orange; 100 nM), NE (dilution 1:200; antibody listed above), and/or MPO (dilution 1:100; antibody listed above). Slides were embedded in Vectashield Mounting Medium (Brunschwig Chemie, Cat. No. H-1200, Amsterdam, The Netherlands), and pictures were obtained with a Zeiss fluorescence microscope with Axiovision software (Sliedrecht, The Netherlands).

Statistical Analyses

Values are expressed as mean \pm SEM. Significance was either determined by Student's *t*-test or one-way ANOVA followed by Bonferroni correction using GraphPad Prism 5.0 (La Jolla, CA, USA). *p* values less than 0.05 were considered as statistically significant.

RESULTS

Neutrophils Selectively Release NETs in Response to Different LPS Structures

To evaluate whether LPS is capable of inducing NETosis, and whether there are species- and/or serotype-specific differences in the capacity of LPS to induce NETosis, purified neutrophils were exposed to seven different LPS structures (at a concentration of 8 pg LPS per neutrophil, which is equivalent to ~ 10 μ g/ml LPS) under platelet- and serum-free conditions (thereby largely approaching tissue circumstances), after which NETosis was quantified by measuring DNA release. Extracellular DNA was only detected when neutrophils were exposed to LPS-O128 and LPS-PA, whereas the other LPS serotypes did not induce DNA release (**Figure 1A**, left panel). For LPS-O128 and LPS-PA, the amount of extracellular released DNA approached ~ 40 – 50% of the total cellular DNA, as determined in total cell lysates, indicating that approximately half of the neutrophils were lysed and released NETs. Measurement of extracellular elastase activity in the same culture supernatants revealed that only LPS-O128 and LPS-PA were able to induce the release of elastase (**Figure 1A**, right panel). The release of both extracellular DNA and elastase clearly suggested that NETs were released. Indeed, the presence

of NETs could be confirmed by immunofluorescence imaging, where typical DNA filaments (“spread” NETs) as well as “diffused” NETs could be observed (**Figure 1B**). In sum, LPS-induced NETosis is under tissue circumstances species- and serotype-dependent and is, among the seven LPS structures investigated here, limited to LPS-O128 and LPS-PA.

LPS-O128 and LPS-PA Induce NETosis Only When Present above a Threshold Value

Next, the NET-inducing capacity of LPS-O128 and LPS-PA was tested at lower concentrations under the same serum- and platelet-free circumstances. Intriguingly, lowering LPS concentrations did not result in a gradual decrease of the number of neutrophils undergoing NETosis, but instead all neutrophils remained unaffected below concentrations of 8 pg of LPS-O128 and LPS-PA per neutrophil (**Figure 2A**). This indicates that a certain minimum quantity of LPS-O128 and LPS-PA is required to exceed a threshold value that induces NETosis in neutrophils. It can be hypothesized that a pro-inflammatory milieu, i.e., the presence of pro-inflammatory cytokines, might lower the threshold for the induction of NETosis by LPS-O128 and LPS-PA. To test this, neutrophils were preincubated for 1 h with the cytokines TNF- α (10 ng/ml), IL-6 (10 ng/ml), or IFN- α (100 ng/ml), or a mixture of these cytokines, after which LPS was added at a concentration just below the threshold (6 pg LPS per neutrophil). The pro-inflammatory cytokines neither induced NETosis themselves (**Figure 2B**, top left panel) nor lowered the threshold for NETosis induced by LPS-O128 and LPS-PA (**Figure 2B**, middle panels). In addition, LPS-O111 did not gain NET-inducing capacity by priming neutrophils with pro-inflammatory cytokines (**Figure 2B**, bottom left panel). Also, the pro-inflammatory cytokines did not enhance NETosis by LPS-O128 and LPS-PA at a concentration of 8 pg LPS per neutrophil (**Figure 2B**, right panels). In summary, LPS-O128 and LPS-PA trigger NETosis when they are present above a certain threshold value, which is independent from a pro-inflammatory milieu.

LPS-O128 and LPS-PA Induce ROS- and Autophagy-Dependent “Suicidal” NETosis

As outlined, NETs can be generated in several ways, i.e., through cell death-associated “suicidal” NETosis or through “vital” NETosis (5). Since the viability of neutrophils stimulated with LPS-O128 and LPS-PA under serum- and platelet-free conditions (i.e., tissue circumstances) appeared heavily altered (**Figure 1B**), as witnessed by altered lobulated nuclei and decondensed chromatin, we tested the hypothesis that LPS-O128 and LPS-PA trigger the canonical cell death-associated pathway of “suicidal” NETosis, which is dependent on reactive oxygen species (ROS) and autophagy and usually takes hours to complete (7). Indeed, LPS-induced NETosis typically occurred after 3 h (**Figure 3A**) and could be fully prevented by inhibition of autophagy (using wortmannin) or ROS [using diphenyleneiodonium (DPI)] (**Figures 3B,C**). Since LPS signaling is heavily dependent on TLR4, and arguably to a lesser extent on TLR2, we assessed

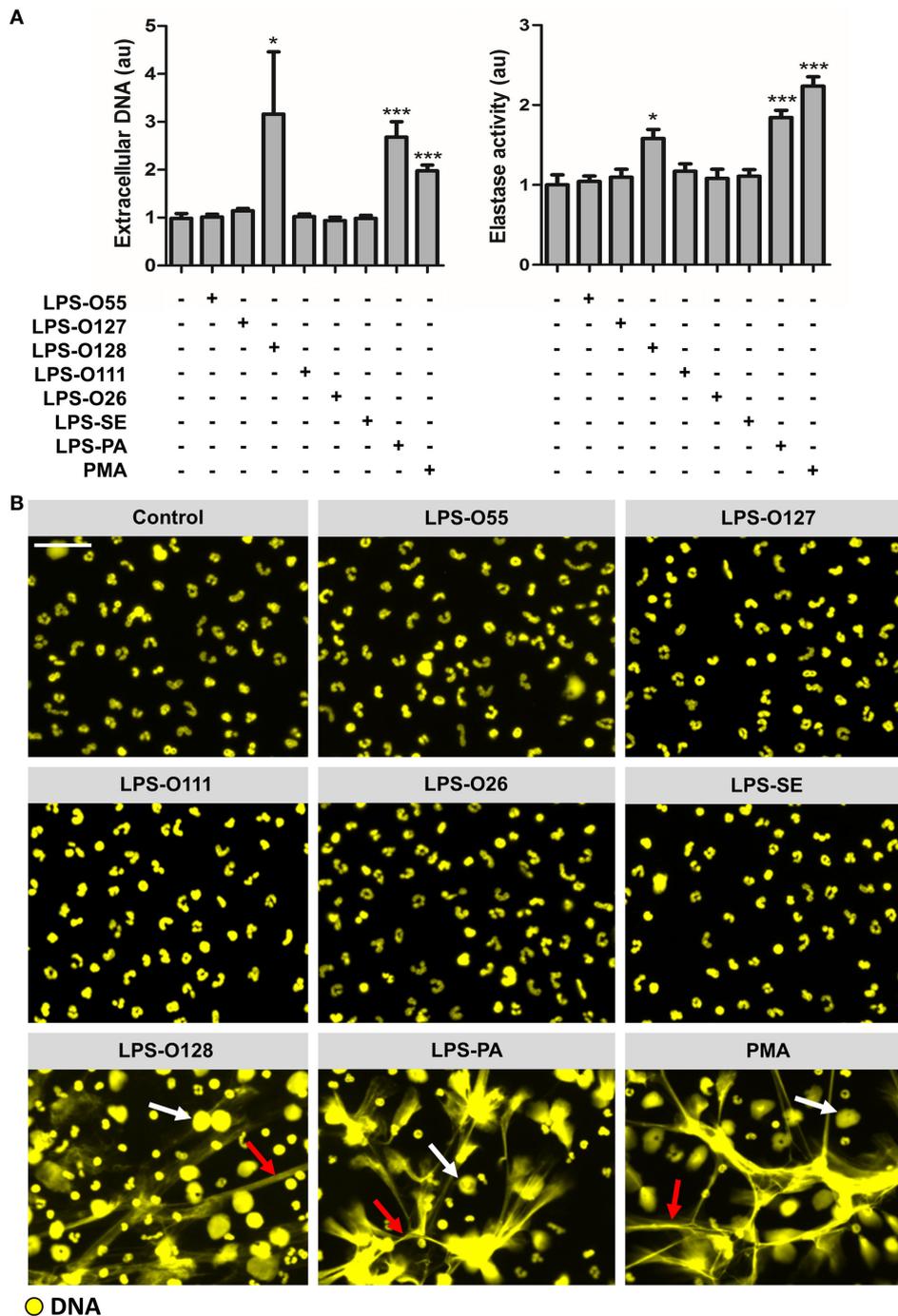


FIGURE 1 | Neutrophils selectively release NETs in response to LPS structures. (A) LPS-O128 and LPS-PA stimulate neutrophils to release NETs after 180 min of incubation in platelet-free cell cultures (left panel), as measured by fluorometry. The release of DNA by LPS-O128 and LPS-PA coincides with extracellular activity of neutrophil elastase (right panel). **(B)** NET release in response to LPS-O128 and LPS-PA was confirmed by immunofluorescence microscopy, in which DNA was stained with 100 nM Sytox Orange (yellow). Both “spread” NETs (red arrows) and “diffused” NETs (white arrows) were observed. NET release in response to 100 nM PMA served as positive control. LPS was used at a concentration of 8 pg LPS per neutrophil. Scale bar: 30 μ m. * p < 0.05 and *** p < 0.001, when compared to control. Data represent mean values \pm SEM of at least three experiments.

whether blockade of TLR2 and TLR4 could prevent NET release in response to LPS-O128 and LPS-PA. However, inhibition of TLR2 and TLR4 did not alter NET release in response to either

LPS structure, suggesting that LPS-induced NETosis occurs in a TLR4 and TLR2-independent manner (**Figure 3B**). Collectively, these data indicate that LPS-PA and LPS-O128 induce “suicidal”

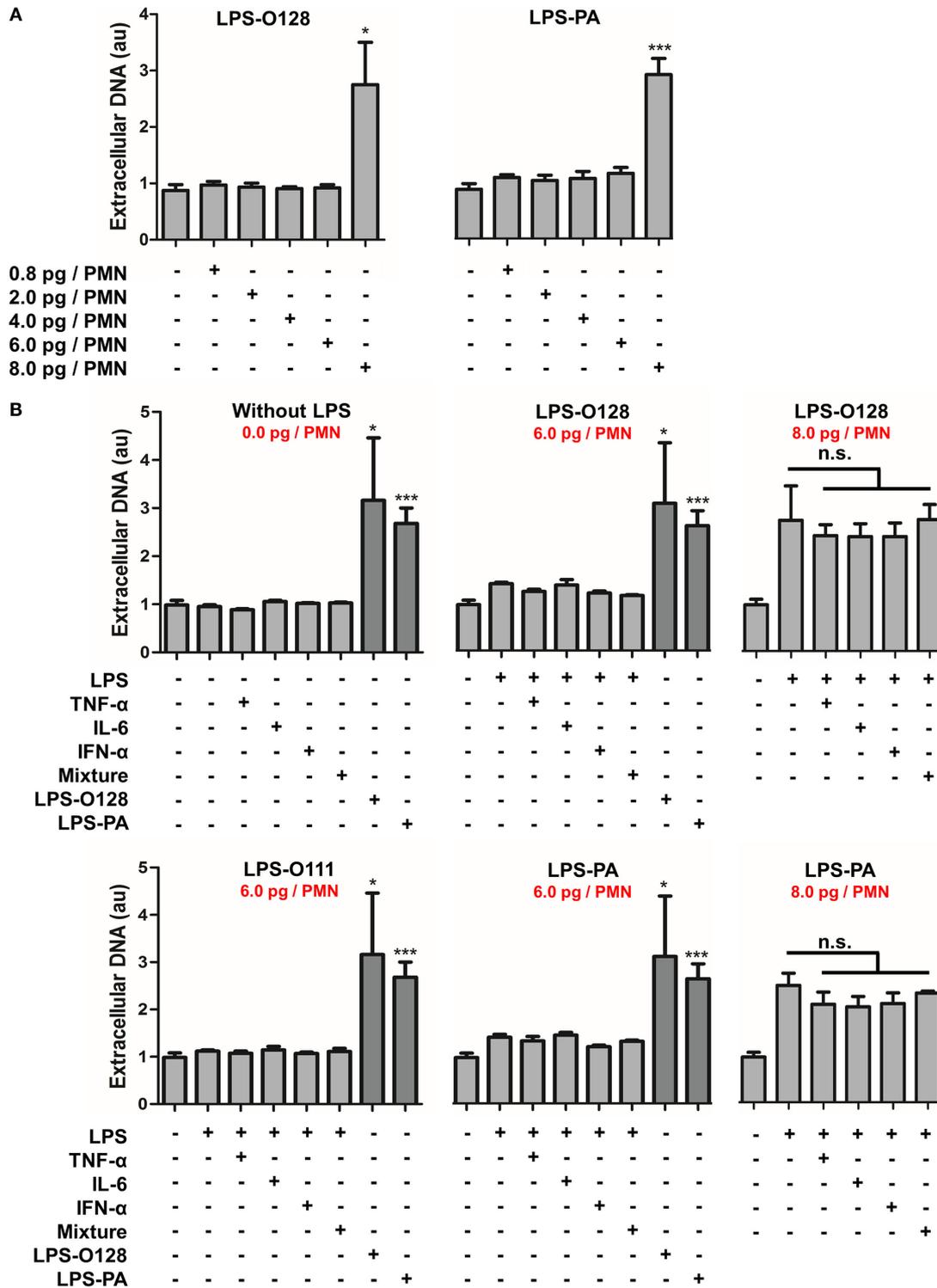
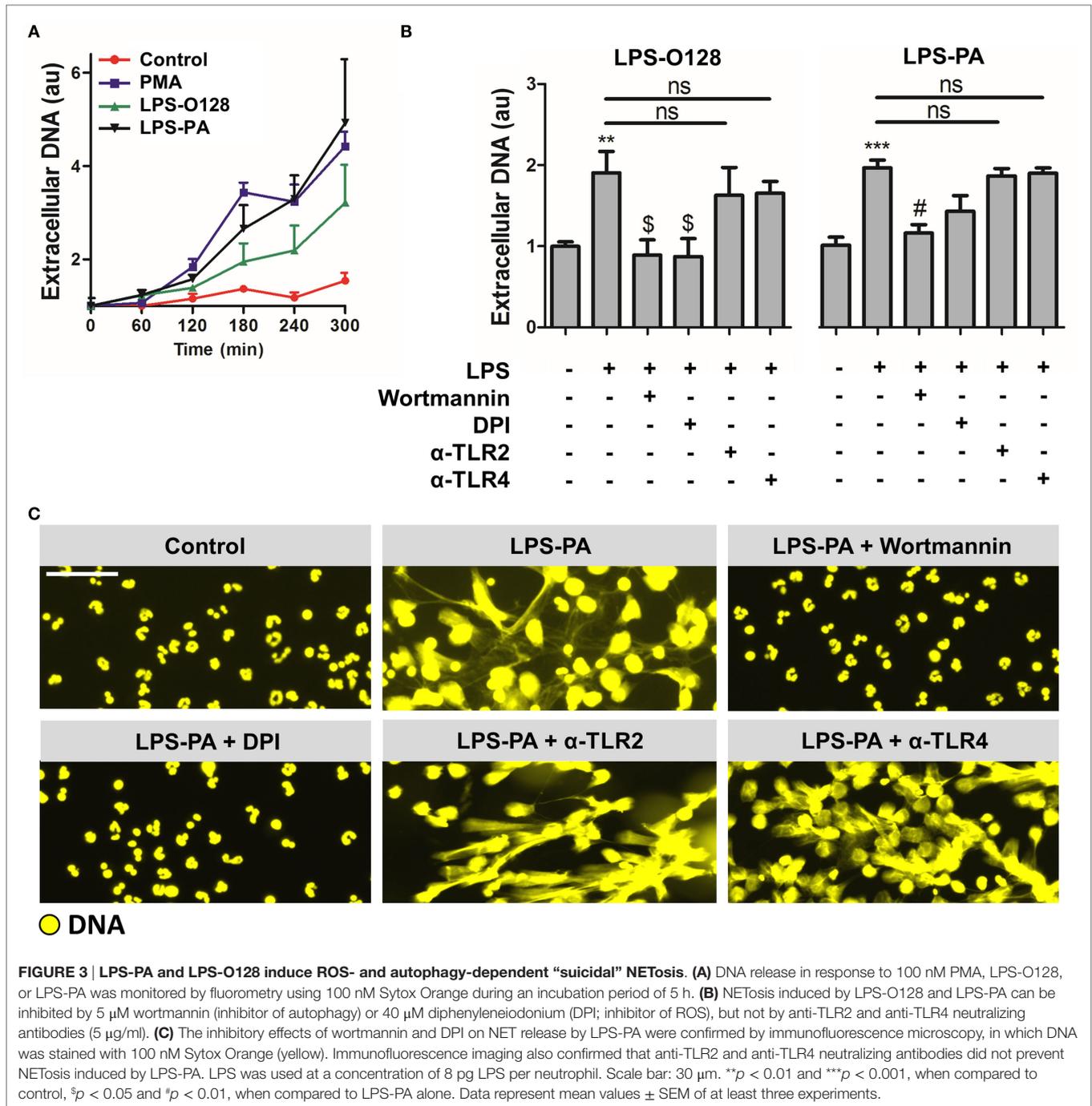


FIGURE 2 | LPS-O128 and LPS-PA induce NETosis when present above a threshold value. (A) LPS-O128 and LPS-PA induce NET release only at high LPS concentrations of 8 pg LPS per neutrophil (PMN). **(B)** Priming of neutrophils for 1 h with recombinant TNF- α (10 ng/ml), IL-6 (10 ng/ml), IFN- α (100 ng/ml), or a mixture of all, does not promote NET release nor prime neutrophils for LPS-induced NETosis by LPS-O111 (bottom left), LPS-O128 (top middle), and LPS-PA (bottom middle) at a concentration of 6 pg LPS per neutrophil (PMN). In these graphs, NETosis induced by LPS-O128 and LPS-PA at 8 pg LPS per neutrophil is shown in dark as positive controls. Cytokines do not enhance NETosis by LPS-O128 or LPS-PA at LPS concentrations of 8 pg LPS per neutrophil (top and bottom right). Quantifications of NET release were performed by fluorometry, as outlined. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, when compared to control. Data represent mean values \pm SEM of at least three experiments.



ROS- and autophagy-dependent NETosis under tissue circumstances, which does not require TLR2 or TLR4.

LPS Induces “Vital” NETosis in the Presence of Platelets

It was recently demonstrated that LPS-activated platelets induce “vital” NETosis during sepsis (4, 5). This form of NET release is fundamentally different from “suicidal” NETosis; hence, “vital” NETosis occurs much faster, is not dependent

on autophagy or ROS, and is not associated with direct lytic cell death. Therefore, the NET-inducing capacity of all seven LPS structures was retested in the presence of platelets. Intriguingly, in whole blood *ex vivo*, thus in the presence of platelets, NET-like DNA lattices could be identified in response to all seven LPS structures (Figure 4A). An *in vitro* coculture setting of neutrophils and isolated platelets indeed revealed NETosis in response to LPS structures that previously failed to induce NETosis under platelet-free conditions, for instance LPS-O111

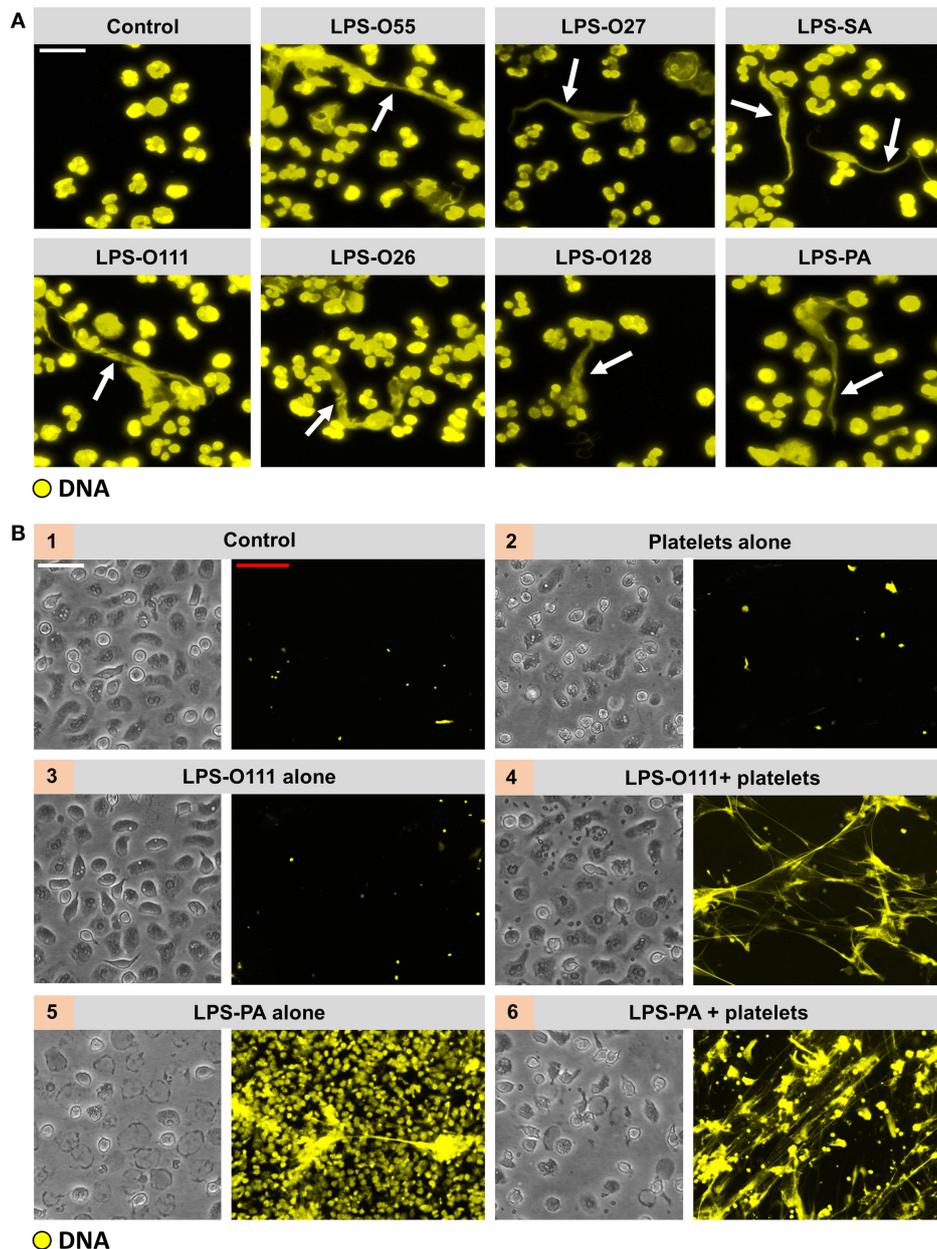


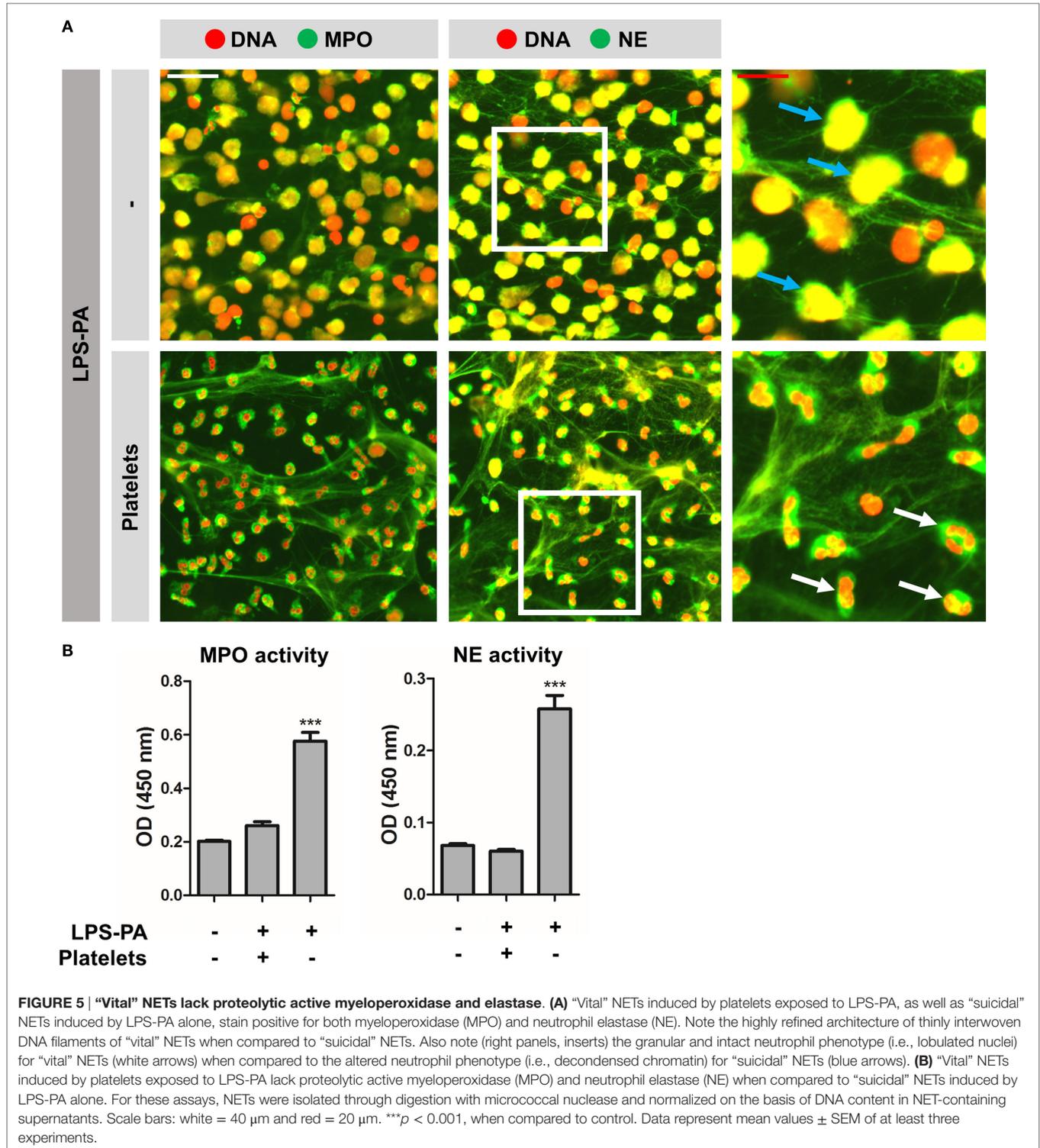
FIGURE 4 | All LPS structures induce “vital” NETosis in the presence of platelets. (A) Typical extracellular DNA filaments (white arrows) were observed in whole blood cultures *ex vivo* after 180 min of incubation with the different LPS serotypes. **(B)** Platelets (panel 2) or LPS-O111 (panel 3) alone does not induce NETosis after 180 min of incubation with neutrophils, whereas the combination of both (panel 4) stimulates NET formation without neutrophil (lytic) cell death. Massive neutrophil cell death is observed in response to LPS-PA alone (panel 5), based on the failure to exclude the vital dye Sytox Orange (yellow), which can be largely prevented by the addition of platelets (panel 6). LPS was used at a concentration of 8 pg LPS per neutrophil. Notably, representative light microscopy images are shown to visualize neutrophil morphology after stimulation and do not correspond in terms of “field of view” to the adjacent representative immunofluorescence images. Scale bars: white = 20 μm and red = 40 μm .

(**Figure 4B**). Whereas platelets alone or LPS-O111 failed to trigger NET release, the combination of platelets and LPS-O111 resulted in robust NET formation (**Figure 4B**, panels 2–4). Platelet-dependent LPS-induced NETs were observed already within 60 min of incubation, and the majority of neutrophils retained the capacity to exclude the vital dye Sytox Orange,

which is indicative for “vital” NETosis (**Figure 4B**, panel 4). On the contrary, neutrophils stimulated with LPS-PA only did not exclude Sytox Orange anymore, which is indicative for “suicidal” NETosis (**Figure 4B**, panel 5). Importantly, “suicidal” NETosis induced by LPS-PA could be largely prevented by the addition of platelets (**Figure 4B**, panels 5 and 6). Finally, to confirm that

the extracellular DNA fibers in response to LPS-exposed platelets represent “vital” NETs, double stainings for DNA with either MPO or elastase (NE) were performed. Indeed, extracellular DNA co-localized with both MPO and NE when neutrophils were stimulated with platelets exposed to LPS-PA (**Figure 5A**; single channel images as Figure S1 in Supplementary Material),

thereby confirming that the observed DNA structures are “vital” NETs. However, the proteolytic activity of both MPO and NE within these “vital” NETs appeared to be lower when compared to “suicidal” NETs induced by LPS-PA alone (**Figure 5B**). In conclusion, LPS-exposed platelets mediate “vital” NETosis independent from the bacterial origin of LPS.



Platelet TLR4 and CD62P Are Required for LPS-Induced “Vital” NETosis

The mechanisms involved in platelet-dependent LPS-induced “vital” NETosis may involve platelet TLR4, which was previously shown to be required for “vital” NETosis (11). Indeed, anti-TLR4 neutralizing antibodies could inhibit NET release in response to

LPS-O111 to a large extent (Figures 6A,B). The inhibition of ROS (with DPI) or autophagy (with wortmannin) did not influence “vital” NETosis induced by LPS-O111 (Figures 6A,B; single channel images as Figure S2 in Supplementary Material). Besides promoting “vital” NETosis, platelets apparently also exerted inhibitory effects on “suicidal” NETosis induced by LPS-PA

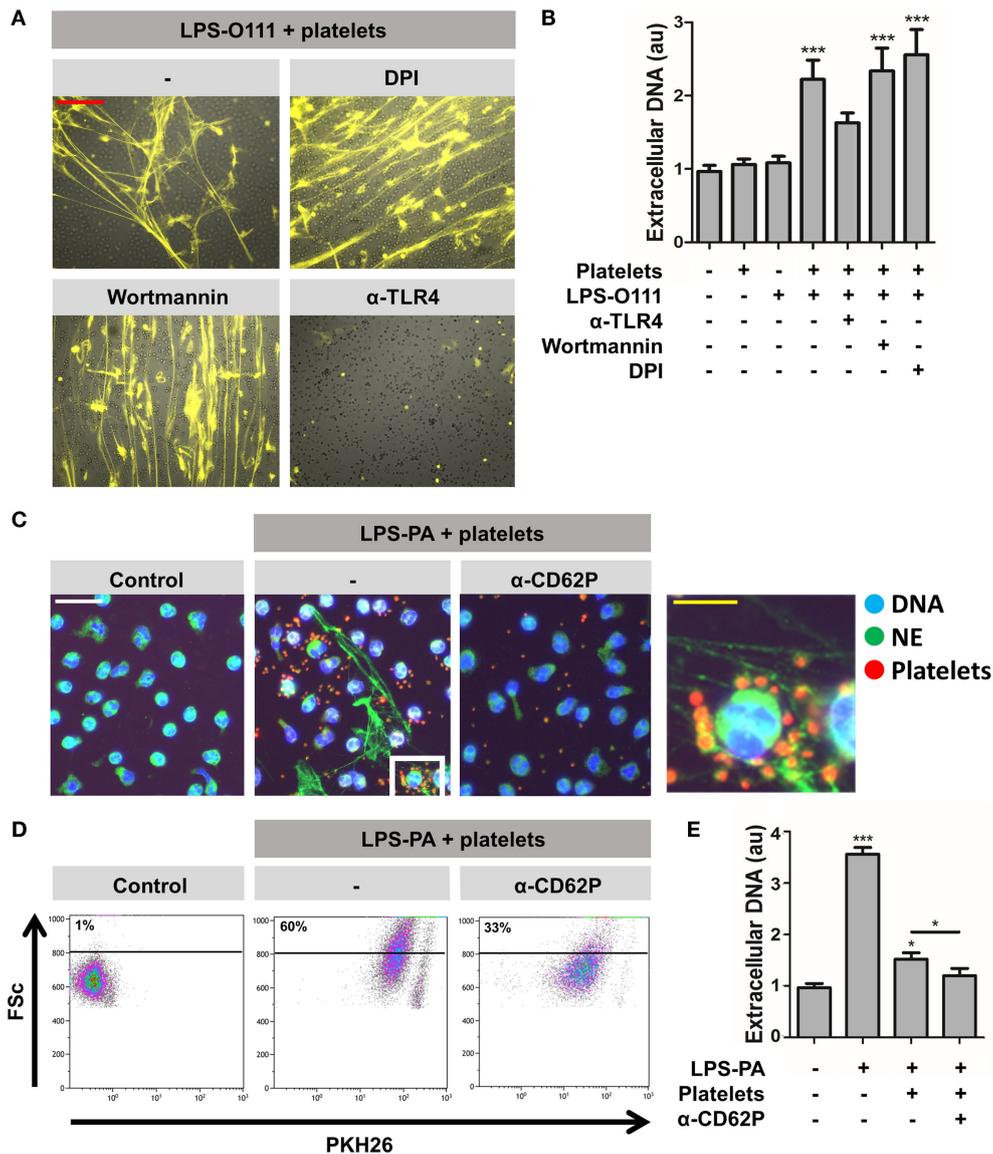


FIGURE 6 | Platelet-mediated “vital” NETosis requires platelet TLR4 and CD62P. (A,B) “Vital” NETosis induced by LPS-O111 and platelets is insensitive to inhibition by 5 μM wortmannin (inhibitor of autophagy) or 40 μM diphenyleneiodonium (DPI; inhibitor of ROS) but can be prevented by pretreatment of platelets with anti-TLR4 neutralizing antibodies (5 μg/ml). Representative images are merged pictures of extracellular DNA (yellow, as stained with 100 nM Sytox Orange) and neutrophils (brightfield channel). The images show abundant NETs despite exclusion of Sytox Orange by neutrophils, indicating cell death-independent NET release (i.e., “vital” NETosis). Quantification of NET release (B) was performed by fluorometry, as outlined. (C) PKH26-labeled platelets (red) stimulated with LPS-PA form aggregates with neutrophils (NE, neutrophil elastase) after 30 min of incubation. This aggregate formation is inhibited by anti-CD62P-neutralizing antibodies. (D) Flow cytometry analysis confirms the formation of platelet–neutrophil aggregates, since cells within the predefined neutrophil gate increase in size (FSc, forward scatter) and stain positive for PKH26-labeled platelets after LPS-PA stimulation. (E) Anti-CD62P-neutralizing antibodies decrease platelet-mediated “vital” NETosis induced by LPS-PA, as measured by fluorometry. LPS was used at a concentration of 8 μg LPS per neutrophil. Scale bars: red = 100 μm; white = 30 μm; yellow = 10 μm. **p* < 0.05 and ****p* < 0.001, when compared to control, where not indicated. Data represent mean values ± SEM of at least three experiments.

(Figure 4B, panels 5 and 6). In the presence of platelets, LPS-PA hardly promoted cell death, while NETs remained present to the same extent as for neutrophils exposed to LPS-PA in the absence of platelets. Thus, platelets seem to be important mediators in directing the neutrophil's fate during NET release, whereas LPS-PA induces "vital" NETosis in the presence of platelets, "suicidal" NETosis is induced when platelets are absent. As an explanation for this observation, it can be hypothesized that LPS-exposed platelets are internalized by neutrophils, thereby delivering NE to phagosomes and sequestering NE from the nucleus, which in turn inhibits "suicidal" NETosis since nuclear elastase is required in this pathway (18, 19). To test this hypothesis, PKH26-labeled platelets exposed to LPS-PA were cocultured with neutrophils, after which PKH26-labeled platelets and NE were visualized by immunofluorescence imaging. Apparently, platelets were not internalized by neutrophils, but instead formed large aggregates with neutrophils (Figure 6C, middle panel; single channel images as Figure S3 in Supplementary Material). The platelet–neutrophil aggregates coincided with the presence of NETs, consisting of extracellular filaments of DNA and NE (Figure 6C, insert right panel). The formation of platelet–neutrophil aggregates was further analyzed by flow cytometry, which revealed an increased forward scatter and PKH26-positivity in the neutrophil population (Figure 6D). Since CD62P is crucial for platelet–neutrophil interactions (20), PKH26-labeled platelets exposed to LPS-PA were cocultured with neutrophils in the presence of CD62P-neutralizing antibodies. Indeed, CD62P-neutralizing antibodies could largely prevent the formation of LPS-PA-induced platelet–neutrophil aggregates (Figures 6C,D) and decreased NET release (Figure 6E). Collectively, these data demonstrate a dominant role for platelets in LPS-induced "vital" NETosis, which occurs in an autophagy- and ROS-independent, but platelet TLR4- and platelet CD62P-dependent manner.

DISCUSSION

This study shows that neutrophils are able to discriminate between LPS of different bacterial sources and thereby selectively release NETs. Under serum- and platelet-free conditions, thereby mimicking tissue circumstances, neutrophils released NETs in response to two out of seven tested different LPS structures, of which LPS derived from *P. aeruginosa* (LPS-PA) appeared particularly potent. *P. aeruginosa* is a pathogen that typically infects the respiratory and urinary tract, as well as chronic wounds, and is a significant cause of morbidity and mortality in hospitalized patients (21). It is well-accepted that neutrophils comprise a pivotal component of host protection against *P. aeruginosa* (22). It was recently reported that *P. aeruginosa* is a robust instigator of NET formation *in vitro* and *in vivo* within the lungs, which contribute to the pathogenesis of airway changes in patients with bronchiectasis and cystic fibrosis (23–26). It remains unelucidated why LPS-PA is particularly potent in eliciting NET release when compared to the LPS derived from other bacterial species. Since *P. aeruginosa* is generally perceived as a non-virulent opportunist, it is unclear why this microorganism would elicit an antimicrobial defense mechanism (i.e., NETosis) that is associated with

collateral tissue damage (6), rather than triggering conventional phagocytosis. Reasoning otherwise, the potent NET-inducing capacity of LPS-PA could explain why this bacterium is actually perceived as an opportunist; hence, NETosis represents an extremely powerful strategy of constraining bacterial traits (27). An explanation for the NET-inducing capacity of LPS-PA may lie in the fact that *P. aeruginosa* is well-known for its ways to circumvent phagocytosis, for example through the formation of biofilms (28). Thus, NETosis may provide an immune response to those bacteria that have evolved strategies to circumvent phagocytic killing. Indeed, multiple other pathogens notorious for their attempts to evade phagocytosis, such as *Streptococcus pneumoniae* (29), *Haemophilus influenzae* (30), and *Klebsiella pneumoniae* (31), are potent stimuli for NETosis. Thus, phagocytosis and NETosis may have a complementary role, whereby the failure to phagocytose may elicit NET release. In line with this is the observation that NETosis is triggered by fungi that are too large for phagocytosis, whereas small hyphae become phagocytosed without inducing NETosis (18).

In addition to LPS-PA, LPS from *E. coli* (serotype O128:B12; LPS-O128) induced NETosis under tissue circumstances. Of note, four other tested LPS serotypes of *E. coli* (serotypes O55:B5, O127:B8, O111:B4, and O26:B6) did not elicit NET release. Thus, LPS-induced NETosis is not only bacterial species-specific but also serotype-specific. Growing evidence supports the notion that inflammatory responses triggered by LPS vary among serotypes. For instance, in a murine model of infection-induced preterm labor, four different *E. coli* LPS serotypes yielded highly variable outcomes (32). Our data suggest that subtle changes in the sugar composition of the O-antigen can impact NET release in response to LPS. It has been shown that modulation of the O-antigen composition alters the recognition and consecutive phagocytosis of LPS molecules by macrophages (33), which may also hold for neutrophils. In line with the reciprocal relationship between NETosis and phagocytosis described above, NETosis may thus be triggered by certain O-antigens that facilitate bypassing of phagocytosis.

Notably, the selectivity of NET release in response to LPS structures in our study was lost when neutrophils were cocultured with platelets. Also, extracellular chromatin fibers typical for NETs could be identified in whole blood *ex vivo* in response to all seven different LPS structures. Thus, in the presence of platelets, there is no selectivity of NET release in response to the different LPS structures. We observed that neutrophil–platelet interactions induced a rapid release of NETs (<60 min) in response to all LPS structures, which occurred in a ROS-independent manner and preceded without evident lysis of neutrophils. This form of rapid NET release could largely be inhibited through the blockade of platelet CD62P. Indeed, it has previously been shown that CD62P promotes NETosis in mice (34). The binding of CD62P, also known as P-selectin, to P-selectin glycoprotein ligand-1 (PSGL-1) on neutrophils thus causes signaling events that result in NETosis. However, the exact molecular mechanisms underlying CD62P-induced NET release remain elusive. Nevertheless, many downstream pathways of PSGL-1, such as the Src/Syk, PI3K/Akt, and p38 MAPK pathways, have in other contexts already been shown to be involved in NET release (35–37).

Our observations that LPS-exposed platelets rapidly induce NET release independent from ROS and independent from neutrophil lysis closely resemble a form of NETosis, which has previously been referred to as “vital” NETosis (5). However, the existence of a non-cell death “vital” NETosis program has been doubted by some critics, who question whether a neutrophil can still live and function without an intact nucleus (38, 39). Nevertheless, there is evidence that anuclear granulocytes are in fact metabolically active and able to perform cellular functions, such as transmigration (40) and phagocytosis (41). Simultaneously, some researchers have been critical toward fundamental aspects of “suicidal” NETosis, who perceive “suicidal” NETosis rather harmful than beneficial for the host due to its robust nature (42). Furthermore, it remains controversial whether “suicidal” NETosis is truly a unique form of cell death or whether it in fact reflects other forms of cell death, such as necroptosis (38, 43). Nonetheless the above, we have adapted the terms “suicidal” and “vital” NETosis in this manuscript, since these terms have been defined in previously published work in which the reported observations correspond to our findings. However, we agree that at least the term “vital” NETosis is a *contradictio in terminis*, since the “osis” of NETosis implies death

and “vital” implies alive. Although our data support the coexistence of both “suicidal” and “vital” NETosis, whereby platelets ultimately direct the neutrophil’s fate, further investigation is required to fully understand “vital” and “suicidal” processes and to assess the reciprocal relationship between both. Moreover, there should be international consensus about the terminology applied to describe the different forms of NET release as well as neutrophil cell death.

The structure of “vital” NETs induced by LPS-exposed platelets appeared highly refined and sophisticated, forming much larger structures of thinly interwoven DNA filaments when compared to “suicidal” NETs. Furthermore, we found that peroxidase and elastase activity was lacking in “vital” NETs, in contrast to “suicidal” NETs. These two characteristics of “vital” NETs (i.e., the complex web-like structure and the lack of proteolytic activity) makes “vital” NETs highly suitable for trapping and encapsulating pathogens, but presumably not for direct extracellular killing. However, since neutrophils appear to remain viable during “vital” NETosis, subsequent phagocytosis of entrapped pathogens may follow, and this combination of NET release and phagocytosis may provide an efficient strategy to combat pathogens in the bloodstream during sepsis without inducing protease-mediated

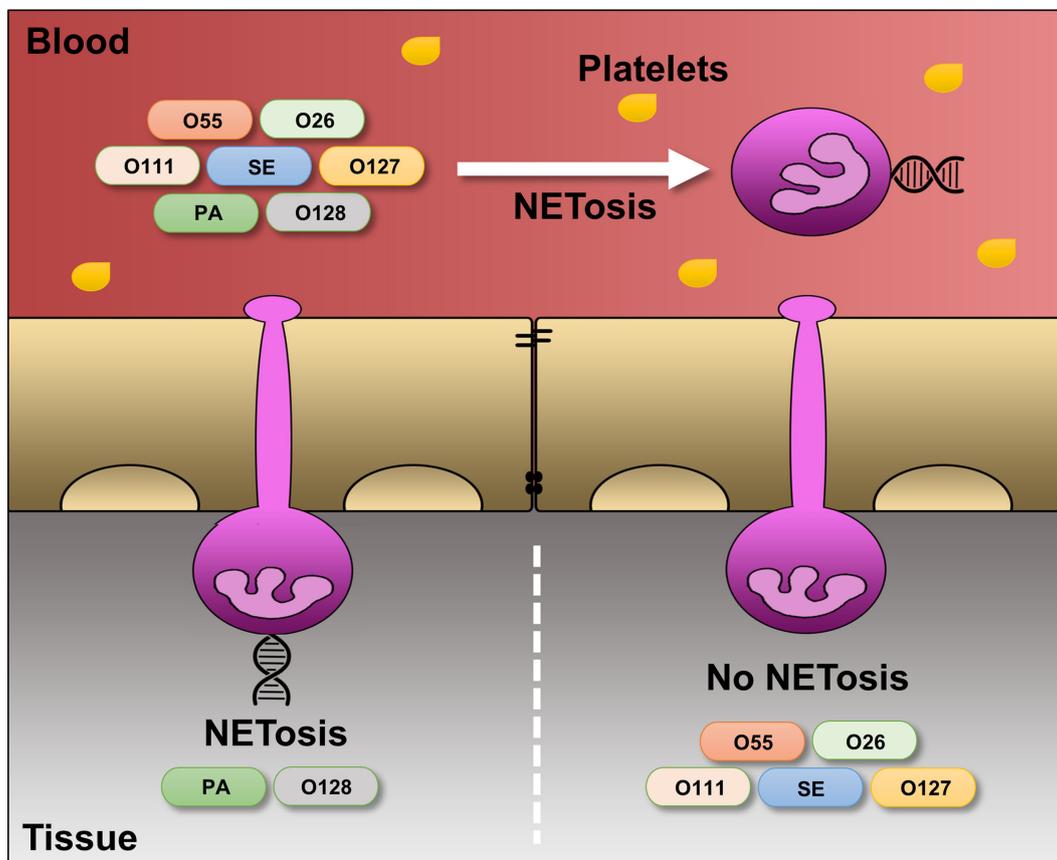


FIGURE 7 | Differential regulation of LPS-induced NET release under platelet-free and platelet-rich circumstances. Under serum- and platelet-free conditions, mimicking tissue circumstances, LPS-PA and LPS-O128 trigger ROS- and autophagy-dependent “suicidal” NET release in extravasated neutrophils, whereas other LPS structures (LPS-O26, LPS-O55, LPS-SE, LPS-O127, and LPS-O111) do not. In the presence of platelets, mimicking blood circumstances, neutrophils do no longer discriminate between LPS structures and release “vital” NETs in response to all LPS structures.

collateral tissue damage to the vessel lumen. Thus, aside from the idea that NETosis may occur when phagocytosis is impaired, there is also evidence that NETosis and phagocytosis by neutrophils can occur simultaneously and complement each other during septic conditions (44).

In addition to the biological relevance, our data may also have technical experimental implications. Since many studies addressing the contribution of NETosis to disease conditions (i.e., systemic lupus erythematosus) depend on the *in vitro* induction and isolation of NETs for downstream assays, the choice for LPS as an inducer of NET release should be selected carefully in light of the current data (6, 45–47).

Taken together, our data reveal a complex interplay between neutrophils and LPS, which can induce both “suicidal” and/or “vital” NETosis, depending on the quantity and structure of the LPS and the presence or absence of platelets (Figure 7). Although the present study compares the effects of a single molecule (LPS) from different bacterial sources, it is tempting to speculate that similar results may be obtained for whole microbes. This could be addressed in future research.

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AUTHOR CONTRIBUTIONS

EP designed and performed research, analyzed and interpreted data, and wrote the manuscript; NR and CY performed research and analyzed data; LH interpreted data and wrote the manuscript; and JV designed and supervised research, interpreted data, and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at <http://journal.frontiersin.org/article/10.3389/fimmu.2016.00484/full#supplementary-material>.

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Transforming Growth Factor- β -Activated Kinase 1 Is Required for Human Fc γ R111b-Induced Neutrophil Extracellular Trap Formation

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Neutrophils (PMNs) are the most abundant leukocytes in the blood. PMN migrates from the circulation to sites of infection where they are responsible for antimicrobial functions. PMN uses phagocytosis, degranulation, and formation of neutrophil extracellular traps (NETs) to kill microbes. Several stimuli, including bacteria, fungi, and parasites, and some pharmacological compounds, such as Phorbol 12-myristate 13-acetate (PMA), are efficient inducers of NETs. Antigen-antibody complexes are also capable of inducing NET formation. Recently, it was reported that Fc γ R111b cross-linking induced NET formation similarly to PMA stimulation. Direct cross-linking of Fc γ R111A or integrins did not promote NET formation. Fc γ R111b-induced NET formation presented different kinetics from PMA-induced NET formation, suggesting differences in signaling. Because Fc γ R111b also induces a strong activation of extracellular signal-regulated kinase (ERK) and nuclear factor κ B-1, and the transforming growth factor- β -activated kinase 1 (TAK1) has recently been implicated in ERK signaling, in the present report, we explored the role of TAK1 in the signaling pathway activated by Fc γ R111b leading to NET formation. Fc γ R111b was stimulated by specific monoclonal antibodies, and NET formation was evaluated in the presence or absence of pharmacological inhibitors. The antibiotic LL Z1640-2, a selective inhibitor of TAK1 prevented Fc γ R111b-induced, but not PMA-induced NET formation. Both PMA and Fc γ R111b cross-linking induced phosphorylation of ERK. But, LL Z1640-2 only inhibited the Fc γ R111b-mediated activation of ERK. Also, only Fc γ R111b, similarly to transforming growth factor- β -induced TAK1 phosphorylation. A MEK (ERK kinase)-specific inhibitor was able to prevent ERK phosphorylation induced by both PMA and Fc γ R111b. These data show for the first time that Fc γ R111b cross-linking activates TAK1, and that this kinase is required for triggering the MEK/ERK signaling pathway to NETosis.

Keywords: immunoglobulin, immunoreceptor, inflammation, neutrophil, DNA, TAK1, ERK

INTRODUCTION

Neutrophils are innate immune cells that migrate from the circulation to sites of inflammation or infection. Classically, neutrophils are considered the first line of defense since they are the first cells to appear at the affected site, and they display important antimicrobial functions (1). Neutrophils use phagocytosis, degranulation, and formation of neutrophil extracellular traps (NETs) to kill microbes (2, 3). NETs are formed through a unique cell death program named “NETosis” that involves first degradation of the nuclear membrane and chromatin expansion into the cytosol, while the cell membrane remains intact. Later, 3 or 4 h after stimulation, the cell membrane breaks, and the chromatin fibers get expelled outside the cell, creating a net-like structure. NET fibers are composed of chromatin covered with histones (4) and antimicrobial proteins derived from the neutrophil granules, such as the bactericidal/permeability-increasing protein (BPI), elastase, myeloperoxidase, lactoferrin, and metalloprotease 9 (2, 5). NETs prevent further spread of pathogens because they function as a physical barrier where microorganisms get trapped and because they bring antimicrobial proteins in close proximity of pathogens. Thus, NETs can kill microorganisms extracellularly and independently of phagocytosis (6).

Human neutrophils express constitutively two IgG antibody receptors: FcγRIIa (CD32a) and FcγRIIIb (CD16b) (7). FcγRIIa consists of a single polypeptide chain bearing an ITAM on its cytoplasmic domain (8). This ITAM confers on FcγRIIa the ability to initiate signaling events that regulate cell responses, including phagocytosis, cytokine production, and antibody-dependent cell-mediated cytotoxicity (9). FcγRIIIb is present exclusively on neutrophils, and it is a glycosylphosphatidylinositol (GPI)-linked receptor, lacking transmembrane and cytoplasmic domains (10). The signaling mechanism for this receptor is still unknown, since possible signaling molecules directly associated with it remain unidentified. However, several reports show that FcγRIIIb can initiate signaling events leading to various cell responses including increase in calcium concentration (11), activation of integrins (12), and activation of NF-κB (13, 14).

FcγRIIIb cross-linking induced efficient NET formation similarly to Phorbol 12-myristate 13-acetate (PMA) stimulation (15). This NET formation was dependent on NADPH-oxidase and extracellular signal-regulated kinase (ERK) activation (15). But, the mechanism linking FcγRIIIb to ERK is not known. Previously, we reported that FcγRIIIb cross-linking led to activation of NF-κB (13); while others have reported that transforming growth factor-β-activated kinase 1 (TAK1) was associated to the IκB kinase complex, both in the nucleus and cytoplasm of human neutrophils favoring NF-κB activation (16). More recently, we also found that FcγRIIIb induced a robust activation of ERK and also of the transcription factor Elk-1 (17), but we could not identify the molecule responsible for ERK activation. Similarly, others have reported that, in human neutrophils, TAK1 acted upstream of MEK (ERK kinase) and ERK signaling pathway (18, 19). Thus, in this report, we explored the possibility that TAK1 is functionally coupled to FcγRIIIb leading to NETosis *via* ERK activation. FcγRIIIb was stimulated by specific monoclonal antibodies, and

the NET formation was evaluated in the presence or absence of pharmacological inhibitors. The antibiotic LL Z1640-2, a selective inhibitor of TAK1 prevented FcγRIIIb-induced, but not PMA-induced NET formation. Both PMA and FcγRIIIb cross-linking induced phosphorylation of ERK. But, LL Z1640-2 only inhibited the FcγRIIIb-mediated activation of ERK. Also, a MEK-specific inhibitor was able to prevent ERK phosphorylation induced by both PMA and FcγRIIIb. These data show for the first time that FcγRIIIb cross-linking activates TAK1, and that, this kinase is required for triggering the MEK/ERK signaling pathway to NETosis.

MATERIALS AND METHODS

Neutrophils

Neutrophils were isolated from the peripheral blood collected from adult healthy volunteers following a protocol that was approved by the Bioethics Committee at Instituto de Investigaciones Biomédicas – UNAM. All volunteers provided a written informed consent for their blood donation. The procedure for neutrophil isolation was exactly as previously described (14).

Reagents

Bovine serum albumin (BSA) was from F. Hoffmann-La Roche Ltd. (Mannheim, Germany). Piceatannol, a spleen tyrosine kinase (Syk) inhibitor was from Acros Organics (NJ, USA). PD98059 and U0126, MEK (ERK kinase) inhibitors were obtained from New England Biolabs (Beverly, MA, USA) and from Promega (Madison, WI, USA), respectively. The antibiotic LL Z1640-2 [also known as (5Z)-7-Oxozeaenol; cas 66018-38-0] (catalog no. sc-202055) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). GÖ6983, a protein kinase C (PKC) inhibitor, SB 203580, a p38 MAP kinase inhibitor (catalog number 559389), and 3-(1-methyl-1H-indol-3-yl-methylene)-2-oxo-2,3-dihydro-1H-indole-5-sulfonamide (iSyk), another Syk inhibitor (catalog no. 574711) were from Calbiochem/EMD Millipore (Billerica, MA, USA). Recombinant Human TGF-β1 (catalog No. 100-21) was from Peprotech (Rocky Hill, NJ, USA). The cComplete™ protease inhibitor cocktail (catalog No. 11697498001) and PhosSTOP™ phosphatase inhibitor cocktail (catalog No. 04906845001) were from Roche Diagnostics (Basel, Switzerland). PMA and all other chemicals were from Sigma Aldrich (St. Louis, MO, USA). The following antibodies were used: anti-human FcγRI (CD64) mAb 32.2 (ATCC® HB-946™) and anti-human FcγRIIa (CD32a) mAb IV.3 (20) (ATCC® HB-217™) were from American Type Culture Collection (Manassas, VA, USA). The anti-human FcγRIIIb (CD16b) mAb 3G8 (21) was donated by Dr. Eric J. Brown (University of California in San Francisco, San Francisco, CA, USA). The anti-β1 integrin mAb TS2/16 was donated by Martin Hemler (Dana Farber Cancer Research Institute, Boston, MA, USA). Monoclonal antibodies were purified as previously described (15). Rabbit polyclonal anti-ERK 1 (catalog no. sc-94), rabbit polyclonal anti-phospho-ERK 1/2 (pTyr204) (catalog no. sc-101761), and rabbit polyclonal anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (catalog no. sc-25778) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). F(ab')₂ fragment goat anti-mouse IgG (catalog No. 115-006-072) was

from Jackson Immuno Research Laboratories Inc. (West Grove, PA, USA). HRP-conjugated F(ab')₂ goat anti-mouse IgG (catalog No. 0855572) and HRP-conjugated F(ab')₂ goat anti-rabbit IgG (catalog No. 0855686) were from MP Biomedicals (Santa Ana, CA, USA). Rabbit polyclonal anti-phospho-TAK1 (T187) (catalog No. ab192443) was from Abcam plc. (Cambridge, UK).

NET Formation Kinetics

Neutrophil extracellular trap formation was quantified by detecting DNA release spectrophotometrically with the DNA-binding dye SYTOX[®] Green (22–24). For PMA stimulation, neutrophils were resuspended at 1×10^6 cell/ml in RPMI-1640 medium (Gibco[®]; Grand Island, NY, USA) containing 500 nM SYTOX[®] Green (Molecular Probes, Inc.; Eugene, OR, USA), and 100 μl of this cell suspension (1×10^5 PMN) were added to each well of the 96-well plate. The plate was then incubated at 35°C for 20 min in a microplate reader model Synergy HT from BioTek Instruments (Winooski, VT, USA). Next, 20 μl of 120 nM PMA dissolved in the same RPMI/SYTOX medium were added to each well for a final concentration of 20 nM. After that, the plate was incubated for up to 4 h, reading the fluorescence from the bottom of the plate, using the 485 nm excitation and 528 emission filters, every 5 min. For FcγR stimulation, neutrophils were resuspended at 0.5×10^7 cell/ml in RPMI/SYTOX medium containing 10 μg/ml of the corresponding anti-FcγR antibody and incubated in ice for 20 min. After one wash in PBS, cells were resuspended in the same volume of RPMI/SYTOX medium, and 20 μl of this cell suspension (1×10^5 PMN) were added to each well of the 96-well plate. The plate was then incubated at 35°C for 20 min in a microplate reader. Next, 100 μl of 45 μg/ml goat anti-mouse IgG in RPMI/SYTOX medium were added to each well (final concentration 37.5 μg/ml). Finally, the plate was incubated for up to 4 h, reading the fluorescence every 5 min. For TAK1 inhibition, cells were treated with 10 nM LL Z1640-2 for 30 min before stimulation.

Neutrophil Stimulation

PMNs were stimulated by cross-linking Fc receptors with specific mAbs as follows: PMN were resuspended in PBS at 1×10^7 cells/ml, and 200 μl of the cell suspension were placed in Eppendorf tubes. The corresponding mAb was then added at 10 μg/ml, and the cells were incubated on ice for 30 min. Next, cells were washed twice with 500 μl of PBS. Receptor cross-linking was then induced by resuspending the cells in 100 μl of PBS containing 37 μg/ml of F(ab')₂ goat anti-mouse IgG and incubating them at 37°C for 15 min. For PMN stimulation with PMA or TGF-β, PMN were incubated at 37°C for 15 min with 20 nM PMA or 5 ng/ml TGF-β. In assays where pharmacological inhibitors were used, PMN were pretreated with 10 nM LLZ 640-2 or only with the solvent dimethyl sulfoxide (DMSO) on ice for 30 min before adding the first mAb.

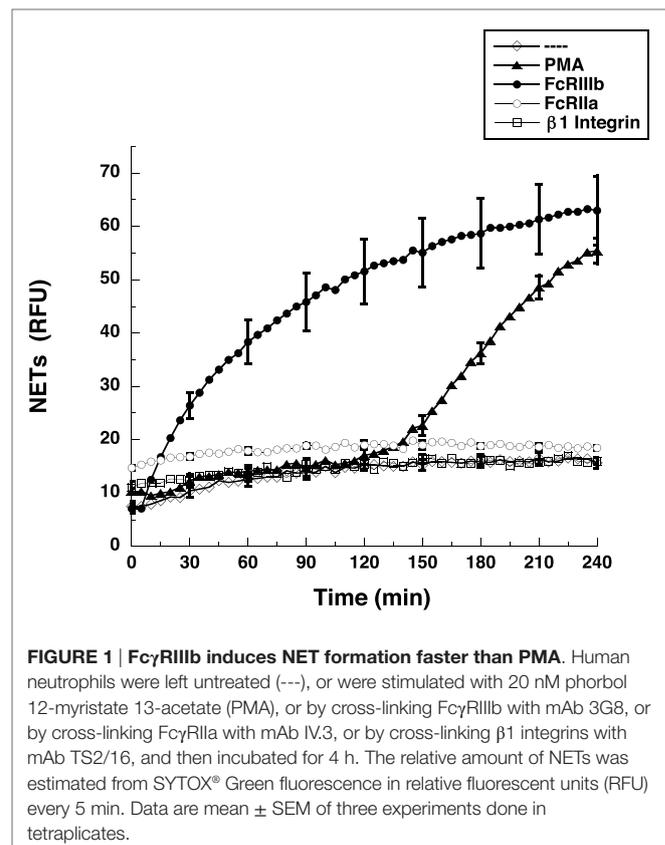
Protein Extraction and Western Blotting

Total protein extracts were obtained by lysing the cells in cold RIPA lysis buffer (150 mM NaCl, 5 mM EDTA, 50 mM Hepes, 0.5% sodium deoxycholate, 1% Non-idet P-40, 50 mM NaF, and 1 mM sodium orthovanadate, pH 7.5) supplemented with 1× protease

inhibitor cocktail and 1× phosphatase inhibitor cocktail, which were added just before lysing the cells. Cell lysates were incubated on ice for 20 min, then cleared by centrifugation, and proteins resolved on SDS 10% PAGE. Proteins were then electrotransferred onto polyvinylidene fluoride (PVDF) membranes (Immobilon-P; Millipore, Bedford, MA, USA). Membranes were incubated in blocking buffer (1% BSA, 5% non-fat dry milk) (Carnation; Nestle, Glendale, CA, USA) and 0.1% Tween 20 in Tris-buffered saline (TBS: 50 mM Tris-HCl, 150 mM NaCl, pH = 7.5) overnight at 4°C. Membranes were subsequently probed with the corresponding antibody in blocking buffer for 1 h at room temperature. Anti-phospho-ERK 1 (1/1000 dilution) or anti-phospho TAK1 (1/2000 dilution). Membranes were washed with TBS-Tween six times and incubated with a 1/3000 dilution of HRP-conjugated F(ab')₂ goat anti-rabbit IgG for 1 h at room temperature. After washing six more times, the membrane was developed with Immobilon Western chemiluminescent HRP substrate (catalog No. WBKLS0100) from EMD Millipore (Billerica, MA, USA) according to the manufacturer's instructions. Afterward, membranes were stripped with 0.2 M NaOH and reprobed with anti-ERK 1 (1/2000 dilution) or anti-GAPDH (1/1000 dilution) to assess protein loading in PAGE gels.

Statistical Analysis

Quantitative data were expressed as mean ± SEM. Single variable data were compared by paired-sample Student's *t*-tests using the computer program KaleidaGraph[®] version 3.6.2 for Mac (Synergy



Software; Reading, PA, USA). Differences were considered statistically different at a value $p < 0.05$.

RESULTS

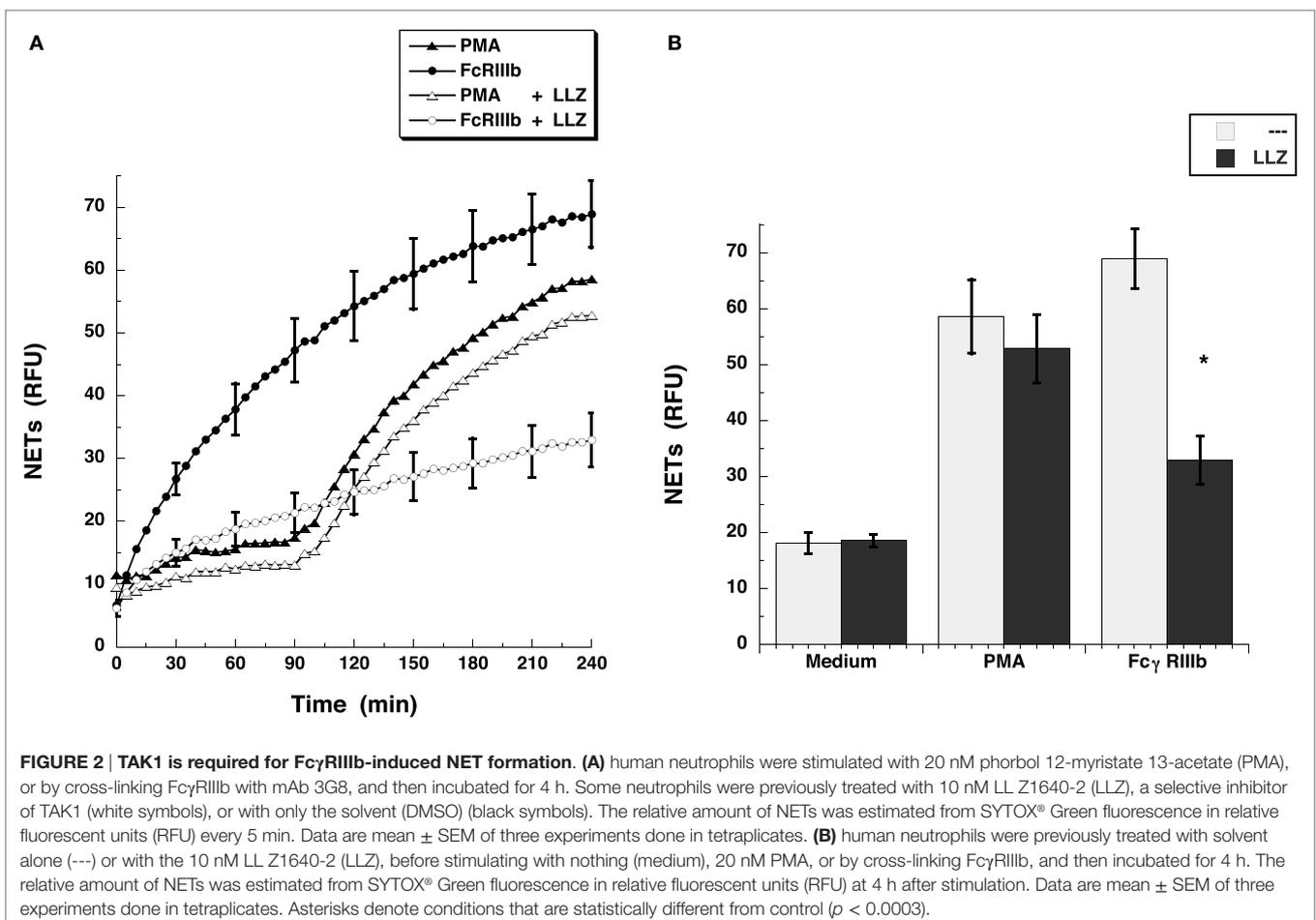
FcγRIIIb-Mediated NETosis Presents a Different Kinetics from PMA-Induced NETosis

Most studies on NETs have used PMA, a potent activator of PKC, to induce the formation of NETs (2). Direct cross-linking of FcγRIIIb also leads to a robust activation of NET formation (15). However, the kinetics of these responses is different. When human neutrophils were stimulated by PMA, NETosis (5) is observed as a late event with NETs (extracellular DNA fibers) detected after 2.5 h of stimulation (Figure 1). Complete NET formation was seen, as previously described, by 4 h after stimulation (Figure 1). In contrast, stimulation of FcγRIIIb with mAb 3G8, induced NETosis with a much faster kinetics. By 30 min after receptor cross-linking, NETs could already be detected (Figure 1). By 2 h, about half of the total amount of NETs had already been formed, and by 4 h, NETs reached a level similar to that induced by PMA (Figure 1). In order to confirm that the mAb 3G8 (IgG1) was specifically targeting (cross-linking) FcγRIIIb, neutrophils were also stimulated by

the isotypic control antibody TS2/16 (IgG1) that binds to β1 integrins, and by the mAb IV.3 (IgG2b) that binds FcγRIIa. Neither mAb IV.3 nor mAb TS2/16 induced NET formation (Figure 1), strengthening the point that FcγRIIIb is the receptor responsible for induction of NETosis. These data indicated that cross-linking FcγRIIIb is an efficient stimulus for NET formation with a faster response than the one induced by PMA. This difference in response kinetics led us to explore the signaling pathway from FcγRIIIb to NETosis.

TAK1 Is Involved in FcγRIIIb-Mediated NETosis

Others and we have seen that the MEK/ERK signaling pathway is required for both PMA- (25) and FcγRIIIb-induced NETosis (15, 23). Because the transforming growth factor-β-activated kinase 1 (TAK1) is a known activator of MAP kinase signaling pathways in various immune cells (26), and in human neutrophils, TAK1 was also reported to act upstream of ERK (18), we explored the possibility that TAK1 is involved in FcγRIIIb-mediated NETosis. The antibiotic LL Z1640-2, a selective inhibitor of TAK1 prevented FcγRIIIb-induced NET formation (Figure 2A), but not PMA-induced NET formation (Figure 2A). The inhibitory effect was maximum at 4 h after stimulation when the amount of NETs from FcγRIIIb-stimulated neutrophils was



reduced by half (**Figure 2B**). This result indicated for the first time that indeed TAK1 is involved in NET formation after cross-linking Fc γ RIIIb.

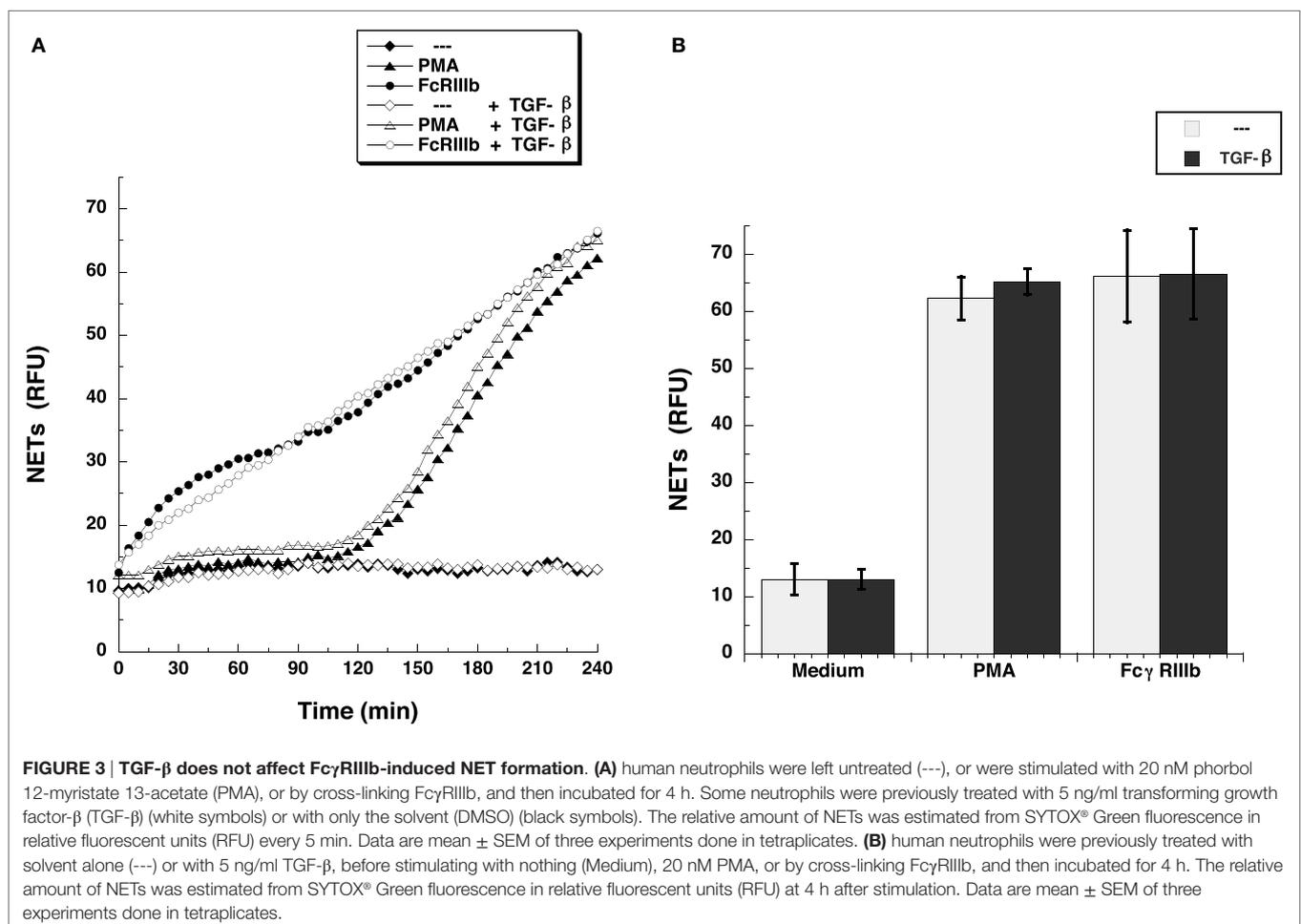
Transforming growth factor- β -activated kinase 1 was initially identified as a regulator of MAPK in response to TGF- β (27), thus, we explored whether TGF- β could have an effect on NETosis. Treatment of neutrophils with TGF- β did not change the kinetics nor the amount of NET formation induced either by Fc γ RIIIb cross-linking or PMA stimulation (**Figure 3**). This lack of effect on NETosis was not due to failure of TGF- β to activate TAK1. Neutrophils treated with TGF- β presented a robust phosphorylation of TAK1 (**Figure 4A**) indicating that the axis TGF- β /TAK1 was functional in these cells. Moreover, cross-linking of Fc γ RIIIb also led to phosphorylation of TAK1 (**Figure 4A**). This phosphorylation in Thr-187 is indicative of activation of TAK1 (28). The Fc γ RIIIb-mediated phosphorylation of TAK1 was detectable at 5 min, reached a maximum at 15 min, and was almost gone by 30 min after receptor cross-linking (**Figure 4B**). Thus, this time was used in all other experiments to detect TAK1 phosphorylation. Opposite to this result, treatment with PMA did not induce any phosphorylation of TAK1 (**Figure 4B**).

Human neutrophil expresses constitutively two low-affinity Fc γ receptors, Fc γ RIIa and Fc γ RIIIb, and after interferon- γ ,

they can upregulate Fc γ RI. Previously, it has been reported that Fc γ RIIIb is the receptor responsible for NET formation (15, 23). Therefore, we explored the possibility that each of these Fc receptors could activate TAK1 after cross-linking each receptor with the corresponding specific monoclonal antibody. Treating the cells with monoclonal antibody 32.2 (anti-Fc γ RI) did not induce TAK1 phosphorylation (**Figure 4C**). This was an expected result since Fc γ RI is not expressed in resting neutrophils. Similarly, cross-linking with monoclonal antibody IV.3 (anti-Fc γ RIIa) also did not cause any TAK1 phosphorylation (**Figure 4C**). In contrast, cross-linking of Fc γ RIIIb with the monoclonal antibody 3G8 efficiently induced TAK1 phosphorylation (**Figure 4**). Together, these data suggested that, indeed, Fc γ RIIIb signaling in human neutrophils requires TAK1 activation for induction of NET formation.

Syk Is Required for Fc γ RIIIb-Mediated TAK1 Activation

After establishing a role for TAK1 in Fc γ RIIIb-mediated NET formation, we explored a possible connection from Fc γ RIIIb to TAK1. Neutrophils were stimulated by Fc γ RIIIb cross-linking in the presence or absence of two Syk inhibitors. Fc γ RIIIb-induced TAK1 phosphorylation and also ERK 1 phosphorylation were



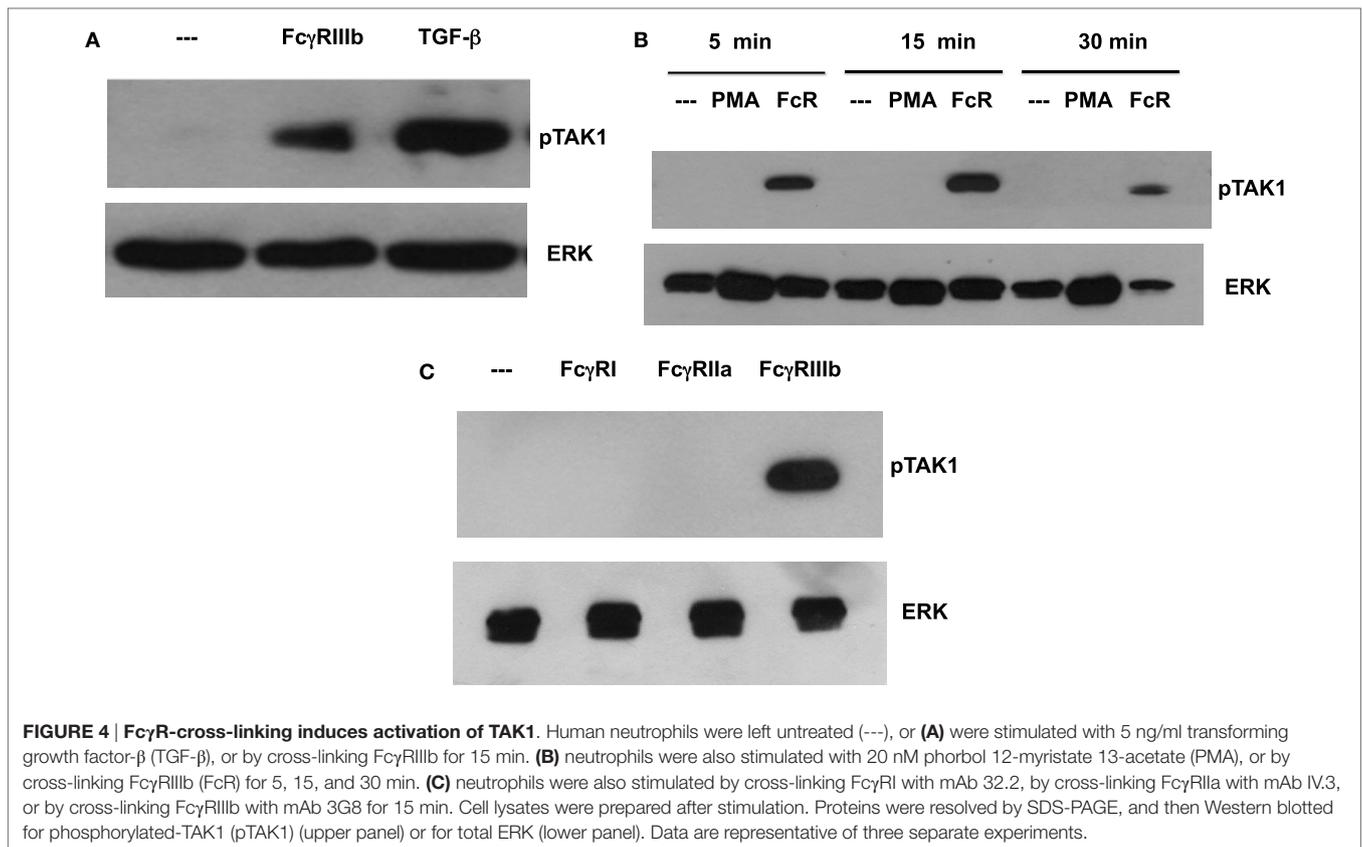


FIGURE 4 | FcγR-cross-linking induces activation of TAK1. Human neutrophils were left untreated (---), or (A) were stimulated with 5 ng/ml transforming growth factor-β (TGF-β), or by cross-linking FcγRIIIb for 15 min. (B) neutrophils were also stimulated with 20 nM phorbol 12-myristate 13-acetate (PMA), or by cross-linking FcγRIIIb (FcR) for 5, 15, and 30 min. (C) neutrophils were also stimulated by cross-linking FcγRI with mAb 32.2, by cross-linking FcγRIIa with mAb IV.3, or by cross-linking FcγRIIIb with mAb 3G8 for 15 min. Cell lysates were prepared after stimulation. Proteins were resolved by SDS-PAGE, and then Western blotted for phosphorylated-TAK1 (pTAK1) (upper panel) or for total ERK (lower panel). Data are representative of three separate experiments.

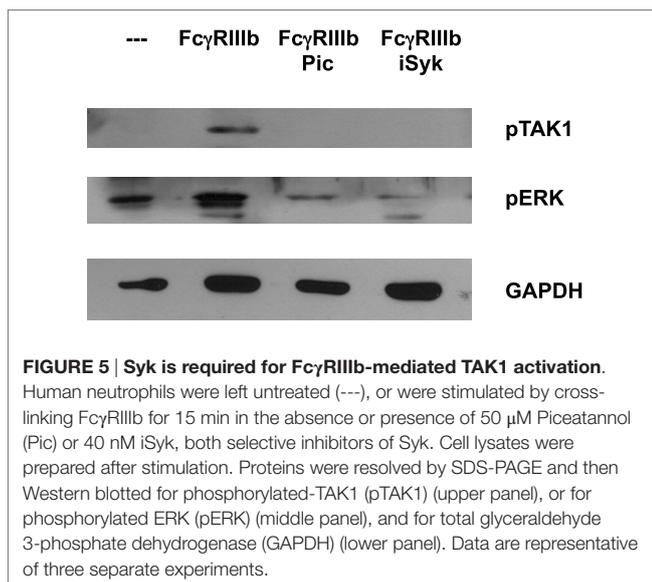


FIGURE 5 | Syk is required for FcγRIIIb-mediated TAK1 activation. Human neutrophils were left untreated (---), or were stimulated by cross-linking FcγRIIIb for 15 min in the absence or presence of 50 μM Piceatannol (Pic) or 40 nM iSyk, both selective inhibitors of Syk. Cell lysates were prepared after stimulation. Proteins were resolved by SDS-PAGE and then Western blotted for phosphorylated-TAK1 (pTAK1) (upper panel), or for phosphorylated ERK (pERK) (middle panel), and for total glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (lower panel). Data are representative of three separate experiments.

efficiently blocked by both Syk inhibitors (Figure 5). This result suggested that FcγRIIIb connects to TAK1 activation *via* Syk.

TAK1 Is Required for FcγRIIIb-Mediated ERK Activation

Next, we explored the signaling pathway from TAK1 to ERK. Neutrophils were stimulated by PMA or FcγRIIIb cross-linking

in the presence or absence of the TAK1 inhibitor, and ERK 1 activation was detected by Western blotting. First, we confirmed that LL Z1640-2 was inhibiting TAK1 phosphorylation (Figure 6A). Under the same conditions, PMA induced ERK phosphorylation (Figure 6B) as previously reported (15). This ERK phosphorylation was not affected by the TAK1 inhibitor (Figure 6B). In contrast, FcγRIIIb cross-linking also induced ERK phosphorylation, but this ERK phosphorylation was efficiently blocked by the TAK1 inhibitor (Figure 6B). This result strongly indicated that TAK1 activation is required for ERK activation after FcγRIIIb cross-linking, but not after PMA stimulation.

In most situations, MEK activation leads to ERK activation, as the former phosphorylates the latter. In order to confirm that this is also the case in the case of FcγRIIIb- or PMA-induced NETosis, neutrophils were treated with the MEK inhibitor PD98059 prior to stimulation. As shown before, cross-linking of FcγRIIIb clearly activated ERK, and this activation was completely blocked by the MEK inhibitor (Figure 7A). Similarly, this MEK inhibitor also blocked ERK activation induced by PMA (Figure 7B). Neither PD98059 nor UO126, another potent MEK inhibitor, was able to block TAK1 activation induced by FcγRIIIb (Figure 7C). This last result confirms that TAK1 is upstream of MEK/ERK signaling module in the case of FcγRIIIb signaling. These data also suggest that the signaling pathways initiated by both FcγRIIIb and PMA converge at the level of PKC or MEK to activate ERK leading to downstream NETosis.

p38 MAPK Is Not Required for FcγRIIIB-Mediated NET Formation

Because it is well known that TAK1 functions upstream of p38 MAPK pathway rather than ERK (26, 29) in many cell types, we examined whether blockade of p38 MAPK affected FcγRIIIB-induced NET formation. The specific p38 MAPK inhibitor SB203580 blocked phosphorylation of p38 MAPK induced by TGF-β (Figure 8A). As expected, neutrophils treated with PMA in the presence of SB203580 produced NETs as efficiently as the neutrophils with no inhibition of p38 MAPK (Figure 8B). Similarly, inhibition of p38 MAPK did not affect NET formation

induced by cross-linking FcγRIIIB (Figure 8B). These data strongly suggest that FcγRIIIB activates TAK1 to connect with the MEK/ERK pathway in order to activate NET formation.

DISCUSSION

The MAP3K, TAK1, is activated by different stimuli including cytokines such as tumor necrosis factor (TNF), interleukin (IL)-1, and IL-18, or TLR ligands such as LPS in various cell types (26, 30–34) and participates in activating several signaling pathways. In this study, we report for the first time that, in human

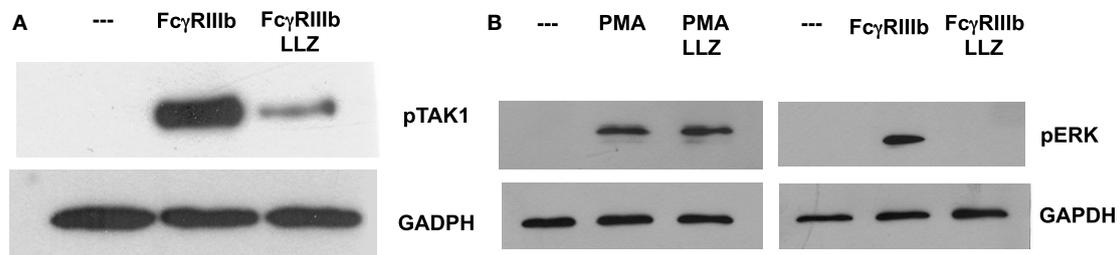


FIGURE 6 | TAK1 is required for FcγRIIIB-mediated ERK activation. Human neutrophils were left untreated (---), or were stimulated by cross-linking FcγRIIIB for 15 min, or by 20 nM phorbol 12-myristate 13-acetate (PMA). Some neutrophils were previously treated with 10 nM LLZ 1640-2 (LLZ), a selective inhibitor of TAK1. Cell lysates were prepared after stimulation. Proteins were resolved by SDS-PAGE, and then, Western blotted for (A) phosphorylated-TAK1 (pTAK1) (upper panel) and for total glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (lower panel); or for (B) phosphorylated ERK (pERK) and total GAPDH (lower panel). Data are representative of three separate experiments.

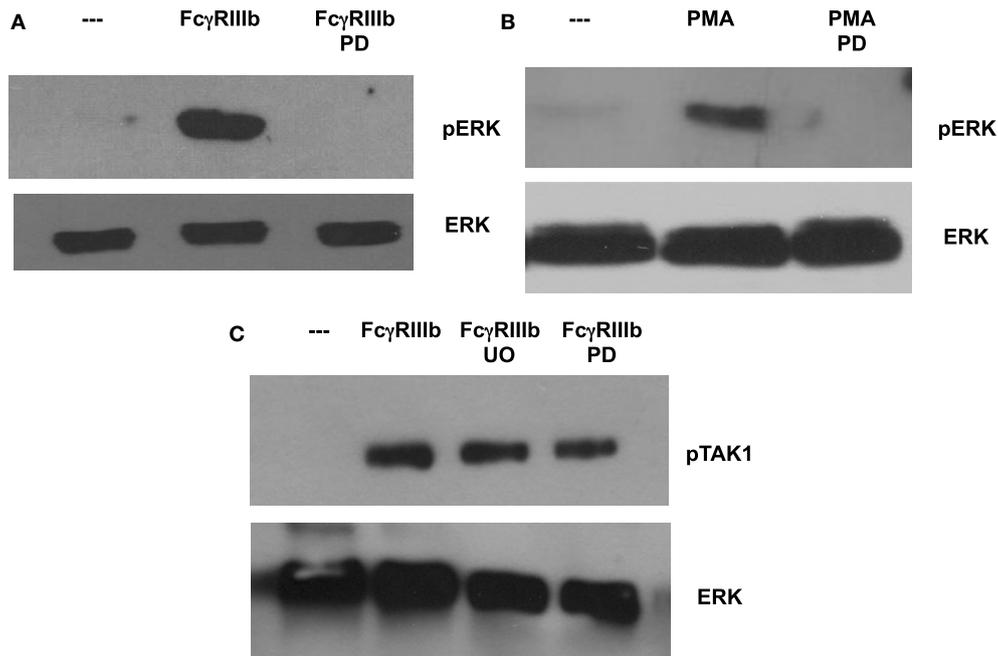
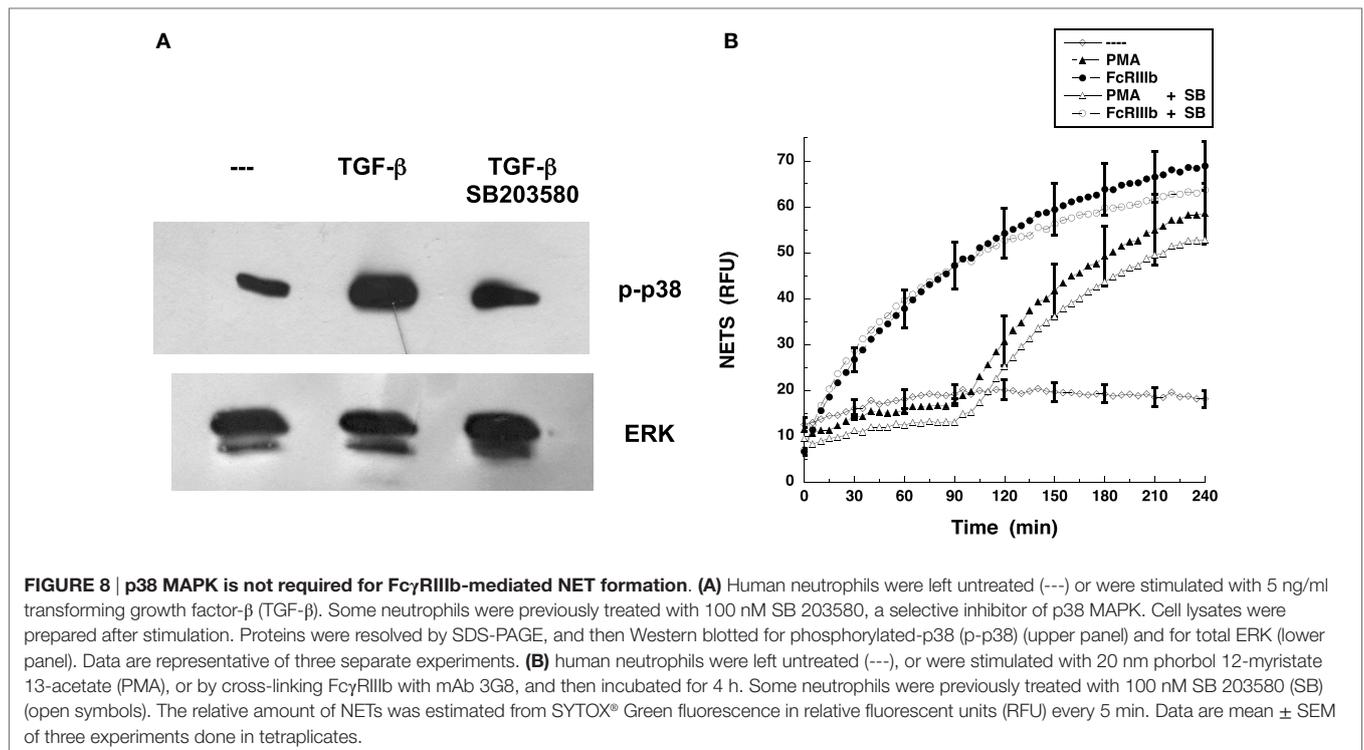


FIGURE 7 | MEK1 is required for FcγRIIIB-mediated ERK activation. Human neutrophils were left untreated (---), or were stimulated by cross-linking FcγRIIIB for 15 min, or by 20 nM phorbol 12-myristate 13-acetate (PMA). Some neutrophils were previously treated with 50 μM PD98059 (PD) or with 50 μM UO126 (UO), both selective inhibitors of MEK. Cell lysates were prepared after stimulation. Proteins were resolved by SDS-PAGE, and then Western blotted for (A,B) phosphorylated ERK (pERK) (upper panel) and for total ERK (lower panel); or for (C) phosphorylated-TAK1 (pTAK1) and for total ERK (lower panel). Data are representative of three separate experiments.



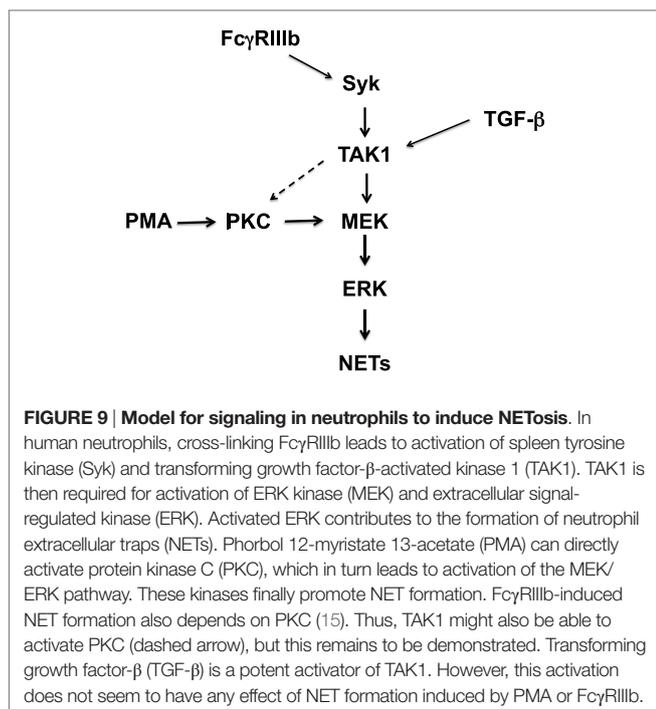
neutrophils, TAK1 can also be activated in response to stimulation of antibody receptor FcγRIIb. We also show that TAK1 is required for induction of NETosis by this receptor *via* the MEK/ERK signaling cascade.

Neutrophil activation is required for the initiation of the several defense mechanisms, including phagocytosis, respiratory burst, release of various microbicidal molecules by degranulation (35), and the formation of NETs (3). Many pathogens, including virus, bacteria, fungi, and parasites are known to induce NET formation (6). These microorganisms must be recognized by pattern recognition receptors (PRRs) such as TLRs. In fact, TLR-4 has been identified as an important receptor for NET formation (36–38). In addition, receptors for the Fc portion of antibody molecules have recently been identified as potent inducers of NET formation. In particular, the receptor for IgA FcαRI (CD89) (39) and the receptor for IgG FcγRIIb (CD16b) (15, 23) are the only Fc receptors known to induce NETosis.

FcγRIIb is present exclusively on human neutrophils, and it is a GPI-linked receptor, lacking transmembrane and cytoplasmic domains (10). Despite the fact that the initial signaling mechanism for this receptor remains to be described, it is clear that it can activate several signaling pathways leading to various cell responses including increase in calcium concentration (11), activation of integrins (12), activation of the transcription factors NF-κB (13) and Elk-1 (17), and induction of NET formation (15, 23). In our previous publication, we described that FcγRIIb can activate ERK, and this activation is important for NET formation (15). However, we could not identify how the MEK/ERK signaling cascade was engaged. Here, we now report for the first time, as far as we know, that the transforming growth factor-β-activated kinase 1 (TAK1) is activated upon FcγRIIb engagement, and

that this kinase is required both for NET formation and MEK/ERK activation. Our findings are similar to those reported for chemoattractant and growth factor stimulation of neutrophils where TAK1 is also activated and acts upstream of the MEK/ERK pathway (19). Still, the manner in which FcRIIb activates TAK1 remains elusive. Possible activators include Syk or TRAF6. We addressed the involvement of Syk by blocking its activity with two different specific inhibitors. Both Piceatannol and iSyk prevented activation (phosphorylation) of both TAK1 and ERK. These data clearly indicate that Syk functions upstream of TAK1 after FcγRIIb engagement. However, how this receptor lacking a cytoplasmic tail can connect with Syk remains an unsolved problem for future studies.

Although, both stimuli PMA and FcγRIIb cross-linking initiate signaling that seems to converge at the level of MEK (Figure 9), an important difference in NETosis induced by PMA or by FcγRIIb was found in this study. PMA release of DNA fibers was detected at later times just as described before (2, 40), more than 2½ h after stimulation, and reached a maximum around 4 h (Figure 1). In contrast, FcγRIIb-induced NETosis liberated DNA fibers rapidly in less than 1 h (Figure 1). Mechanistically, we do not know the reason for this difference, but it is possible that another pathway in addition to the ERK pathway is involved. Previously, Syk was also found to participate in NET formation induced by insoluble immune complexes (23) and by PMA (23). We also found that inhibition of Syk by Piceatannol blocked the release of NETs induced either by PMA or by FcγRIIb (15). In addition, we have observed inhibition of FcγRIIb-mediated TAK1 phosphorylation by Piceatannol and by iSyk (Figure 5). This suggests as mentioned above that Syk is required for TAK1 activation to deliver a signal for NET formation after FcγRIIb



engagement. Yet, activation of Syk by PMA has also been previously described in neutrophils. PMA induced PKC-dependent phosphorylation of Syk (41). However, we do not think that this pathway is involved in this case because inhibition of PKC did not prevent FcγRIIb-induced TAK1 phosphorylation (our unpublished data). Thus, TAK1 acts downstream of FcγRIIb and upstream (or independently) of PKC (Figure 9). In contrast, inhibition of PKC leads to reduced FcγRIIb-induced NET formation (15). Hence, it would seem that TAK1 connects to PKC for activation of the MEK/ERK signaling cascade. In support of this idea, another receptor has been recently reported to activate Syk and TAK1 together with PKC. The innate decoy receptor CEACAM3, also exclusively expressed by human neutrophils, triggers a Syk-, PKCδ-, and TAK1-dependent signaling cascade that results in activation of NF-κB (42). In another even more recent report, TAK1 was clearly shown to activate the MEK/ERK pathway (19). Unfortunately, in this study, the involvement of PKC was not investigated. Whether TAK1 connects to MEK directly of *via* PKC remains unsolved (Figure 9). Also, the difference in

kinetics for NET formation might be due, at least in part, to the selective activation of TAK1 by FcγRIIb (Figure 9). This idea is attractive, since, when neutrophils were treated with TGF-β, a stronger phosphorylation of TAK1 was detected (Figure 4A). Yet, no difference in NET formation was observed in cells pretreated with TGF-β. The mechanism responsible for the faster kinetics in FcγRIIb-mediated NET formation remains to be elucidated.

In several cell types, TAK1 functions upstream of p38 MAPK pathway rather than ERK (26, 29). In contrast, in human neutrophils, it has been reported that chemotactic and growth factors induce TAK1 activation leading to the MEK/ERK pathway independently of p38 MAPK (19). In the case of FcγRIIb-induced NET formation, we also found that inhibition of p38 MAPK with the inhibitor SB203580 did not affect NETosis (Figure 8B). Thus, our data also support the hypothesis that, in human neutrophils, TAK1 connects to MEK/ERK and not to p38 MAPK or JNK.

In conclusion, to our knowledge, this is the first demonstration that TAK1 can be activated by FcγRIIb in human neutrophils, and that this kinase is required for triggering the MEK/ERK signaling pathway to NETosis.

AUTHOR CONTRIBUTIONS

OA performed most of the experiments for NET formation and analyzed the data. NM prepared the cells and performed Western blots. RC-V generated some of the Western blot data. EU-Q helped with the first draft, performed the statistical analysis, organized the references, and prepared all figures. CR designed the research, mentored all other authors, and wrote the final version of the paper.

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The Selective Estrogen Receptor Modulator Raloxifene Inhibits Neutrophil Extracellular Trap Formation

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Raloxifene is a selective estrogen receptor modulator typically prescribed for the prevention/treatment of osteoporosis in postmenopausal women. Although raloxifene is known to have anti-inflammatory properties, its effects on human neutrophils, the primary phagocytic leukocytes of the immune system, remain poorly understood. Here, through a screen of pharmacologically active small molecules, we find that raloxifene prevents neutrophil cell death in response to the classical activator phorbol 12-myristate 13-acetate (PMA), a compound known to induce formation of DNA-based neutrophil extracellular traps (NETs). Inhibition of PMA-induced NET production by raloxifene was confirmed using quantitative and imaging-based assays. Human neutrophils from both male and female donors express the nuclear estrogen receptors ER α and ER β , known targets of raloxifene. Similar to raloxifene, selective antagonists of these receptors inhibit PMA-induced NET production. Furthermore, raloxifene inhibited PMA-induced ERK phosphorylation, but not reactive oxygen species production, pathways known to be key modulators of NET production. Finally, we found that raloxifene inhibited PMA-induced, NET-based killing of the leading human bacterial pathogen, methicillin-resistant *Staphylococcus aureus*. Our results reveal that raloxifene is a potent modulator of neutrophil function and NET production.

Keywords: neutrophils, raloxifene, MRSA, host-pathogen interactions, extracellular traps

INTRODUCTION

Neutrophil extracellular traps (NETs), first described in 2004 (1), are DNA-based structures decorated with antibacterial components (e.g., histones, antimicrobial peptides, and myeloperoxidase) that form during a specialized cell death pathway (NETosis) to ensnare and kill pathogens. Since their initial discovery, NETs have been shown to play an important role in host defense; however, the molecular mechanisms that control NET production remain a topic of active investigation. Although NET production is typically driven in concert with the generation of reactive oxygen species (ROS) and activation of PKC, additional pathways that facilitate NET production (e.g., ERK activation, histone citrullination, and intracellular ceramide accumulation) have been identified in recent years (2–4). Identification of novel pathways that modulate NETosis could reveal new therapeutic targets to enhance antibacterial innate immunity or inhibit aberrant neutrophil-mediated inflammatory responses.

Recently, we demonstrated that tamoxifen, an estrogen receptor modulator classically described as an estrogen receptor antagonist, is a potent NET inducer (4). Our results indicated that tamoxifen-induced NET production is largely driven by an accumulation of intracellular ceramide resulting from inhibition of glucosylceramide synthase. This finding prompted us to investigate whether other selective estrogen receptor modulators (SERMs) with pharmacological properties distinct from tamoxifen may also modulate neutrophil function/NET production. Of immediate interest was raloxifene, an FDA-approved compound typically prescribed for the prevention or treatment of osteoporosis in postmenopausal women (5). Similar to tamoxifen, the most well-characterized pharmacological targets of raloxifene are the nuclear estrogen receptors ER α and ER β (6). Although raloxifene has been described to exhibit potent anti-inflammatory effects (7), our understanding of how it affects neutrophil function remains limited (8), with no studies having directly assessed its effects on human neutrophils. Intriguingly, however, raloxifene treatment reduces plasma neutrophil myeloperoxidase levels *in vivo* (9). Because myeloperoxidase is released into the plasma following NET induction (10), this association suggested that raloxifene may inhibit NET production. Here, we provide evidence that raloxifene prevents neutrophil cell death in response to treatment with the NET inducer phorbol 12-myristate 13-acetate (PMA). Building on these results, we use both quantitative and imaging-based approaches to define the effects of raloxifene on NET production and probe the molecular mechanisms underlying this effect.

MATERIALS AND METHODS

Materials

Raloxifene hydrochloride, MPP dihydrochloride, and PHTTP were purchased from Tocris Bioscience (Bristol, UK). PMA, micrococcal nuclease from *Staphylococcus aureus*, the small molecule library used for screening and all other compounds were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Cell Viability Screen

An image-based screen, described in detail in Ref. (2), was used in conjunction with the Sigma LOPAC (Library of Pharmacologically Active Compounds; Sigma-Aldrich, St. Louis, MO, USA) library of ~1280 pharmacologically active small molecules. Following exposure to compounds from this library (each at a final concentration of 100 μ M), cell viability in response to 4-h PMA treatment was quantified by fixing cells with 2% paraformaldehyde, staining nuclei with the fluorescent DNA stain Sytox Green (2 μ M; Life Technologies, Carlsbad, CA, USA), and imaging using an Ascent Fluoroskan MTP reader (Thermo Scientific).

Neutrophil Isolation

Venous blood was collected from healthy volunteers according to an approved protocol. Heparin was used as an anticoagulant. Neutrophils were isolated using Polymorphprep density gradient medium (Axis-Shield, Dundee, Scotland) according to the manufacturer's protocol.

Fluorescence Microscopy/ NET Visualization

To visualize NETs, cells were seeded in Nunc Lab-Tek II Chambered Coverglass slides (Thermo Fisher, Waltham, MA, USA) at a density of 2×10^5 cells/well. Following incubation with indicated antagonist compounds, NET production was induced *via* addition of 25 nM PMA prior to a 2-h incubation at 37°C with 5% CO₂. Cells were fixed by addition of paraformaldehyde (4% final) for 10 min at 24°C. Following three washes with PBS, neutrophils were permeabilized *via* incubation in Triton X-100 solution (0.1%) for 10 min at 24°C. After an additional three washes with PBS, DNA was stained with 2 μ M Sytox Green (Life Technologies, Carlsbad, CA, USA) for 10 min at 24°C; slides were then washed a final three times with PBS prior to imaging using a Zeiss AxioObserver D1 microscope equipped with an LD A-Plan 20X/0.35 Ph1 objective (Carl Zeiss AG, Oberkochen, Germany).

Imaging of intracellular ceramide was performed by permeabilizing cells with 0.25% Triton X-100 prior to fixation with paraformaldehyde, blocking with PBS containing 2% bovine serum albumin (2% PBS-BSA), and 2% donkey serum for 1 h. Cells were subsequently incubated for 1 h with mouse anti-ceramide primary antibody (1:300 in 2% PBS-BSA; Sigma-Aldrich, St. Louis, MO, USA) and 45 min (protected from light) with Alexa Fluor 488 donkey anti-mouse IgG secondary antibody (1:500 in 2% PBS-BSA; Life Technologies, Carlsbad, CA, USA). Representative images shown were collected the Zeiss AxioObserver D1 microscope and objective described above, with exposure and gain settings kept consistent during collection of control and raloxifene-treated images.

PicoGreen NET Quantification Assay

All incubations were performed at 37°C and 5% CO₂ unless otherwise noted. Isolated neutrophils were plated on 96-well tissue culture plates at 2×10^5 cells/well. Cells were pretreated with estrogen receptor antagonists (e.g., raloxifene) for 30 min, then incubated an additional 2 h with PMA (25 nM) to induce NET production. Micrococcal nuclease was then added at a final concentration of 500 mU/ml for 10 min to allow digestion of extracellular DNA. Following addition of 5 mM EDTA, plates were centrifuged at $200 \times g$ for 8 min; supernatant samples (100 μ l) were then collected and transferred to a 96-well plate. DNA was quantified using a Quant-iT PicoGreen[®] dsDNA Assay Kit from Life Technologies (Carlsbad, CA, USA).

Quantification of Estrogen Receptor Expression

Freshly isolated neutrophils were harvested and 1×10^6 cells resuspended in TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA, USA). Total RNA was extracted using a Zymo Research Direct-zol RNA kit (Irvine, CA, USA) following the manufacturer's protocol (including DNase treatment). Following quantification of RNA using a Thermo Fisher Scientific Nanodrop Spectrophotometer (Waltham, MA, USA), quantitative PCR was performed on an ABI 7000 platform using USB[®] Veriquest[™] Probe One-Step qRT-PCR Master Mix (2 \times). Predesigned human TaqMan[®] Gene Expression Assays (Life Technologies, Carlsbad,

CA, USA) were used to probe for expression of ESRRG, ESR2, and GAPDH. An equal input of total RNA was used for each assay.

Transwell Chemotaxis Assay

Neutrophils, pre-incubated for 20 min at 37°C in HBSS alone or HBSS with 10 μ M raloxifene, were seeded in 6-mm transwell permeable supports (3- μ m pore size; Corning Inc., Corning, NY, USA) that were placed in 24-well plates; lower chambers contained either HBSS alone or 100 nM f-Met-Leu-Phe (fMLP). Following a 45-min incubation at 37°C, inserts were removed, and cells were lysed by addition of Triton X-100 (0.1% final, 10 min, 24°C). To determine the relative levels of migration to the lower well, the colorimetric elastase substrate *N*-methoxysuccinyl-Ala-Ala-Pro-Val *p*-nitroanilide was added to lysed cell samples (10 mM final); after a 30-min incubation at 24°C, absorbance at 405 nm was measured using a SpectraMAX Gemini EM fluorescence reader (Molecular Devices, Sunnyvale, CA, USA).

Phagocytosis Assay

Following a 30-min incubation at 37°C in the presence or absence of 10 μ M raloxifene, neutrophils were combined in a 96-well plate (2×10^5 cells/well) with pHrodo Red *S. aureus* Bioparticles (Life Technologies, Carlsbad, CA, USA) as specified by the manufacturer. Plates were incubated at 37°C, and phagocytosis was assessed by measuring fluorescence intensity (560 nm excitation, 585 nm emission) at 15 min intervals using a SpectraMAX Gemini EM fluorescence reader (Molecular Devices, Sunnyvale, CA, USA).

ROS Production Assays

Neutrophils were incubated in HBSS supplemented with 10 mM 2',7'-dichlorofluorescein diacetate (DCFDA) for 20 min at 37°C with gentle agitation. Neutrophils were then centrifuged at $400 \times g$ for 5 min, washed with HBSS, and centrifuged again using the same settings before being counted and resuspended in HBSS at a concentration of 5×10^6 cells/ml. Samples (100 μ l) of cell suspension were then added to a 96-well plate (5×10^5 cells/well) and incubated with HBSS or raloxifene for 30 min prior to addition of PMA. Fluorescence intensity (485 nm excitation, 530 nm emission) was measured at 15-min intervals using a SpectraMAX Gemini EM fluorescence reader over 2 h; between reads, plates were incubated at 37°C while protected from light.

Total/Phospho-ERK ELISA

Relative levels of total/phospho-ERK were determined using InstantOne ELISA kits (eBiosciences, San Diego, CA, USA) according to the manufacturer's protocol, with minor modifications (11). Total/phospho ERK was quantified following PMA stimulation (25 nM) for 45 min in the presence or absence of raloxifene (10 μ M).

NET Killing Assay

Neutrophils suspended in serum-free RPMI (SF-RPMI) were added to 48-well plates at a density of 4×10^5 cells/well. SF-RPMI or raloxifene (10 μ M final) was added to applicable wells, and cells were incubated for 30 min at 37°C with 5% CO₂ (identical

wells containing no neutrophils were also prepared). Following addition of PMA to applicable wells, cells were incubated for a further 4 h at 37°C with 5% CO₂. Overnight cultures of USA300 MRSA (strain UAMS 1182) bacteria were resuspended in RPMI containing 10% 70°C heat-inactivated fetal bovine serum (FBS) to achieve a density of 8×10^5 colony forming units (CFUs)/ml. Fifty microliters of bacterial suspension were added to each well, resulting in a 2% final concentration of FBS and a multiplicity of infection (MOI) of 0.1. Following a centrifugation at 1600 rpm for 5 min, mixed neutrophil/bacterial cultures were incubated for 15 min at 37°C with 5% CO₂. Samples from each well were then collected, serially diluted in sterile H₂O, and plated on Todd Hewitt Agar for enumeration of CFUs.

Statistical Analysis

All statistical analyses described in the figure legends were performed using GraphPad Prism version 7.0.

RESULTS

Raloxifene Inhibits PMA-Induced NET Production

An image-based cell viability screen (2) using the fluorescent DNA dye Sytox Green identified raloxifene as one of the small molecules from the ~1200 pharmacologically active known compounds tested to have enhanced the survival of neutrophils when exposed to PMA, a potent NET inducer (Figure 1A), suggesting that raloxifene treatment may inhibit NET production.

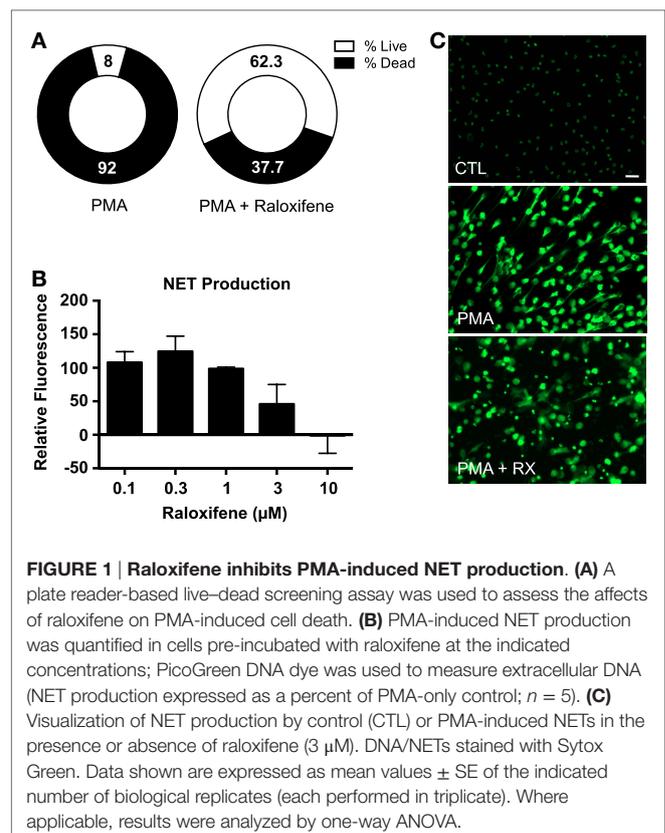


FIGURE 1 | Raloxifene inhibits PMA-induced NET production. (A) A plate reader-based live–dead screening assay was used to assess the affects of raloxifene on PMA-induced cell death. **(B)** PMA-induced NET production was quantified in cells pre-incubated with raloxifene at the indicated concentrations; PicoGreen DNA dye was used to measure extracellular DNA (NET production expressed as a percent of PMA-only control; $n = 5$). **(C)** Visualization of NET production by control (CTL) or PMA-induced NETs in the presence or absence of raloxifene (3 μ M). DNA/NETs stained with Sytox Green. Data shown are expressed as mean values \pm SE of the indicated number of biological replicates (each performed in triplicate). Where applicable, results were analyzed by one-way ANOVA.

To directly assess the effect of raloxifene on NET production, we quantified release of DNA using the fluorescent DNA dye PicoGreen, finding a concentration-dependent inhibition of PMA-induced NET production by the drug (Figure 1B). These results were confirmed *via* fluorescence microscopy of SYTOX Green-stained cells, which revealed a reduction in the number of NET-positive cells and an increase in the number of intact, condensed nuclei (Figure 1C).

Raloxifene Does Not Affect Neutrophil Chemotaxis or Phagocytosis but Reduces Intracellular Ceramide

We previously demonstrated that tamoxifen, in addition to stimulating NET production, enhances both chemotaxis in a chemoattractant gradient and phagocytosis of bacteria-labeled bioparticles (4). Tamoxifen treatment also increases intracellular ceramide levels in neutrophils *via* inhibition of glucosylceramide synthase, a key enzyme in the sphingolipid synthesis pathway that converts ceramide to glucosylceramide. Tamoxifen-induced NET production is dependent on this increase of intracellular ceramide (4). In contrast to our observations with tamoxifen,

we found that raloxifene had no statistically significant effect on either chemotaxis in a gradient of the chemoattractant fMLP (Figure 2A) or phagocytosis of *S. aureus*-labeled bioparticles (Figure 2B). Furthermore, immunostaining of raloxifene-treated neutrophils revealed that, in contrast to tamoxifen, raloxifene reduces intracellular ceramide levels (Figures 2C,D).

Selective Estrogen Receptor Antagonists Inhibit PMA-Induced NET Production

Quantitative PCR-based analysis of ER α and ER β expression in neutrophils from 12 healthy donors (6 male and 6 female) revealed that circulating neutrophils express both nuclear receptors (Figure 3A). Fluorescence-based quantification of NET production indicated that the selective estrogen receptor antagonists MPP (ER α inhibitor) and PHTPP (ER β inhibitor) mimicked the raloxifene-mediated inhibition of PMA-induced NET production (Figure 3B). Imaging of SYTOX Green-stained cells revealed some residual NET production by neutrophils pre-incubated with MPP and PHTPP, suggesting the potential involvement of multiple estrogen receptors in raloxifene-mediated inhibition of NETosis (Figure 3C).

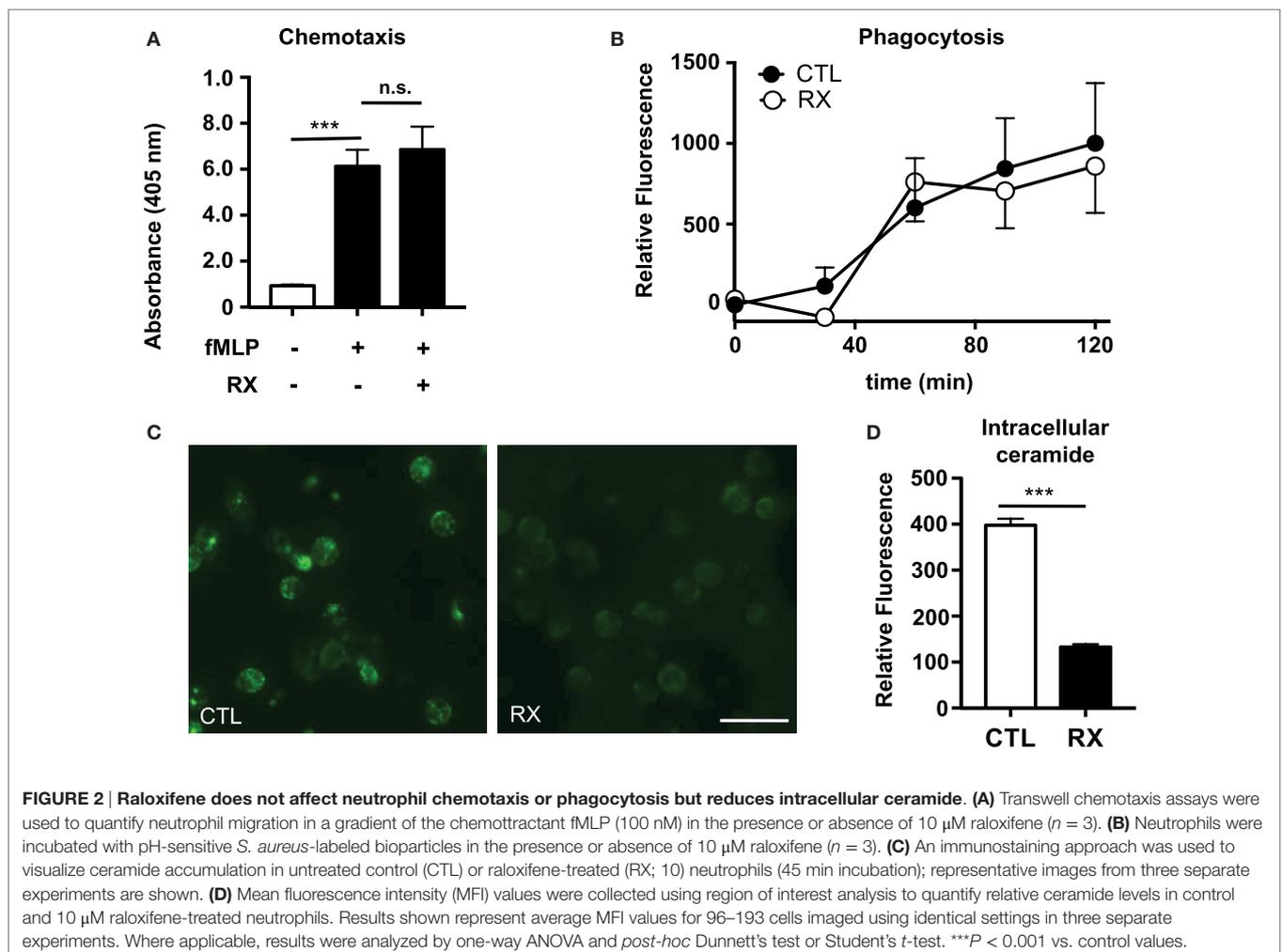


FIGURE 2 | Raloxifene does not affect neutrophil chemotaxis or phagocytosis but reduces intracellular ceramide. (A) Transwell chemotaxis assays were used to quantify neutrophil migration in a gradient of the chemoattractant fMLP (100 nM) in the presence or absence of 10 μ M raloxifene ($n = 3$). (B) Neutrophils were incubated with pH-sensitive *S. aureus*-labeled bioparticles in the presence or absence of 10 μ M raloxifene ($n = 3$). (C) An immunostaining approach was used to visualize ceramide accumulation in untreated control (CTL) or raloxifene-treated (RX; 10) neutrophils (45 min incubation); representative images from three separate experiments are shown. (D) Mean fluorescence intensity (MFI) values were collected using region of interest analysis to quantify relative ceramide levels in control and 10 μ M raloxifene-treated neutrophils. Results shown represent average MFI values for 96–193 cells imaged using identical settings in three separate experiments. Where applicable, results were analyzed by one-way ANOVA and *post-hoc* Dunnett's test or Student's *t*-test. *** $P < 0.001$ vs. control values.

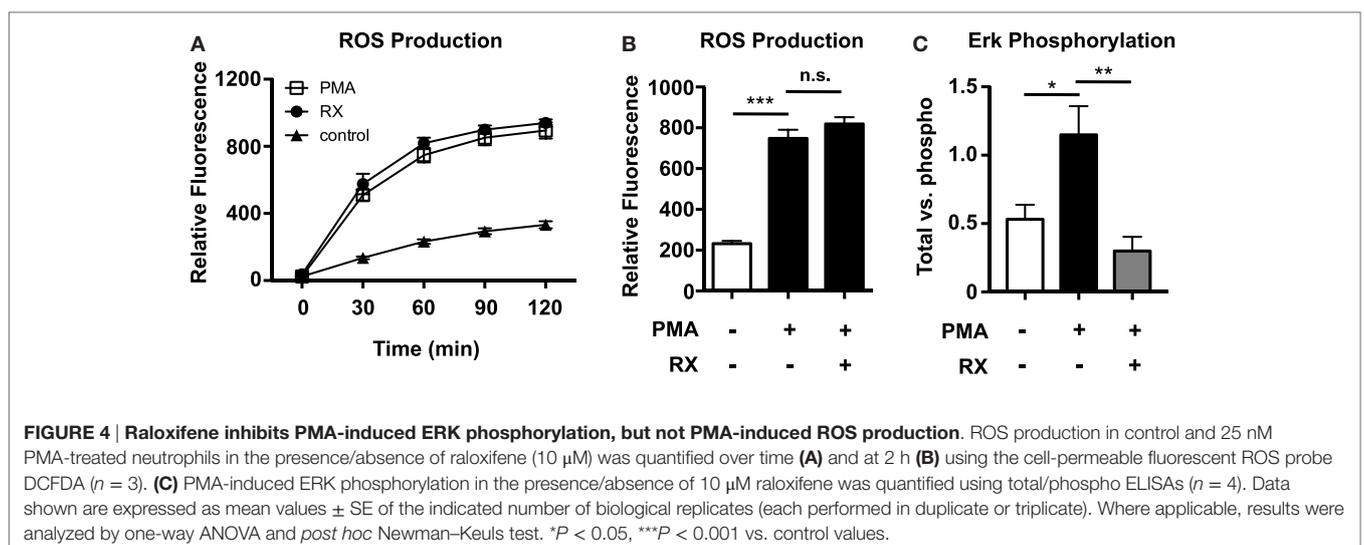
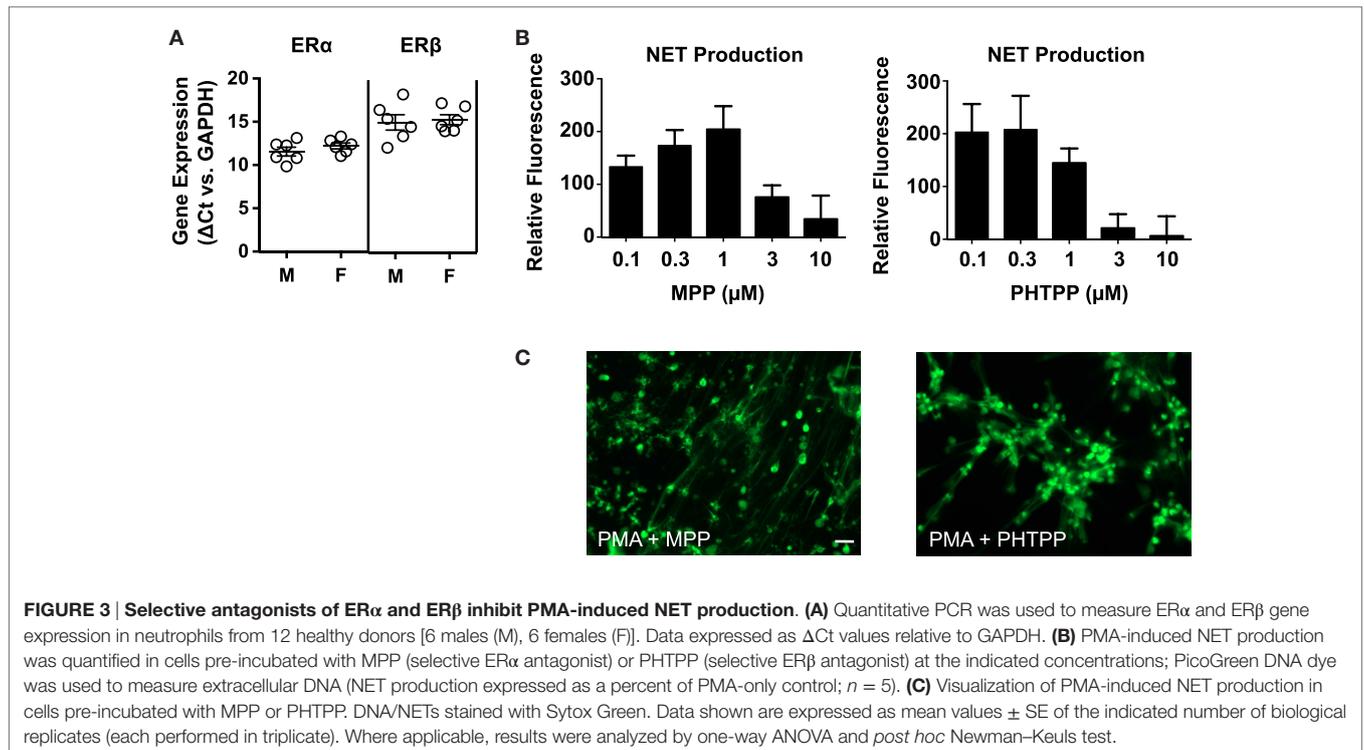
Raloxifene Inhibits PMA-Induced ERK Phosphorylation but Not PMA-Induced ROS Production

Reactive oxygen species production was quantified using the membrane-permeable fluorescent ROS probe 2',7'-dichlorofluorescein diacetate (DCFDA). Although PMA-induced NET production is known to be ROS dependent (1), raloxifene did not inhibit PMA-induced ROS production (Figures 4A,B). However, ELISA-based quantification of ERK phosphorylation revealed that raloxifene significantly inhibited PMA-induced

ERK activation (Figure 4C), a critical signaling event that has been shown to play a role in NET production (2).

Raloxifene Inhibits NET-Mediated Killing of Methicillin-Resistant *Staphylococcus aureus*

To determine whether raloxifene affects NET-based bactericidal activity of neutrophils, we performed a NET-based killing assay in which neutrophils were pre-stimulated with PMA to induce NET production prior to challenge with methicillin-resistant



Staphylococcus aureus (MRSA; MOI 0.1). Stimulation of NETs via 4 h incubation with 25 nM PMA produced a statistically significant reduction in bacterial survival in neutrophil/MRSA cocultures (Figure 5). Incubation with raloxifene 30 min prior to addition of PMA reversed this effect.

DISCUSSION/CONCLUSION

Here, we show evidence that the SERM raloxifene inhibits PMA-induced NET production. Unlike tamoxifen, an estrogen receptor modulator that induces NET production (4), raloxifene reduces intracellular ceramide levels. Neutrophils from both male and female donors express the nuclear estrogen receptors ER α and ER β , the best characterized targets of raloxifene. Selective antagonists of either receptor inhibited PMA-induced NETosis, suggesting that raloxifene may act through multiple receptors to inhibit NET production, and further indicating that tamoxifen-induced NET production is largely dependent on estrogen

receptor-independent, non-specific effects (e.g., inhibition of glucosylceramide synthase). We find that raloxifene inhibits PMA-induced ERK phosphorylation, a signal transduction event known to be critical for NETosis (2); thus, raloxifene likely modulates NET production via regulation of ERK signaling.

Our results suggest that raloxifene may have beneficial therapeutic effects in contexts where excessive NETosis is undesirable and associated inflammatory changes cause damage to the host (12). NETs appear to play a role in the pathophysiology of rheumatoid arthritis (13); notably, raloxifene has been shown to exhibit significant antiarthritic properties in a murine model of this disease (14). The incidence of rheumatoid arthritis in raloxifene-treated postmenopausal women (and specifically the contribution of NETs to disease pathophysiology) represents an important area for further investigation; however, our results also suggest that raloxifene therapy may come at some consequence to patients at high risk of infection, as raloxifene inhibits NET-mediated bacterial killing.

These findings provide new insight into the effects of raloxifene on neutrophil function, which at present are poorly understood. Such effects hold important clinical implications, given the large number of patients receiving this drug. Further exploration of the pathways mediating the anti-NETosis activity of raloxifene may reveal new therapeutic targets and facilitate development of therapies that can more selectively regulate NET production in appropriate physiological contexts.

ETHICS STATEMENT

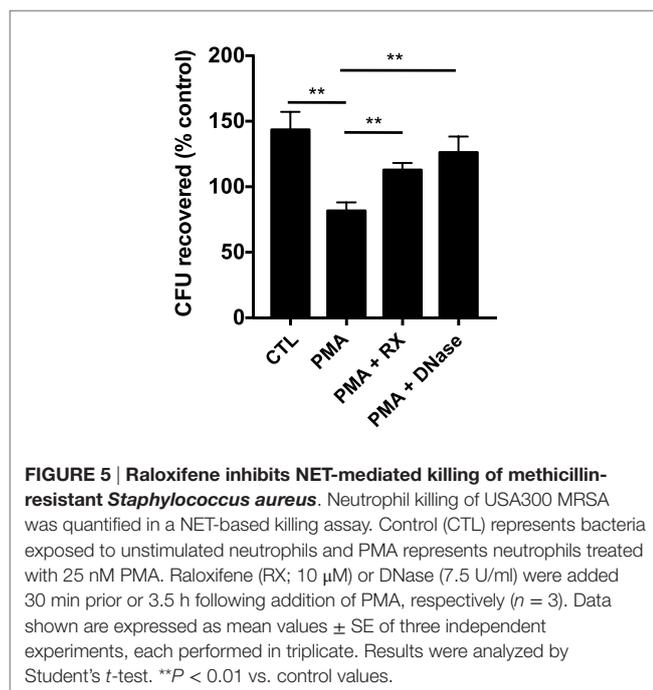
All studies described here were reviewed and approved by the local (UCSD) Institutional Review Board.

AUTHOR CONTRIBUTIONS

RC and VN conceptualized and led the project. RC, RF, SD, CS, and AH designed and performed experiments and interpreted data. RC, RF, and VN wrote the manuscript.

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Conflict of Interest Statement: The 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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Multimodal Regulation of NET Formation in Pregnancy: Progesterone Antagonizes the Pro-NETotic Effect of Estrogen and G-CSF

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Human pregnancy is associated with a mild pro-inflammatory state, characterized by circulatory neutrophil activation. In order to explore the mechanism underlying this alteration, we examined NETosis during normal gestation. Our data indicate that neutrophils exhibit a pro-NETotic state, modulated in a multimodal manner during pregnancy. In general, circulatory granulocyte colony-stimulating factor, the levels of which increase during gestation, promotes neutrophil extracellular trap (NET) formation. Early in pregnancy, NETosis is enhanced by chorionic gonadotropin, whereas toward term is stimulated by estrogen. A complex interaction between estrogen and progesterone arises, wherein progesterone restrains the NETotic process. In this state, extensive histone citrullination is evident, yet full NETosis is inhibited. This coincides with the inability of neutrophil elastase to translocate from the cytoplasm to the nucleus and is regulated by progesterone. Our findings provide new insight concerning gestational and hormone-driven pathologies, since neutrophil recruitment, activation, and NET release could be associated with excessive endothelial and placental injury.

Keywords: neutrophils, NETs, pregnancy, sex hormones, G-CSF

INTRODUCTION

Pregnancy presents a unique challenge for the maternal immune system, which is modulated in such a manner that the mother accepts her semi-allogeneic fetus, yet is still capable of mounting an effective response against infections (1, 2). Although a considerable body of knowledge has been accumulated regarding the role of T lymphocytes, regulatory T cells, or natural killer cells in tolerance promotion or tissue modification during gestation, the role of the innate immune response and the involvement of neutrophils in particular has not been examined to the same extent in

Abbreviations: citH3, citrullinated histone H3; DAPI, 4',6-diamidino-2-phenylindole; E2, β -estradiol; E3, estriol; EIA, enzyme immunoassay; G-CSF, granulocyte colony-stimulating factor; hCG, human chorionic gonadotropin; MPO, myeloperoxidase; NADPH, nicotinamide adenine dinucleotide phosphate oxidase; NE, neutrophil elastase; NET, neutrophil extracellular trap; P4, progesterone; PAD4, peptidyl arginine deiminase IV; PMA, phorbol myristate acetate; RA, rheumatoid arthritis; ROS, reactive oxygen species; SLE, systemic lupus erythematosus.

normal pregnancy (3). There is a need to address this, since a comprehensive body of evidence implicates aberrant neutrophil activity in severe pregnancy complications such as preeclampsia (PE), recurrent fetal loss, or poor pregnancy outcome due to autoimmune conditions such as systemic lupus erythematosus (SLE) (4–8). In part, these pathologies appear to entail an excessive alteration of the moderately increased inflammatory activity of circulatory neutrophils in normal pregnancy (4, 9). Such overt neutrophil activity is proposed to contribute to increased neutrophil extracellular traps (NETs) in preeclamptic placentae or, as recently shown, those affected by SLE (10–12).

Neutrophils play a key role in the immediate response to infections, employing an array of weapons including phagocytosis, release of toxic granular enzymes and of reactive oxygen species (ROS), or the generation of NETs to dispose of harmful microorganisms (13, 14). NETs are a rather unique innate immunity tool, being formed by the extrusion of nuclear DNA into the extracellular environment, where they ensnare a wide array of microorganisms, ranging from bacteria and fungi to parasites (15). Aberrant NET formation may induce damage or cell death of neighboring tissues and has been implicated in a number of pathologies including rheumatoid arthritis (RA), SLE, small vessel vasculitis, or coagulopathies (16). The underlying signal-transducing pathway initiating NETosis involves calcium mobilization, generation of ROS by NADPH oxidase, nuclear transfer of neutrophil elastase (NE), myeloperoxidase (MPO), and peptidylarginine deiminase 4 (PAD4), and histone citrullination by the latter (17–21). These events contribute to chromatin unfolding, a prerequisite for efficient DNA extrusion (21–23).

To date, the generation of NETs and the regulation of their release have not been studied during the three trimesters of normal human pregnancy (7, 12). Our data indicate that neutrophils from normal healthy pregnancies exhibit a distinctive pro-NETotic phenotype, which increases toward term. It was determined that granulocyte colony-stimulating factor (G-CSF) not only contributes to increasing neutrophil counts during gestation but also to progressively enhanced NETosis. Early in gestation, NETosis is augmented by the action of human chorionic gonadotropin (hCG), while toward term the steroid sex hormones estradiol (E2) and progesterone (P4) modulated neutrophil activity in a complex manner. While E2 acts to promote NET formation, P4 acts as an antagonist, by retaining neutrophils in an advanced primed state, thus hindering progression of NETosis. Our findings suggest that the regulatory mechanism evoked by P4 involves the prevention of NE transfer from the cytoplasm to the nucleus, a step previously demonstrated to be vital for effective NET formation (18).

MATERIALS AND METHODS

Human Subjects

Pregnant women were recruited at the time of their routine examination at the end of the first (median gestational age: 12 weeks and 4 days – $n = 15$; median age: 34.1 years) and second trimesters (median gestational age: 24 weeks and 3 days – $n = 25$; median age: 34.1 years) and at the time of elective cesarean section toward the end of the third trimester (median gestational age

at delivery: 38 weeks and 4 days – $n = 35$; median age: 34.1 years) (Table S1 in Supplementary Material). Healthy non-pregnant controls, matched for age ($n = 45$; median age: 33.5 years), were recruited at the Blood Bank of the Swiss Red Cross, Basel (Table S1 in Supplementary Material). Inclusion criteria for non-pregnant controls were fair general condition, female sex, age ≥ 25 and ≤ 45 years, and for blood donors fulfilling national criteria for blood donation. Exclusion criteria were current or previous systemic autoimmune disease, asthma, reconvalescence after major illness, surgery, current medication with corticosteroids, immunosuppressive agents and malignant neoplasia, or chemotherapy within 5 years before recruitment for the study. Exclusion criteria for pregnant subjects included any major complication of pregnancy or coincident disease, such as PE, pre- or post-term labor (< 37 or > 42 weeks), intrauterine growth retardation, and viral, bacterial, or parasitic infections. Informed, written consent was obtained from all subjects prior to inclusion in the study, which was approved by the Ethical Review Board of Basel/Basel-Land, Switzerland.

Blood Cell Count and Preparation of Plasma and Serum

Whole blood was collected into EDTA- and silicone-coated tubes (Sarstedt), and 25 μ l of blood was analyzed by a Hemavet 950FS (Drew Scientific) for complete blood cell counts. Plasma and serum was collected and processed as described previously (24). Samples were studied immediately or stored at -80°C until analyzed.

Human Neutrophil Isolation

Neutrophils were isolated by Dextran–Ficoll density centrifugation (25). Cell viability was assessed by trypan blue dye exclusion via a hemocytometer and was measured routinely 96–98% with a purity of over 95%. Neutrophils were directly seeded in 24-well or 96-well plates and allowed to settle for 15 min at 37°C under 5% CO_2 prior to further experimentation. Time-points of measurements are given in the figure legends.

Stimulation and Neutralization Studies

For *in vitro* incubation studies, 2.5×10^4 neutrophils from healthy women were treated with 3% serum or 6% plasma derived from non-pregnant controls and pregnant donors during the first, second, and third trimesters of gestation. All experiments were carried out over 3 h in four to six replicates.

Neutrophils from healthy controls were incubated with ascending concentrations of hCG, E2, E3, P4, or G-CSF, which covered the physiological plasma concentrations during gestation, individually or in different combinations.

For the two-step stimulation *in vitro* experiments, neutrophils were pretreated with hormones or G-CSF as a primary stimulus for 60 min, and then exposed to the secondary stimulus (PMA or G-CSF) for another 120 min, for a total time of 3 h.

To neutralize sex hormones' activity, pooled sera or plasma from the study groups of interest were pretreated for 30 min with fulvestrant (10 $\mu\text{g/ml}$, Sigma) and mifepristone (10 $\mu\text{g/ml}$, Sigma) and used for a 3-h treatment of control neutrophils for the inhibition of estrogen and progesterin receptors, respectively.

To neutralize G-CSF, pooled sera or plasma from the study groups of interest were pretreated with anti-G-CSF antibody (0.2 µg/ml, Peprotech) for 30 min.

Fluorimetric Quantification and Fluorescence Microscopy

NETs were quantified by SytoxGreen fluorimetry (10, 20, 26). 2.5×10^4 freshly isolated neutrophils were cultured in the presence of 0.2 µM SytoxGreen (Invitrogen, Life Technologies) in a 96-well dark microtiter plate at 37°C under 5% CO₂ and left untreated or stimulated with the aforementioned agents over 3 h. PMA (25nM) was used as the positive control. Fluorescence (excitation 485 nm, emission 535 nm) was measured in a Biotek Synergy H1 Hybrid Reader (Biotek) and results given as DNA mean fluorescence intensity (MFI). Photomicrographs in bright field and green fluorescence spectra were assessed with an Olympus IX50 inverted fluorescence microscope coupled to an Olympus XM10 monochromatic CCD camera and analyzed with the Olympus CellSens Dimension software (Olympus).

Neutrophil Viability

Apoptosis was detected by Annexin V/7-aminoactinomycin D (7-AAD) staining (BD BioSciences) according to the manufacturer's instructions. 10^4 cells were counted by flow cytometry using a BD Accuri™ C6 flow cytometer (Becton-Dickinson). The data were analyzed using Flowjo v10 software (Treestar).

Cytokine Proteome Array

Cytokines, chemokines, and acute phase proteins were detected with the Human Cytokine Array Kit (R&D Systems) according to the manufacturer's instructions. Pooled sera collected from control non-pregnant individuals and pregnant donors during the first, second, and third trimesters of gestation were centrifuged and incubated with the pre-coated nitrocellulose membranes. After washing and addition of the detection antibody streptavidin-HRP conjugates, the membranes were exposed to X-ray film (Fuji). The cytokine proteomic array comprised 36 targets spotted in duplicate on the membranes. The intensity of each spot in the captured images was analyzed with ImageJ analysis software (NIH Image Processing).

NE, MPO, Cell-Free Histone/DNA Complex, MPO/DNA Complex, and G-CSF Protein Analysis

The concentrations of NE and MPO were measured in sera and plasma by sandwich ELISA, utilizing, respectively, the Elastase/a1-PI Complex ELISA Kit (Calbiochem) and the human MPO ELISA Kit. Histone/DNA complexes in sera and plasma were measured using the Human Cell Death Detection ELISA^{PLUS} (Roche Diagnostics); nucleosomes in cell culture supernatants were detected similarly after incubation with DNase I (10 U for 5 min) (Roche Diagnostics). To identify NET-associated MPO/DNA complexes, a modified capture ELISA was utilized (27). NET-associated MPO in culture supernatant was captured using the coated 96-well plate of the human MPO ELISA Kit (Hycult Biotech), and the NET-associated DNA backbone was detected

using the anti-DNA-POD antibody of the Human Cell Death Detection ELISA^{PLUS} (Roche Diagnostics). G-CSF serum and plasma protein concentrations were assessed with the Human G-CSF Quantikine ELISA Kit (R&D Systems).

Oxidative Burst Analysis

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-mediated ROS production was measured either by a 2',7'-dichloro dihydrofluorescein diacetate (DCFH-DA) plate assay (28) or a luminol-based chemiluminescence microtiter plate assay (29, 30). Also, 2.5×10^4 neutrophils per well were incubated without or with stimulants mentioned above in dark 96-well microtiter plates with 25 µM DCFH-DA (Sigma-Aldrich), which reacts with ROS species produced in intracellular compartments (granules or phagosomes). Fluorescence was recorded immediately in a Biotek Synergy H1 Hybrid plate Reader (Biotek) for 30 min. The response was expressed as relative fluorescence units (RFU). Similarly, 2.5×10^4 neutrophils per well were incubated without or with the aforementioned stimulants in white 96-well microtiter plates with 60µM luminol (5-amino-2,3-dihydro 1,4-phthalazinedione). Chemiluminescence was recorded every 5 min over a period of 30 min in a Biotek Synergy H1 Hybrid plate Reader (Biotek), and the response was expressed as relative luminescence units (RLU).

Immunohistochemistry, Morphometric Analysis, and Confocal Microscopy

The 1×10^5 neutrophils were seeded on poly-L-lysine-coated glass coverslips (BD Biosciences) in 24-well tissue-culture plates and allowed to settle prior to stimulation as described above. Coverslips were rinsed with ice-cold HBSS and the cells fixed with 4% paraformaldehyde and blocked overnight (HBSS with 10% FBS, 0.1% Tween20, and 2mM EDTA) at 4°C. NETs were detected with rabbit anti-NE (Abcam), rabbit anti-MPO (Dako), and rabbit anti-citrullinated histone H3 (citH3, Abcam). Secondary antibodies were goat anti-rabbit IgG AF555, goat anti-rabbit IgG AF488 (Invitrogen Life Technologies), and goat anti-mouse IgG AF647. DNA was stained with 4',6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich). NETs were visualized by using a Zeiss Axioplan 2 Imaging fluorescence microscope in conjunction with a Zeiss AxioCam MRm monochromatic CCD camera and analyzed with Axiovision 4.8.2 software (Carl Zeiss). A minimum of 20 fields (at least 1,000 neutrophils) per case was evaluated for MPO/NE and DNA co-staining; nuclear phenotypes and NETs were counted and expressed as percentage of the total number of cells in the fields.

In another setup, NETs were quantified by IHC staining of 2.5×10^4 neutrophils per well in a 96-well plate with mouse anti-human MPO antibody (Abcam) and rabbit anti-human citH3 antibody (Abcam), or the respective isotype controls, followed by incubation with goat anti-mouse IgG AF555 and goat anti-rabbit IgG AF488 (Invitrogen Life Technologies). DNA was counterstained with DAPI (Sigma-Aldrich). NETs were visualized by using an Olympus IX81 motorized epifluorescence microscope (Olympus) in conjunction with an Olympus XM10 monochromatic CCD camera (Olympus) and analyzed with the

Olympus CellSens Dimension software (Olympus). A minimum of 20 fields at 10× magnification (at least 500–1,000 neutrophils) per sample were evaluated for MPO/citH3 and DNA co-staining through ImageJ analysis software (NIH); nuclear phenotypes and NETs were determined, counted, and expressed as percentage of the total area of cells in the fields (31). Images were captured on a Nikon A1R inverted microscope (Nikon) coupled to a Visitron CSU-W1 spinning disk confocal microscopy module (Visitron) and a Thor ablation laser (Thor Labs) using an UPL APO 60×/1.40 oil objective lens with the Visiview Cell Analyser software (Visitron Systems, Version 3.1.2.2).

Phagocytosis Activity

Neutrophil phagocytic activity was examined by the uptake of latex beads coated with FITC-labeled rabbit IgG into cells (Cayman Chemical) according to the instruction manual. The 1×10^5 untreated neutrophils exposed to various stimulants were resuspended in 200 μ l phagocytosis buffer to which FITC-labeled beads (1:100) were added and incubated for 2 h at 37°C. The amount of phagocytosis was determined by flow cytometry utilizing the BD Accuri™ C6 flow cytometer (Becton-Dickinson) and analyzed by Flowjo v10 software (Treestar). The uptake of the beads into neutrophils was additionally captured with an Olympus IX50 inverted fluorescence microscope and phagocytosis quantified as described above with ImageJ analysis software.

RNA Isolation and Quantitative Real-time PCR

Total RNA was isolated from 3×10^6 neutrophils by using the RNeasy Mini Kit (Qiagen). TaqMan real-time quantitative RT-PCR was performed utilizing the Applied Biosystems StepOne Plus cyler (Applied Biosystems) and TaqMan Gene Expression Assay primer and probe sets (Applied Biosystems) for *ELANE* (HS00236952_m1), *MPO* (HS00924296_m1), *PAD4* (HS00202612_m1), *IL-6* (HS00202612_m1), *IL-8* (HS00202612_m1), and *TNF* (HS00202612_m1). Data were normalized to the housekeeping gene *B2M* (HS99999907_m1), after a selection procedure from six different endogenous reference genes, as suggested in the MIQE guidelines (32). Relative values were calculated with $2^{-\Delta\Delta C_t}$ analysis (33).

Protein Isolation and Western Blot Analysis for PAD4 and Citrullinated Histone H3

Total protein was isolated by NucleoSpin TriPrep kit (Macherey-Nagel) from 5×10^6 neutrophils. All protein concentrations were determined with the MN Protein Quantification Assay (Macherey-Nagel). Western blotting was performed utilizing AnykD Mini-PROTEAN TGX Gels (Biorad) and nylon/nitrocellulose membranes (Biorad). Primary and secondary antibodies utilized were rabbit anti-MPO (Cell Signalling Technologies), rabbit anti-PAD4 (Abcam), rabbit anti-citH3 (Abcam), mouse anti- β -Actin (Sigma), anti-rabbit HRP (Santa Cruz), and anti-mouse HRP (Santa Cruz). HRP activity was detected by using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). Equal loading was verified using beta-actin. Western

blots of citrullinated H3 (citH3) protein were prepared as described previously (34). Gel documentation, densitometric analysis, and protein quantification of the western blots was performed using the ChemiDoc XRS+ imaging system (Biorad) with the ImageLab 4.1 image analysis software (Biorad).

Statistical Analysis

All data are presented as mean \pm SE. Descriptive statistics for continuous parameters consisted of median and range, and categorical variables were expressed as percentages. Comparisons between patients and healthy controls were carried out by the Mann-Whitney *U* test with a Welch post-test correction. Statistical significance in multiple comparisons was by one-way analysis of variance (ANOVA) with a Dunn's post-test correction. *P* values under <0.05 were considered statistically significant. Data were processed in GraphPad Prism version 6.0 for MacOSX (GraphPad Software Inc.¹). Professional statistical assistance was provided by Andreas Schoetzau.²

RESULTS

Pregnancy Is Associated with a Pro-NETotic State That Increases with Advancing Gestation

An initial examination of circulatory neutrophils isolated from normal maternal blood samples with SytoxGreen, a cell impermeable DNA dye, indicated that they exhibited an increased tendency to form NETs *in vitro*, when compared to matching non-pregnant control donors (Figure 1A; Video S1 in Supplementary Material). This was most evident after culturing for 3 h, as confirmed by immunocytochemical staining (Figure 1B). To verify that these filamentous structures stained by SytoxGreen were indeed NETs, we used immunofluorescence microscopy (Figure 1B) and detection of MPO/cell-free DNA complexes in culture supernatants (Figure S1A in Supplementary Material). This confirmed the increased tendency for neutrophils to undergo increased NETosis with advancing gestational age. While the numbers of neutrophils increased during pregnancy, the proportion undergoing NETosis was greater than the increment in neutrophil numbers (Figure S1B in Supplementary Material).

Analysis of nuclear shape indicated that a significant proportion of the circulatory neutrophils in pregnant women were in a primed pro-NETotic state, evident by a delobulated diffused staining pattern (Figure 1C). This enhanced pro-NETotic activity was mirrored by an increase in ROS production (Figure 1D), as well as by MPO expression and histone H3 citrullination, likely driven by enhanced PAD4 expression (Figure 1E), key signaling events required for efficient NET generation. This pro-NETotic phenotype was further substantiated by monitoring the response to other stimuli, such as that to phorbol ester (PMA), where a hyperresponsive increase in NET generation was observed, particularly evident in samples collected close to term (Figure 1F; Figures S1C,D in Supplementary Material).

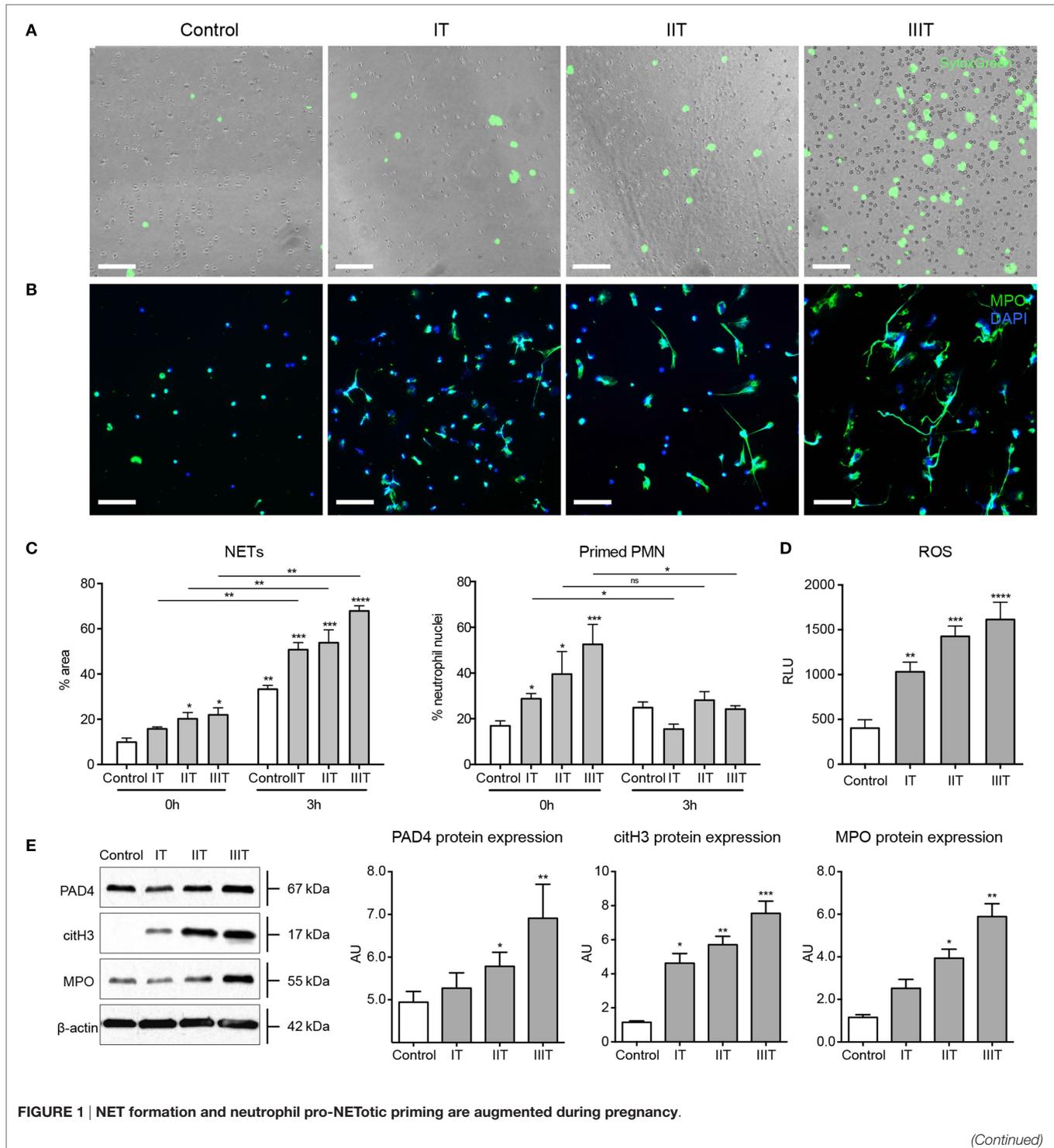
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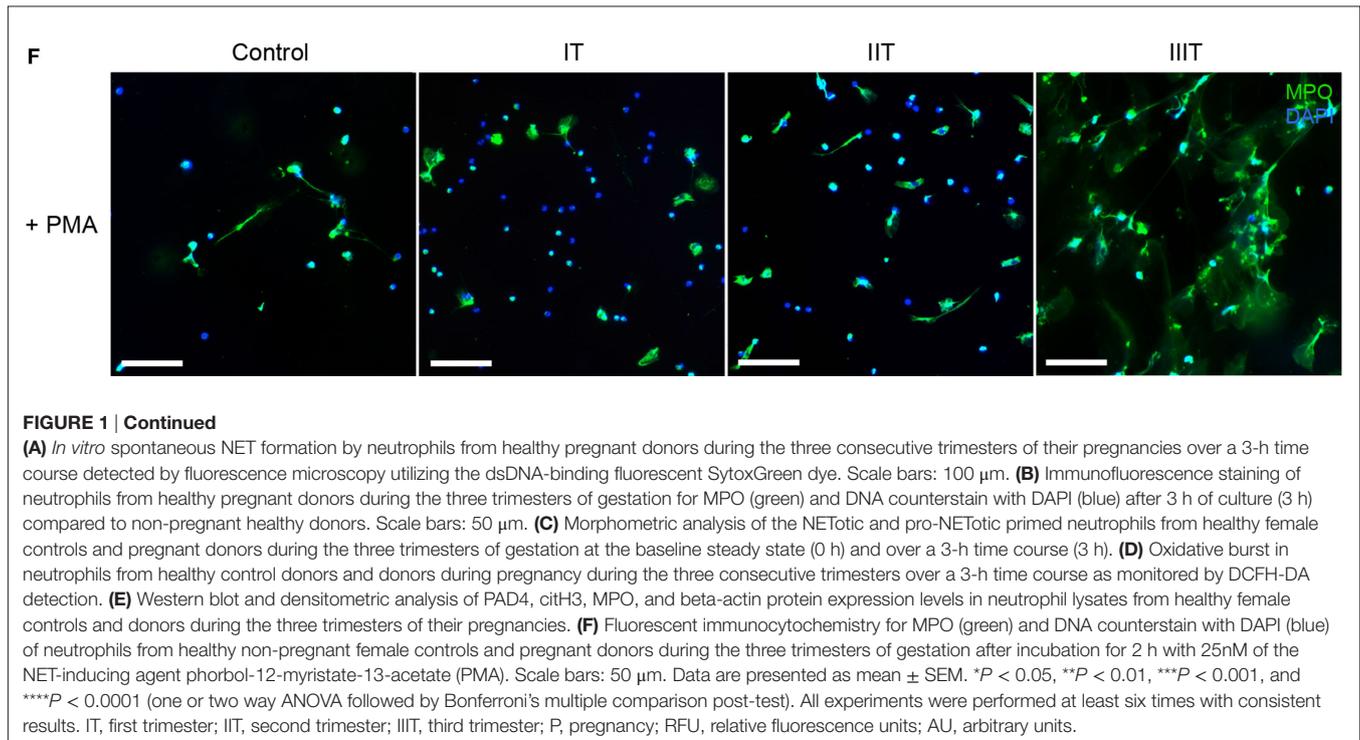
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G-CSF Promotes a Pro-NETotic Status during Pregnancy

In order to assess which circulatory factors could contribute to the pro-NETotic state, we examined the effect of sera from pregnant women on control neutrophils, where we observed that such treatment augmented NET formation (Figure 2A), and that

in a gestational age-dependent manner (Figure 2B). G-CSF was determined to be significantly elevated in a cytokine proteome array analysis (Figure S2 in Supplementary Material), a feature confirmed by specific enzyme immunoassay (EIA) (Figure 2C). This piqued our interest, as this cytokine has recently been implicated to predisposing neutrophils toward NETosis in a





murine tumor model (35). Circulatory G-CSF appears to be an important contributor to the pro-NETotic state present in pregnancy, as a G-CSF blocking antibody diminished NET formation induced by plasma from pregnant women (Figures 2D,E). This was greatest with advanced gestation, indicating increased quantities of circulatory G-CSF close to term (Figure 2C). On the other hand, treatment of maternal plasma samples with the anti-G-CSF antibody led to a step-wise decrease in ROS production inversely proportional to advancing gestational age (Figure 2F). The pro-NETotic role of circulatory G-CSF during pregnancy was investigated by assessing the effect of different concentrations of recombinant human (rh)G-CSF on isolated normal neutrophils. rhG-CSF increased NET formation in a concentration-dependent manner (Figure 2G; Figure 2H, left panel), while 12.5 mg/ml, which corresponds to the physiologic range in pregnancy, triggered the highest primed pro-NETotic state (Figure 2H, right panel) that was highly responsive to a secondary stimulus by PMA (data not shown). Furthermore, increasing doses of rhG-CSF led to concordant increases in ROS production (Figure 2I), while physiological concentrations led to increases in key NETosis signaling components, namely PAD4, citH3, and MPO (Figure 2J). These data suggest that G-CSF plays a vital role in promoting a pro-NETotic phenotype in pregnancy.

The Gestational Hormonal Milieu Regulates NET Formation

The immune-modulatory role of hCG, estrogen, and progesterone during pregnancy is well documented. hCG levels peak at the end of the first trimester, while those of estrogen and

progesterone reach a maximum at term (36–38). Accordingly, we examined their influence, focusing on the interplay between estrogen and progesterone, as would occur physiologically in preparation for parturition. As shown in Figures 3A,B, both hCG and estrogen promote a pro-NETotic state, as well as increased ROS production (Figure 3C) and elevations in citH3 protein (Figure 3D), important hallmarks of ensuing NETosis. Remarkably, progesterone had a pronounced anti-NETotic effect (Figures 3A,B), significantly diminishing the pro-NETotic influence of estrogen (Figures 3A,B). Though progesterone curbed ROS production, PAD4, and MPO protein expression induced by estrogen (Figure 3C; Figure S3A in Supplementary Material), citH3 levels remained unchanged (Figure 3D), suggesting a differential effect on signaling leading to NETosis. A detailed appraisal of the interaction between the two hormones in modulating NETosis is presented in Figure 3E, where it is evident that physiological concentrations of progesterone hindered the degree of NETosis attained by applications of estrogen alone. Progesterone was also effective in reducing NETosis triggered by the powerful stimulant PMA (Figure 3F; Figures S3B,C in Supplementary Material).

To further analyze the modulatory role of circulatory estrogen and progesterone on NETosis during pregnancy, we added specific inhibitors of the estrogen and progesterone receptors, fulvestrant and mifepristone (RU486), respectively, to pregnancy plasma samples and assayed NET formation (Figure 3G; Figures S3D,E in Supplementary Material). NETosis was reduced by the estrogen antagonist fulvestrant (Figure 3G; Figures S3D,E in Supplementary Material). This effect is presumably due to the unopposed action of progesterone, since gestational plasma

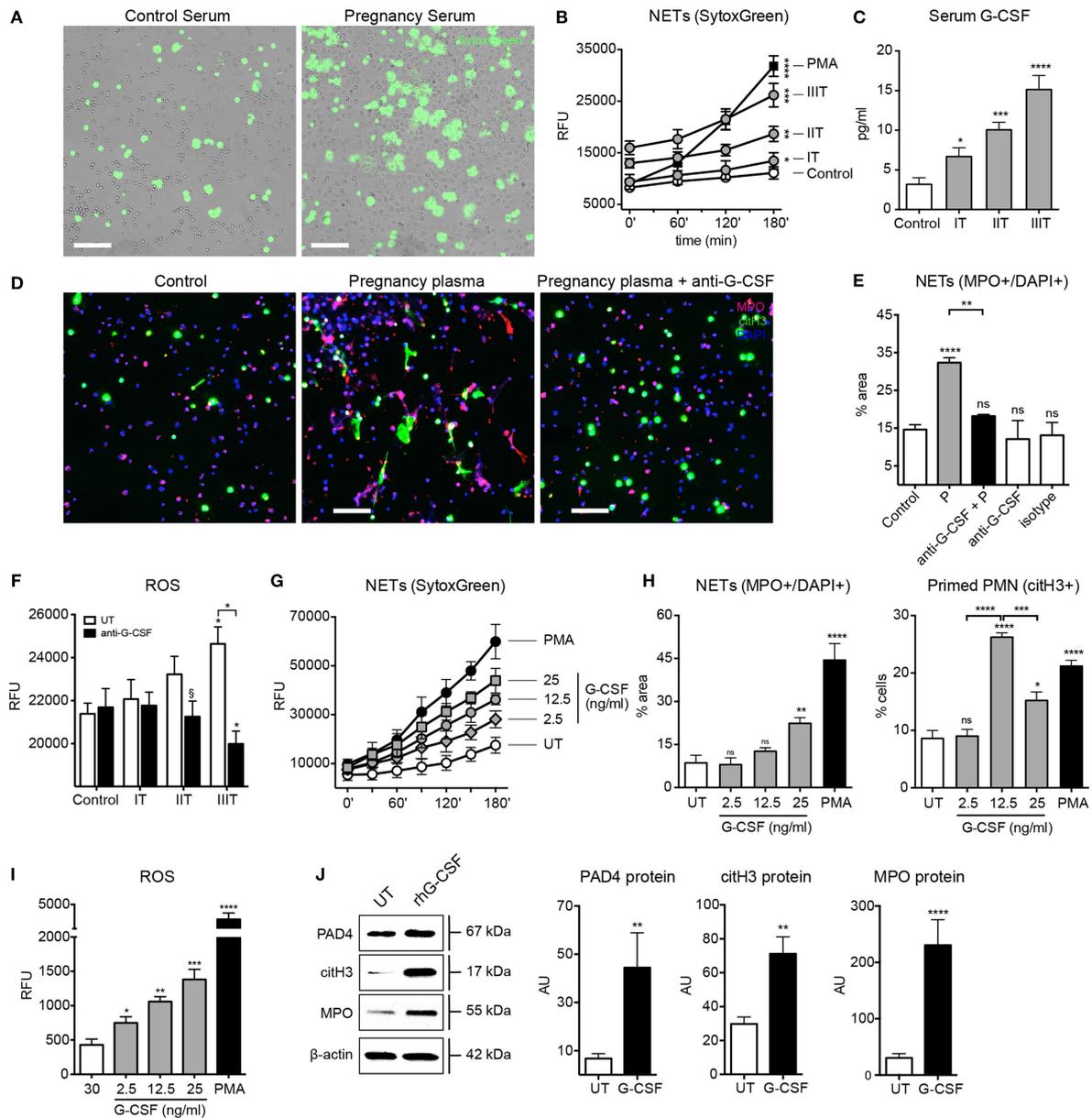
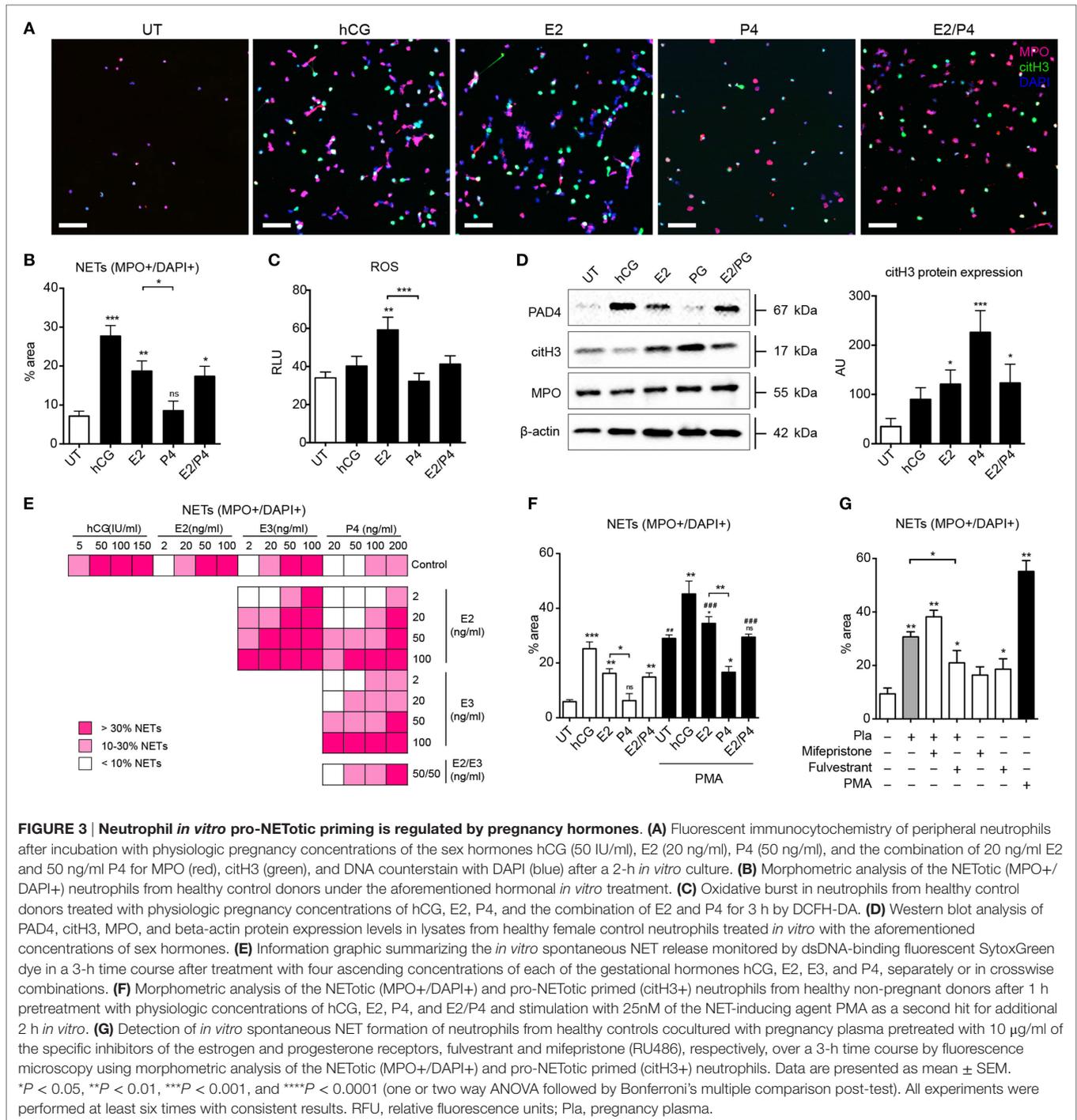


FIGURE 2 | G-CSF leads to neutrophil pro-NETotic priming during the course of pregnancy. (A) *In vitro* spontaneous extracellular DNA release monitored over a 3-h time course by SytoxGreen dye fluorescence microscopy after treatment of control neutrophils with pooled sera from women during their pregnancy. Scale bars: 100 μ m. (B) Fluorimetric quantification of extracellular DNA release from neutrophils treated *in vitro* with serum from the three trimesters of pregnancy using dsDNA-binding SytoxGreen dye compared to the NET-inducing phorbol ester PMA (25nM). (C) Detection of G-CSF levels in sera from donors during the three trimesters of pregnancy by EIA. (D) Fluorescent immunostaining for MPO (red), citH3 (green), and DNA (blue) depicting the formation of NETs after *in vitro* treatment of control neutrophils with 6% pooled plasma from women during their pregnancy and 30 min pretreatment of pregnancy plasma with 50 ng/ml anti-G-CSF neutralizing antibody compared to plasma from control non-pregnant individuals. Scale bars: 50 μ m. (E) Quantitative morphometric analysis of the NETotic (MPO+/DNA+) positive and pro-NETotic primed (citH3+) neutrophils from healthy non-pregnant donors under the aforementioned experimental setup. (F) Intracellular ROS generation from neutrophils cocultured *in vitro* with 6% plasma derived from donors during the three trimesters of pregnancy and 50 ng/ml anti-G-CSF neutralizing antibody monitored with DCFH-DA. (G) Evaluation of rhG-CSF activity by *in vitro* titration experiments using fluorimetric analysis of control neutrophils after *in vitro* incubation with 2.5, 12.5, and 25 ng/ml rhG-CSF compared to untreated (UT) and treated with PMA (25nM) neutrophils. (H) Morphometric analysis of the NETotic (MPO+/DNA+) and pro-NETotic primed (citH3+) neutrophils from healthy control donors under rhG-CSF treatment *in vitro* at the indicated concentrations compared to untreated (UT) and PMA-treated neutrophils. (I) Oxidative burst in neutrophils from healthy control donors treated *in vitro* with rhG-CSF at the indicated concentrations monitored by DCFH-DA detection and compared to untreated (UT) and PMA-treated neutrophils. (J) Western blot and densitometric analysis of PAD4, citH3, MPO, and beta-actin protein expression in neutrophil lysates from healthy female controls treated *in vitro* with the physiological concentrations of rhG-CSF (12.5 ng/ml). Data are presented as mean \pm SEM. * P < 0.05, ** P < 0.01, *** P < 0.001, and **** P < 0.0001 (one or two way ANOVA followed by Bonferroni's multiple comparison post-test). All experiments were performed at least six times with consistent results. IT, first trimester; IIT, second trimester; IIIT, third trimester; RFU, relative fluorescence units.



samples contain an assortment of sex hormones. On the other hand, in samples treated with mifepristone, thereby inhibiting progesterone, NETosis was significantly increased. In this instance, the effect would be attributable to unopposed estrogen in the plasma sample.

These results show that NET formation is tightly regulated by the gestational hormonal milieu.

Progesterone Confines NETosis of Neutrophils at an Advanced State of Priming

During our experiments utilizing the blocking anti-G-CSF antibody in maternal plasma, we noted that such treatment not only led to a substantial reduction in NET formation (Figure 2E) but also to a significant increase in the number of primed citH3+ cells (Figure 4A). Since the anti-NETotic effect of G-CSF was

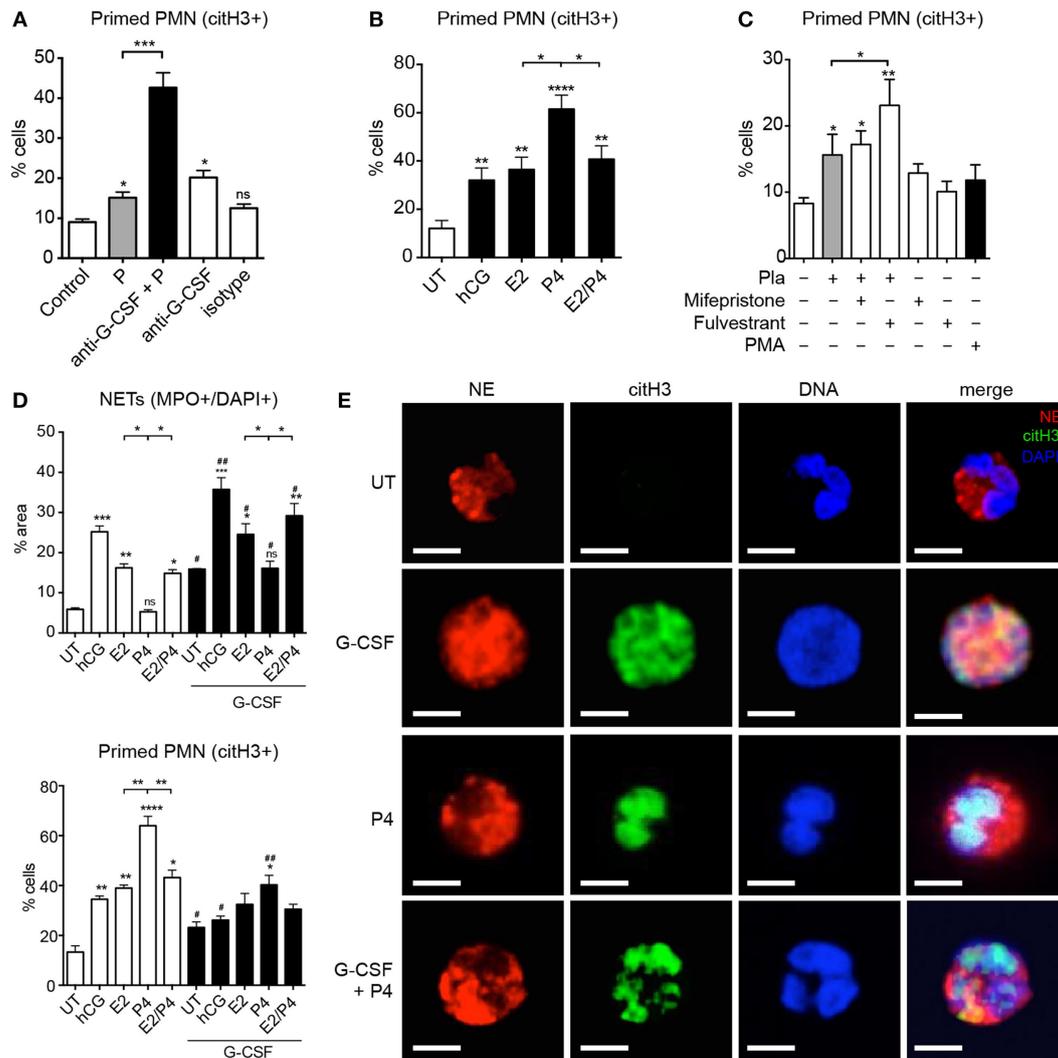
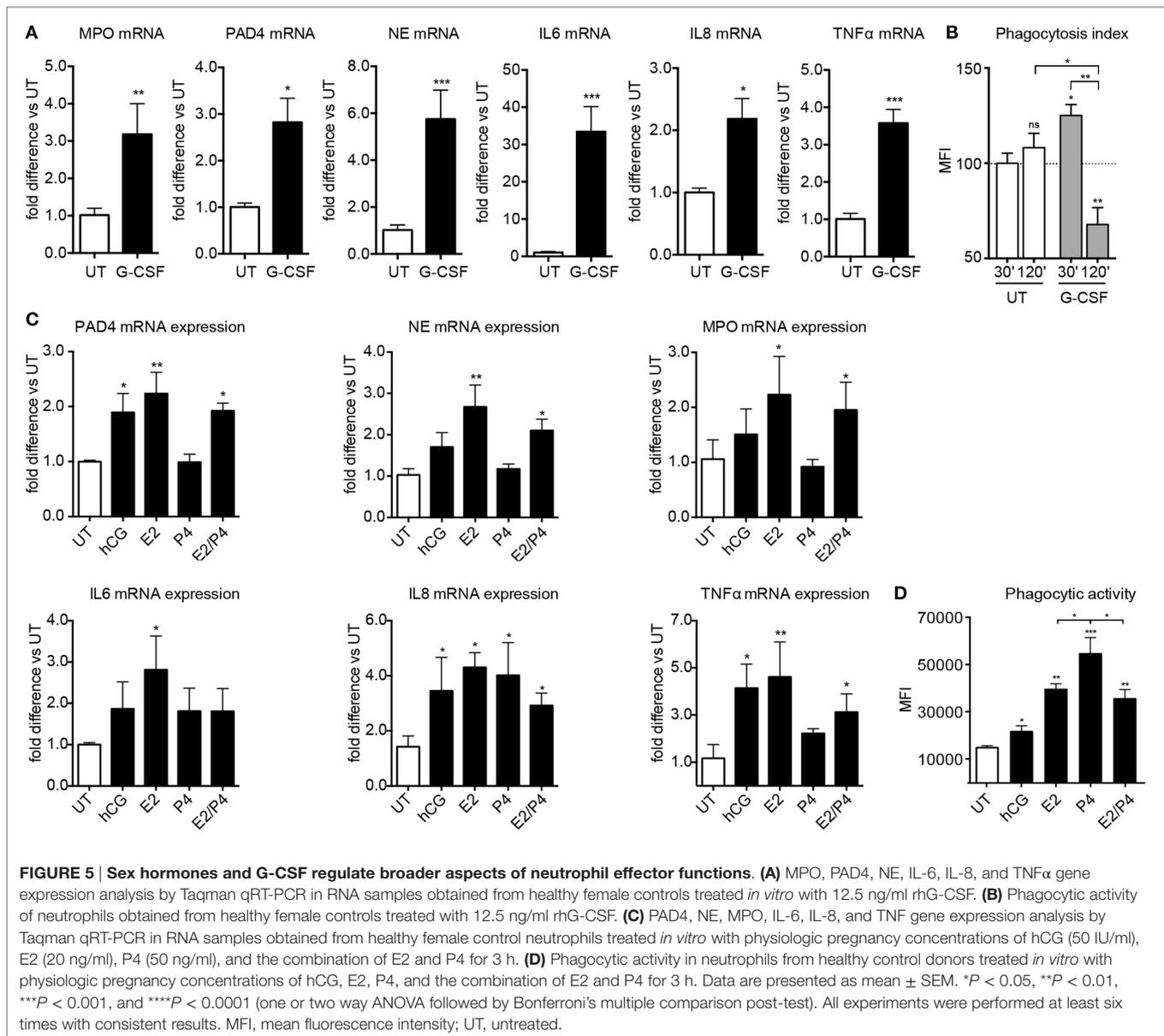


FIGURE 4 | Sex hormones differentially modulate G-CSF-driven neutrophil pro-NETotic priming. (A) Morphometric analysis of the pro-NETotic primed (citH3+) neutrophils from healthy non-pregnant donors after *in vitro* incubation of control neutrophils with pooled pregnancy plasma with and without *in vitro* pretreatment with anti-G-CSF neutralizing antibody compared to the priming activity of control plasma. (B) Morphometric analysis of the pro-NETotic primed (citH3+) neutrophils from healthy non-pregnant donors after *in vitro* incubation of control neutrophils with physiologic pregnancy concentrations of hCG (50 IU/ml), E2 (20 ng/ml), P4 (50 ng/ml), and the combination of E2 and P4 for 3 h. (C) Morphometric analysis of the pro-NETotic primed citH3+ neutrophils from healthy non-pregnant donors under *in vitro* hormonal treatment and after *in vitro* preincubation of control neutrophils with the sex hormone inhibitors mifepristone and fulvestrant. (D) Morphometric analysis of the NETotic (MPO+/DAPI+) and pro-NETotic primed (citH3+) neutrophils from healthy non-pregnant donors without and with pretreatment with 12.5 ng/ml rhG-CSF for 1 h (lower panel) and stimulation with the gestational hormones hCG, E2, P4, and the combination of E2 and P4 for additional 2 h *in vitro*. (E) Fluorescent immunostaining and confocal microscopy for NE (red), citH3 (green), and DNA (blue) after a 3 h *in vitro* coculture of control neutrophils with rhG-CSF and addition of P4 compared to untreated control neutrophils. Scale bars: 10 μ m. Data are presented as mean \pm SEM. * P < 0.05, ** P < 0.01, *** P < 0.001, and **** P < 0.0001 (one or two way ANOVA followed by Bonferroni's multiple comparison post-test). All experiments were performed at least three times with consistent results.

greatest in samples collected close to term (Figure 2F), we speculated that this could be modulated by the pronounced anti-NETotic activity of progesterone (Figures 3A,B), concentrations of which are at their peak late in pregnancy (37). We indeed determined that the anti-NETotic effect of progesterone was associated with an increase in primed neutrophils, as identified by citH3 staining in the nucleus (Figure 4B). When neutrophils were exposed to gestational plasma samples treated with the

estrogen antagonist fulvestrant (Figure 3G; Figures S3D,E in Supplementary Material), this was also evident.

Under physiological conditions, the combined action of G-CSF and estrogen appeared to limit the anti-NETotic effect of progesterone (Figure 4D; Figures S4A–C in Supplementary Material), while in the absence of estrogen, progesterone effectively limited NET formation initiated by G-CSF (Figure 4E). Once again, this anti-NETotic effect was coupled with a pronounced



elevation in the number of primed neutrophils, as detected by citH3 staining (Figures 4C,D; Figure S4 in Supplementary Material).

Although the anti-NETotic action of progesterone appeared to involve reduced ROS production (Figure 3C), this mechanism in itself could not explain the high proportion of primed neutrophils characterized by citH3 staining (Figure 3A) or protein levels (Figure 3D). Rather, these data indicated that progesterone-treated neutrophils had undergone activation, involving nuclear translocation of PAD4 and histone citrullination, and yet were unable to proceed with NETosis.

Previous studies have indicated that cleavage of linker histones by NE present in the nucleus is required for the unfolding of chromatin and subsequent NETosis (18). Upon examining this facet by confocal microscopy, we noted diffuse nuclear staining for NE

in cells treated with G-CSF (Figure 4E). This was in stark contrast to progesterone-treated cells, where nuclear translocation of NE did not occur (Figure 4E). Though these cells were citH3+, they also retained a distinctly lobulated nuclear structure, unlike the G-CSF treated cells, in which the chromatin had decondensed and dispersed into the cytoplasm.

Further Aspects of Neutrophil Activity Modulated by Sex Hormones and G-CSF

In agreement with previous reports, we observed that pregnancy was associated not only with the upregulation of NET related molecules, such as PAD4, MPO, and NE (Figure S5A in Supplementary Material), but also with an increased predisposition to phagocytosis (Figure S5B in Supplementary Material) and degranulation (Figure S5D in Supplementary Material). In

parallel with the results for NETosis, both became more pronounced with advancing gestation. Accordingly, we studied the influence of G-CSF, estrogen, and progesterone on these aspects of neutrophil activity. Apart from promoting NETosis, G-CSF enhanced the expression of the granular proteins MPO and NE (Figure 5A) and promoted phagocytosis (Figure 5B). G-CSF also enhanced the expression of pro-inflammatory cytokines such as IL-6, IL-8, and TNF- α by neutrophils (Figure 5A).

The effect of estrogen and progesterone was more differentiated, with the former significantly enhancing the expression of MPO and NE, as well as that of IL-6 and TNF- α , in contrast to the rather dampened effect mediated by the latter (Figure 5C). Interestingly, both estrogen and progesterone trigger enhanced IL-8 expression, an important chemokine for neutrophil recruitment, degranulation, and NETosis (10, 39). This finding is important, since neutrophil expression of IL-8 progressively increases with advancing gestational age (Figure S5C in Supplementary Material), thereby indicating an important role of this pivotal cytokine in regulating neutrophil activity in pregnancy. In general, the influence of hCG was similar to that of estrogen, albeit to a lower level (Figures 5C,D). Furthermore, phagocytic activity was significantly more enhanced by progesterone treatment than by estrogen alone or by the combination of both (Figure 5D). These findings suggest that by blocking NETosis, progesterone may shift neutrophil antimicrobial activity toward other pathways, such as phagocytosis.

DISCUSSION

A crucial contribution to our understanding of PE, a highly inflammatory condition during gestation, was the seminal observation that it involved an excessive maternal inflammatory response to pregnancy (4). This was based on the finding that human pregnancy is associated with a subliminal inflammatory condition, characterized by enhanced neutrophil activation, which is most extreme in PE (3, 40, 41). The possible involvement of overtly activated neutrophils in the etiology of PE was further supported by the detection of large numbers of NETs in the intervillous space of affected placentae (10). Furthermore, data from animal models suggested that tissue-resident proangiogenic decidual neutrophils (42) and activation of circulatory neutrophils via the complement cascade may contribute to PE-like conditions or those associated with fetal loss (43, 44). The latter is supported by recent reports that antiphospholipid antibodies, which are frequently detected in cases with recurrent fetal loss, trigger NETosis [reviewed in Ref. (7)].

Unfortunately, the prominent focus on the role of neutrophils in pathological conditions has yielded few advances in the knowledge of their behavior during normal pregnancy (7, 12). As an understanding of the NETotic response in normal pregnancy could provide better insight into pathological subversion, it was the focus of our current study. Our data indicate that (i) circulatory neutrophils from pregnant women exhibit an enhanced pro-NETotic response, which increases with advancing gestation; (ii) pro-NETotic priming is mediated largely by G-CSF; (iii) NETosis is modulated in an opposing fashion by estrogen and progesterone; and (iv) in that progesterone locks neutrophils in

a highly pro-NETotic state by hindering NE migration to the nucleus. Since neutrophil NETs were originally described as an antimicrobial mechanism (39), our data suggest that this operative arm of the innate immune response is highly proactive in human pregnancy. In this manner, by being in a highly primed pro-NETotic state, such pre-activated neutrophils could react immediately to a threat by pathogens.

Early in gestation, enhanced NETosis seems to be largely driven by hCG, which plays a pivotal role during this phase of pregnancy, with a minimal contribution by G-CSF. Interestingly, *in vitro* application of hCG leads to a significant increase in PAD4 expression, without a major increment in citH3 levels. Furthermore, ROS production, a further key element in the NETotic cascade, was not significantly elevated by hCG treatment, underlining a possible ROS-independent mechanism driving the generation of NETs as described recently (45). As gestation advances, it becomes apparent that a discrete interaction between G-CSF and steroid sex hormones modulates pro-NETotic activity. In this regard, elevated concentrations of G-CSF during pregnancy not only serve to increase the pool of circulatory neutrophils but also to promote a primed pro-NETotic phenotype. This is achieved by triggering increased PAD4 expression and concomitant histone H3 citrullination, essential steps in the NETotic signaling cascade (21, 22).

The most intriguing aspect of our analysis was the antagonistic interplay between estrogen and progesterone in regulating NETosis. While a host of literature supports the contrary influence of progesterone on estrogen-mediated effects (46), our observation is novel in that progesterone appears to exploit key signaling events to prevent the extrusion of NETs. Previous studies have shown that the triggering of NET formation requires several key steps, including ROS production, calcium mobilization, and activation of PKC and MAP kinases. On the other hand, untangling of chromatin, an *a priori* requirement for decondensation of the nuclear chromosomal DNA and its subsequent expulsion into the extracellular milieu, has been shown to involve citrullination of histones by PAD4 (21, 22), as well as the proteolytic clipping of histones (notably H4) by NE (18). Upon activation of neutrophils, ROS production leads to the release of NE from azurophilic granules into the cytosol. It then translocates to the nucleus, where it cleaves histones to extensively decondense chromatin (18). MPO, also originating from the azurophilic granules, consumes H₂O₂ to generate HOCl and other oxidants and is required for translocation of NE to the nucleus during NETosis by regulating actin dynamics (18, 23). Additionally, phagocytosis seems to negatively regulate NETosis (23). Our data indicate that progesterone downregulates ROS production in neutrophils, which is in concordance to previous studies showing that progesterone is effective in reducing ROS and NO generation driven by estradiol (47, 48). The observed inhibition of ROS production occurs most probably in favor of a significant shift towards the involvement of calcium signaling and PAD4 mobilization, which most probably promotes phagocytosis rather than degranulation or formation of NETs (20). The latter might lead to the excessive histone citrullination detected both *in vitro* and *ex vivo*, needed for the formation of NETs under certain stimuli, bringing NE and MPO into the nucleus. This translocation signifies the crucial event leading to the extrusion of NETs from the activated neutrophils.

In pregnancy, the antagonistic effect of progesterone is manifested by hindering translocation of NE to nucleus, thereby preventing terminal nuclear decondensation and disruption of the nuclear membrane, with subsequent intermingling between nuclear and cytoplasmic contents. Progesterone seems to permit histone citrullination by PAD4, thus effectively locking neutrophils in a highly primed pro-NETotic state, till triggered by a second stimulus that overcomes this transitory impediment.

Our data also extend upon previous reports indicating that circulatory neutrophils exhibit an increased propensity for degranulation and phagocytosis during pregnancy (4, 9), shedding new light on their regulation by the action of G-CSF and sex hormones. In this regard, phagocytosis is enhanced by treatment with G-CSF. However, this is most pronounced in short-term cultures of 30 min, as it is drastically diminished after 2 h. In the instance of the hormones, the enhancement of phagocytosis is slower than that for G-CSF, requiring an incubation period of 2 h for maximal effect. Of interest is that phagocytosis was most markedly affected by progesterone, while it is reduced when using estrogen in combination with progesterone under physiological concentrations. This suggests that progesterone may promote antibacterial activity involving phagocytosis rather than NETosis, while in combination with estrogen, this effect is diminished. It is also clear that the interaction between G-CSF and sex hormones influences a broader scope of neutrophil activity, including

expression of key components of the NETotic cascade, such as PAD4, NE, and MPO, as well as pro-inflammatory cytokines and chemokines, such as TNF- α and IL-8. The interplay between these diverse facets may be important in ensuring that neutrophils contribute to an effective innate immune response during pregnancy.

In the same token, it is feasible that aberrances in this system may contribute to the pathogenesis of severe pregnancy-related conditions such as PE. This hypothesis is supported by observations that PE is associated with elevated levels of hCG (49), G-CSF (50), as well as indications of reduced progesterone levels (51), while therapy with synthetic P4 ameliorates PE symptoms in an animal model (52). It is therefore possible that such an imbalance may trigger an enhanced pro-NETotic response, which is exacerbated by the presence of inflammatory placental micro-debris (4), abundant in this condition. On the other hand, our data may provide a novel insight into autoimmune conditions such as SLE, which is associated with reduced levels of progesterone, both during the menstrual cycle, as well as during pregnancy (53). Since NETosis is altered in cases with SLE (54), it is possible that the negative feedback loop hindering NET formation provided by progesterone contributes to this phenomenon. As estrogen levels are normal in these patients, the altered ratio between the two sex steroid hormones could lead to a more facile triggering of the NETotic cascade. It is, hence, possible that this phenomenon

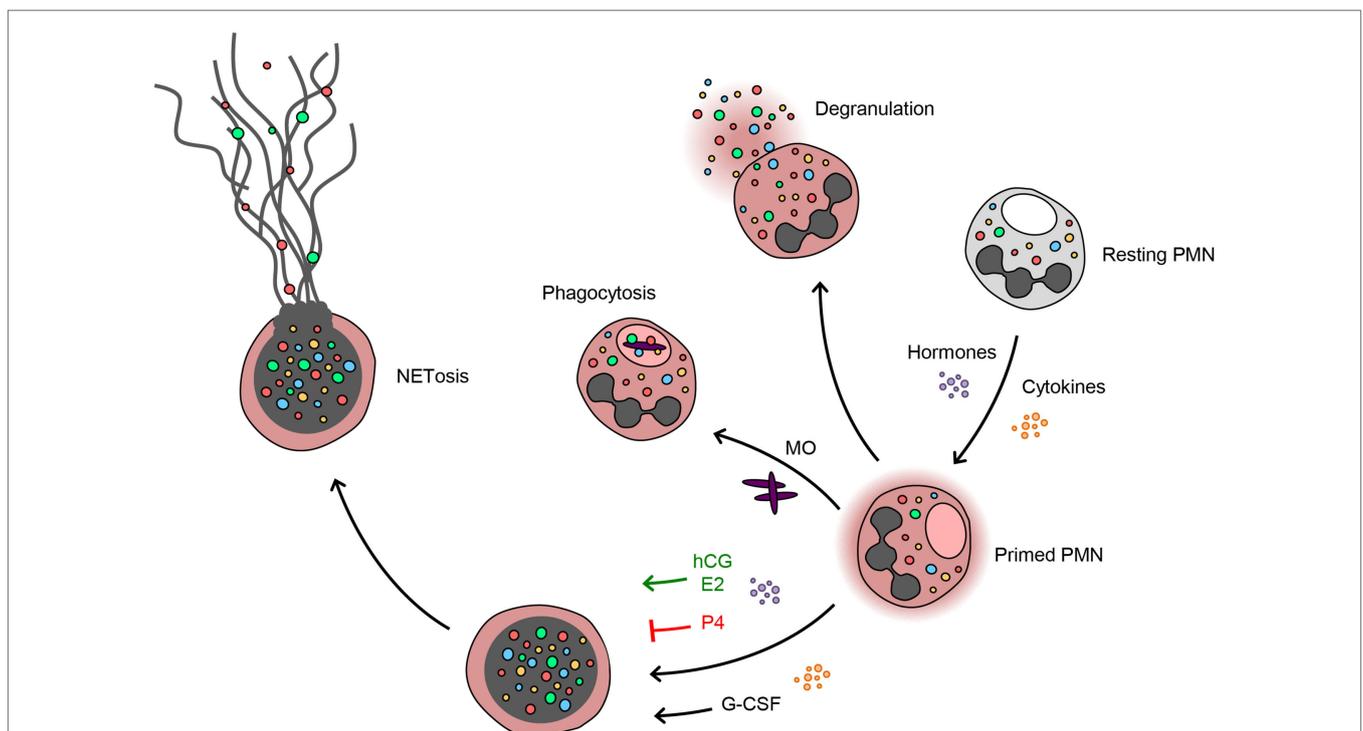


FIGURE 6 | Progesterone antagonizes the estrogen and G-CSF-driven neutrophil extracellular trap formation during pregnancy. Neutrophils during pregnancy lie under the increased influence of cytokines, e.g., G-CSF, and sex hormones. This specific milieu appears to poise the neutrophils in a pro-NETotic primed state. Depending on the stimulus, neutrophils react by phagocytosis or degranulation. When a different NETotic stimulus is present, such as systemic infection or excessive placentally derived plasma microparticles (MP) in preeclampsia (10), primed neutrophils react with overt NET release. Pro-NETotic combinations of hormones and cytokines are given in green, inhibitory combinations are given in red.

may contribute to the PE-like symptoms frequently observed in pregnant women affected by SLE.

In summary, the present study demonstrates that during pregnancy neutrophils exhibit a pro-NETotic primed state and enhanced propensity to release NETs. This activity is modulated at several key levels. First, G-CSF seems to be a major signal for neutrophil release from the bone marrow throughout gestation, providing an important mechanism increasing the levels of circulating neutrophils and promoting pro-NETotic priming. Second, the degree of NET formation seems to be finely tuned by the balance of the sex steroid hormones, which are produced exclusively by the placenta and reach their peak concentrations toward term. Finally, NETs are released under the presence of specific gestational secondary stimuli (**Figure 6**).

Our findings regarding neutrophil responses during normal pregnancy provide new insight concerning gestational and hormone-driven pathologies, since neutrophil recruitment, activation, and NET release could be associated with excessive endothelial and placental injury. Based on these premises, we suggest that the proportions of the sex steroid hormones unique to each individual condition, physiologic or pathologic, tune the innate immune system and neutrophils in particular into a specific functional mode of action. When this balance is lost or absent, neutrophils react with overt NET formation, which in turn leads to tissue injury. With regard to pregnancy, it is plausible that in extreme conditions of NETosis, such as shown previously in PE, these diverse features might become detrimental and promote a strong procoagulant state, possibly resulting in placental infarction.

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AUTHOR CONTRIBUTIONS

SG and MS performed all experiments, except as noted below; CC performed part of the ELISA and immunoblot analysis; GS performed part of the cell isolations and IHC stainings; FG performed part of the cell isolations; IH and OL provided advice for and contributed to the recruitment of the sample donors; SG and SH devised and directed the study; SR provided advice and contributed to the revision of the manuscript; and SG, PH, and SH wrote the manuscript.

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Ménage-à-Trois: The Ratio of Bicarbonate to CO₂ and the pH Regulate the Capacity of Neutrophils to Form NETs

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In this study, we identified and characterized the potential of a high ratio of bicarbonate to CO₂ and a moderately alkaline pH to render neutrophils prone to undergo neutrophil extracellular trap (NET) formation. Both experimental settings increased the rate of spontaneous NET release and potentiated the NET-inducing capacity of phorbol esters (phorbol-2-myristate-13-acetate), ionomycin, monosodium urate, and LPS. In contrast, an acidic environment impaired NET formation both spontaneous and induced. Our findings indicate that intracellular alkalinization of neutrophils in response to an alkaline environment leads to an increase of intracellular calcium and neutrophil activation. We further found that the anion channel blocker DIDS strongly reduced NET formation induced by bicarbonate. This finding suggests that the effects observed are due to a molecular program that renders neutrophils susceptible to NET formation. Inflammatory foci may be characterized by an acidic environment. Our data indicate that NET formation is favored by the higher pH at the border regions of inflamed areas. Moreover, our findings highlight the necessity for strict pH control during assays of NET formation.

Keywords: NET, pH, inflammation, bicarbonate, CO₂, neutrophils, neutrophil extracellular traps, calcium

INTRODUCTION

Neutrophils are the most abundant leukocyte subset in the human blood and constitute the first line of defense during infection (1, 2). A central effector function of neutrophils involves the release of decondensed chromatin decorated with cytoplasmic and granular proteins (3, 4). Since these structures may trap and degrade pathogens extracellularly inside their meshwork, they are referred to as neutrophil extracellular traps (NETs) and the accompanying process is termed NET formation (3). A variety of stimuli have been reported to induce formation of NETs, among them are bacteria, fungi, and microbial products (3, 5, 6). Other physiological stimuli include

monosodium urate, immune complexes, apoptotic cells, or integrin-mediated signals at high cellular density (7–10). In experimental settings, chemicals with defined mechanisms of action such as phorbol-2-myristate-13-acetate (PMA) or ionomycin also induce NET formation (4). We have recently reported that occlusion of the pancreatic ducts by aggregated NETs is a driving factor of pancreatitis (11). In this study, we identified bicarbonate present in the pancreatic juice as a potent inducer of NET formation. In this manuscript, we aim to characterize more closely the influence of the triangular relationship of bicarbonate, CO₂, and pH on NET formation. We observed that both a low pH and a high CO₂ to bicarbonate ratio decrease the capacity of neutrophils to release NETs. Inflammatory foci may be characterized by an acidic microenvironment. Our data indicate that NET formation is favored at the border regions of inflamed areas and the beginning of inflammation. Furthermore, NET release may be favored by the restitution of physiological pH in ischemia–reperfusion situations. These observations will impact the understanding of multiple inflammatory diseases.

MATERIALS AND METHODS

Chemicals

4,4'-Diisothiocyanatostilbene-2,2'-disulfonic acid disodium salt hydrate (DIDS) and PI were from Sigma (Crailsheim, Germany). Hoechst 33342 was obtained from Thermo Fisher Scientific (Frankfurt, Germany).

Isolation of Polymorphonuclear Leukocytes

All analyses of human material were performed in accordance to the institutional guidelines and with the approval of the ethical committee of the University Hospital Erlangen (permit # 193 13B). Written informed consent was given by each donor. Twenty milliliter of heparinized blood (20 U/ml) were taken from each normal healthy donor. Fifteen milliliter of PBS without calcium and magnesium (Thermo Fisher Scientific) were added and the suspension was gently applied on top of 15 ml of Ficoll (Bio-Rad, Dreieich, Germany). Cells were centrifuged at 1,400 rpm for 30 min at room temperature with lowest acceleration. Centrifuge was allowed to spin out without break. Suspension above the buffy coat was removed, and the white layer containing the PMNs on the top of the red blood cells was collected. To remove contaminating erythrocytes, PMNs were subjected to short cycles of hypotonic lysis with deionized water. Normal osmolality was restituted after 30 s with 10× PBS. PMNs with purity higher than 95% were adjusted to a concentration of 2×10^6 cells/ml in PBS without calcium and magnesium (Thermo Fisher Scientific) and stored at room temperature until further use.

Buffers and Culture Conditions

Isolated neutrophils were adjusted to a concentration of 6×10^6 cells/ml in Ringer's solution (Deltaselect, Pfullingen, Germany). Twenty-five microliter of these solutions were added to 175 µl of indicated medium containing 2.5 µM Sytox Green

(Thermo Fisher Scientific) with or without 10 ng/ml PMA (Sigma, Darmstadt, Germany), 1 µg/ml ionomycin (Sigma), 300 pg/cell monosodium urate crystals, or 2.5 µg/ml LPS from *Klebsiella pneumoniae* (L4268, Sigma) or *Salmonella enterica* serotype enteritidis (L6143, Sigma), respectively. Assays were performed either in 96-well cell plates (Greiner Bio-One, Frickenhausen, Germany) or 8-well Nunc chamber slides (VWR, Darmstadt, Germany). Plates and chamber slides were preincubated at 37°C and respective concentrations of CO₂ at least 30 min prior to addition of 25 µl of cells in Ringer.

Platereader-Based Quantification of NET Formation

Plates containing PMN cultures were analyzed under the conditions described above for 4 h on an infinite[®] 200 pro plate reader (TECAN, Crailsheim, Germany). Excitation was performed at 485 nm and emission was detected at 535 nm. Relative fluorescence units were calculated as the 100-fold ratio of the fluorescence at the indicated time point and time point $t = 0$ min.

Immunohistochemistry

After addition of the cells, the chamber slides were incubated under these conditions for 3 h. Subsequently, 1% paraformaldehyde (Merck, Darmstadt, Germany) in PBS (Thermo Fisher Scientific) was added to each well and the preparations were incubated for 18 h at 4°C. Samples were blocked with 10% FCS (Biochrome, Berlin, Germany) in PBS (Thermo Fisher Scientific) for 1 h at room temperature. Cells were permeabilized with 0.1% Triton X-100 in water for 10 min. Primary antibody for neutrophil elastase (NE) (Abcam, United Kingdom, ab21595) 1:200 or citrullinated histone H3 (citH3) (Abcam, ab5103) 1:200 were added in 10% FCS in PBS for 18 h at 4°C. Slides were washed three times with PBS, and secondary anti-rabbit IgG antibody conjugated with Cy[®]5 (Jackson ImmunoResearch, Suffolk, United Kingdom, 111-175-144) 1:400 was added for 1.5 h at room temperature in the dark. Slides were washed with PBS. Staining solution containing 2.5 µM Sytox Green in PBS was added for 15 min at room temperature. Slides were washed with H₂O and samples were embedded in DAKO fluorescent mounting medium (BIOZOL, Eching, Germany). Slides were analyzed on a BZ-X710 microscope (Keyence, Neu-Isenburg, Germany). Maximum intensity projection of Z-stacks and gamma correction were performed to increase depth of field and to allow proper display of NETs and nuclei on these images. Post-processing of pictures was performed with Photoshop CS5 (Adobe, München, Germany). Images were not used for quantification.

Live Cell Imaging

Chamber slides containing PMN cultures under the conditions described above were analyzed on a BZ-X710 microscope (Keyence, Neu-Isenburg, Germany) or an Axio Observer.Z1 microscope (Zeiss, Oberkochen, Germany) using a time-lapsed shooting sequence. Maximum intensity projection of Z-stacks and gamma correction were performed to increase depth of field and to allow proper display of NETs and nuclei on the same

image, respectively. Post-processing of pictures was performed with Photoshop CS5 (Adobe, München, Germany) and ZEN pro 2012 (Zeiss).

Intracellular Calcium Measurement

Isolated neutrophils were suspended at a concentration of 10×10^6 cells/ml in PBS without calcium and magnesium and loaded with 3 μ M Fluo-3 AM (Thermo Fisher Scientific) and 6 μ M Fura-red AM (Thermo Fisher Scientific). Cells were incubated for 20 min at room temperature and followed by incubation at 37°C for 10 min. Cells were washed twice with PBS without calcium and magnesium and suspended in same medium at final concentration of 10×10^6 cells/ml. Fifty microliter of cell suspension was added to 450 μ l of PBS with 0.4 mM CaCl_2 and measured for 1 min by flow cytometry. Then, 2.5 ml of respective medium with calcium was added, which was preincubated at 37°C and 5% CO_2 and fluorescence was measured for 15 min. To study the effect of extracellular acidification/alkalinization on calcium mobilization, PMN loaded with Fluo-3 AM and Fura-red AM was measured by flow cytometry in respective preincubated medium for 1 min. Then predetermined amount of HCl or NaOH was added to achieve respective extracellular pH followed by measurement for 15 min. Beckman Coulter's Epics XL-MCL™ and software Kaluza 1.5 (Beckmann Coulter) were used for measurement and analysis, respectively. Original data file of cell events was divided in time-based gates and ratio-metric fluorescence FL1–FL3 was used to determine intracellular calcium levels. The radiometric calcium levels were normalized to first time point level.

Measurement of Intracellular pH

Isolated neutrophils (10×10^6 cells/ml in PBS without calcium and magnesium) were loaded with 10 μ M carboxy-SNARF-1-AM (Thermo Fisher Scientific) and incubated at room temperature for 20 min followed by incubation at 37°C for 10 min. Cells were washed twice with PBS without calcium and magnesium and suspended in same buffer at 10×10^6 cells/ml. Intracellular pH of neutrophils with different concentrations of bicarbonate in RPMI was recorded using Gallios Flow Cytometer (Beckman Coulter, USA). To determine the change in intracellular pH in response to extracellular pH of the medium, 100 μ l of the cell suspension was added to 2.9 ml of respective medium preincubated at 37°C and 5% CO_2 and fluorescence was recorded for 1 min using Gallios Flow Cytometer (Beckman Coulter, USA) and respective extracellular pH was attained using predetermined volume of HCl and NaOH followed by flow cytometry measurement for 15 min. The change in intracellular pH was determined by ratio of FL6–FL2 in Beckman Coulter analysis software Kaluza 1.5.

Data Presentation and Statistical Analysis

Results are displayed as means \pm SEM of the indicated number of biological replicates. If not indicated otherwise, an analysis of variance was used for statistical analysis. In case of multiple comparisons, Tukey's correction was performed. Statistical significance is indicated with *, **, *** and ****. The respective confidential intervals are $p < 0.05$, $p < 0.01$, $p < 0.001$, and

$p < 0.0001$. Statistical analysis was performed with the software GraphPad Prism 6.0 (GraphPad Software, USA).

RESULTS

Freshly isolated PMNs were cultured in HBSS containing various amounts of bicarbonate. Quantification of DNA release in a fluorescence-based assay revealed that bicarbonate time- and dose-dependently induces an increase in Sytox Green signal (Figure 1A). Immunocytochemistry revealed that increasing amounts of bicarbonate induced the formation of thread-like DNA structures positive for NE (Figure 1B; Figure S1 in Supplementary Material). Live cell imaging further revealed that excessive bicarbonate induces chromatin externalization from neutrophils (Video S1 in Supplementary Material). Together, these data indicate that bicarbonate is a potent determinant of whether culture media induce NET formation. Since the ratio of bicarbonate to CO_2 influences the pH of the medium, we analyzed the impact of the extracellular pH on formation of NETs. Therefore, the medium was supplemented with 5% CO_2 . Indeed, we observed an ameliorative effect of CO_2 supplementation, indicating an important role of the pH in bicarbonate-induced NET release (Figure 1C; Figure S2 in Supplementary Material).

Many processes during NET formation, such as citrullination of histone H3, are dependent on calcium. Elevated levels of calcium are likely to render neutrophils more prone to release NETs. Intracellular alkalinization of neutrophils is reportedly accompanied by intracellular calcium increase. We observed that bicarbonate dose-dependently induces intracellular alkalinization and intracellular increase of calcium (Figure 2A). These effects were reduced when HBSS was supplemented with CO_2 , further highlighting the importance of the pH in bicarbonate-induced formation of NETs (Figure 2B).

We next analyzed whether pH-dependent effects are also observed independently of the bicarbonate/ CO_2 axis. Therefore, we deployed RPMI medium buffered with 20 mM HEPES. We tested a variety of pH values ranging from pH 6.6 to 7.8 and observed that with increasing alkalinity of the medium, more NET formation was observed as identified by immunocytochemistry and live cell imaging displaying the typical morphological characteristics of NETs (Figures 3A,B; Figure S3 in Supplementary Material; Video S2 in Supplementary Material). Importantly, Sytox Green binding to DNA was not influenced by the pH (Figure S4 in Supplementary Material). We further tested whether intracellular alkalinization and increase in Ca^{2+} concentration occur in the absence of bicarbonate and CO_2 . Via addition of hydrochloric acid and sodium hydroxide, the extracellular pH was adjusted to 6 and 7.8, respectively. Strikingly, such a manipulation was ineffective in influencing the intracellular pH (Figure 3C). In line with this, also the intracellular calcium concentration remained stable after acidification or alkalinization (Figure 3C).

So far, we had identified the bicarbonate/ CO_2 axis and the extracellular proton concentration as an important determinant of neutrophil behavior. These data highlight the necessity of strict CO_2 control in media containing bicarbonate (i.e., HBSS and RPMI). Per example, cultivation of neutrophils in regular RPMI containing 24 mM bicarbonate for 30 min in the absence

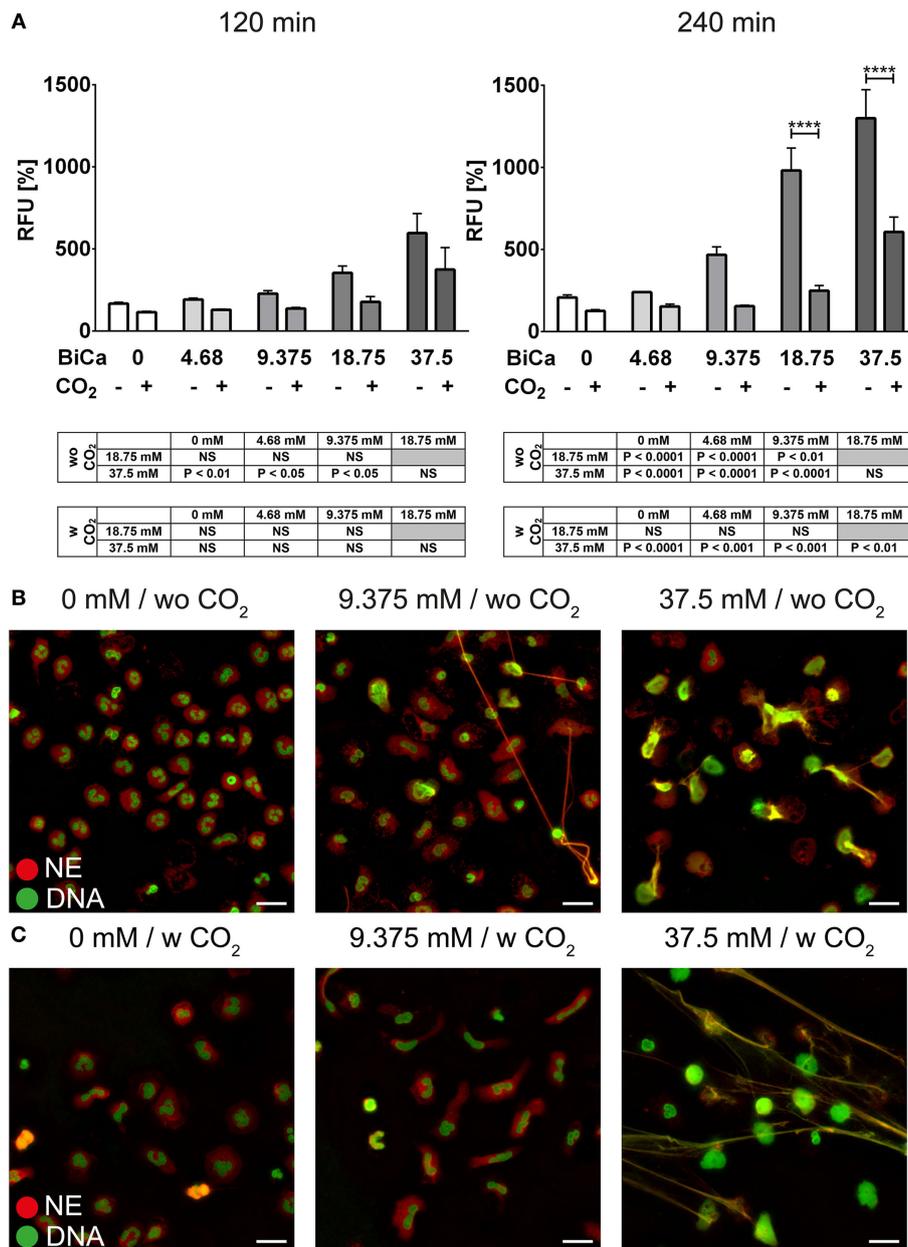


FIGURE 1 | Bicarbonate induces formation of neutrophil extracellular traps in HBSS. HBSS was supplemented with various concentrations of bicarbonate either in the absence (–) or presence (+) of 5% CO₂. **(A)** Fluorescence-based quantification of DNA externalization in response to various concentrations of bicarbonate after 120 min (left) and 240 min (right). *n* = 3–5. **(B)** Immunocytochemical analysis of neutrophils incubated in the presence of various concentrations of bicarbonate. Signal for DNA is depicted in green, and signal for neutrophil elastase (NE) is displayed in red. The scale bar represents 20 μm. **(C)** Immunocytochemical analysis of neutrophils incubated in the presence of various concentrations of bicarbonate in the presence of 5% CO₂. Signal for DNA is depicted in green, and signal for NE is displayed in red. The scale bar represents 20 μm.

of proper CO₂ control, induces robust formation of NET-like structures, which are not present if 5% CO₂ is supplied (Figure S5 in Supplementary Material).

We wondered whether the extracellular pH was also able to modify the response of neutrophils toward known inducers of NET release such as PMA and ionomycin. In order to study the modulation of the intracellular pH by the bicarbonate/CO₂ axis

under more physiologic conditions, we deployed RPMI buffered with various concentrations of bicarbonate under 5% CO₂ atmosphere. NET release in the absence of added inducers was increased at higher concentrations of bicarbonate (Figures 4A,B; Figure S6 in Supplementary Material; Video S3 in Supplementary Material) and we observed dose-dependent alkalization and intracellular calcium increase (Figure 4C). Importantly, sustained elevation

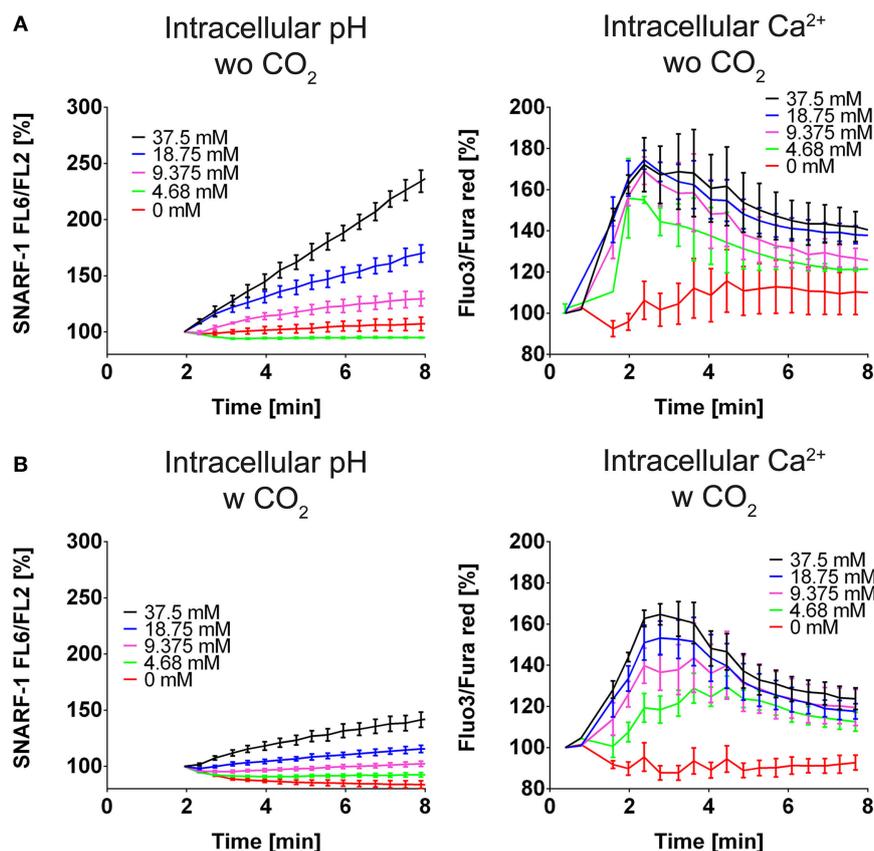


FIGURE 2 | A high ratio of bicarbonate to CO₂ induces cytosolic alkalinization and intracellular calcium increase in HBSS. HBSS was supplemented with various concentrations of bicarbonate either in the absence (A) or presence (B) of 5% CO₂. (A) Flow cytometric determination of intracellular pH (left) and cytosolic Ca²⁺ concentration (right) of neutrophils at various concentrations of bicarbonate. *n* = 3. (B) Flow cytometric determination of intracellular pH (left) and cytosolic Ca²⁺ concentration (right) of neutrophils at various concentrations of bicarbonate. *n* = 3.

of intracellular calcium was only observed after intracellular alkalinization, whereas a mild and transient increase was also observed in response to acidification (Figure 4C).

The bicarbonate/CO₂ axis and the pH were also able to modify the effect of PMA or ionomycin on neutrophils. At low concentrations of bicarbonate, NET formation induced by PMA (Figure 5A; Video S4 in Supplementary Material) or ionomycin (Figure 6A) was significantly reduced compared to 24 and 48 mM bicarbonate, respectively. Immunocytochemistry for NE and citH3 demonstrated increased amounts of NETs positive for NE and/or citH3 in the presence of bicarbonate. Using live cell imaging, we analyzed the morphological changes in neutrophils under these stimulatory conditions and observed the markedly increased chromatin externalization and decondensation in the presence of bicarbonate. Overall, these observations were in line with the results of the quantitative fluorescence-based assay (Figures 5B and 6B; Figures S7–S10 in Supplementary Material).

In order to further analyze the influence of bicarbonate in the media in response to potential physiological inducers of NET formation, we deployed lipopolysaccharides from *K. pneumoniae* and *S. enterica*, respectively (Figures 7A,B). LPS-stimulated NET

formation was reduced under conditions of relative hypercapnia as highlighted by fluorescence-based quantification of DNA externalization (Figure 7) and immunocytochemistry (Figures S11 and S12 in Supplementary Material). As reported by Pieterse and colleagues, LPS from *S. enterica* is a poor inducer of NET formation in the absence of platelets (12). Confirming this data, we observed only a twofold increase as compared to baseline NET formation at 24 mM bicarbonate/5% CO₂. Similarly, NET formation was also not pronounced at relative hypocapnia, further highlighting the necessity of platelets for the induction of NET formation by this particular subtype of LPS (12). In addition, we tested the influence of the bicarbonate to CO₂ ration on the NET-inducing potential of monosodium urate crystals (MSU). NET release in response to monosodium urate was decreased at a low bicarbonate to CO₂ ratio (Figures 8A,B; Video S6 in Supplementary Material). Taken together, our findings indicate an important role of the extracellular pH and the bicarbonate/CO₂ axis in the signal integration of NET formation.

We considered direct effects of the change in pH on phospholipid membrane integrity in neutrophils. Strikingly, the anion channel inhibitor 4,4'-diisothiocyanato-2,2'-stilbenedisulfonic

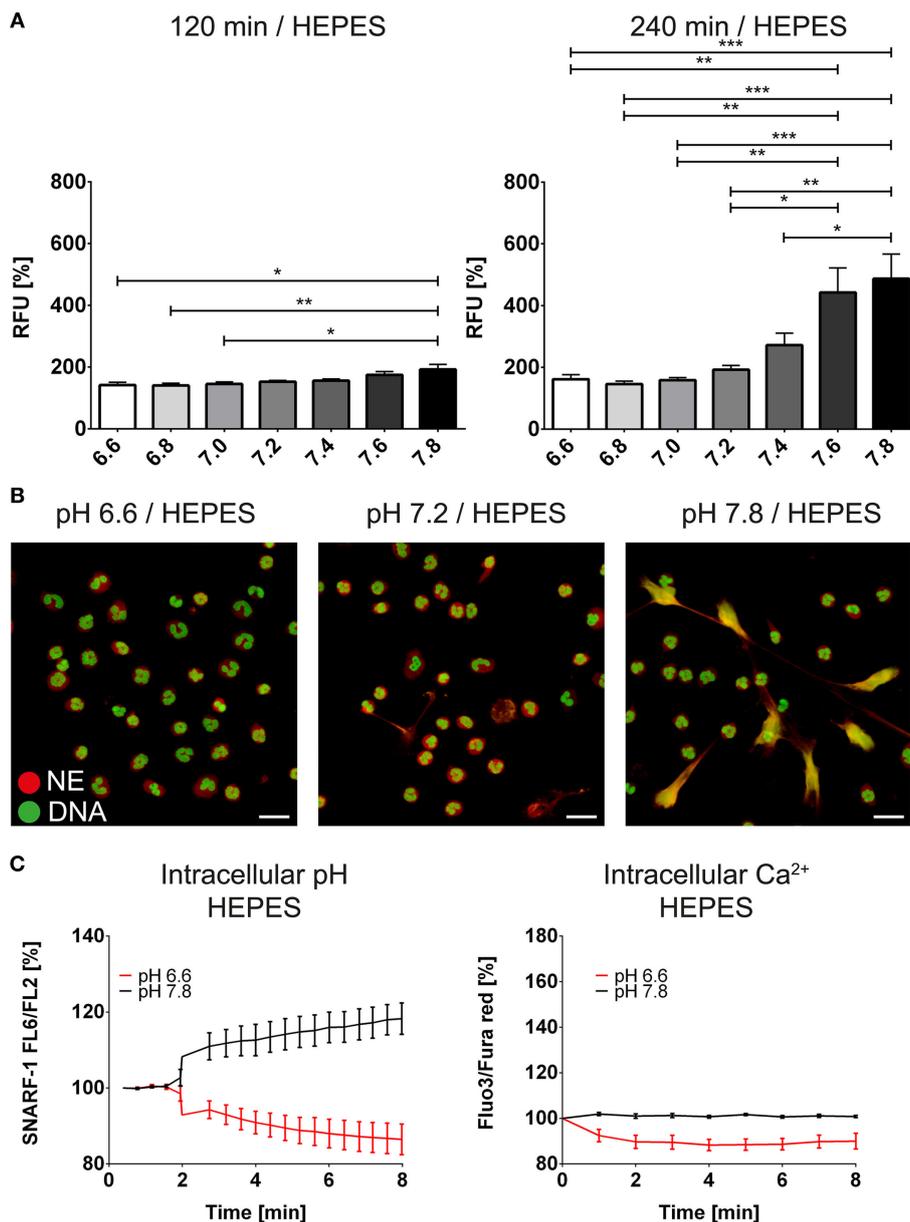


FIGURE 3 | High extracellular pH values induce formation of neutrophil extracellular traps in the absence of bicarbonate/CO₂ in RPMI. RPMI was supplemented with 20 mM HEPES, and the pH was adjusted to values reaching from 6.6 to 7.8 with hydrochloric acid and sodium hydroxide, respectively. **(A)** Fluorescence-based quantification of DNA externalization in response to various extracellular pH values after 120 min (left) and 240 min (right). **(B)** Immunocytochemical analysis of neutrophils incubated at a medium pH of 6.6, 7.2, and 7.8, respectively. Signal for DNA is depicted in green, and signal for neutrophil elastase is displayed in red. The scale bar represents 20 μ m. **(C)** Flow cytometric determination of intracellular pH (left) and cytosolic Ca²⁺ concentration (right) of neutrophils at pH values of 6.6 and 7.8. $n = 3-4$.

(DIDS) drastically reduced DNA externalization (**Figure 7A**). Microscopic analysis revealed that NET formation was strongly reduced/delayed by DIDS (**Figure 7B**; Video S5 in Supplementary Material). DIDS was also able to inhibit NET formation in response to PMA, ionomycin, and MSU at 24 mM bicarbonate/5% CO₂ (**Figure 9**; Figure S13 in Supplementary Material). Since DIDS specifically inhibits anion channels, a protein-dependent mechanism is likely to be causative in pH

control of NET formation. It is likely that alterations of the pH trigger a molecular program that increases the susceptibility of neutrophils to release NETs.

DISCUSSION

Our data indicate that the triangular relationship of CO₂, bicarbonate and pH strongly influences the capacity of neutrophils

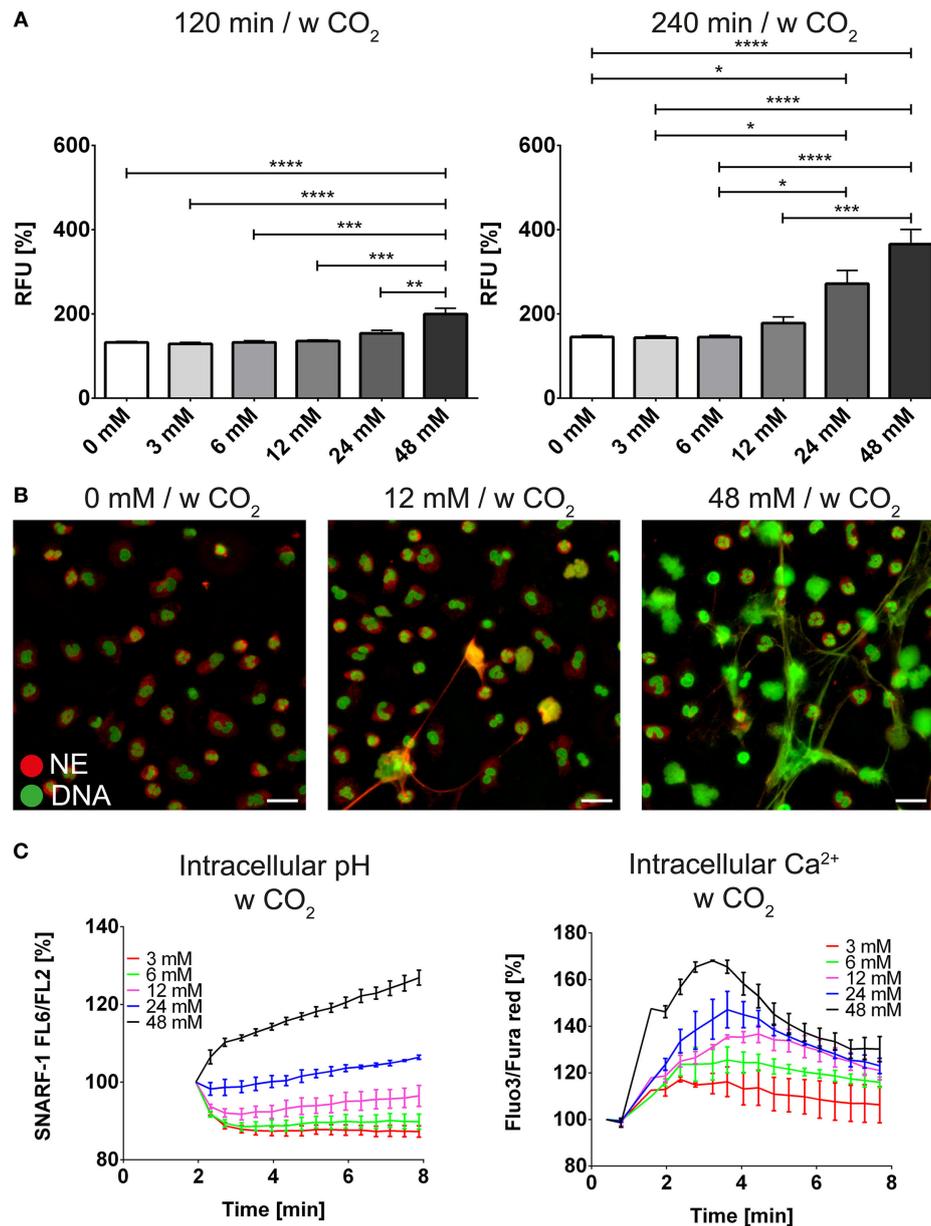


FIGURE 4 | The bicarbonate to CO₂ ratio influences formation of neutrophil extracellular traps in RPMI. RPMI was supplemented with various concentrations of bicarbonate in the presence of 5% CO₂. **(A)** Fluorescence-based quantification of DNA externalization in response to various concentrations of bicarbonate after 120 min (left) and 240 min (right). *n* = 5–11. **(B)** Immunocytochemical analysis of neutrophils incubated in the presence of various concentrations of bicarbonate. Signal for DNA is depicted in green, and signal for neutrophil elastase is displayed in red. The scale bar represents 20 μm. **(C)** Flow cytometric determination of intracellular pH (left) and cytosolic Ca²⁺ concentration (right) of neutrophils at various concentrations of bicarbonate. *n* = 3.

to form NETs. We observed that NET formation is decreased in conditions with a high ratio of CO₂ to bicarbonate even in the presence of biochemical NET-inducers. A low ratio resulted in NET formation in the absence of added inducers and augmented the NET-inducing potential of multiple triggers including PMA, ionomycin, monosodium urate, and lipopolysaccharides. This effect was observed both in HBSS and RPMI, yet markedly enhanced in the absence of extracellular amino acids as in HBSS. In the absence of CO₂ and bicarbonate in HEPES-buffered media,

the independent contribution of the pH was assessed. These studies indicate that the extracellular pH has an important influence on the capacity of neutrophils to release NETs but can only partially account for the drastic NET formation in the presence of bicarbonate/CO₂.

Our data is in line with findings from other groups that observed that neutrophil function is depending on extracellular pH. Trevani and colleagues reported that extracellular acidification enhances specific functions of human neutrophils (13). They observed that

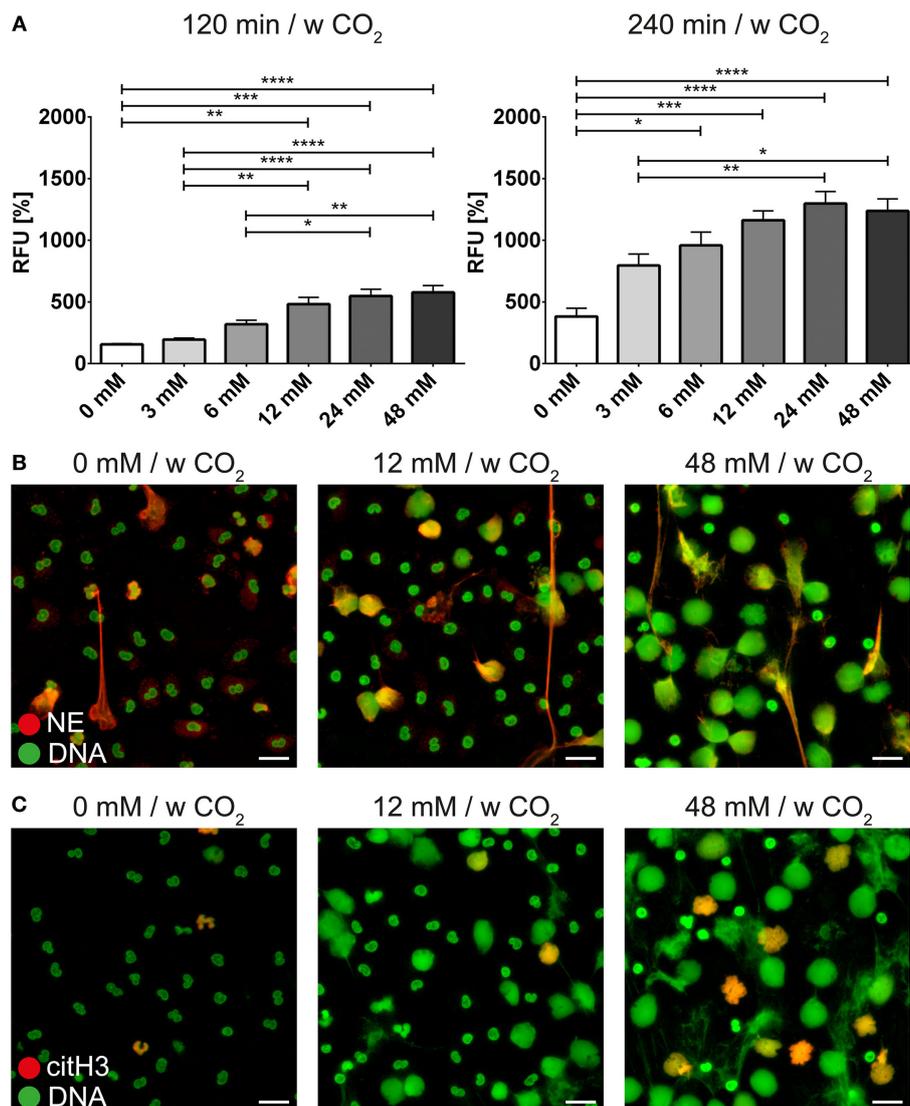


FIGURE 5 | The bicarbonate to CO₂ ratio influences the neutrophil extracellular trap-inducing potential of phorbol-2-myristate-13-acetate (PMA) in RPMI. RPMI was supplemented with various concentrations of bicarbonate in the presence of 5% CO₂. PMA was used at a concentration of 10 ng/ml. **(A)** Fluorescence-based quantification of DNA externalization in response to various concentrations of bicarbonate after 120 min (left) and 240 min (right) in the presence of PMA. $n = 5-11$. **(B)** Immunocytochemical analysis of neutrophils incubated in the presence of various concentrations of bicarbonate in the copresence of PMA. Signal for DNA is depicted in green, and signal for neutrophil elastase is displayed in red. The scale bar represents 20 μm . **(C)** Immunocytochemical analysis of neutrophils incubated in the presence of various concentrations of bicarbonate in the copresence of PMA. Signal for DNA is depicted in green, and signal for citrullinated histone H3 is displayed in red. The scale bar represents 20 μm .

extracellular acidosis transiently increases intracellular calcium and results in upregulation of the adhesion-mediating surface marker CD18. The stimulatory effects of conventional agonists were markedly increased by a low pH and the neutrophils responded with more production of H₂O₂ and increased release of myeloperoxidase (13). A more recent publication confirms these results, indicating that a pH of 6.0 prolongs neutrophil survival and increases phagocytosis of bacteria; however, phagolysosomal killing is decreased (14). Neutrophils cultivated in alkaline conditions show decreased survival compared to neutral or acidic environments (15).

Importantly, the effects of pH on immune and cellular functions are very broad and affect a multitude of cellular signaling molecules (16). Assay conditions may therefore strongly affect the specific results but may not necessarily depict the *in vivo* situation. Trevani and colleagues also highlighted the role of CO₂ and bicarbonate to neutrophil function, since intracellular acidification in response to extracellular hydrochloric acid challenge was less pronounced in bicarbonate free medium (13, 16). The importance of the choice of the acidifying agent is highlighted by findings showing that hydrochloric acid induces an inflammatory response in stimulated RAW 264.7 cells, whereas

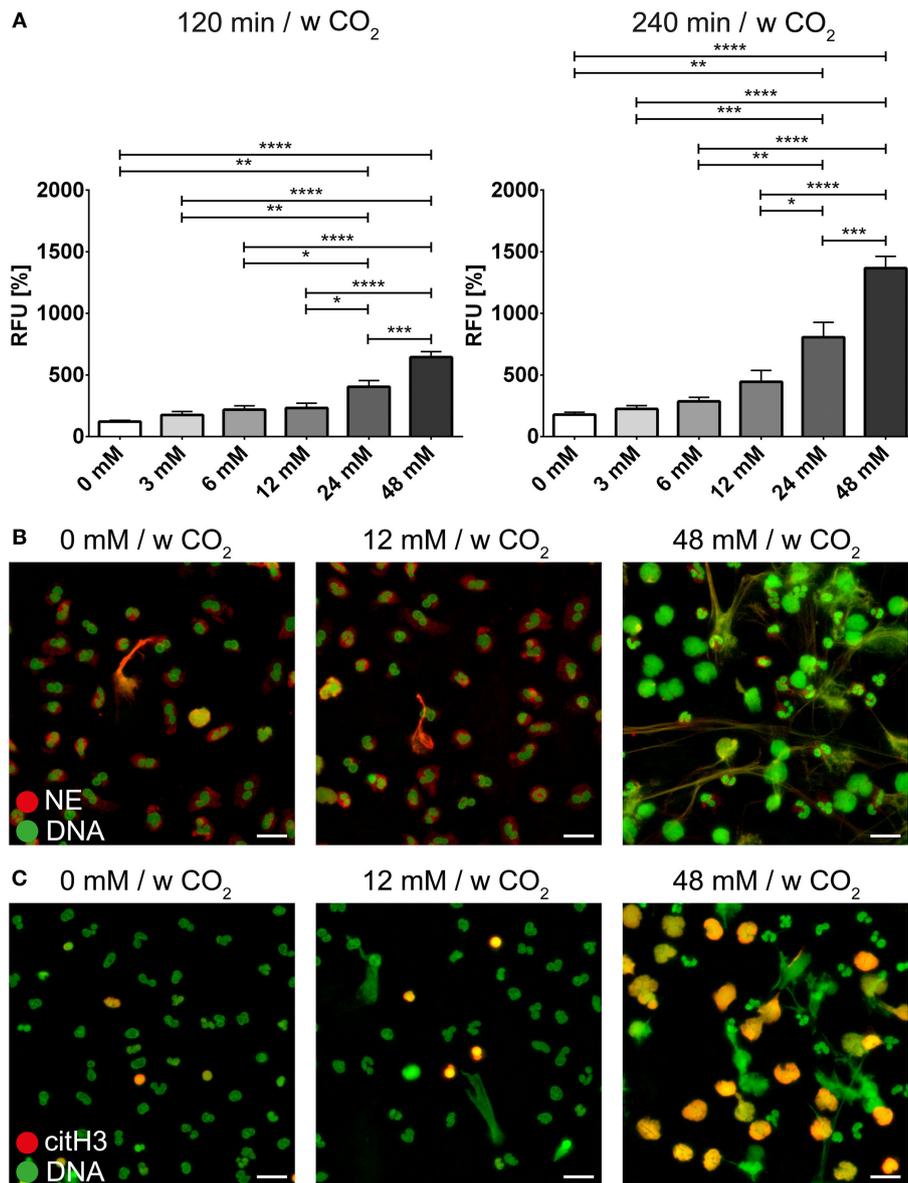
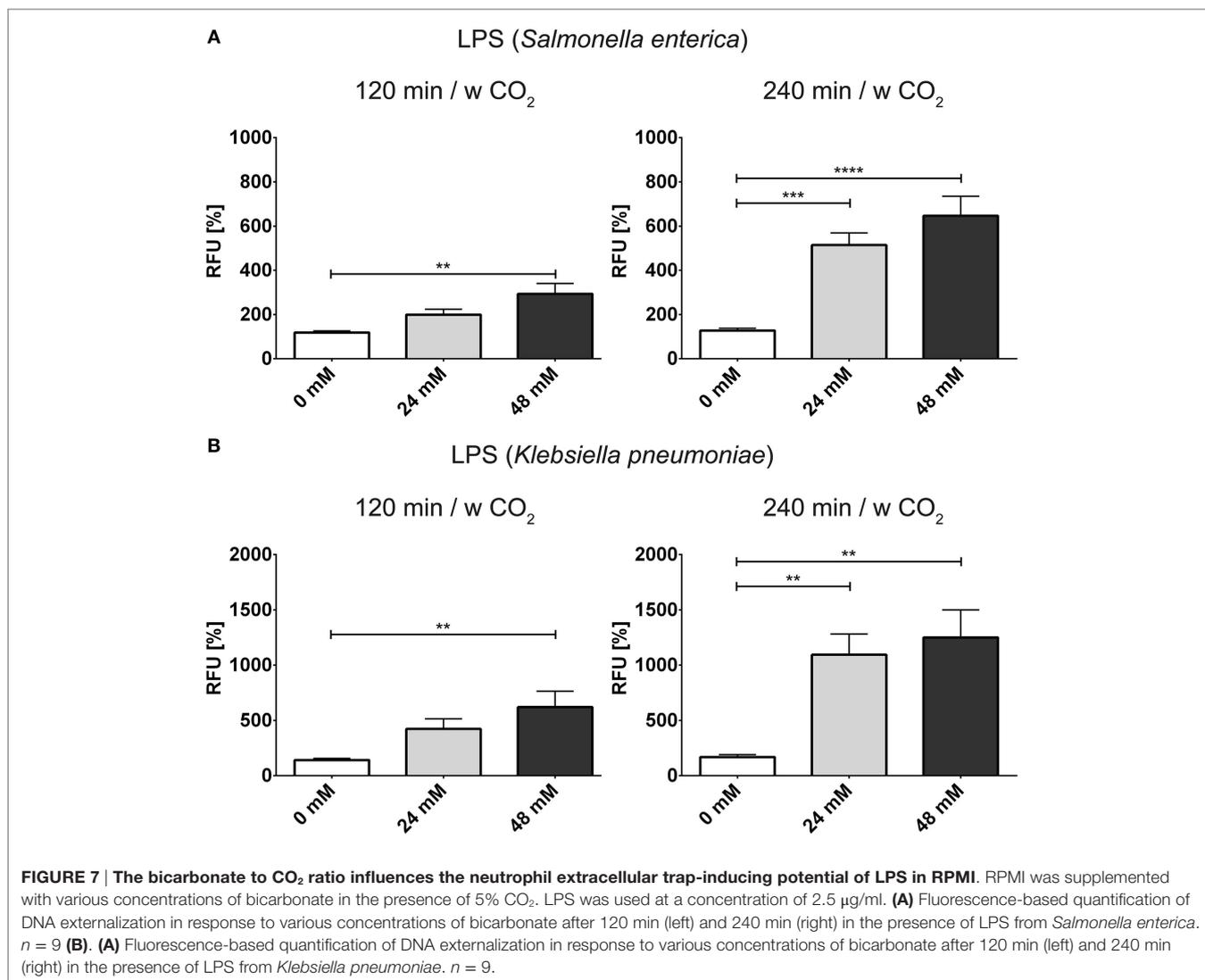


FIGURE 6 | The bicarbonate to CO₂ ratio influences the neutrophil extracellular trap-inducing potential of ionomycin in RPMI. RPMI was supplemented with various concentrations of bicarbonate in the presence of 5% CO₂. Ionomycin was used at a concentration of 1 μM. **(A)** Fluorescence-based quantification of DNA externalization in response to various concentrations of bicarbonate after 120 min (left) and 240 min (right) in the presence of ionomycin. *n* = 5–11. **(B)** Immunocytochemical analysis of neutrophils incubated in the presence of various concentrations of bicarbonate in the copresence of ionomycin. Signal for DNA is depicted in green, and signal for neutrophil elastase is displayed in red. The scale bar represents 20 μm. **(C)** Immunocytochemical analysis of neutrophils incubated in the presence of various concentrations of bicarbonate in the copresence of ionomycin. Signal for DNA is depicted in green, and signal for citrullinated histone H3 is displayed in red. The scale bar represents 20 μm.

acidification with lactate leads to an anti-inflammatory phenotype (17). However, the buffering agent is not solely responsible for the diverse effects reported through the years as indicated by inconsistent results even among studies using one and the same buffering system (13, 14, 16, 18).

We have observed a decrease of intracellular pH in conditions with a high ratio of CO₂ to bicarbonate and an increase of intracellular pH in alkaline conditions. The change of pH in both directions was accompanied by an increase in intracellular

calcium; however, elevated levels of the ion were only observed in response to alkalinization. An increase of cytosolic pH in neutrophils has been reported for a variety of stimuli, including PMA, ionomycin, and platelet-activating factor (19–21). We hypothesize that intracellular alkalinization by a high extracellular pH renders neutrophils more responsive to NET-inducing agents. Intracellular alkalinization likely triggers the same signals that follow stimulus-induced alkalinization. The concomitant increase in calcium is in line with this hypothesis,



since calcium is required for several enzymes involved in NET formation.

The intracellular pH of neutrophils is thought to be mainly regulated by Na⁺/H⁺ and Cl⁻/HCO₃⁻ antiporters. Recently a Na⁺/HCO₃⁻ cotransporter has been identified (22, 23). Efflux of chloride is commonly accompanying activation of neutrophils (24). Substitution of chloride with glucuronate leads to an outward flux of chloride (25). A high extracellular concentration of bicarbonate is likely recapitulating a similar effect. Interestingly, the broadly acting anion channel inhibitor DIDS was able to inhibit bicarbonate-induced NET release in a time and dose-dependent manner. The altered behavior of neutrophils at different pH is of special interest in the setting of cystic fibrosis (CF). The airway mucus and intestinal and pancreatic fluid in patients with CFTR mutations are strongly altered in the pH. Additionally, alterations in neutrophil intracellular pH homeostasis have been implicated in CF (26). It is tempting to speculate that these pH changes influence NET formation observed in patients with CF (27).

Our results give an interesting perspective on the role of the pH in inflammatory processes. We have observed that NET formation is substantially decreased in an acidic environment. In inflamed areas, pH values of as low as 5.5 have been reported (16). Although the observations presented here are limited to *in vitro* experiments, we will discuss possible implications for the *in vivo* situation (Figure 10).

In areas of hypoxia, such as an inflammatory focus or ischemic tissue, anaerobic glycolysis will lead to an increased formation of lactic acid and to tissue acidification. The border of the inflammatory areas is characterized by a steep increase in oxygen saturation as well as a gradient from acidic to neutral pH (Figure 10). We hypothesize that neutrophils detect the border of the inflamed area in part by this pH gradient. NET formation at elevated pH values might then primarily function as a barrier to wall-off infected or inflamed areas and to prevent invasion of the organism with pathogens or to prevent spreading of danger-associated molecular patterns from necroinflammatory areas (28). The pH might also serve as an indicator for neutrophils to sense the

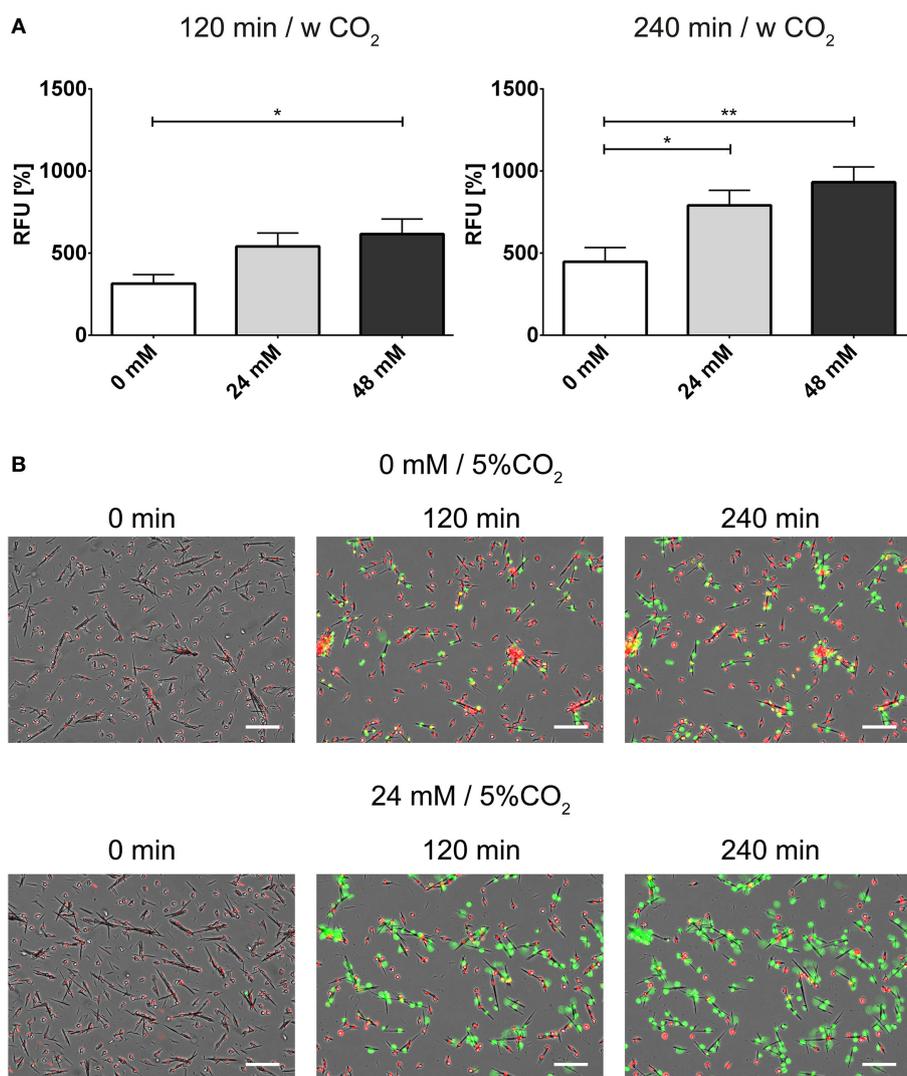
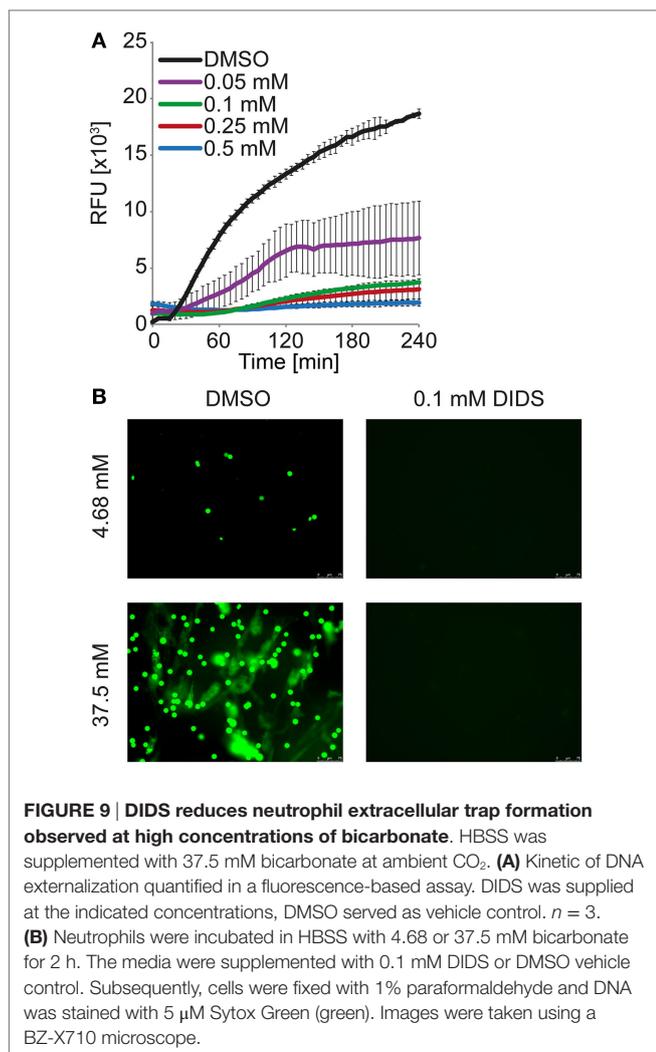


FIGURE 8 | The bicarbonate to CO₂ ratio influences the neutrophil extracellular trap-inducing potential of monosodium urate crystals (MSU) in RPMI. RPMI was supplemented with various concentrations of bicarbonate in the presence of 5% CO₂. MSU was used at a concentration of 300 pg/cell. **(A)** Fluorescence-based quantification of DNA externalization in response to various concentrations of bicarbonate after 120 min (left) and 240 min (right) in the presence of MSU. $n = 8-10$. **(B)** Still movie of PMNs coincubated with MSU either under relative hypercapnia (0 mM bicarbonate/5% CO₂) or 24 mM bicarbonate/5% CO₂. Signal for DNA of cells with intact plasma membrane integrity is depicted in red (Hoechst 33342), and signal for extracellular DNA and inside of necrotic cells is depicted in green. The numbers indicate the time in min after addition of MSU, the scale bar represents 20 μ m.

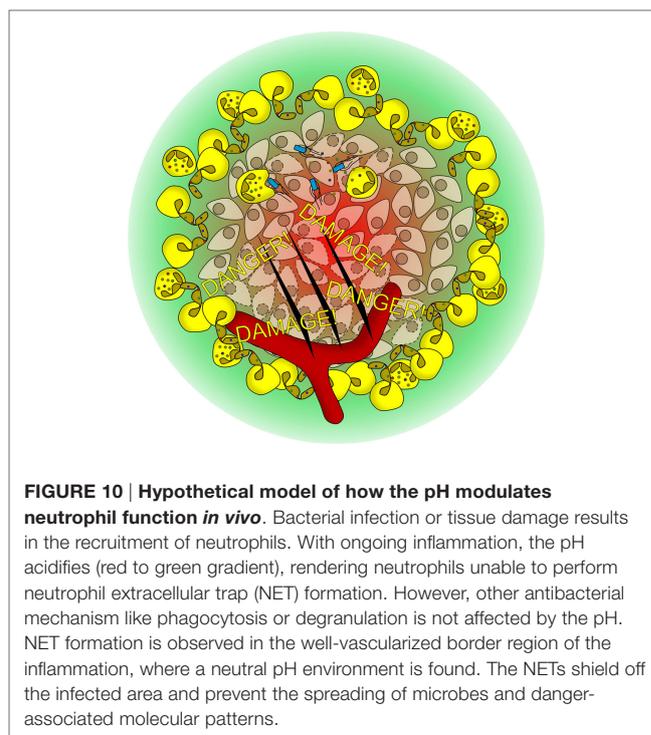
progress of inflammation. In the acidic center of the inflammatory focus, pathogens might still be traced and phagocytosed whereas in the periphery, the strategy is to shield the non-inflamed tissue (Figure 10). Restitution of serum pH in the setting of reperfusion may then promote NET formation and contribute to reperfusion injury.

The tumor microenvironment can technically be considered a necroinflammatory area, with hypoxic, necrotic cores, and areas of hypervasculation due to aberrant neoangiogenesis. In a similar manner, also the tumor environment is characterized by steep intratumoral and peritumoral pH gradients. Besides the presence of tumor-associated neutrophils, tumor-bearing mice exert a prothrombotic phenotype. Recent studies have

causally implicated to increased NET formation as a hypercoagulable state (29–31). Moreover, NETs reportedly promote metastasis. The existence and the role of NETs in the microenvironment surrounding the tumor are far less examined. Following our hypothesis, we would expect neutrophils to undergo NET formation in contact to necrotic tumor tissue spatially influenced by the local pH. It will be the focus of future studies to determine the biological role of NETs adjacent to tumors, as these NETs could temporarily prevent expansion of the tumor or be hijacked by the malignancy to promote its spread. Such complexity is in line with recent reports about neutrophils being involved in both the initiation and resolution of inflammation (32–34).



Apart from these thoughts on the role of the pH on neutrophil function *in vivo*, our results have far-reaching technical implications for the multitude of studies which currently examine the effect of chemical interference with NET formation. Experimentators need to be aware of the *ménage-à-trois* of bicarbonate, CO₂, and pH. Even mild changes in the ratio of CO₂ to bicarbonate and the pH may severely impact the outcome of an experiment. An inhibitory effect of a compound *in vitro* might solely be due to pH alterations of the media. In a standard lab incubator, simply opening the door to enter a well plate profoundly changes the incubator atmosphere for up to 15 min. Therefore, using a gas-control module in a microplate reader will significantly improve the quantification of DNA externalization. pH effects are especially pronounced for ionomycin, which shows increasing binding affinity to Ca²⁺ with increasing medium pH (35). Ionomycin induces a calcium and PADI4-dependent subroutine of NET release and is therefore in the center of therapeutic research (36–39). Small variations of the extracellular pH may easily mask the effects of drugs targeting the potential of neutrophils to externalize chromatin.



Likewise, the strong inter- and intra-individual variations in the formation of NETs could at least in part be related to unstable experimental settings (40).

Altogether, our findings highlight the necessity of optimized pH control in NET assays. A close attention to pH-related issues will increase the validity of experiments and allow higher reproducibility and detection of more subtle changes.

AUTHOR CONTRIBUTIONS

CM, AM, and ML planned and performed most of the experiments, conducted data analysis, and wrote the manuscript. SP, SG, JH, DK, and MB performed experiments and conducted data analyses. LM, CB, and GS provided scientific input and wrote the manuscript. PT and RF performed microscopy and scientific input. ML and MH supervised the project, planned and conducted experiments, analyzed data, and wrote the manuscript. All the authors read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at <http://journal.frontiersin.org/article/10.3389/fimmu.2016.00583/full#supplementary-material>.

FIGURE S1 | Bicarbonate induces formation of neutrophil extracellular traps in HBSS (full). Immunocytochemical analysis of neutrophils incubated in the presence of various concentrations of bicarbonate at ambient CO₂. Signal for DNA is depicted in green, and signal for neutrophil elastase is displayed in red. Single channel full images and overlays of the sections from **Figure 1B** are depicted. The scale bar represents 80 μm.

FIGURE S2 | Formation of neutrophil extracellular traps in HBSS is reduced at 5% CO₂ (full). Immunocytochemical analysis of neutrophils incubated in HBSS containing various concentrations of bicarbonate in the presence of 5% CO₂. Signal for DNA is depicted in green, and signal for neutrophil elastase is displayed in red. Single channel full images and overlays of the sections from **Figure 1C** are depicted. The scale bar represents 80 μm.

FIGURE S3 | Sytox Green binding to DNA is not affected by medium pH. Calf thymus DNA was diluted to the indicated concentrations in RPMI containing various concentrations of bicarbonate and 2.5 μM Sytox Green. Fluorescence was assessed with quantitative fluorimetry similar to the detection of DNA externalization. Depicted is the mean of two independent measurements.

FIGURE S4 | High extracellular pH values induce formation of neutrophil extracellular traps in the absence of bicarbonate/CO₂ in RPMI (full). Immunocytochemical analysis of neutrophils incubated in RPMI containing 20 mM HEPES adjusted to various pH values. Signal for DNA is depicted in green, and signal for neutrophil elastase is displayed in red. Single channel full images and overlays of the sections from **Figure 3B** are depicted. The scale bar represents 80 μm.

FIGURE S5 | Bicarbonate in RPMI induces chromatin externalization in the absence of proper CO₂ control. Isolated neutrophils were incubated for 30 min either at ambient CO₂ (bottom) or in the presence of 5% CO₂ (top). Cells were fixed with 1% paraformaldehyde for 30 min and stained with 5 μM Sytox Green and analyzed on a BZ-X710 microscope. Maximum intensity projection of Z-stacks and gamma correction were performed to increase depth of field and to allow proper display of neutrophil extracellular traps and nuclei on the same image, respectively. Post-processing of pictures was performed with Photoshop CS5. The scale bar represents 40 μm.

FIGURE S6 | Bicarbonate-induced formation of neutrophil extracellular traps in RPMI (full). Immunocytochemical analysis of neutrophils incubated in RPMI containing various concentrations of bicarbonate in the presence of 5% CO₂. Signal for DNA is depicted in green, and signal for neutrophil elastase is displayed in red. Single channel full images and overlays of the sections from **Figure 4B** are depicted. The scale bar represents 80 μm.

FIGURE S7 | The bicarbonate to CO₂ ratio influences the neutrophil extracellular trap-inducing potential of PMA in RPMI – neutrophil elastase (NE) staining (full). Immunocytochemical analysis of neutrophils incubated in RPMI containing various concentrations of bicarbonate in the presence of 5% CO₂ and 10 ng/ml PMA. Signal for DNA is depicted in green, and signal for NE is displayed in red. Single channel full images and overlays of the sections from **Figure 5B** are depicted. The scale bar represents 80 μm.

FIGURE S8 | The bicarbonate to CO₂ ratio influences the neutrophil extracellular trap-inducing potential of PMA in RPMI – citrullinated histone H3 (citH3) staining (full). Immunocytochemical analysis of neutrophils incubated in RPMI containing various concentrations of bicarbonate in the presence of 5% CO₂ and 10 ng/ml PMA. Signal for DNA is depicted in green, and citH3 is displayed in red. Single channel full images and overlays of the sections from **Figure 5C** are depicted. The scale bar represents 80 μm.

FIGURE S9 | The bicarbonate to CO₂ ratio influences the neutrophil extracellular trap-inducing potential of ionomycin in RPMI – neutrophil

elastase (NE) staining (full). Immunocytochemical analysis of neutrophils incubated in RPMI containing various concentrations of bicarbonate in the presence of 5% CO₂ and 1 μg/ml ionomycin. Signal for DNA is depicted in green, and signal for NE is displayed in red. Single channel full images and overlays of the sections from **Figure 6B** are depicted. The scale bar represents 80 μm.

FIGURE S10 | The bicarbonate to CO₂ ratio influences the neutrophil extracellular trap-inducing potential of ionomycin in RPMI – citrullinated histone H3 (citH3) staining (full). Immunocytochemical analysis of neutrophils incubated in RPMI containing various concentrations of bicarbonate in the presence of 5% CO₂ and 1 μg/ml ionomycin. Signal for DNA is depicted in green, and signal for citH3 is displayed in red. Single channel full images and overlays of the sections from **Figure 6C** are depicted. The scale bar represents 80 μm.

FIGURE S11 | The bicarbonate to CO₂ ratio influences the neutrophil extracellular trap-inducing potential of LPS from *Salmonella enterica* in RPMI – neutrophil elastase (NE) staining. Immunocytochemical analysis of neutrophils incubated in RPMI containing various concentrations of bicarbonate in the presence of 5% CO₂ and 2.5 μg/ml LPS. Signal for DNA is depicted in green, and signal for NE is displayed in red. The scale bar represents 80 μm.

FIGURE S12 | The bicarbonate to CO₂ ratio influences the neutrophil extracellular trap-inducing potential of LPS from *Klebsiella pneumoniae* in RPMI – neutrophil elastase (NE) staining. Immunocytochemical analysis of neutrophils incubated in RPMI containing various concentrations of bicarbonate in the presence of 5% CO₂ and 2.5 μg/ml LPS. Signal for DNA is depicted in green, and signal for NE is displayed in red. The scale bar represents 80 μm.

FIGURE S13 | DIDS interferes with neutrophil extracellular trap formation. Upper left: fluorescence-based quantification of DNA externalization in response to 100 ng/ml PMA either in the presence (PMA + DIDS) or absence (PMA) of 0.1 mM DIDS. Unstimulated (US) controls and DIDS without inducer of neutrophil extracellular trap (NET) formation (US + DIDS) are included additionally. *n* = 3. Upper right: fluorescence-based quantification of DNA externalization in response to 1 μg/ml ionomycin either in the presence (IONO + DIDS) or absence (IONO) of 0.1 mM DIDS. US controls and DIDS without inducer of NET formation (US + DIDS) are included additionally. *n* = 3. Lower left: fluorescence-based quantification of DNA externalization in response to 300 pg/cell monosodium urate crystals (MSU) either in the presence (MSU + DIDS) or absence (MSU) of 0.1 mM DIDS. US controls and DIDS without inducer of NET formation (US + DIDS) are included additionally. *n* = 3. Depicted is the mean ± SD.

VIDEO S1 | Bicarbonate induces formation of neutrophil extracellular traps in HBSS. Neutrophils were cultivated in isotonic HBSS medium containing 75 mM of bicarbonate and 1% of BSA. Cells were stained with 0.1 μg/ml Hoechst 33342 (green) and 500 ng/ml propidium iodide (red). Images were captured every 30 s for 30 min. Microscopy was performed on an Axio Observer. Z1 microscope using a time-lapse shooting sequence. Time is indicated in seconds and the scale bar represents 20 μm.

VIDEO S2 | High extracellular pH values induce formation of neutrophil extracellular traps in the absence of bicarbonate/CO₂ in RPMI. Neutrophils were cultivated in RPMI containing 20 mM HEPES. pH was adjusted either to pH 6.6 (top) or pH 7.8 (bottom). Cells were stained with 5 μM Sytox Green (green). Images were captured every 10 min for 240 min. Microscopy was performed on a BZ-X710 microscope using a time-lapse shooting sequence. Time is indicated in minutes and the scale bar represents 20 μm.

VIDEO S3 | The bicarbonate to CO₂ ratio influences formation of neutrophil extracellular traps in RPMI. Neutrophils were cultivated in RPMI containing either 0 mM (top) or 48 mM (bottom) bicarbonate. Medium was supplied with 5% CO₂. Cells were stained with 5 μM Sytox Green (green). Images were captured every 10 min for 240 min. Microscopy was performed on a BZ-X710 microscope using a time-lapse shooting sequence. Time is indicated in minutes and the scale bar represents 20 μm.

VIDEO S4 | The bicarbonate to CO₂ ratio influences the neutrophil extracellular trap-inducing potential of PMA in RPMI. Neutrophils were

cultivated in RPMI containing either 0 mM (top) or 48 mM (bottom) bicarbonate in the copresence of 10 ng/ml PMA. Medium was supplied with 5% CO₂. Cells were stained with 5 μM Sytox Green (green). Images were captured every 10 min for 240 min. Microscopy was performed on a BZ-X710 microscope using a time-lapse shooting sequence. Time is indicated in minutes and the scale bar represents 20 μm.

VIDEO S5 | Bicarbonate-induced formation of neutrophil extracellular traps is delayed by DIDS. Neutrophils were cultivated in isotonic HBSS medium containing 75 mM of bicarbonate and 1% of BSA either in the absence (top) or presence (bottom) of 0.1 mM DIDS. Cells were stained with 0.1 μg/ml Hoechst 33342 (green) and 500 ng/ml propidium iodide (red). Images were captured every 30 s for 30 min. Microscopy was performed on an Axio Observer.

Z1 microscope using a time-lapse shooting sequence. Time is indicated in seconds and the scale bar represents 20 μm.

VIDEO S6 | The bicarbonate to CO₂ ratio influences the neutrophil extracellular trap-inducing potential of monosodium urate crystals (MSU) in RPMI. Neutrophils were cultivated in RPMI containing either 0 mM (top) or 24 mM (bottom) bicarbonate in the copresence of 300 pg/ml MSU. Medium was supplied with 5% CO₂. Cells were stained with 5 μM Sytox Green (green) and 0.05 μg/ml Hoechst 33342 (red). Hoechst 33342 (red) stains nuclei of cells with intact plasma membrane, whereas Sytox Green (green) only stains the nuclei of necrotic cells or extracellular DNA. Images were captured every 10 min for 240 min. Microscopy was performed on a BZ-X710 microscope using a time-lapse shooting sequence. Time is indicated in minutes and the scale bar represents 20 μm.

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Formation of Neutrophil Extracellular Traps under Low Oxygen Level

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Since their discovery, neutrophil extracellular traps (NETs) have been characterized as a fundamental host innate immune defense mechanism. Conversely, excessive NET-release may have a variety of detrimental consequences for the host. A fine balance between NET formation and elimination is necessary to sustain a protective effect during an infectious challenge. Our own recently published data revealed that stabilization of hypoxia-inducible factor 1 α (HIF-1 α) by the iron chelating HIF-1 α -agonist desferoxamine or AKB-4924 enhanced the release of phagocyte extracellular traps. Since HIF-1 α is a global regulator of the cellular response to low oxygen, we hypothesized that NET formation may be similarly increased under low oxygen conditions. Hypoxia occurs in tissues during infection or inflammation, mostly due to overconsumption of oxygen by pathogens and recruited immune cells. Therefore, experiments were performed to characterize the formation of NETs under hypoxic oxygen conditions compared to normoxia. Human blood-derived neutrophils were isolated and incubated under normoxic (21%) oxygen level and compared to hypoxic (1%) conditions. Dissolved oxygen levels were monitored in the primary cell culture using a Fibox4-PSt3 measurement system. The formation of NETs was quantified by fluorescence microscopy in response to the known NET-inducer phorbol 12-myristate 13-acetate (PMA) or *Staphylococcus (S.) aureus* wild-type and a nuclease-deficient mutant. In contrast to our hypothesis, spontaneous NET formation of neutrophils incubated under hypoxia was distinctly reduced compared to control neutrophils incubated under normoxia. Furthermore, neutrophils incubated under hypoxia showed significantly reduced formation of NETs in response to PMA. Gene expression analysis revealed that mRNA level of *hif-1 α* as well as *hif-1 α* target genes was not altered. However, in good correlation to the decreased NET formation under hypoxia, the cholesterol content of the neutrophils was significantly increased under hypoxia. Interestingly, NET formation in response to viable *S. aureus* wild-type or nuclease-deficient strain was retained under hypoxia. Our results lead to the conclusion that hypoxia is not the ideal tool to analyze HIF-1 α in neutrophils. However, the data clearly suggest that neutrophils react differently under hypoxia compared to normoxia and thereby highlight the importance of the usage of physiological relevant oxygen level when studying neutrophil functions.

Keywords: neutrophils, innate immunity, neutrophil extracellular traps, hypoxia, HIF-1 α , cholesterol

INTRODUCTION

Neutrophils belong to the first line of defense of the innate immune system against various pathogens including bacteria, fungi, and protozoa. Besides degranulation and the intracellular killing of pathogens, neutrophils are able to entrap and kill pathogens by the release of extracellular structures, so-called neutrophil extracellular traps (NETs) (1). NETs are formed upon activation in response to a wide range of stimuli, like interferon- α , interleukin-8 (IL-8), the pharmacological agent phorbol 12-myristate 13-acetate (PMA), as well as numerous microbes and their products [reviewed by von Köckritz-Blickwede and Nizet (2)]. The formation of NETs is characterized by the disruption of the nuclear membrane, chromatin decondensation, and the mixing of nuclear contents with cytoplasmic and granular proteins. As a final step, the nuclear and granular components are released into the extracellular space (3). The fibrous DNA functions as a backbone in which histones, proteases [e.g., myeloperoxidase (MPO) and elastase], and antimicrobial peptides (AMPs) (e.g., cathelicidins) reside mediating their antimicrobial activity (4). The transcriptional regulation and intracellular signaling pathways of NET generation has not yet been fully investigated.

Generation of reactive oxygen species (ROS) is a key event for NET formation, and NET-based antimicrobial activity crucially depends on the formation of ROS through the membrane-bound NADPH oxidase enzyme complex as well as MPO (3, 5–9). Consequently, the most frequently used pathway to induce NETs is triggered by PMA, a protein kinase C (PKC) activator (1, 10, 11). That, in turn, activates the NADPH oxidase complex-subunit p47^{phox} (12) and thus strongly supports its activation. The produced superoxide anions serve as a starting product for additional ROS (13). Blocking the NADPH oxidase inhibits ROS generation and NET-release, respectively (14).

The downstream effects of ROS are extremely broad and range from the induction of NF- κ B signaling (15), to peroxidation of phospholipids (16), or activation of the cell death receptor (17). Another interesting target of ROS is the hypoxia-inducible factor 1 α (HIF-1 α) (18).

HIF-1 α was initially known to act as a transcriptional activator functioning as a master regulator of cellular and systemic oxygen homeostasis. Nowadays, HIF-1 α was additionally shown to play a role in the production of defense factors and to improve the bactericidal activity of myeloid cells (19, 20). Peyssonnaud and colleagues demonstrated in 2005 for the first time that HIF-1 α expression regulates the antibacterial capacity of phagocytes focusing on neutrophils and macrophages. HIF-1 α was induced by different bacterial pathogens including *Staphylococcus* (*S.*) *aureus* and *Streptococcus pyogenes*, even under normal oxygen levels (normoxia), and regulated the production of key immune effector molecules. Although the full spectrum of HIF-1 α downstream targets remains to be determined, the expression of a number of molecular effectors of host defense, including AMPs, TNF- α , and the granule proteases cathepsin G and elastase, significantly correlated with HIF-1 α levels (19). Mice lacking HIF-1 α in their myeloid cell lineage showed decreased bactericidal activity and were not able to restrict a systemic spread of an infection from its initial tissue (19).

It was already shown that HIF-1 α is crucial in the formation of extracellular traps in mast cells [mast cell extracellular traps (MCETs)]. Augmentation of HIF-1 α -activity resulted in a boosting of the antimicrobial activity of human and murine mast cells by inducing extracellular trap formation (21). At the same time, HIF-1 α -deficient mast cells exhibited reduced antimicrobial activity and ability to form extracellular traps. Recently, it was reported that the mammalian target of rapamycin (mTOR), a highly conserved PI3K-like serine/threonine kinase and a posttranscriptional regulator of HIF-1 α protein expression, regulates the formation of NETs (22). As mTOR kinase is known as a key regulator of autophagy in many mammalian cells including neutrophils, it is hypothesized that mTOR plays a regulatory role in NET-release by regulating autophagic activity (23). Interestingly, McInturff et al. (22) also demonstrated that the iron chelating HIF-1 α agonist cobalt chloride (CoCl₂) triggered NET formation (22). Several authors discussed that the regulation of ROS generation could be a key factor in these HIF-1 α - and mTOR-mediated processes (22–24). Our own data confirm the hypothesis that HIF-1 α might be involved in the formation of NETs: the HIF-1 α -agonist desferoxamine enhanced the release of extracellular traps in human and bovine neutrophils in a ROS-dependent manner (25).

Although the importance of HIF-1 α in the formation of NETs has already been stated, the impact of hypoxia on NET generation still needs to be clarified. Hypoxia was found to be able to enhance bactericidal activities of human neutrophils, increase their chemotactic, phagocytic, and respiratory burst capacities, and protect them from apoptosis (19, 26–29). Based on the described literature it may be hypothesized that NET formation increases under low oxygen conditions similarly to that shown by HIF-1 α stabilizing agents.

MATERIALS AND METHODS

Bacterial Strains

Staphylococcus aureus strain USA 300 wildtype (wt) (LAC AH 1263) and its nuclease mutant (Δ nuc) (LAC AH 1680) (30) were used in this study. *S. aureus* was grown in brain heart infusion (BHI) medium at 37°C with shaking. Fresh overnight cultures were diluted 1:100 in BHI and then grown to mid-exponential growth phase (OD₆₀₀ = 0.7) until usage. Heat inactivation was performed for 30 min in 95°C hot water.

Neutrophil Isolation

Human blood-derived neutrophils were isolated from healthy donors in agreement with the local ethical board by density gradient centrifugation at 500 \times g using PolymorphPrep (Axis-Shield, Oslo, Norway) as previously described (31). Then, neutrophils were resuspended in RPMI 1640 (PAA, Freiburg, Germany) and plated on poly-L-lysine (# P4707, Sigma-Aldrich) coated coverslips at a concentration of 2 \times 10⁵ cells/well in 48-well plates or 5 \times 10⁵ cells/well in 24-well plates (Nunc, Germany).

Oxygen Measurements

Oxygen measurements were performed as previously described (32) using a Fibox4-PSt3 measurement system in 24-well plates

(Nunc, Germany). Importantly, oxygen was measured non-invasively and was not consumed during the process of measurement. Freshly isolated neutrophils from human blood were adapted to hypoxia. Using optical sensors (placed on the bottom of the wells in the medium), the dissolved oxygen level in the cell culture media was measured based on the oxygen-dependent quenching of phosphorescent probes (32–34). Oxygen measurements were performed over a time period of 5 h while the cells were incubated under hypoxic (7 mmHg, 1% O₂) or normoxic (159 mmHg, 21% O₂) conditions, respectively.

NET Induction and Visualization

Neutrophils were preincubated under normoxic or hypoxic conditions for 2 h before they were subsequently infected with living or heat-inactivated bacteria (MOI 2) and incubated at 37°C and 5% CO₂ for 3 h under the respective oxygen condition. As a positive control to stimulate NET formation, 25 nM PMA (Sigma, Hamburg, Germany) was used, while untreated neutrophils served as a negative control. Neutrophils were treated with 10 µg/ml diphenyleneiodonium (DPI) to block NADPH oxidase activity. Finally, cells were fixed with 4% paraformaldehyde (PFA; Roth, Germany).

Neutrophil extracellular traps were stained with an antibody against the histone–DNA complex (Millipore, mouse monoclonal anti DNA/Histone1 MAB 3864). Briefly, after blocking and permeabilization [2% BSA PBS + 0.2% Triton X-100 for 45 min at room temperature (RT)], samples were incubated for 1 h at RT with the primary antibody (2.2 mg/ml, diluted 1:5000 in PBS containing 2% BSA, 0.2% Triton X-100). An Alexa Fluor 488-conjugated goat anti-mouse antibody (Thermo Scientific; diluted 1:1000 in PBS containing 2% BSA, 0.2% Triton X-100) was used as a secondary antibody. Slides were embedded in ProlongGold® antifade with DAPI (P36931, Molecular Probes) and analyzed by confocal fluorescence microscopy using a Leica TCS SP5 confocal microscope with a HCX PL APO 40×0.75–1.25 oil immersion objective. Settings were adjusted in accordance to control preparations using the respective isotype control antibody. The total number of neutrophils and the number of neutrophils releasing NETs per field of view were counted in six representative images per sample.

Lipid Isolation and Analysis

A total of 5 × 10⁶ neutrophils were incubated in a 1.5 ml reaction tube for 3 h at either 1 or 21% oxygen. Samples were washed twice with PBS, resuspended in chloroform–methanol (1:1), and lysed by passing cells through a 45 mm cannula syringe 15 times. Subsequent lipid isolation was performed as previously described (35).

Cholesterol content was analyzed with a Hitachi Chromaster HPLC using a Chromolith® HighResolution RP-18 endcapped 100–4.6 mm column coupled to a 5–4.6 mm guard cartridge and heated to 32°C. Methanol was used as the mobile phase at a flow rate of 1 ml/min at 22 bar, and a UV detector measuring at 202 nm to determine the amount of cholesterol in each sample. The results were quantified against an external standard ranging from 0.05 to 2 mg/ml cholesterol and expressed as nanogram cholesterol per 1 × 10⁶ neutrophils.

Triglycerides, free fatty acids, monoacylglycerols, and phospholipids were analyzed by thin layer chromatography (TLC) based on a method described previously (35). Briefly, isolated lipid samples were loaded on silica gel plates (Merck, Germany) and separated based on polarity. Lipids were visualized by copper sulfate solution and the band intensities subsequently analyzed by CP Atlas (Lazer Software). Lipids were identified against a known standard. Each sample was analyzed in repetition.

RNA Expression Analysis

RNA was extracted from 5 × 10⁵ neutrophils after incubation under normoxia or hypoxia for 2 or 3 h, with the RNeasy Micro Kit (Qiagen) as described in the user's manual. RNA quality was tested with a bioanalyzer (RNA 6000 Pico Kit, Agilent) following the manufacturer's instructions. Real-time PCR of reverse transcribed RNA (RT-qPCR) was designed to analyze expression of genes of interest and the housekeeping gene *rps9*. The respective primers are given in **Table 1**. The RT-qPCR was conducted as previously described (36) with the following modified program: initial denaturation at 95°C for 20 min and 40 cycles of denaturation at 95°C for 25 s, annealing at 58°C for 30 s, and amplification at 72°C for 20 s using an AriaMX Real-Time PCR system. Products were verified by melting curve analysis and 1.5% agarose gel electrophoresis. Data were normalized to a non-regulated housekeeping gene (*rps9*). The relative ΔCT values were determined for expression of the genes *hif-1 α* , *il-37*, and *vegf*. CT is the cycle number at the chosen amplification threshold, $\Delta\text{CT} = \text{CT}_{\text{gene}} - \text{CT}_{\text{reference (rps9)}}$ and $\Delta\Delta\text{CT} = \Delta\text{CT}_{\text{sample}} - \Delta\text{CT}_{\text{calibrator}}$. The fold change in expression ($2^{-\Delta\Delta\text{CT}}$) was calculated as the read-out parameter. The calibrator was neutrophils under normoxia.

Statistical Analysis

All experiments were performed at least three independent times unless indicated otherwise. Data were analyzed using Excel 2010 (Microsoft) and GraphPad Prism 6.0 (GraphPad Software). Differences between two or more groups were analyzed by using a two-way ANOVA with Sidak's multiple comparisons test if not otherwise stated. The significance is indicated as follows: n.s., not significant, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, and **** $p < 0.0001$.

TABLE 1 | Oligonucleotide primers used in RT-qPCR.

Gene	Primer sequence (5'-3') forward	Primer sequence (5'-3') reverse	Amplicon length (bp)
<i>rps9</i>	CTGACGCTTGATGAGAAGGAC	CTCATCCAGCACCCCAAT	87
<i>hif-1α</i>	GATGGAAGCACTAGACAAAGTTCA	ATCAGTGGTGGCAGTGGTAGTG	360
<i>il-37</i>	GCCAGGTCCTCAGCTACAAG	TGGTTGAGGGTCACTGTCCCC	260
<i>vegf</i>	ATGAACITTTCTGCTGTCTTGGGT	TGGCCTGGTGGAGGTTTGATCC	344

RESULTS AND DISCUSSION

Low Oxygen Levels in *In Vitro* Neutrophil Suspension Culture

At sites of infection and inflammation, the environmental oxygen concentration decreases and can drop to 1% due to invading pathogens and translocating immune cells, including neutrophils, with consequences for cellular functions (34, 37–39). To study the effect of hypoxia, freshly isolated human neutrophils were seeded and incubated under normoxic compared to 1% hypoxic conditions, and the oxygen level in the wells was measured over a time period of 5 h as described above. When cultured under normoxia, the neutrophil cultures maintained a constant oxygen level that reflected the atmospheric condition at around 165 mmHg ($22.3 \pm 0.46\%$ O₂). In contrast, hypoxic incubation decreased the dissolved oxygen level in the culture to less than 37 mmHg ($4.9 \pm 0.2\%$ O₂) within 45 min and resulted in an equilibration lower than 13.3 mmHg ($1.79 \pm 0.03\%$ O₂) within 5 h (Figure 1). Our results indicate that the applied experimental settings by incubating neutrophils under hypoxia decrease the oxygen level and reflect physiological oxygen conditions that may occur in infected tissue.

Spontaneous and PMA-Induced Net Formation under Hypoxia Compared to Normoxia

To study the effect of hypoxia on the spontaneous and PMA-induced NET formation, neutrophils were preincubated for 2 h at the defined oxygen concentration (normoxia and hypoxia, as shown in Figure 1). After an additional 3 h of incubation in the

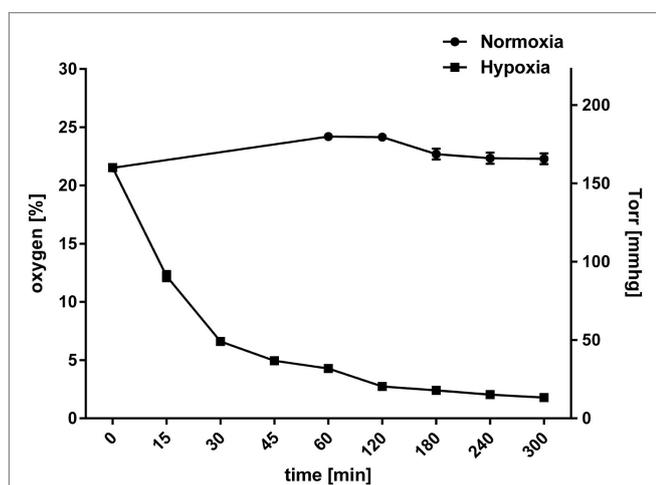


FIGURE 1 | Oxygen levels in neutrophil suspension cultures.

Neutrophils were incubated under normoxia (21%) or hypoxia (1%). Dissolved oxygen levels in neutrophil suspension culture were monitored for 5 h. Plotted values represent mean \pm SEM and are displayed as % oxygen on the left y-axis and mmHg oxygen on the right y-axis. Starting at $21.5 \pm 0.14\%$ (normoxia) and $21.5 \pm 0.21\%$ (hypoxia) after 5 h, the oxygen level dropped to $1.79 \pm 0.03\%$ under hypoxia, while it stayed relatively constant around $22.3 \pm 0.46\%$ under normoxia.

presence or absence of PMA, the neutrophils were analyzed for their NET-releasing capability.

An amount of $11.6 \pm 1.0\%$ of untreated neutrophils spontaneously formed NETs under normoxia (Figure 2). Interestingly, incubation under hypoxia distinctly reduced the number of spontaneously NET forming neutrophils to $7.1 \pm 0.7\%$ (Figures 2A,B).

However, as previously published (1, 3) and confirmed here (Figure 2), approximately 90% of cells were found to release NETs after incubation at an atmospheric oxygen concentration in response to PMA. Importantly, hypoxic pretreatment of neutrophils completely abolished the PMA-induced NET formation seen at normoxic conditions (Figure 2). It is well known, that the PMA-dependent NET formation is based on the generation of ROS (1, 7). In good correlation to these findings, Kirchner et al. (40) recently confirmed that the formation of ROS is blocked at 2% O₂ and also leads to an abrogated NET-release. Furthermore, a broad spectrum of antioxidative substances such as flavonoids, vitamin C, and aminosalicic acid were also shown to inhibit NET formation through the reduction of ROS (41).

Gene Expression of *hif-1 α* and Target Genes

As already mentioned in the Section “Introduction,” HIF-1 α is an essential regulator to modulate cellular stress responses to low oxygen conditions and also has been shown to modulate the formation of NETs (22, 25). Therefore, the mRNA expression of this transcription factor as well as two of its target genes (*vegf* and *il-37*) (42–44) were investigated in neutrophils incubated under normoxia versus 1% hypoxia with RT-qPCR.

As depicted in Figure 3, the expression level of *hif-1 α* or its target genes did not change neither after 2 h (Figure 3A) nor after 3 h (Figure 3B) incubation under hypoxia compared to normoxia. All $\Delta\Delta$ CT values remained around 1 meaning that there were no differences in the gene expression levels of the neutrophils that were cultivated under normal oxygen level in comparison to those cultivated under low oxygen levels. Therefore, short-time treatment at 1% hypoxia for 2 or 3 h might not be the optimal model to study the HIF-1 α -dependent response of neutrophils. Moreover, HIF-1 α might not be responsible for the altered NET-phenotype under hypoxia shown in Figures 2A,B. It seems that neutrophils need an additional trigger to induce the expression of *hif-1 α* as hypoxia alone does not alter the expression level under the selected conditions (Figures 3A,B). In good correlation to our RT-qPCR results, Kirchner et al. (41, 40) also exhibited that hypoxia alone did not stabilize HIF-1 α protein level. Interestingly, those authors demonstrated that HIF-1 α is stabilized under hypoxia as well as under normoxia by stimulating neutrophils with PMA (41).

Lipid Alterations

Previous data revealed that lipid alterations modulate the formation of NETs (45): decreased level of cholesterol mediated by methyl- β -cyclodextrin in primary blood-derived neutrophils led to increased spontaneous NET formation. Furthermore, pharmacological treatment of neutrophils with statins that

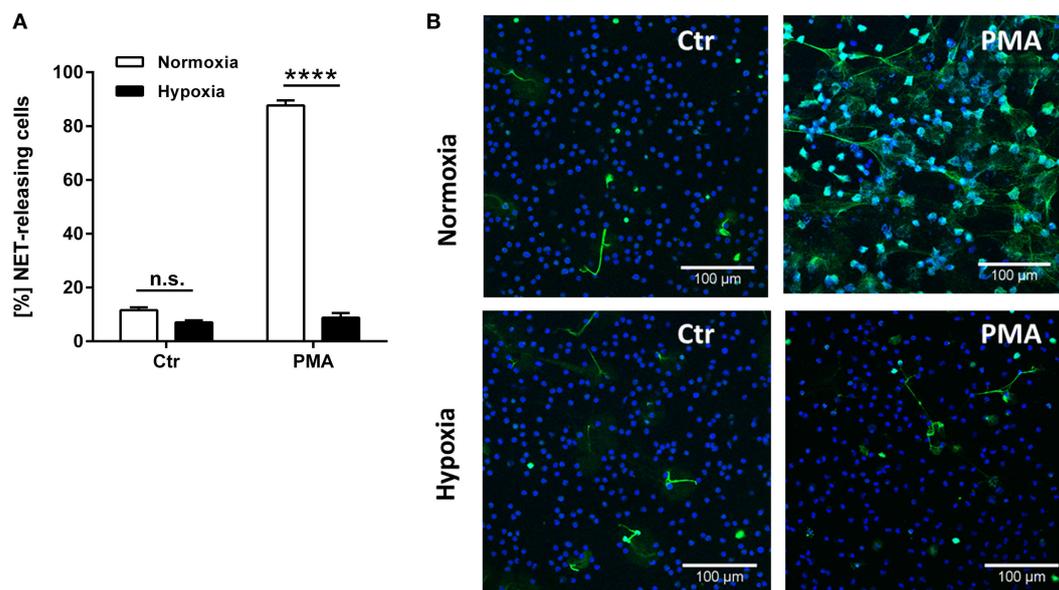


FIGURE 2 | Spontaneous and PMA-induced NET-release is decreased under hypoxia. Neutrophils were incubated for 2 h under normoxia or hypoxia before treatment with 25 nM PMA for additional 3 h. Formation of NETs was visualized using a monoclonal antibody against histone–DNA complexes (green) in combination with DAPI to stain the nuclei (blue). **(A)** Under normoxia NET-release was stimulated with PMA in $87.7 \pm 1.8\%$ of the cells, while $11.6 \pm 1.0\%$ of the neutrophils formed NETs spontaneously. Incubation under hypoxia decreased the spontaneous NET-release to $7.1 \pm 0.7\%$ and abrogates the PMA-stimulatory effect to a rate of $8.8 \pm 1.7\%$. **(B)** Representative fluorescence micrographs depicting NET-release of neutrophils. Statistical analysis of was performed with one-tailed paired Student's *t*-test.

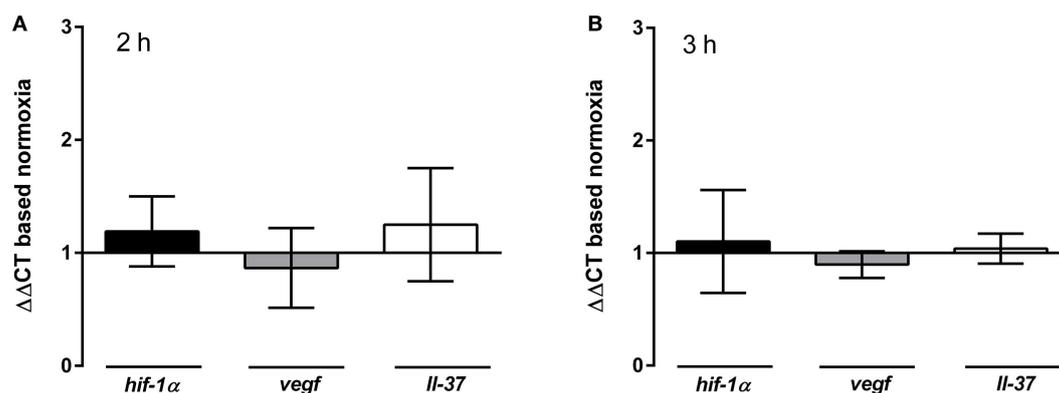


FIGURE 3 | Gene expression of *hif-1α* and target genes. Neutrophils were incubated under normoxia or hypoxia for 2 h **(A)** and 3 h **(B)** before RNA was isolated and gene expression of *hif-1α* and its target genes *vegf* and *Il-37* was analyzed by RT-qPCR. Data were normalized to the non-regulated housekeeping gene *rps9*. The *x*-fold changes of the values from samples incubated under hypoxia were calculated against the normoxic samples. Independent of the time point, there was neither a regulation of *hif-1α* nor of its target genes *vegf* and *Il-37* detectable, as all $\Delta\Delta\text{CT}$ s remain below 2 [*hif-1α*: 1.19 ± 0.3 (2 h) and 1.01 ± 0.5 (3 h); *vegf*: 0.87 ± 0.4 (2 h) and 0.9 ± 0.1 (3 h); *Il-37*: 1.25 ± 0.5 (2 h) and 1.0 ± 0.1 (3 h)].

block cholesterol synthesis also induce formation of NETs (46). Therefore, we here compared the lipid composition of the neutrophils when incubated under normoxia versus hypoxia by TLC. The data shown in **Figure 4A** demonstrate substantial lipid alterations comparing hypoxic and normoxic conditions, e.g., cholesterol. To verify the results, HPLC was used to quantify the cholesterol level in the cells. In good correlation to these data, a significant higher cholesterol level was found in cells after incubation under hypoxia compared to normoxia (**Figure 4B**)

at the same time when decreased spontaneous NET formation was detectable in control cells (**Figures 2A,B**). Thus, it may be speculated that hypoxia-mediated changes in lipid content, e.g., cholesterol accumulation in the cell may contribute to altered spontaneous NET formation under hypoxia.

***S. aureus*-Induced NET Formation**

Even though the RT-qPCR results reveal no exclusive link of HIF-1 α to hypoxia, our data are consistent: the absence of sufficient

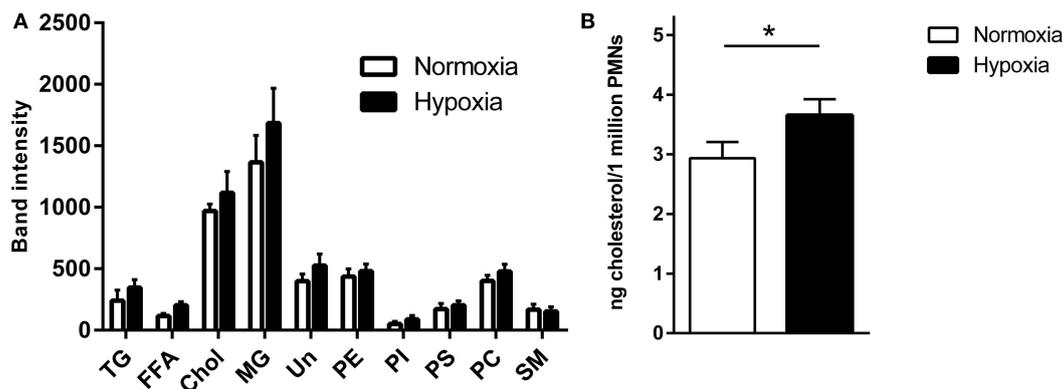


FIGURE 4 | The cellular cholesterol content increased under hypoxia. Neutrophils were incubated in either 1 or 21% oxygen, and their lipid composition was analyzed by TLC (A): triglycerides (TG), free fatty acids (FFA), cholesterol (chol), monoacylglycerols (MG), unknown (UN), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphoserine (PS), phosphatidylcholine (PC), and sphingomyelin (SM). Furthermore, cholesterol content was analyzed via HPLC (B). Results are expressed as nanogram cholesterol per 1×10^6 neutrophils. A significant higher cellular cholesterol level was detectable after incubation under hypoxia compared to normoxia. Statistical analysis was performed with one-tailed paired Student's *t*-test.

oxygen combined with the lack of HIF-1 α signaling and increased cholesterol level hamper the formation of extracellular traps in neutrophils.

Nevertheless, we were interested if a bacterial stimulus could affect NET-release even under the above described condition. Therefore, it was of special interest to analyze the *S. aureus*-induced NET formation at low oxygen levels in more detail. The *S. aureus* wt strain and its nuclease-deficient Δ nuc mutant were used in parallel, since the Δ nuc mutant enables better visualization of full length NETs, which are not shortened by *S. aureus* nuclease. However, both strains are known to induce NET formation at similar level (30). Similar to the PMA-stimulated approach, neutrophils were incubated under hypoxia or normoxia for 2 h before bacteria were added, and the cells were further cocultivated with *S. aureus* for 3 h under consistent oxygen conditions.

Interestingly, the amount of NET-releasing cells was not abrogated in the absence of O₂: living bacteria induced comparable amounts of NET-releasing cells under normoxia as well as under hypoxia (Figure 5A: *S. aureus* wt: $33.9 \pm 10.8\%$ normoxia compared to $36.6 \pm 8.0\%$ hypoxia; Figure 5B: *S. aureus* Δ nuc: $43.6 \pm 4.96\%$ normoxia compared to $43.6 \pm 12.3\%$ hypoxia). These results confirm the existing literature and emphasize that living *S. aureus* have the capacity to stimulate NET formation independent of oxygen concentration (47, 48). In contrast to viable *S. aureus*, h.i. *S. aureus* was no longer able to induce NET formation under hypoxia with the same efficiency compared to normoxia, indicating a different oxygen-dependent mechanism triggered by dead *S. aureus* (Figure 5C: $31.8 \pm 4.3\%$ normoxia compared to $20 \pm 2.8\%$ hypoxia).

So far, different bacterial factors that are released by viable bacteria have been described to trigger the formation of NETs in response to *S. aureus* infections: the N-terminal ArgD peptides (49), leukotoxin GH (47), and Panton-Valentin leukocidin (PVL) (48). Importantly, those exotoxins trigger formation of NETs by completely different mechanisms. The PVL-mediated NET formation is described as a vesicular release of nuclear DNA by

a mechanism independent of NADPH oxidase. In contrast, the leukotoxin GH and ArgD peptides-mediated NET formation is associated with cytolysis (no vesicular release of nuclear DNA), which is in accordance with the initial data from Fuchs et al. (3), who described the NADPH-oxidase-dependent formation of NETs in response to *S. aureus* as a novel cell death later called "NETosis" (50).

To further characterize the role of NADPH oxidase-mediated production of ROS in the *S. aureus*-mediated NET formation under 1% oxygen, we treated neutrophils with DPI to block NADPH oxidase. As shown in Figure 5D, treatment of neutrophils with DPI significantly decreased the *S. aureus* Δ nuc-mediated formation of NETs under hypoxia and normoxia as also previously shown by Fuchs et al. (3) under normoxia. These data indicate that under hypoxic as well as normoxic conditions, NADPH oxidase contribute to the formation of NETs in response to *S. aureus*. Since residual NET formation is still detectable in the presence of DPI (Figure 5D), additional pathways might also contribute to the phenotype as described by Pilszczek et al. (48). Under consideration of the lipid data shown in Figure 4, it might also be speculated that lipid alterations found under hypoxia lead to altered susceptibility of the neutrophil to bacterial exotoxins and/or oxidative stress as also shown by Chow et al. (46), and that these cellular changes modulate the neutrophil ability to release NETs. However, further studies are needed to prove this hypothesis.

Interestingly, our data obtained from the PMA- or *S. aureus*-stimulated neutrophils revealed a highly specific response, namely a characteristic NET formation level, to a distinct stimulus under various oxygen conditions. Whereas the NET formation mediated by viable *S. aureus* remains preserved under hypoxia (Figures 5A,B), the NET formation in response to PMA (Figures 1 and 5D) or dead *S. aureus* (Figure 5C) is reduced under hypoxia. However, the data shown here highlight that neutrophils can react completely different to the same trigger under hypoxia compared to normoxia. Since neutrophils are prominent immune

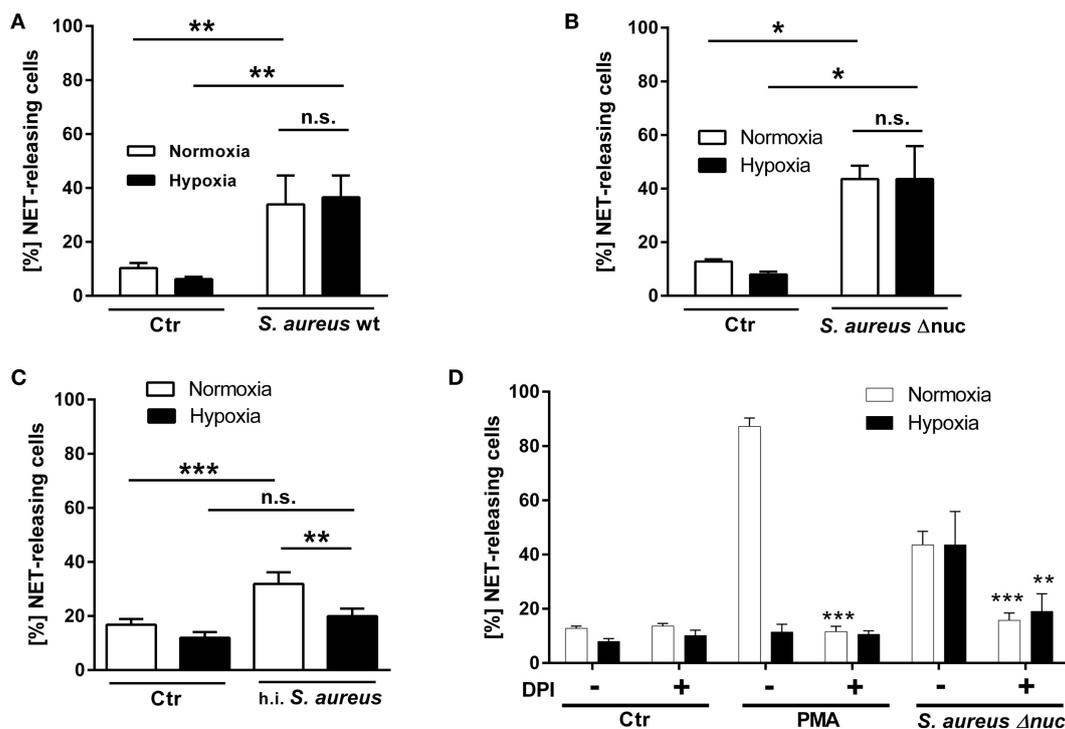


FIGURE 5 | *S. aureus*-induced NET formation. Neutrophils were preincubated under normoxic or hypoxic conditions for 2 h and further co-incubated for 3 h under the respective oxygen concentration with *S. aureus* wt (living and h.i.) and *S. aureus* Δnuc. Formation of NETs was microscopically analyzed and calculated as (%) NET-releasing cells. Neutrophils stimulated with *S. aureus* wt (A) and *S. aureus* Δnuc (B) release significantly more NETs compared to the amount of spontaneous NET formation both under normoxia as well as under hypoxia (normoxia: *S. aureus* wt: $33.9 \pm 10.8\%$ and ctr: $10.3 \pm 1.9\%$ /*S. aureus* Δnuc: $43.6 \pm 5\%$ and ctr: $12.8 \pm 0.9\%$, hypoxia: *S. aureus* wt: $36.9 \pm 12.8\%$ and ctr: $8 \pm 1.1\%$ /*S. aureus* Δnuc: $43.6 \pm 12.3\%$ and ctr: $8 \pm 1.1\%$). (C) The h.i. *S. aureus* caused significantly higher numbers of NET-releasing cells compared to untreated control under normoxia, while this difference was highly decreased under hypoxic conditions (normoxia: h.i. *S. aureus*: $31.8 \pm 4.3\%$ and ctr: $16.8 \pm 2.1\%$, hypoxia: h.i. *S. aureus*: $20 \pm 2.8\%$ and ctr: $12.3 \pm 2.1\%$), $n = 2$ independent experiments with each $n = 6$ images (total $n = 12$). (D) Neutrophils were treated with $10 \mu\text{g/ml}$ DPI to block NADPH oxidase activity. Formation of NETs was statistically analyzed compared to respective control in the absence of DPI by one-tailed unpaired Student's *t*-test.

cells found in inflamed tissue associated with low oxygen levels, hypoxia mimics the physiologic situation during an inflammation or infection much more accurately (34, 51). The described model should therefore be preferably used instead of the standard *in vitro* models, including the standardized NET induction assay, which are performed under normoxic conditions. Finally, in light of these results it should also be discussed if data obtained from studies performed under normoxia are really physiologically relevant. This is especially interesting since the formation of NETs was also implicated in the development of tumor-related diseases (52). Recently, Tohme et al. showed that NETs promote the development and progression of liver metastases after surgical stress. Importantly, in growing metastatic tumors, the authors found that intratumoral hypoxia accentuated NET formation (53). In line of this study, Alfaro et al. (54) demonstrated that tumor-produced IL-8 leads to extrusion of NETs in human myeloid-derived suppressor cells, which are considered an important T-cell immunosuppressive component in cancer-bearing hosts (54). Based on these data, it may be suggested that the elimination of NETs or pharmacological blocking of NET formation may reduce risks of tumor relapse. Thus, for a better understanding of the neutrophil

biology as a target for new therapeutic interventions it is urgently needed to study its activity under specific physiological relevant oxygen conditions.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Medizinische Hochschule Hannover Ethics Committee (Ethics Statement No. 3295-2016).

AUTHOR CONTRIBUTIONS

MK-B, LV, and HN: conceived and designed the experiments; LV, KB-H, DH, NB, SB, FR, and GB: performed the experiments; LV, MK-B, KB-H, NB, and GB: analyzed the data; HM, KB-H, and MK-B: wrote the paper.

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Capability of Neutrophils to Form NETs Is Not Directly Influenced by a CMA-Targeting Peptide

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During inflammatory reaction, neutrophils exhibit numerous cellular and immunological functions, notably the formation of neutrophil extracellular traps (NETs) and autophagy. NETs are composed of decondensed chromatin fibers coated with various antimicrobial molecules derived from neutrophil granules. NETs participate in antimicrobial defense and can also display detrimental roles and notably trigger some of the immune features of systemic lupus erythematosus (SLE) and other autoimmune diseases. Autophagy is a complex and finely regulated mechanism involved in the cell survival/death balance that may be connected to NET formation. To shed some light on the connection between autophagy and NET formation, we designed a number of experiments in human neutrophils and both in normal and lupus-prone MRL/lpr mice to determine whether the synthetic peptide P140, which is capable of selectively modulating chaperone-mediated autophagy (CMA) in lymphocytes, could alter NET formation. P140/Lupuzor™ is currently being evaluated in phase III clinical trials involving SLE patients. Overall our *in vitro* and *in vivo* studies established that P140 does not influence NET formation, cytokine/chemokine production, or CMA in neutrophils. Thus, the beneficial effect of P140/Lupuzor™ in SLE is apparently not directly related to modulation of neutrophil function.

Keywords: NET formation, neutrophils, autophagy, murine models of lupus, systemic lupus erythematosus, P140/Lupuzor

INTRODUCTION

Systemic lupus erythematosus (SLE) is an autoimmune disorder characterized by chronic reactivity against components of the cell nucleus that results in the formation of antinuclear autoantibodies and multiorgan involvement. It is thought that the immune system in lupus is sensitized to intracellular antigens following extended exposure to chromatin under inflammatory conditions. This can be caused by insufficient removal of cells having undergone apoptosis or the formation of neutrophil extracellular traps (NETs) (1–3). NETs contain a complex network made of processed chromatin bound to granular and selected cytoplasmic proteins. Due to the large amounts of nuclear antigens freely accessible in NETs, they are prime candidates for the

initiation or enhancement of autoimmunity and organ damage in lupus (4). Blockade of NET formation by pharmacological inhibition of peptidylarginine deiminases, which are essential for certain forms of NET formation (5), is therefore considered a promising strategy to ameliorate the clinical course of SLE (6, 7).

There is currently no cure for lupus but in the list of molecules that show clear benefits in patients with SLE are antimalarials, such as hydroxychloroquine/Plaquenil™, a therapeutic that is generally prescribed in combination with steroids or other compounds. This molecule, which unfortunately induces undesirable side effects that can be dramatic (retinopathy), directly influences lysosomal pathways. Other drugs acting at the level of the lysosome through known or assumed modulation of autophagic pathways include rapamycin, bortezomib/Velcade™ (8), 15-deoxyspergualin/Gusperimus™ that however displays serious adverse effects (most notably leukopenia), and P140/Lupuzor™ (9–11). The P140 peptide was shown to ameliorate lupus in the MRL/lpr murine model of the disease and to significantly delay mortality (12, 13). Moreover, treatment of patients with SLE with P140/Lupuzor™ convincingly improved the biological and clinical status of patients in a multicenter, randomized, placebo-controlled phase IIb trial and was considered efficacious and safe for the treatment of SLE (14). P140/Lupuzor™ recently entered into multicenter, double-blind, placebo-controlled phase III clinical trials in the US, Europe, and countries of the West Indian Ocean. The 21-mer peptide P140 encompasses residues 131–151 of the spliceosomal U1-70K protein, containing a phosphoserine at position 140 (hence its name). In MRL/lpr mice, P140 was shown to work via inhibition of autophagy, particularly chaperone-mediated autophagy (CMA), which we discovered to be hyperactivated in lymphocytes in this mouse model (10, 11, 15). In the lysosomes of MRL/lpr B cells, the phosphorylated peptide P140, but interestingly not the non-protective unphosphorylated peptide (termed 131-51), is supposed to compromise CMA by disruption of the luminal HSPA8 heterocomplexes containing HSP90 as it does *in vitro* (11). This inhibitory effect on CMA modulates autoantigen loading to MHCII molecules and therefore results in a diminished priming of autoreactive T cells. Consequentially, the proliferation of autoreactive B cells and their differentiation into deleterious autoantibody-secreting plasma cells is reduced.

Previous studies have stated that phorbol myristate acetate (PMA)-induced formation of NETs requires autophagy (16). Autophagy was also claimed to be involved in various neutrophil functions and in neutrophil-mediated inflammation (17, 18). We therefore wondered whether the effect of P140/Lupuzor™ on lupus features could be related to an influence on neutrophil function.

MATERIALS AND METHODS

Peptides

P140, scrambled P140 (ScP140), and the non-phosphorylated peptide 131-151 were synthesized and purified as described

previously (19). Homogeneity of peptides was checked by analytical high-performance liquid chromatography, and their identity was assessed by MALDI-TOF mass spectrometry.

Mice

C3H/HeOuj (thereafter named C3H) and MRL/MpJ-*Fas^{lpr}/2J* (thereafter named MRL/lpr) mice were bought from Charles River. MRL/lpr mice were also kindly given by Prof. Lars Nitschke, Division of Genetics, Department of Biology, Erlangen-Nuremberg University. Mice were kept at 12 h light/dark cycles in polystyrene cages in the animal facilities of the University of Erlangen-Nuremberg and CNRS in Strasbourg and were fed with standard rodent chow and water *ad libitum*. Experiments, which were performed with the investigators blinded to group allocation, were approved by the local ethical committees (Regierung von Unterfranken, Germany, and Comité Régional d’Ethique en Matière d’Expérimentation Animale de Strasbourg, respectively).

In Vivo Treatment with Peptides

P140, ScP140, and 131-51 peptides were resuspended in distilled water to a concentration of 10 mg/mL and further diluted in 0.9% (w/v) NaCl to a concentration of 100 µg/100 µl. Each mouse received two intravenous injections of 100 µg P140 or control peptides at days 1 and 4. Twenty-four hours after the last injection, blood was collected in heparinized collection tubes (Sarstedt) and processed and described as below.

Isolation of Human and Mouse Neutrophils

A total of 20 mL heparinized blood were taken from each human normal healthy donor. A total of 15 mL of phosphate-buffered saline (PBS) without calcium and magnesium (Thermo Fisher Scientific) were added and polymorphonuclear leukocytes (PMNs) were isolated by standard density gradient centrifugation using Ficoll (Bio-Rad). To remove contaminating erythrocytes, PMNs were subjected to short cycles of hypotonic lysis with deionized water. Finally, PMNs were adjusted to a concentration of 2×10^6 cells/mL in PBS without calcium and magnesium (Thermo Fisher Scientific).

For isolation of mouse neutrophils, single-cell suspensions were prepared from spleens of mice by squeezing through a 70 µm cell strainer. After hypotonic lysis of erythrocytes, neutrophils were isolated by negative selection using the EasySep™ mouse neutrophil enrichment kit (Stemcell Technologies) according to the manufacturer’s instruction.

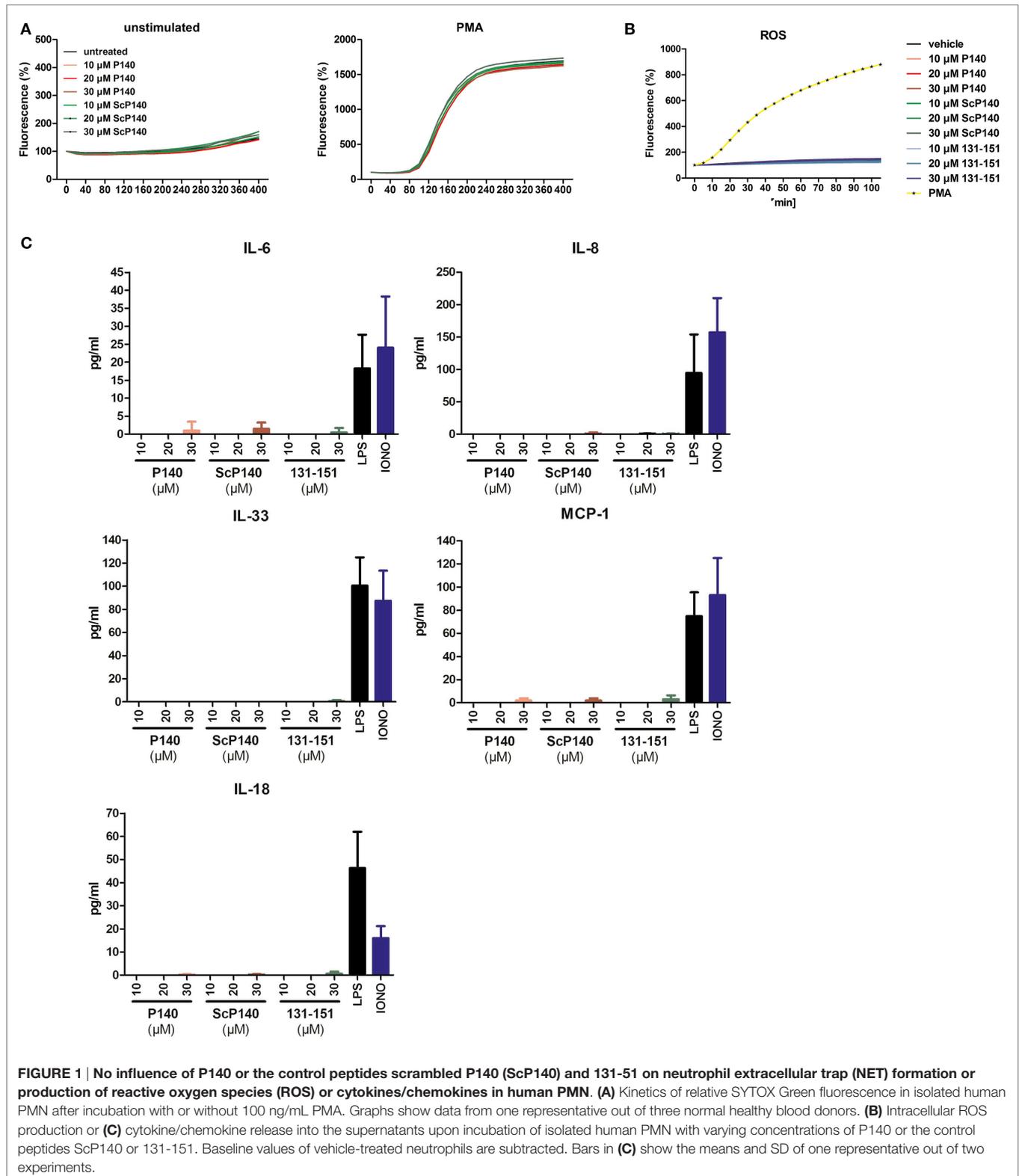
Purity of isolated neutrophils was checked by flow cytometry and was above 95% (human neutrophils) or 85% (mouse neutrophils), respectively. All experiments were approved by the ethical committee of the University of Erlangen-Nuremberg.

Plate Reader-Based Quantification of NET Formation

Isolated neutrophils were adjusted to a concentration of 2×10^6 cells/mL in Hanks’ balanced salt solution (HBSS; Thermo

Fisher Scientific). Cell suspension (100 μ L) was pipetted into each well of a 96-well cell plate. A total of 100 μ L of HBSS and 5 μ M Sytox Green (Thermo Fisher Scientific) containing either PMA (200 ng/mL; Sigma), ionomycin (2 μ g/mL; Sigma), or vehicle

control were added to the cells. The plate was tightly sealed and analyzed in an infinite[®] 200 pro plate reader (TECAN). Relative fluorescence units were normalized to the starting values and the respective vehicle control.



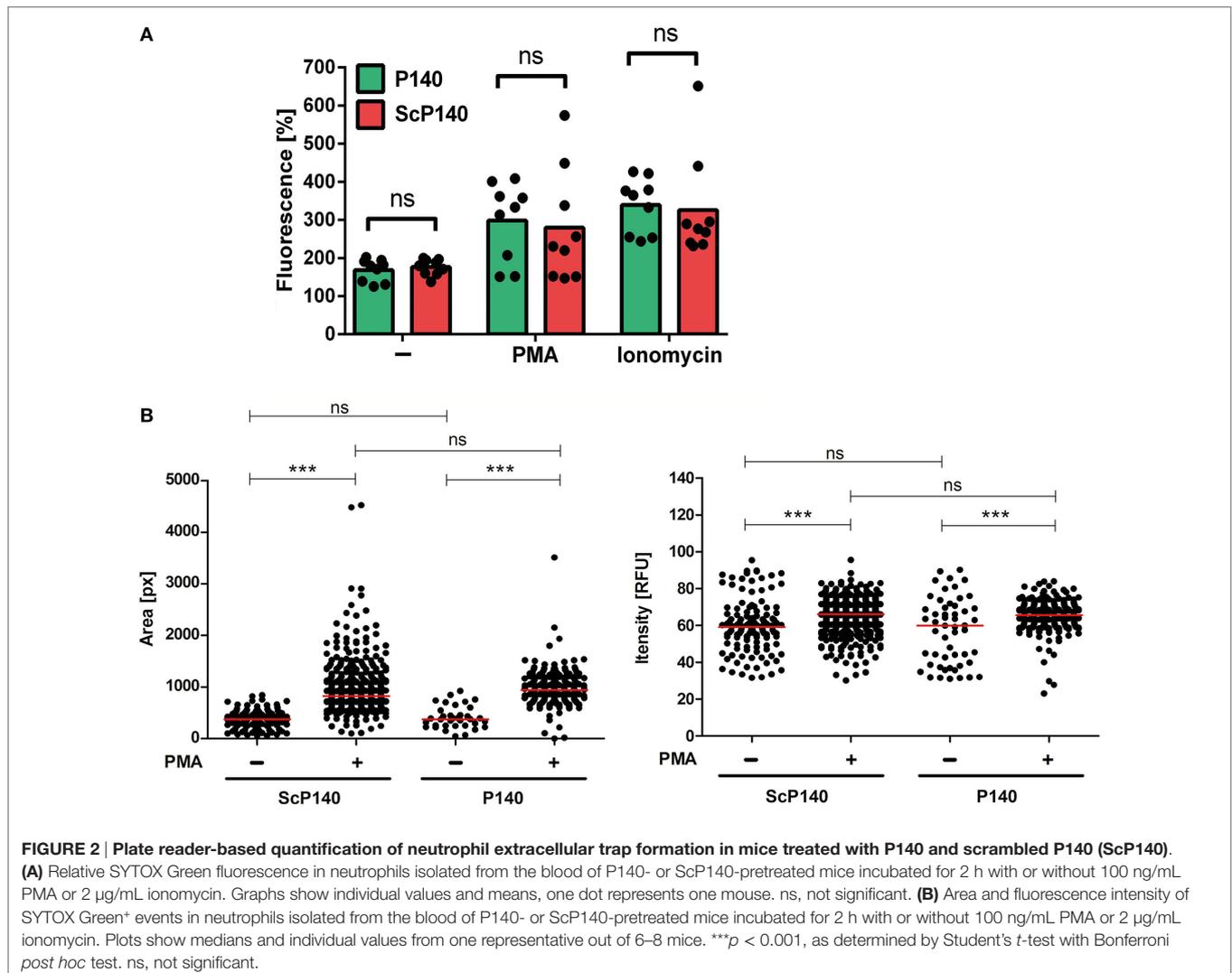
Immunohistochemical Analysis of NET Formation

Isolated neutrophils were adjusted to a concentration of 2×10^6 cells/mL in HBSS containing calcium and magnesium. A total of 100 μ L of cell suspension was added to each well of an 8-well cell chamber slide (Thermo Fisher Scientific). A total of 100 μ L of HBSS containing either 200 ng/mL PMA, 2 μ g/mL ionomycin, or vehicle control were added to the cells. The chamber slide was incubated at 37°C and 5% CO₂ for 2 h. Subsequently, 1% (v/v) paraformaldehyde (Merck) was added to each well and the preparations incubated for 18 h at 4°C. Samples were blocked with 10% (v/v) fetal calf serum (FCS; Biochrome)/2% (w/v) bovine serum albumin (BSA) in PBS for 1 h at room temperature. Primary antibody for neutrophil elastase (NE) (Abcam ref. ab21595; 1:200) or citrullinated histone H3 (Abcam ref. ab1503; 1:100) were added in 10% FCS/2% BSA in PBS for 18 h at 4°C. Slides were washed three times with PBS and secondary Cy5-conjugated anti-rabbit IgG antibody (Jackson ImmunoResearch) was added for 1.5 h at room temperature in the dark. Slides were

washed with PBS. Staining solution containing 2.5 μ M SYTOX Green in PBS was added for 15 min at room temperature. Slides were washed with H₂O and samples were embedded in mounting medium (BIOZOL). Slides were analyzed the same day on a BZ-X710 microscope (Keyence). Events positive for SYTOX Green were analyzed with regard to area and mean intensity by Photoshop CS5 software. Percentage of NETs was defined as PI/NE double-positive events with >3-fold mean nuclear size on three random slide sections.

Analysis of Autophagy

Spleens were homogenized through a 70 μ M strainer, washed, and seeded at a concentration of 4×10^6 cells/well in a 48-well plate with 1 mL of complete culture media in the presence or absence of lysosomal protein inhibitors pepstatin A (5 μ g/mL; ref. P5318; Sigma) and E64d (5 μ g/mL; ref. E8640; Sigma). After 4 h at 37°C under 5% CO₂, cells were washed, lysed by adding 160 μ L/sample of Laemmli buffer (ref. 161-0737; Bio-Rad) containing 5% (v/v) β -mercaptoethanol, and finally boiled at 95°C



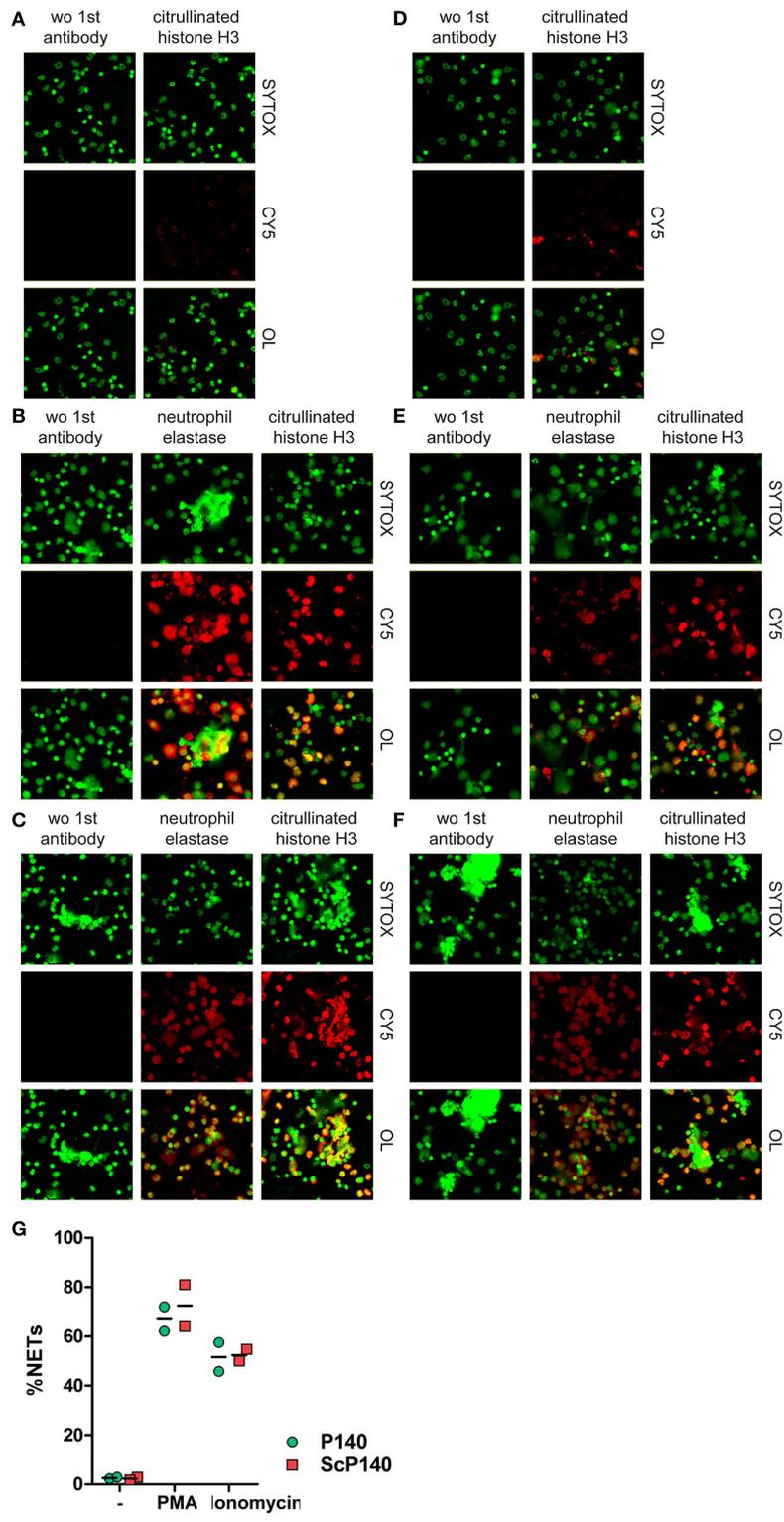


FIGURE 3 | Morphological analysis and quantification of neutrophil extracellular traps (NETs) in P140 and scrambled P140 (ScP140)-treated mice by fluorescence microscopy. Representative immunofluorescence images of SYTOX Green-, neutrophil elastase (NE)-, and citrullinated histone H3 (citH3)-stained neutrophils isolated from ScP140- (**A–C**) and P140- (**D–F**) treated mice and incubated without external stimulus (**A,D**), with PMA (**B,E**) or with ionomycin (**C,F**). Left panels show staining controls incubated without (wo) primary antibody to NE or citH3. CY5 stands for control Cyanin 5 and OL stands for overlay. (**G**) Quantitative analysis of NETs. Scatter plots show individual values and mean of %NETs (defined as % PI⁺/NE⁺ cells with >3-fold mean nuclear size) from two mice.

(5 min) before loading (20 μ L samples, equivalent to 0.5×10^6 cells) on 4–20% SDS-PAGE gradient gels for analysis. For western immunoblotting, the following antibodies were used: rabbit SQSTM1/p62 (0.5 μ g/mL; ref. ab109012), rabbit HSPA8 (0.5 μ g/mL; ref. ab51052), and rabbit ATG12/5 (ref. ab155589), all from Abcam, and mouse microtubule-associated-protein light chain 3b (MAP1LC3B) (0.5 μ g/mL; ref. M186-3) from MBL. Secondary antibodies were peroxidase-conjugated goat anti-mouse IgG Fc (50 ng/mL; ref. 115-035-008) and goat anti-rabbit IgG Fc (25 ng/mL; ref. 111-035-008) from Jackson ImmunoResearch. ACTB-reacting antibodies were from Santa Cruz (10 ng/mL; ref. sc47778).

Measurement of Reactive Oxygen Species (ROS) and Inflammatory Mediators

For measurement of ROS in human PMNs, lithium-heparinized blood was incubated with dihydrorhodamine 123 (3 μ g/mL, Molecular Probes) for 15 min at 37°C. Cells were then stained with anti-human CD14-eFluor450 and CD16-APC/Cy7, and ROS production was measured after incubation with peptides (10, 20, or 30 μ M) or PMA (100 ng/mL) for 15 min at 37°C. Before analysis on a Beckman Coulter Gallios™, FACS samples were subjected to hypotonic water lysis.

Cytokines/chemokines in supernatants of human PMNs after 18 h incubation with varying concentrations of peptides, 2.5 μ g/mL LPS, or 1 μ M ionomycin in RPMI medium including 1% autologous serum were analyzed by Legendplex bead technology (BioLegend) and quantified on a Gallios™ cytofluorometer (Beckman Coulter).

Statistical Analysis

For calculation of statistical differences, we used Mann–Whitney *U* test or unpaired Student's *t*-test with Welch's correction, Dunnett's, or Bonferroni's *post hoc* test, where applicable. Adjusted $p < 0.05$ was considered statistically significant. Computations and charts were performed using the GraphPad Prism 6 software.

RESULTS

To investigate a potential direct effect of P140 on NET formation, we pre-incubated isolated human PMNs with increasing concentrations of P140 or control ScP140 peptide (10–30 μ M) for 1 h and measured SYTOX Green fluorescence after treatment with the NET-inducing agent PMA. The concentrations of peptides used for incubation have been previously shown not to be toxic to cells, except that prolonged incubation with concentrations over 20 μ M induces granzyme B-dependent apoptosis in specific T cell subsets (13). Quantitative analysis of NET formation was performed in an established plate reader-based assay. Treatment with P140 or ScP140 did not reveal any effect on the capacity of PMNs to undergo NETosis in response to PMA (Figure 1A). Also neither P140 nor ScP140 directly induced NET formation, production of ROS, or cytokine/chemokine production in these cells (Figures 1B,C).

Isolated PMNs have a very limited lifespan, which precludes extended *in vitro* treatment with P140. To overcome this

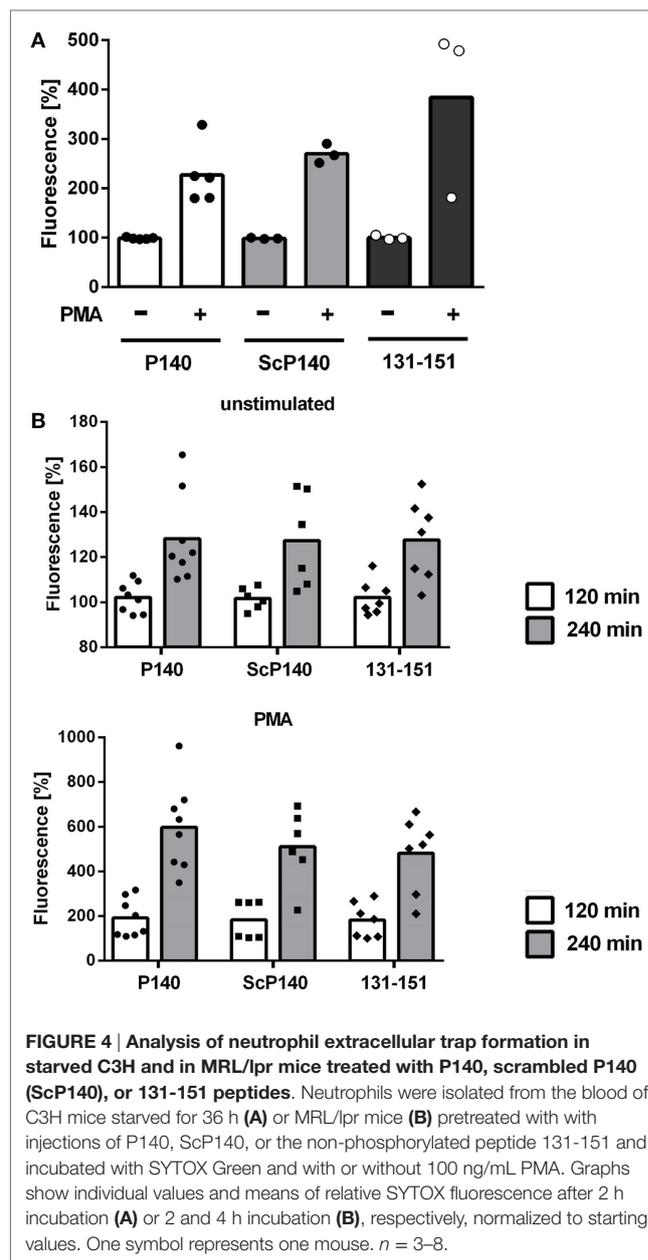


FIGURE 4 | Analysis of neutrophil extracellular trap formation in starved C3H and in MRL/lpr mice treated with P140, scrambled P140 (ScP140), or 131-151 peptides. Neutrophils were isolated from the blood of C3H mice starved for 36 h (A) or MRL/lpr mice (B) pretreated with with injections of P140, ScP140, or the non-phosphorylated peptide 131-151 and incubated with SYTOX Green and with or without 100 ng/mL PMA. Graphs show individual values and means of relative SYTOX fluorescence after 2 h incubation (A) or 2 and 4 h incubation (B), respectively, normalized to starting values. One symbol represents one mouse. $n = 3-8$.

limitation, we administered two intravenous doses of 100 μ g P140 or ScP140, respectively, into mice and sacrificed the mice 1 day later. Injections were performed in C3H mice because this strain harbors elevated number of neutrophils in the circulation (20), which resembles the human situation more closely than other mouse strains. Neutrophils from the blood were then purified from P140/ScP140-treated mice and NET formation was induced with PMA and ionomycin, respectively. Injection of P140 did not significantly influence the amount of total SYTOX fluorescence (Figure 2A). Also the mean area or fluorescence intensity of SYTOX Green⁺ events was not significantly different between cells isolated from P140 and ScP140-treated mice (Figure 2B).

SYTOX Green detects extracellular DNA, which can occur in NETs and other forms of cell death (21). However, DNA

released during necrosis and other forms of cell death is not normally associated with material from neutrophil granules. NETs are defined by co-localization of nuclear content with granule proteins, such as myeloperoxidase, NE, or antimicrobial molecules (22). Furthermore, the citrullination of histones is typical for NET formation in response to some but not all triggers (5, 23, 24). We therefore performed a more thorough investigation of the morphological changes in PMA- and ionomycin-stimulated and unstimulated cells in neutrophils from mice pretreated with P140 or ScP140. This study was conducted by immunofluorescence microscopy for SYTOX Green, NE, and citrullinated histone H3. Irrespective of the marker that was followed, no significant differences were observed

between the groups of C3H mice that had received the P140 and ScP140 peptides (Figure 3). Taken together, these results show that treatment with P140 does not affect NET formation in normal mice.

P140 has been previously shown to be active under conditions of increased autophagic flux (11, 15). Autophagy is activated in response to nutrient deprivation in almost all cell types. We therefore analyzed the effect of P140 in starved C3H mice. Again, NET formation was not significantly influenced upon injection of P140, ScP140, or non-phosphorylated peptide 131-51, as determined by plate reader-based fluorescence assay (Figure 4A) and morphology of immunofluorescence images of isolated mouse neutrophils (not shown).

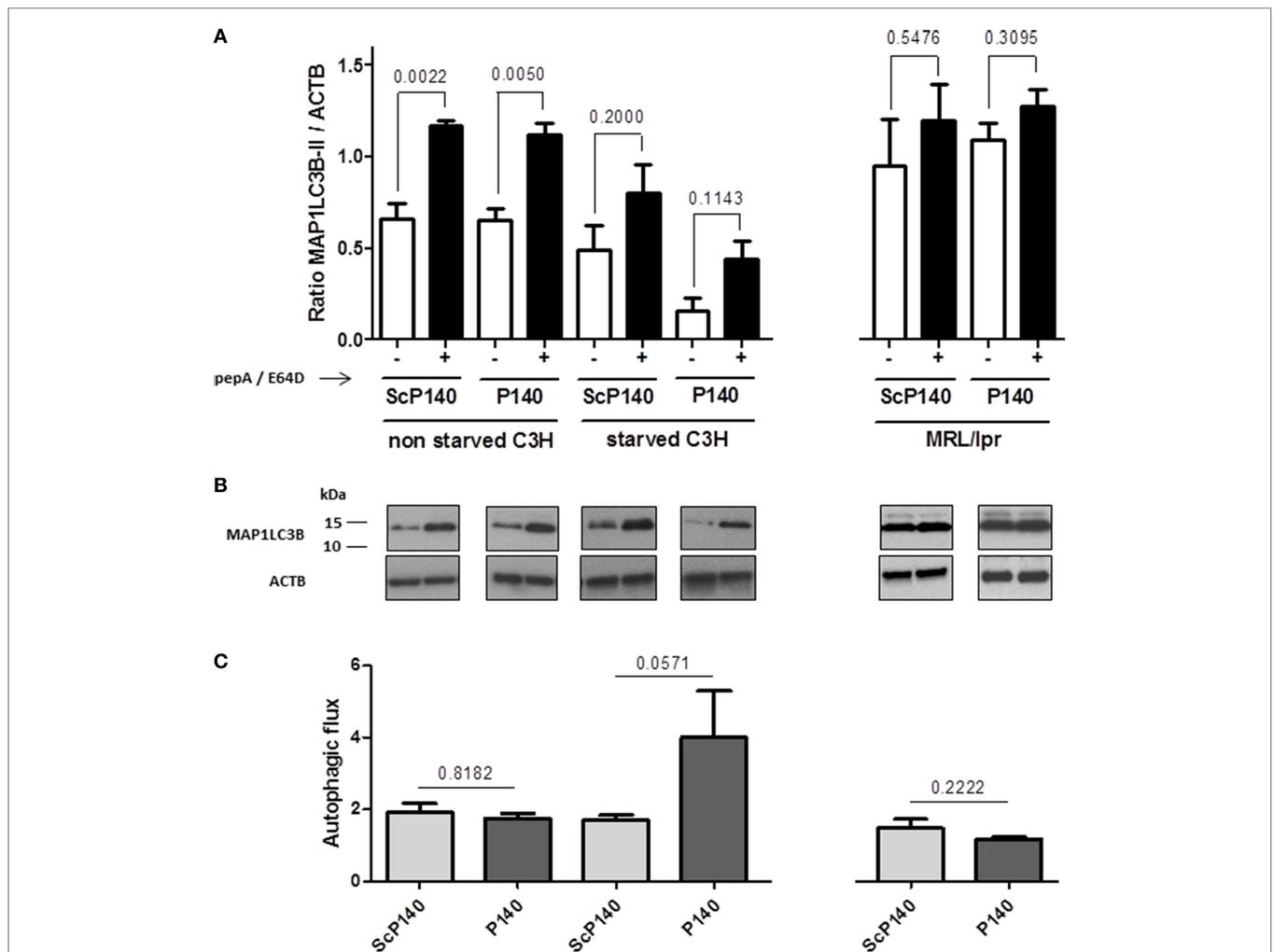


FIGURE 5 | Effect of P140 peptide on autophagic flux evaluated by measuring MAP1LC3B-II levels in total splenocytes from non-starved mice and starved C3H mice and MRL/lpr mice. Cell lysates were resolved by SDS-PAGE, transferred onto polyvinylidene difluoride membranes before staining with anti-microtubule-associated-protein light chain 3b (MAP1LC3B) antibodies. When indicated, cells were treated (+) or not (-) during the last 4 h of the culture with 5 $\mu\text{g}/\text{mL}$ pepstatin A and 5 $\mu\text{g}/\text{mL}$ E64d to block lysosomal degradation. **(A)** MAP1LC3B-II levels were evaluated by densitometry and normalized to ACTB/ β -actin. Histogram bars represent the means of individual experiments with SEM. * $p < 0.05$, using a Mann-Whitney U test to compare the data obtained in the presence or absence of protease inhibitors. **(B)** Representative immunoblot. ACTB was used as loading controls. **(C)** Autophagic flux as measured by comparing values of MAP1LC3B-II in the presence of lysosomal protease inhibitors divided by values of MAP1LC3B-II in the absence of lysosomal protease inhibitors. Histogram bars represent the means of individual experiments with SEM. * $p < 0.05$ using a Mann-Whitney test between the untreated and treated mice, in each condition. The groups were constituted of $n = 6$ for non-starved C3H mice, $n = 4$ for starved C3H mice, and $n = 5$ for MRL/lpr mice.

So far, none of our data indicated that the ameliorative effect of P140/Lupuzor™ observed in patients with SLE and lupus mice could be related to the inhibition of NET formation. However, we had not yet looked at the influence of P140 under conditions of established lupus disease. We therefore analyzed NET formation in P140- and control peptide-treated MRL/lpr mice. Again no differences could be seen between MRL/lpr mice treated with P140, ScP140, or 131-151 peptides (Figure 4B).

The above data show no obvious evidence that the P140 peptide might directly influence neutrophils to undergo NET formation. We then investigated further the effect of P140 peptide on autophagy processes. The autophagic flux was measured by visualizing the expression of a well-established autophagy marker, namely, ATG8/MAP1LC3B, in calibrated conditions, in the presence or absence of lysosome proteases E64d and pepstatin A as described (25). We confirmed the existence of an active autophagic flux in total splenocytes of normal C3H mice, which however, was not altered by P140 peptide (Figures 5A–C, left). In spleen cells from starved C3H mice, however, no active flux was detectable and as expected, therefore, there was no significant effect of P140 peptide (Figures 5A–C). The same result as shown with cells from starved C3H mice was observed in MRL/lpr (activated) splenocytes (Figures 5A–C, right). For the same reasons, no statistically significant effect of P140 peptide was visualized when other markers of macroautophagy and CMA, such as ATG12/5 (that participates to the autophagosome formation), the autophagy substrate SQSTM1/p62, and the heat shock protein HSPA8, were followed in the whole splenocyte population (Figures 6A–C).

The results generated with normal C3H splenocytes, in which autophagic flux is active and showing no effect of P140 peptide, probably result from the fact that in normal (unstressed) cells, the peptide entry notably differs from the one it uses in activated/stressed cells and could not reach the cell compartment where it exerts its function (11).

The absence of P140 effect in total splenocytes from 16-week-old diseased MRL/lpr mice contrasts with earlier results generated with B cells that were purified from the spleen of young (8-week-old) MRL/lpr mice (15). In B cells, P140 could effectively reduce the excessive autophagic flux. To investigate if P140 influences autophagy directly in neutrophils, we isolated splenic neutrophils from MRL/lpr mice treated with either 100 µg P140 or ScP140 (Figure 7). Interestingly, and in contrast to what was previously shown in lymphocytes (11, 15), autophagic flux was not activated in neutrophils from diseased MRL/lpr mice. Accordingly, P140 did not affect expression of autophagy markers as compared to ScP140.

DISCUSSION

Many aspects of neutrophil biology have been implicated in the pathogenesis of SLE (4). Especially the formation of NETs has been in the center of research during the last years (26–30). Typical during PMA-induced NETosis is the generation of many vesicles before plasma membrane rupture (16, 31). These vesicles have a double phospholipid bilayer and are believed to originate from the nuclear envelope, which disintegrates during

NET formation. Finally, but still before plasma membrane permeabilization, nuclear chromatin decondenses and mixes with the contents of the granules; this is essential for formation of functional NETs. These arguments led us to examine if P140/Lupuzor™, a molecule that was shown to modulate autophagy in lymphocytes, could also alter NET formation or other aspects of neutrophil function, such as ROS production and the release of inflammatory mediators, which are important in both certain kinds of NET formation and the pathogenesis of lupus (31–33).

At this stage, however, our experimental data do not allow us to endorse this idea, at least in the experimental setting used in this study (namely, with diseased lupus mice). Treatment with P140 did not induce production by PMNs of ROS, cytokines, or chemokines and did not modulate NET formation in *in vitro* and *in vivo* assays. Consequently, a connection between CMA or macroautophagy and neutrophil functions cannot be drawn, in contrast to B lymphocytes, in which P140 modulates autophagy (11, 34). Several possible explanations can be put forward to explain this lack of effect.

First, and most importantly, we did not observe an increase of autophagic flux in neutrophils from diseased mice, which explains the lack of P140 activity in this cell type, because P140

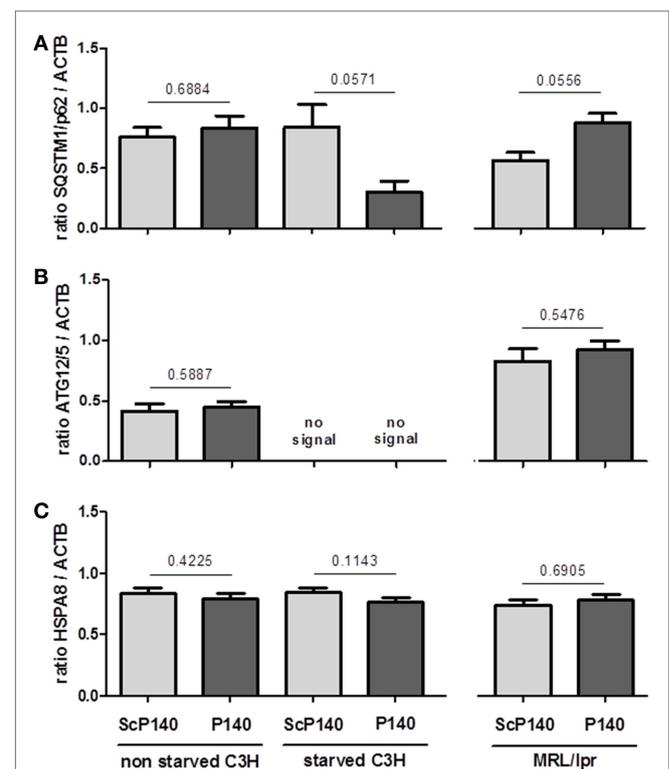


FIGURE 6 | Effect of P140 peptide on autophagy evaluated by measuring SQSTM1, ATG12/5, and HSPA8 levels in total splenocytes from non-starved mice, starved C3H mice, and MRL/lpr mice. Three markers were followed, namely, (A) SQSTM1, (B) ATG12/5, and (C) HSPA8. The groups were constituted of $n = 6$ for non-starved C3H mice, $n = 4$ for starved C3H mice, and $n = 5$ for MRL/lpr mice. Histogram bars represent the means of individual experiments with SEM. * $p < 0.05$ using a Mann–Whitney test between the untreated and treated mice, in each condition.

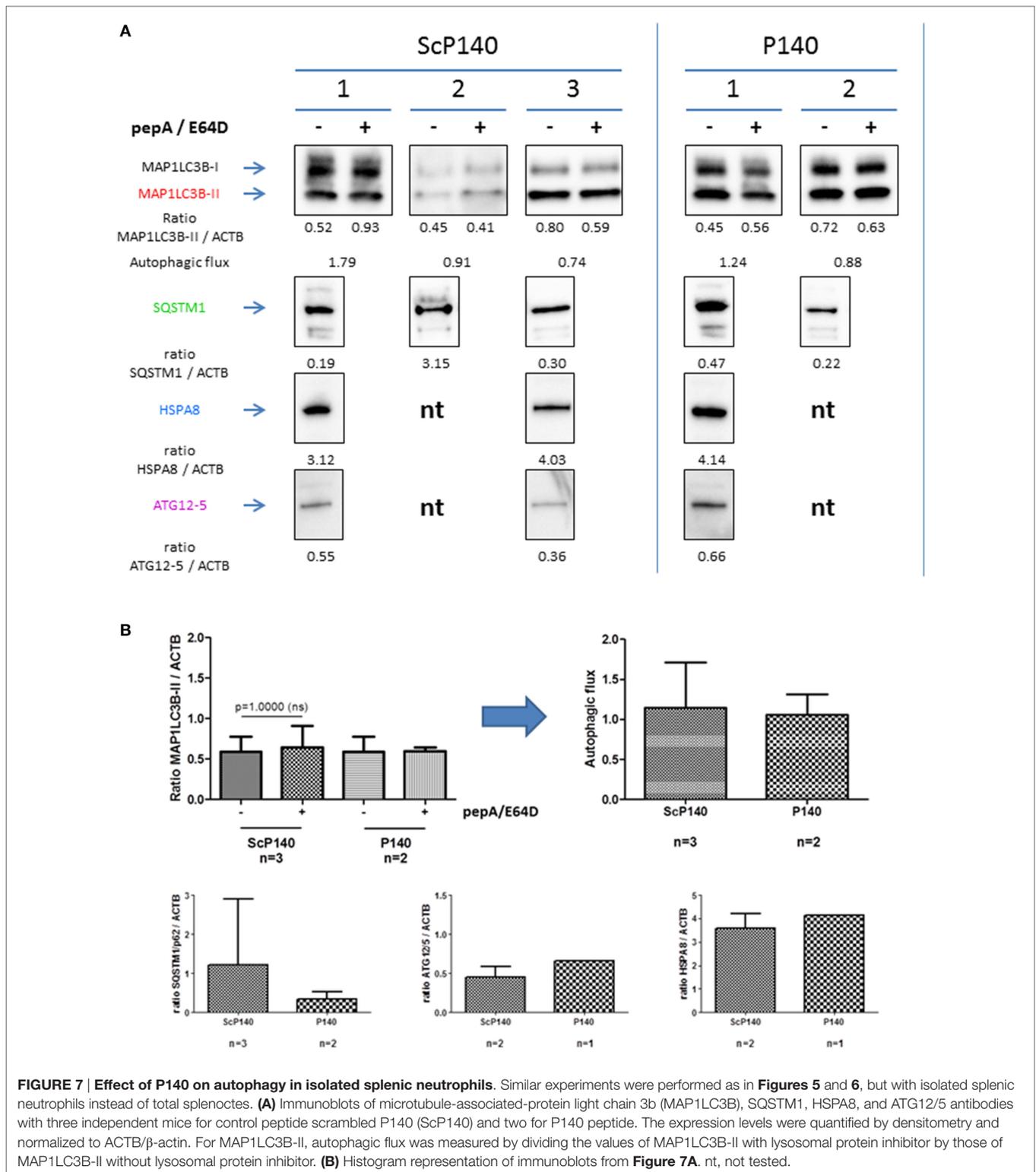


FIGURE 7 | Effect of P140 on autophagy in isolated splenic neutrophils. Similar experiments were performed as in **Figures 5** and **6**, but with isolated splenic neutrophils instead of total splenocytes. **(A)** Immunoblots of microtubule-associated-protein light chain 3b (MAP1LC3B), SQSTM1, HSPA8, and ATG12/5 antibodies with three independent mice for control peptide scrambled P140 (ScP140) and two for P140 peptide. The expression levels were quantified by densitometry and normalized to ACTB/ β -actin. For MAP1LC3B-II, autophagic flux was measured by dividing the values of MAP1LC3B-II with lysosomal protein inhibitor by those of MAP1LC3B-II without lysosomal protein inhibitor. **(B)** Histogram representation of immunoblots from **Figure 7A**. nt, not tested.

is active under conditions of autophagic flux only. Second, no active flux in splenocytes of starved C3H mice could be visualized. It is widely accepted that autophagy is activated in response to nutrient deprivation in a variety of cell types. However, it has to be mentioned that when animals are starved, effects are more

particularly observed in organs like the liver as compared to immune cells, and this feature could explain our results. Third, the *in vivo* administration protocol of P140 chosen in the present work could have influenced the results. An effect of P140 on autophagy markers in MRL/lpr splenocytes was visualized 5 days

after a single injection of P140. This optimized protocol, however, could not be used with splenic neutrophils due to their short life expectancy and higher turn-over in the spleen. Since our experiments were focused on neutrophils, we decided to apply P140 a second time, 1 day before the isolation of neutrophils. It is possible that P140 administered under this alternative protocol we were obliged to apply, would have also no effect in lymphocytes.

Although P140 does not seem to directly influence NET formation, an indirect effect cannot be excluded. Patients with SLE show an increased rate of spontaneous NET formation and this phenotype is likely to be associated with the inflammatory status of the patients. Amelioration of the disease may in turn decrease the pre-activation of neutrophils resulting in less reactive neutrophils and reduced NET formation. To this regard, it is interesting to mention that compared to CBA/J control mice, HSPA8, a receptor of P140, is overexpressed at the cell surface of CD11b⁺ Gr-1⁺ granulocytes collected from the spleen of MRL/lpr mice (15). Upon P140 intravenous administration, granulocytes, as also monocytes and lymphocyte subsets that are over-represented in the peripheral blood of MRL/lpr mice, egress from the blood. Noticeably, contrary to other white blood cells subsets, which recolonize the blood in a few days, granulocytes remain at their basal level for at least 10 days before reappearing in the peripheral blood of P140-treated MRL/lpr mice (Schall et al., unpublished). These findings highlight the fact that P140 might be an attractive tool to target lupus neutrophils.

Since autophagy is often upregulated in response to cellular stress, further studies need to be conducted to analyze the influence of NET formation under such conditions.

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ETHICS STATEMENT

All experiments were approved by Regierung von Unterfranken, Germany, and Comité Régional d'Ethique en Matière d'Expérimentation Animale de Strasbourg. All subjects gave written informed consent.

AUTHOR CONTRIBUTIONS

CM planned and performed *in vitro* and *in vivo* experiments on NET formation, conducted data analysis, and drafted the manuscript. NS performed the experiments on autophagy and conducted data analysis. BG and FM performed experiments on autophagy. JH and DK performed experiments on NET formation. MH and SM planned the experiments, provided scientific input, and wrote the manuscript.

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