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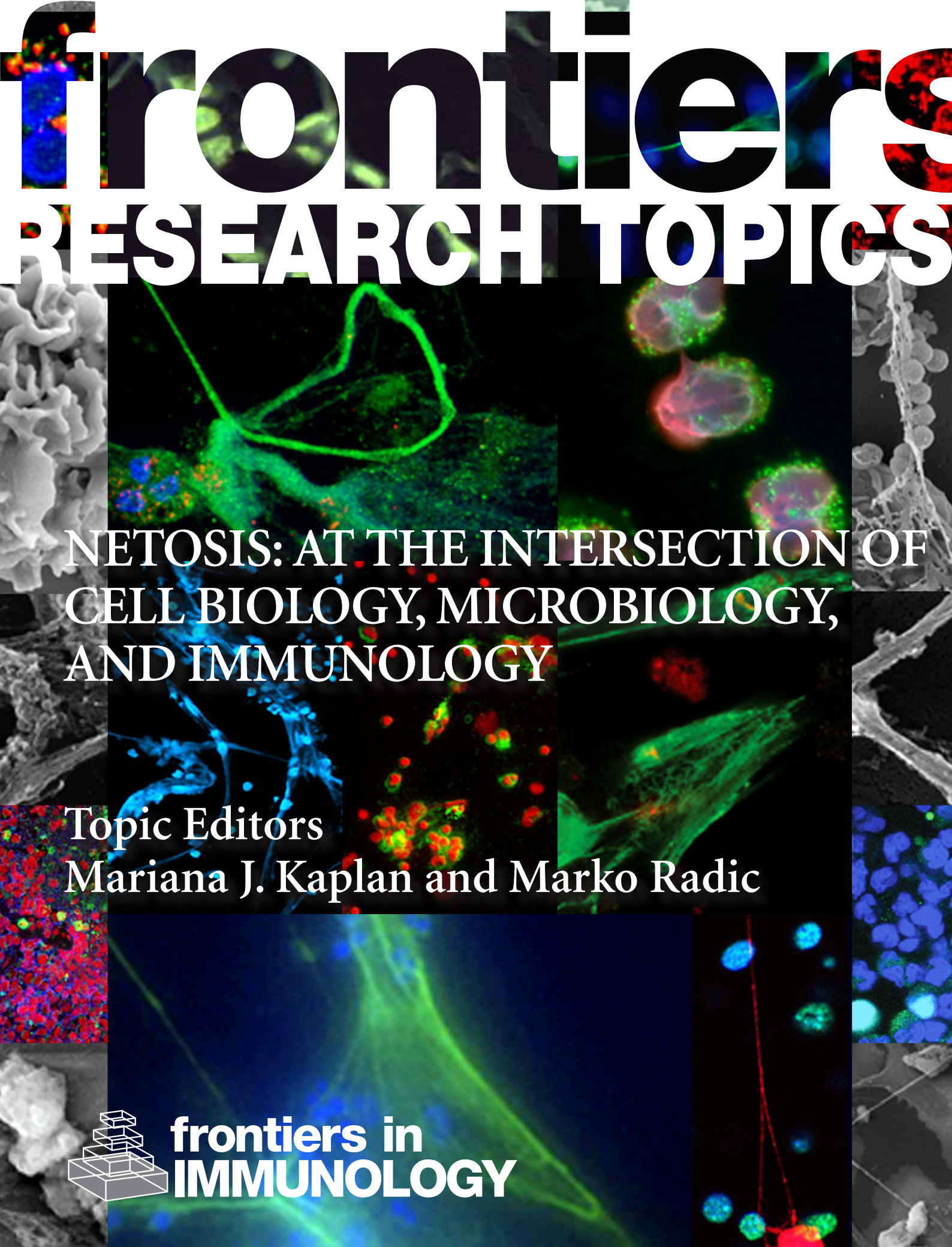

NETOSIS: AT THE INTERSECTION OF CELL BIOLOGY, MICROBIOLOGY, AND IMMUNOLOGY

Topic Editors
Mariana J. Kaplan and Marko Radic

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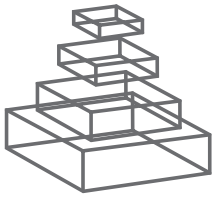

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ISSN 1664-8714
ISBN 978-2-88919-158-1
DOI 10.3389/978-2-88919-158-1

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NETOSIS: AT THE INTERSECTION OF CELL BIOLOGY, MICROBIOLOGY, AND IMMUNOLOGY

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NETosis is a unique form of cell death that is characterized by the release of decondensed chromatin and granular contents to the extracellular space. The initial observation of NETosis placed the process within the context of the innate immune response to infections. Neutrophils, the most numerous leukocytes that arrive quickly at the site of an infection, were the first cell type shown to undergo extracellular trap formation. However, subsequent studies showed that other granulocytes are also capable of releasing nuclear chromatin following stimulation. The extracellular chromatin acts to immobilize microbes and prevent their dispersal in the host. Bacterial breakdown products and inflammatory stimuli induce NETosis and the release of NETs requires enzyme activities. Histones in NET chromatin become modified by peptidylarginine deiminase 4 (PAD4) and cleaved at specific sites by proteases. NETs serve for attachment of bactericidal enzymes including myeloperoxidase, leukocyte proteases, and the cathelicidin LL-37.

While the benefit of NETs in an infection appears clear, NETs also figure prominently at the center of various pathologic states. Therefore, it is important for NETs to be efficiently cleared; else 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. Recent studies identified aberrant NET synthesis and/or clearance in inflammatory/autoimmune conditions such as systemic lupus erythematosus (SLE), psoriasis, ANCA-positive vasculitis, gout and Felty's syndrome. In the case of SLE, for example, it appears that LL-37 exposed in the NETs may be a significant trigger of type I Interferon responses in this disease. Recent evidence also implicates aberrant NET formation in the development of endothelial damage, atherosclerosis and thrombosis.

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 approach the 10-year-anniversary of the initial discovery of NETosis, it is useful and timely to review the so far identified mechanisms and pathways of NET formation, their role in bacterial and fungal defense and their putative importance as inducers of autoimmune responses. We look forward to a rich and rigorous discussion of these and related issues that benefit from interdisciplinary approaches, collaborations and exciting discoveries.

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IMMUNOLOGY

EDITORIAL
published: 24 June 2013
doi: 10.3389/fimmu.2013.00160

Extracellular chromatin traps interconnect cell biology, microbiology, and immunology

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In a scientist's career, the often elusive goal is to discover a biological process that is both unexpected and, once revealed, of obvious importance. In that case, the new observation will attract the attention of others and prove to be of crucial significance in several overlapping areas of science. Nearly a decade after the original discovery of neutrophil extracellular traps (NETs) by Brinkmann et al. (2004), NETs have been firmly established as a fundamental biological mechanism used by neutrophils to respond to infections. Scores of additional reports confirmed and extended the initial observations to provide insights into the contributions of NETs to bacterial, fungal, viral, and protozoan infections (Brinkmann and Zychlinsky, 2012). NET release was shown to be the regulated outcome of a programed cell death process designated NETosis, and NETs were linked to autoimmunity and cardiovascular disease. As Editors, we are pleased by the enthusiastic response of 20 groups of scientists from 4 continents who participated in our effort to summarize, evaluate, and extend understanding of NETosis. This booklet captures some of the excitement that was shared with us by the authors.

Goldmann and Medina (2013) introduce the topic of NETosis by describing aspects that differentiate NETosis from other forms of cell death and by reminding the readers that extracellular traps are produced by eosinophils, mast cells, and even monocytes/macrophages in addition to neutrophils. Thus, NETosis is more accurately called ETosis. In alternative manner, DNA for ETs can be derived from the nucleus or from mitochondria and the DNA can be released without completely inactivating all functions of the cell releasing the DNA. The authors provide a careful summary of steps in ETosis and illustrate the process with electron micrographs, while reminding us that many open questions still remain.

Brinkmann et al. (2013) present a semi-automated method for enumerating cells that traverse sequential stages in NETosis. This useful and easily adaptable method by the original discoverers of NETs relies on dual channel fluorescence and compares binding of anti-chromatin antibodies relative to staining with a DNA-intercalating dye. Image analysis computes the percentage of NETting neutrophils. The authors give examples of Toll-like receptor stimuli, crystals, and cytokines that induce NETosis. The new method should be ideally suited to high throughput screening for drugs that affect the efficiency of NET release.

Additional contributors discuss mechanistic features of NETosis. Rohrbach et al. (2012) focus on the role of peptidylarginine deiminase 4 (PAD4) in the regulation of NETosis. By reviewing PAD4 structure and function, the authors discuss inhibitors of PAD4 and their potential use in suppressing NETosis. PAD4 is a particularly appealing target for inhibition (or enhancement) of NETosis because the reaction pathway of this enzyme is understood in molecular detail.

Leshner et al. (2012) provide an elegant illustration of PAD4's potential to induce NETs. They demonstrate that overexpression of PAD4 in heterologous cells leads to chromatin decondensation and unfolding of NET-like chromatin. Induced PAD4 expression leads to histone deimination and the release of NET-like webs from U2OS and NIH 3T3 cells. Specifically, the authors show that deimination of arginines in the amino terminus of histone H3 reduces the binding of the heterochromatin protein HP1β to the adjacent lysine 9 in H3. The disruption of higher order chromatin packing may provide a molecular switch that regulates NET release in NETosis.

Neeli and Radic (2013) observed that two ancient regulatory enzymes from the protein kinase C (PKC) family exhibit opposite effects on PAD4 activity. The authors used PMA and ionophore, two compounds that induce NETosis, along with inhibitors of PKC activity, to identify PKCα as an inhibitor of PAD4 and PKCζ as a facilitator of PAD4-mediated histone deimination. The authors conclude that evolutionary pressure ensured precise regulation of histone deimination because NETs make important, yet potentially dangerous contributions to innate immunity.

The important question of whether extracellular chromatin traps are involved in autoimmune disease is critically reviewed by two groups of scientists. Darrah and Andrade (2013) present an analytical comparison of different modes of cell death and contrast the potential contributions of apoptosis, necrosis, and NETosis to the release of nuclear autoantigens. The authors survey different possibilities that may link NETosis to the stimulation of the adaptive immune system in systemic autoimmune disorders. The large number of autoantigens that are integral components of NETs provide a compelling argument for NETosis' role in the pathogenesis of autoimmune disorders and the loss of immune tolerance.

Knight et al. (2012) take a broad view to summarize evidence linking NETosis with specific aspects of autoimmune disorders. By highlighting recent findings from studies of small vessel vasculitis, psoriasis, gout, Felty's syndrome, and systemic lupus, the authors suggest two mechanisms for how NETs could participate in disease pathogenesis. One mechanism involves the contribution of NETs to organ damage; the other sees NET proteins as the trigger or exacerbating factor in autoimmunity. In particular, post-translational modifications of NET proteins are highlighted as features that make them into "altered self," autoantigens that may break tolerance and initiate autoimmunity.

Schorn et al. (2012) investigate sterile inflammation that is caused by crystals that form in the joints and kidneys of patients suffering from gout. The crystals consist of monosodium urate (MSU) and induce NETosis in purified neutrophils. The NETosis is dependent on reactive oxygen production, thus it likely follows established NETosis pathways. The authors detect NETs in biopsies of gouty arthritis patients and examine downstream pathways involved in clearance of NETs. Surprisingly, they find that molecules that function in the clearance of apoptotic cells (complement factor C3b, C-reactive protein, and galectin-9) have limited affinity for NET chromatin and thus may not effectively contribute to NET clearance.

Kambas et al. (2012) review evidence implicating neutrophils and NETs in the release of tissue factor (TF), an important regulatory protein that orchestrates initial steps in coagulation and whose excess may directly lead to thrombosis. Even though neutrophils are clearly involved in thrombotic events, the issue of neutrophil production of TF is more controversial. The authors review recent publications in this area and highlight their own data that identify TF as a component of NETs with important implications for thrombosis.

Hahn et al. (2012) summarize evidence for the participation of NETs in reproductive complications, ranging from infertility to preeclampsia and fetal loss. The authors introduce this fascinating topic by reviewing the essential role of neutrophils in the estrus cycle and pregnancy. They shift focus to data suggesting that neutrophils are involved in the complications of preeclampsia, the sudden rise in blood pressure that can jeopardize the life of the mother and fetus. The proposed role of NETosis is supported by an activated, pro-inflammatory state of neutrophils in normal pregnancy, high levels of neutrophil elastase (a NET component) in preeclampsia, and detection of cell-free DNA in circulation. The authors describe reactions between normal neutrophils and highly purified placental micro-debris to argue for the involvement of NETs in preeclampsia.

Parker and Winterbourn (2013) highlight contributions of reactive oxygen products and specific enzymes such as myeloperoxidase (MPO) in facilitating NET release. These authors point to the association of MPO and neutrophil proteases with NET chromatin to connect reactive oxygen functions in NETosis and in bacterial killing. Two major insights arise from these studies. First, different stimuli induce NET release by different mechanisms. Thus, NETosis is MPO-dependent if PMA is the stimulus, whereas MPO is dispensable if *Pseudomonas aeruginosa* induces NETs. Second, bacterial killing by NETs is enhanced if exogenous H_2O_2 is supplied, suggesting that MPO in NETs converts H_2O_2 to more potent bactericidal oxygen species.

Almyroudis et al. (2013) focus on NADPH oxidase contributions to NETosis by highlighting the different effects of NETosis and apoptosis on immune responses and inflammation. The authors argue that for inflammation to be turned off following clearance of an infectious threat, the form of cell death needs to switch from NETosis to apoptosis. The authors review genetic factors that regulate NADPH and NETosis to conclude that both processes provide opportunities for the development of therapeutics.

Hosseinzadeh et al. (2012) present new data on the development and testing of tempol, a compound that has promise as an effective and relatively safe inhibitor of NETosis. Tempol is a low molecular weight compound that easily passes the plasma membrane and acts as a scavenger of reactive oxygen. The authors test tempol by using a real-time fluorescence assay during neutrophil phagocytosis, chemotaxis, and NETosis. Tempol inhibits NETosis induced by PMA or *Candida albicans*, suggesting its potential use in clinical conditions with excessive NET release.

Lu et al. (2012) review NETosis in infectious diseases and stress that much more needs to be learned about the precise benefits of NETosis in bacterial infections. More specifically, the authors remind us that certain bacterial pathogens such as *Staphylococcus aureus* have a plethora of virulence factors that exploit weaknesses in the immune defenses of the host. Possibly, bacteria may take advantage of neutrophil mechanisms that lead to NETosis. The authors further argue that phagocytosis is the most effective way to dispose of infectious bacteria because fusion of phagosomes and granules produces an environment with highly concentrated bactericidal compounds. In contrast, NETosis diffuses these compounds and is thus less efficient. In that light, pathogens such as staphylococci benefit from the lysis of neutrophils and can also escape from NETs by means of a bacterial nuclease. Thus, NETosis may offer only limited benefits in certain infections but may still enhance collateral damage in the host.

Cheng and Palaniyar (2013) examine NETosis in the context of lung infection and inflammation. Infections that induce NETosis in the lungs are contrasted with inflammatory disorders that lead to tissue injury. Among the lung diseases with suspected NET involvement are acute lung injury and acute respiratory distress syndrome. NETs also may be the potential culprits in the development of cystic fibrosis and asthma, thus making the development of therapies for suppressing NETosis a priority.

Daigo and Hamakubo (2012) present a detailed look at the interaction between pentraxin 3, a soluble pattern recognition receptor, and NETs. Because pentraxin 3 was identified as a component of NETs, the authors purify pentraxin 3 complexes from sepsis patients and carry out their proteomic analysis. They discover that several NET components associate with pentraxin 3 and conclude that pentraxin 3 complexes capture circulating pathogens and deliver them for clearance to phagocytes. The detailed analysis of pentraxin 3 complexes should yield new markers for infectious and inflammatory diseases.

Narayana Moorthy et al. (2013) raise the important question whether NETs are protective or detrimental in secondary bacterial infections. Secondary pneumococcal infections are the major cause of serious complications following seasonal flu infections. The authors quantitate the increased severity of infections in mice by using a grading system for NET release in the lungs. Of relevance, the authors report that *Streptococcus pneumoniae* express a nuclease that makes them impervious to the bactericidal effects of NETs and thus account for why NETs are not more effective in suppressing the bacterial infections.

Abi Abdallah and Denkers (2012) summarize recent data indicating that NETs participate in immune responses to protozoan parasites. More specifically, reports link infections by *Toxoplasma gondii*, *Plasmodium falciparum*, *Eimeria bovis*, and *Leishmania parasites* to the production of NETs. In some cases, NETs may limit the mobility and infectivity of the parasites and lead to the killing of the pathogen. However, at least some protozoan species may

have evolved unique mechanisms for escaping neutrophil traps. In addition, protozoan infections may contribute to manifestations of autoimmunity by modifying NET components.

Berger-Achituv et al. (2013) report exciting new findings from a small group of Ewing sarcoma patients in whom tumor-infiltrating neutrophils were detected and analyzed for induction of NETosis. Preliminary data indicate that the release of NETs within or adjacent to the tumors has a negative effect on the outcome of cancer therapy and disease relapse. The topic of tumor-associated neutrophils and inflammation is rapidly gaining momentum in the cell biology of cancer. The discovery of NETs in the vicinity of tumors and their potential role in tumor progression and metastasis will certainly attract growing attention in the near future.

Mohanan et al. (2013) carry out experiments that link obesity with macrophage release of extracellular trap-like chromatin in adipose tissue. Because the death and clearance of adipocytes is implicated in the inflammatory cytokine release by fat tissue macrophages, the authors predicted that macrophage may release extracellular traps upon entering areas of high adipocyte turnover. The results indicate that macrophages within adipose tissues express PAD2 and some of them contain citrullinated histone H4. These data will inspire future studies to determine the role of macrophage traps in adipose tissue.

Nakazawa et al. (2012) complete our collection of articles with a clinical case study that analyzes the cause of death in a patient experiencing microscopic polyangiitis and deep vein thrombosis. In this type of vasculitis, thrombotic complications are common, and the authors expand on previously published data linking NETs to disease severity and progression.

ACKNOWLEDGMENTS

We thank the contributors and many dedicated reviewers for their efforts and generous enthusiasm. We hope that this group of participants and others attracted to the field of NETosis will remain highly productive and we look forward to building additional opportunities for interactions among researchers interested in NET-working.

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Received: 21 May 2013; accepted: 10 June 2013; published online: 24 June 2013.
Citation: Radic M and Kaplan MJ (2013) Extracellular chromatin traps interconnect cell biology, microbiology, and immunology. Front. Immunol. ***4****:160. doi: 10.3389/fimmu.2013.00160*
This article was submitted to Frontiers in Molecular Innate Immunity, a specialty of Frontiers in Immunology.

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IMMUNOLOGY

REVIEW ARTICLE
published: 11 January 2013
doi: 10.3389/fimmu.2012.00420

The expanding world of extracellular traps: not only neutrophils but much more

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The release of extracellular traps (ETs) is a recently described mechanism of innate immune response to infection. Although ETs have been intensely investigated in the context of neutrophil antimicrobial effector mechanisms, other immune cells such as mast cells, eosinophils, and macrophages can also release these structures. The different ETs have several features in common, regardless of the type of cells from which they originated, including a DNA backbone with embedded antimicrobial peptides, proteases, and histones. However, they also exhibit remarkable individual differences such as the type of sub-cellular compartments from where the DNA backbone originates (e.g., nucleus or mitochondria), the proportion of responding cells within the pool, and/or the molecular mechanism/s underlying the ETs formation. This review summarizes the knowledge accumulated in recent years regarding the complex and expanding world of ETs and their role in immune function with particular emphasis on the role of other immune cells rather than on neutrophils exclusively.

Keywords: extracellular traps, neutrophils, mast cells, eosinophils, macrophages/monocytes, etosis

INTRODUCTION

Extracellular traps (ETs) were first described in 2004 in a ground breaking publication by Brinkmann and colleagues who observed the released of web-like structures by neutrophils after stimulation with phorbol myristate acetate (PMA), lipopolysaccharides (LPS), interleukin 8 (IL-8), platelet-mediated neutrophil activation (Clark et al., 2007) and after exposure to Gram-positive or Gram-negative bacteria (Brinkmann et al., 2004). The composition of these structures has been intensively investigated during recent years. Besides the backbone formed by DNA and histones, ETs also comprise a number of molecules which impart an antimicrobial effect including elastase, cathepsin G, proteinases or defensins, bacterial permeability increasing protein (BPI), or myeloperoxidase (MPO; Brinkmann et al., 2004; Papayannopoulos et al., 2010).

In recent years it has become increasingly evident that ETs are not formed exclusively by neutrophils but also by other cell types including, mast cells (von Kockritz-Blickwede et al., 2008), eosinophils (Yousefi et al., 2008), chicken heterophils (Chuammitri et al., 2009), and macrophages/monocytes (Chow et al., 2010). The molecules, microorganisms, and microbial products that are able to induce ETs formation by various cell types are summarized in **Table 1**.

Apart from humans and mice, ETs have also been found to be released by cells from a variety of other animals including ox, horses, fish, cats, and even by invertebrates. In fact, extracellular nucleic acid released by oenocytoid cells has been reported to be an important defense mechanism toward pathogenic microorganisms in insects (Altincicek et al., 2008). ETs are also apparent in plants where they have been demonstrated to play an important role in defense against fungal infections of the root tip (Wen et al., 2009; Hawes et al., 2011). The common feature of ETs released by the different cell types is a backbone composed of DNA decorated with antimicrobial molecules that is capable of snaring and killing a wide spectrum of microbes (Brinkmann et al., 2004; Fuchs et al., 2007; Urban and Zychlinsky, 2007; von Kockritz-Blickwede and Nizet, 2009). Nevertheless, it should be mentioned that ETs arising from different cell types also exhibit unique features, distinct from those originally described for neutrophils.

Much of the research on ETs has been conducted on neutrophils, most probably because these cells were the first to be associated with the production of such extracellular structures. This is also the reason why the mechanism of cell death leading to the formation of ETs was first termed Netosis (Fuchs et al., 2007) and then later generalized to Etosis. The differences between Etosis and the other forms of cell death such as necrosis or apoptosis are summarized in **Table 2**. The intracellular signaling events reported to be involved in the induction of etosis includes the activation of NADPH oxidase with the concomitant formation of reactive oxygen radicals (ROS; Papayannopoulos et al., 2010; Guimaraes-Costa et al., 2012). There are also reports demonstrating that, in addition to chromosomal DNA, mitochondrial DNA could also be used by eosinophils (Yousefi et al., 2008) and neutrophils (Yousefi et al., 2009) to form ETs without induction of cell death. However, the mechanism/s behind this unusual mode of ET formation remains a mystery. Although the primary function of ETs has been attributed to their antimicrobial effect, the overall role of ETs in host defense against pathogens remains a topic of debate.

THE MOLECULAR BASIS OF EXTRACELLULAR TRAPS FORMATION

While significant progress has been made in unraveling the cellular processes that are taking place during the formation of ETs, many

Table 1 | Differences between netosis, apoptosis, and necrosis.

Necrosis	Apoptosis	Netosis
Membrane and organelle disintegration	Membrane blebbing	Vacuolization
Phosphatidylserine exposure during early steps of necrosis	Phosphatidylserine exposure	No exposure to Phosphatidylserine
Cellular swelling and bursting	Nuclear chromatin condensation without disintegration of the nuclear membrane	Nuclear chromatin decondensation with disintegration of the nuclear membrane
Cell damage releasing the intracellular contents	Programmed cell death	Programmed cell death

Table 2 | Cell types shown to release ETs and triggering stimuli.

Cell type	Activating agent	Reference
Neutrophils	IL-8	Ramos-Kichik et al. (2009)
Neutrophils, Mast cells	Lipopolysaccharide (LPS)	Brinkmann et al. (2004), von Kockritz-Blickwede et al. (2008), Ramos-Kichik et al. (2009)
Neutrophils, Mast cells	Phorbol-12-myristate-13-acetate (PMA)	Brinkmann et al. (2004), von Kockritz-Blickwede et al. (2008)
Neutrophils	Platelet via TLR4	Clark et al. (2007)
Neutrophils, Eosinophils	Interferon (IFN) γ + C5a	Yousefi et al. (2008)
Eosinophils	Interferon (IFN) α + C5a	Yousefi et al. (2008)
Eosinophils	Interferon (IFN) + eotaxin	Yousefi et al. (2008)
Neutrophils	GM-CSF + C5a	Martinelli et al. (2004), Yousefi et al. (2009)
Neutrophils	GM-CSF + LPS	Martinelli et al. (2004), Yousefi et al. (2009)
Neutrophils	Lipophosphoglycan	Guimaraes-Costa et al. (2009)
Neutrophils, Mast cells	M1-protein-fibrinogen complex	Lauth et al. (2009), Oehmcke et al. (2009)
Neutrophils, Mast cells, Eosinophils	Hydrogen peroxide	Brinkmann et al. (2004), von Kockritz-Blickwede et al. (2008), Oehmcke et al. (2009)
Neutrophils	Calcium	Wang et al. (2009)
Neutrophils, Mast cells	Glucose oxidase	Fuchs et al. (2007), von Kockritz-Blickwede et al. (2008)
Mast cells	IL-23 and IL-1β	Lin et al. (2011)
Neutrophils, Monocytes/Macrophages	Statins	Chow et al. (2010)
Neutrophils	Tumor necrosis factor (TNF) α	Wang et al. (2009)
Neutrophils	Panton-Valentin leukocidin	Pilsczek et al. (2010)
Neutrophils	Platelet activating factor	Hakkim et al. (2011)

aspects still remain unresolved. ET formation generally begins in stimulated cells with the loss of the tight organization of the nuclei followed by chromatin decondensation. The characteristic shape of the nuclei disappears and a gap between the inner and outer membrane of the nucleus emerges. Formation of vesicles in the nuclear membrane follows leading to widespread membrane disruption. At the same time, disruption of the granular membranes takes place in the cell cytoplasm facilitating the mixing of granular content with the chromatin leaking into the cytoplasm through the disrupted cellular membrane. Finally, eruption of the cell membrane follows and DNA mixed with the granular content is released into the extracellular milieu (Fuchs et al., 2007). This characteristic form of cell death, termed Netosis by Steinberg and Grinstein (2007), was described earlier by Takei et al. (1996) although without showing an association with the release of ETs. Netosis seems to be a process entirely independent of caspases and certain kinases such as RIP-1 and is not affected by the caspase inhibitor zVAD-fmk (Urban et al., 2009; Remijsen et al., 2011). Netosis is not associated with DNA fragmentation or phosphatidylserine (PS) exposure on the outer leaflet of the cellular membrane, which are distinctive aspects of apoptosis. The lack of PS impedes the clearance of cells undergoing netosis by phagocytic cells such as macrophages. An additional feature that distinguishes netosis from apoptosis and necrosis is the fact that both the nuclear as well as the granular membranes undergo fragmentation.

A critical factor involved in etosis and ET formation is the production of ROS. In neutrophils, ROS produced by NADPH oxidases has been reported to inactivate caspase function thereby leading to the blockage of the apoptotic cell death pathway (Fadeel et al., 1998; Hampton et al., 2002). The importance of NADPH

oxidase for ET release was demonstrated by the reduced capacity of neutrophils to form ETs after pharmacological inhibition of this enzyme (Metzler et al., 2011). Furthermore, neutrophils from patients suffering from chronic granulomatous diseases, which are defective in NADPH oxidase function, are unable to form ETs (Fuchs et al., 2007; Bianchi et al., 2009). Etosis is nevertheless a multifactorial process and NADPH oxidase activity is necessary but alone is insufficient to trigger this process. Thus, increased intracellular Ca^{2+} levels after treatment with Thapsigargin has also been shown to induce ET formation in neutrophils (Gupta et al., 2010). The increased Ca^{2+} level induces a Ca^{2+}-dependent PAD4 activity leading to histone citrullination, which constitutes a downstream signaling processes in the formation of ETs (Neeli et al., 2008; Wang et al., 2009). Indeed, PAD4-dependent citrullination of histone H3 is a key molecular event in the formation of ETs (Neeli et al., 2008; Wang et al., 2009; **Figure 1**).

Even though the development of ROS and the activity of NADPH oxidase have been claimed as being essential in the formation of ETs, it has also been reported that microorganisms such as *Staphylococcus aureus* (Pilsczek et al., 2010) or *Leishmania donovani* (Gabriel et al., 2010) are able to induce ET release through a molecular process that is independent of ROS. This adds a further level of complexity to the molecular puzzle of this cellular process.

EXTRACELLULAR TRAPS FORMATION OUTSIDE THE NEUTROPHIL WORLD

As mentioned before, other immune cells including mast cells, eosinophils, and macrophages are also capable of releasing ETs. Although the molecular principles underlying the formation of ETs by mast cells (von Kockritz-Blickwede et al., 2008), eosinophils (Yousefi et al., 2008), and monocytes/macrophages (Chow et al., 2010) share some similarities with those observed for neutrophils, there are some notable disparities. The most remarkable mechanism of ET formation has been described in eosinophils. In these cells, ETs are formed by both nuclear and mitochondrial DNA in a ROS-dependent manner. The presence of several mitochondrial genes including Co1 (cytochrome oxidase subunit 1), ND1 (NADH dehydrogenase subunit 1), or Cyb (cytochrome *b*) in the nucleic acid material released by eosinophils provides clear evidence of its mitochondrial origin (Yousefi et al., 2008). DNA is rapidly expelled by the eosinophils in response to stimulation with LPS, eotaxin, complement factor 5a (C5a) or infection with Gram-negative bacteria after priming with interleukin 5 (IL-5) or IFN-γ (**Figure 1**), which, in this case, was shown to be essential for the explosive release of mtDNA by eosinophils. The time frame reported for the release of eosinophil ETs is seconds and is thus much shorter than the classical ET formation by neutrophils. An additional and interesting characteristic of ETs formed by eosinophils is the lack of cytosolic proteins, although eosinophil granule proteins were shown to be released concurrently with mtDNA (Yousefi et al., 2012). An additional and important feature that differentiates the eosinophil from the classical neutrophil release of ETs is that it is not dependent upon the cell death of the eosinophils. Interestingly, a similar mechanism of ET release that is non-associated with cell death has also been recently described for neutrophils (Pilsczek et al., 2010). This challenges the generalized opinion that ETs are released by dying cells. Nevertheless, it should also be noted that in both cases where ET formation was non-associated with cell death, the cells needed to be primed first before stimulated to form ETs. In the case of neutrophils, cells were initially activated by granulocyte/macrophage stimulating factor (GM-CSF) followed by short-term toll-like receptor 4 (TLR4) or C5a stimulation (**Figure 1**). In these experimental conditions, viable neutrophils were able to release ETs that contained mitochondrial but no nuclear DNA.

DNA-releasing eosinophils have been primarily reported in the context of inflammatory diseases of the intestine (Yousefi et al., 2008) and skin (Simon et al., 2011). They seem to be less prominent, however, in the setting of infectious diseases despite the fact that these structures are also capable of snaring and killing bacteria (Yousefi et al., 2008). Furthermore, while induction of mtDNA associated with eosinophil granules has been reported to contribute to the increased survival of mice (up to 14 days) undergoing cecal ligation puncture (CLP; Yousefi et al., 2008), it is still not clear to what extent eosinophil ET formation contributes to host defense. In this regard, though evidence has been provided that hypereosinophilic transgenic animals are less susceptible to septicemia induced by CLP, the major role of eosinophils has been attributed to host defense against helminths (Blanchard and Rothenberg, 2009; Linch et al., 2009). These granulocytic cells are able to infiltrate the gastrointestinal tract and have been associated with a variety of inflammatory conditions like inflammatory bowl disease (IBD) or eosinophil-associated gastrointestinal disorders (EGIDs; DeBrosse and Rothenberg, 2008; Wedemeyer and Vosskuhl, 2008).

Besides eosinophils, mast cells, which also originate from bone marrow and contain different types of granules enclosing very potent biological effectors molecules, are also capable of releasing ETs following stimulation (**Figure 2**). Mast cells are located in close proximity to the host environment where they are most likely to encounter incoming pathogens. Although mast cells are largely known for their detrimental role in the context of allergic diseases, there is a growing body of evidence that suggests that they are also important contributors to host defense against pathogens (Galli and Wershil, 1996; Bischoff, 2007). Thus, mast cells are not only important for modulating the function of other immune cells (e.g., neutrophils) during infection but they also impart direct antimicrobial effects (Feger et al., 2002). Due to the limited phagocytic activity of mast cells, their antimicrobial activity is largely mediated by extracellular mechanisms including degranulation and the concomitant release of highly potent antimicrobial peptides such as cathelicidins (CRAMP or LL-37), defensins (β-defensins) or proteases (tryptase, chymase). Mast cell degranulation occurs after exposure to pathogens and has been shown to be very efficient in inhibiting the growth of bacteria such as *S. aureus* (Abel et al., 2011). In addition, mast cells are also able to release ETs in a ROS-dependent manner. Mast cell ETs are composed of DNA and histones, which are the general components of most ETs, as well as mast cell-specific granule proteins like tryptase and CRAMP/LL-37 (von Kockritz-Blickwede et al., 2008). In contrast to neutrophils where NETs can be dismantled after treatment with only DNase, the complete disassembling of mast cell ETs requires treatment with DNase as well as the addition of enzymes degrading tryptase (e.g., MPO; von Kockritz-Blickwede

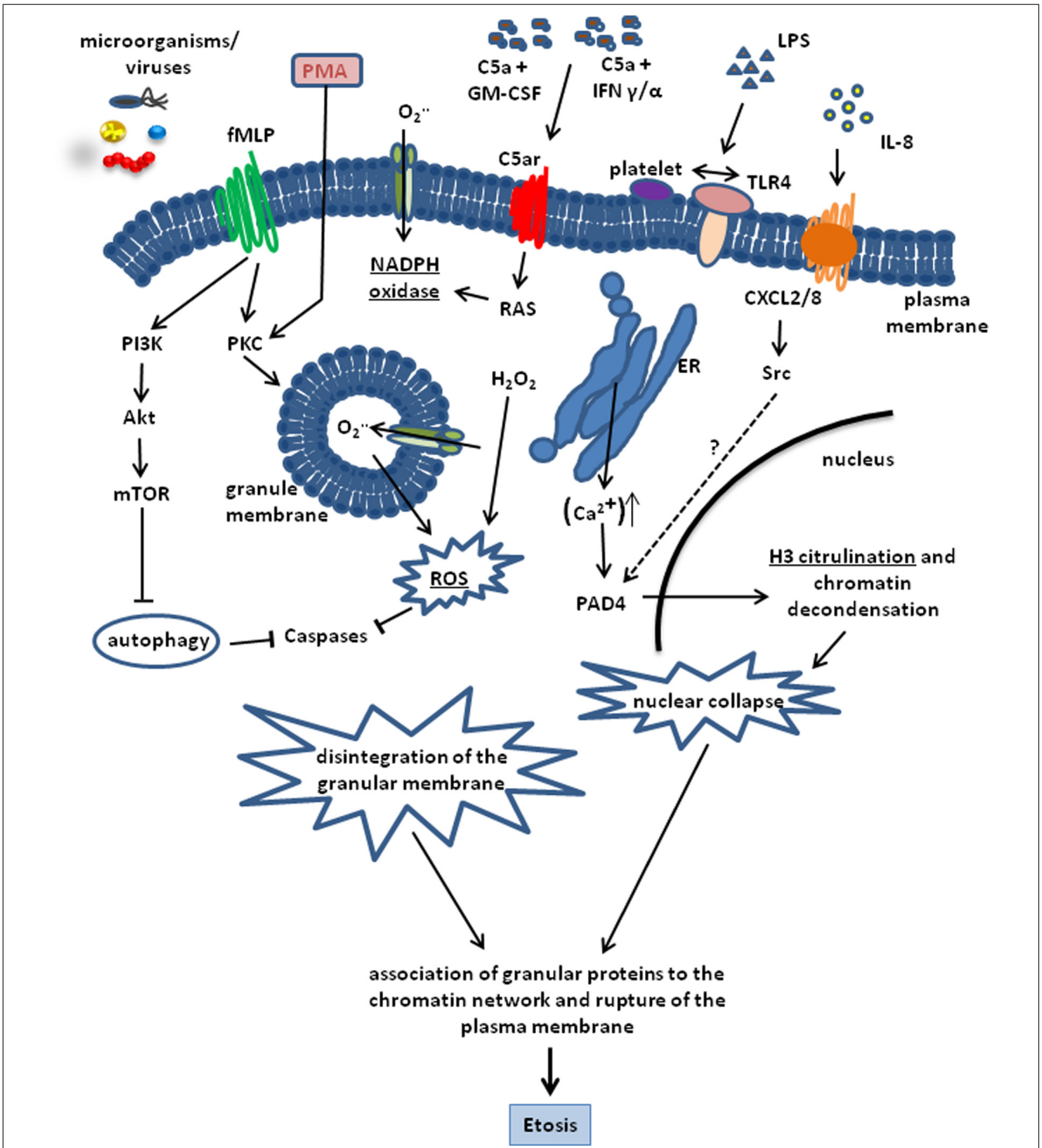

FIGURE 1 | Schematic representation of the cellular processes involved in the formation of ETs. The process can be triggered by a number of stimuli including, PMA, LPS, C5a + GM-CSF, IFN α/γ. LPS, bacteria, and viruses. IL-8 is also able to trigger ET release by interacting with the CXCL2/8 receptor and inducing H3 citrullination through PAD4 activation via Src kinases. Most pathways converge in the activation of the key enzyme NADPH oxidase. This enzyme is highly activated by PMA and formylated peptides. Induction of the fMLP receptor leads to a massive activation of protein kinase C (PKC) and NADPH oxidase activity. On the other hand, fMLP blocks autophagy via PI3K, Akt and mTOR activation, which is able to prevent Etosis. NADPH oxidase activity results in ROS production and H3 citrullination leading to chromatin decondensation and nuclear collapse. Disintegration of the nuclear membrane and adsorption of antimicrobial granular proteins onto the decondensed chromatin network is the final step of Etosis that precedes the release of ETs into the surrounding milieu after rupture of the plasma membrane.

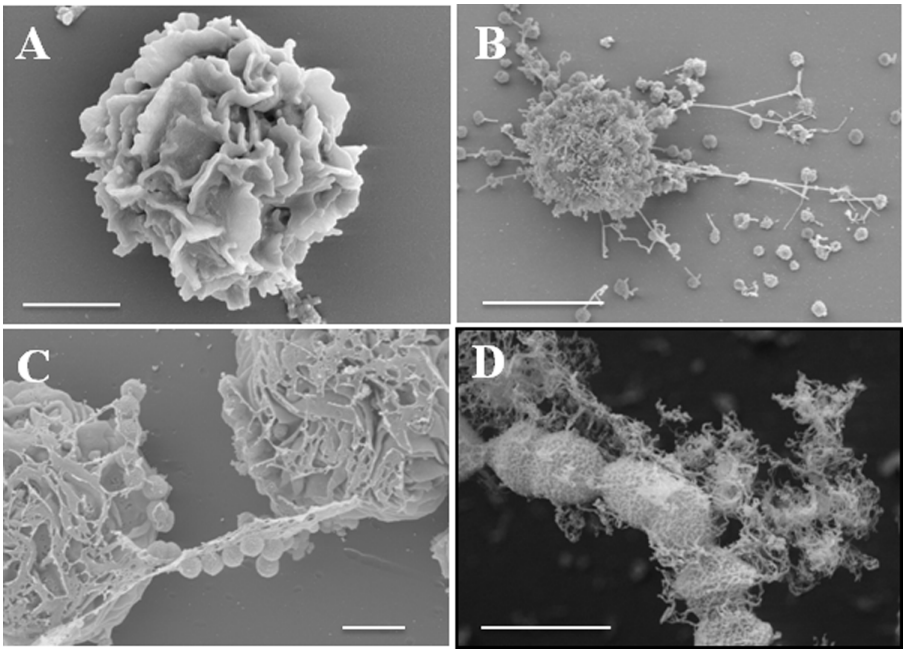

FIGURE 2 | Release of ETs by mast cells after encounter with *S. pyogenes*. **(A)** Field-emission scanning electron microscope (FESEM) images of a resting mast cell (bar, 2 μm). **(B,C)** Mast cells in the process of releasing ETs in response to *S. pyogenes* (**B**, bar, 5 μm and **C**, bar, 2 μm). **(D)** *S. pyogenes* captured in ETs (bar, 1 μm). Provided by M. Rohde, Department of Microbial Pathogenesis, Helmholtz Center for infection Research, Braunschweig, Germany.

et al., 2008). Another interesting feature is the recently reported involvement of the transcriptional hypoxia-inducible factor 1α (HIF-1α) in the modulation of ET release by human and murine mast cells (Branitzki-Heinemann et al., 2012). HIF is a well-known factor for its role in the regulation of the inflammatory and innate immune function of neutrophils and macrophages (Cramer et al., 2003; Peyssonnaux et al., 2005).

Most recently, monocytes/macrophages have also been reported to be capable of releasing ETs (Chow et al., 2010; Aulik et al., 2011). Macrophage ET production has been shown to be boosted by statins, which are inhibitors of the rate-limiting enzyme within the cholesterol biosynthesis 3-hydroxy 3-methyglutaryl coenzyme A (HMG-CoA) reductase. In addition, increased production of ETs release by macrophages has been observed after inhibition of HMG-CoA reductase using siRNA or after treatment of macrophages with the downstream HMG-CoA reductase product mevalonate (Chow et al., 2010). Statins are also capable of inhibiting the release of ETs by neutrophils. The molecular mechanism mediating the effect of statins on phagocytes seems to be linked to the inhibition of the sterol pathway within the cell (Chow et al., 2010). Interestingly, bacterial components such as hemolysins of *Escherichia coli* or leukotoxin of *Mannheimia haemolytica* have been shown to induce the release of ETs by bovine macrophages (Aulik et al., 2011, 2012). However, the extent to which the molecular processes leading to the formation of ETs by monocytes/macrophages is comparable to the mechanisms already described for neutrophils, eosinophils, and mast cells, remains to be elucidated. Although little information is available regarding the molecular basis of ET release by macrophages, it seems that general mechanisms such as NADPH oxidase dependency and oxidative stress are involved (Chow et al., 2010).

THE ANTIMICROBIAL EFFECT OF EXTRACELLULAR TRAPS

Extracellular traps release is thought to be mainly an antimicrobial strategy used by host cells to control and eliminate pathogens (Brinkmann et al., 2004; von Kockritz-Blickwede et al., 2008; Linch et al., 2009; Saitoh et al., 2012). Thus, a number of bacteria, fungi, and parasites have been reported in the literature to be entrapped and killed by ETs (summarized in **Table 3**). Saitoh et al. (2012) provided the first report regarding the involvement of ETs in antiviral immunity. Their study showed that neutrophils are able to produce ETs in response to human immunodeficiency virus-1 (HIV-1) and, more interestingly, that these virus particles can be entrapped and neutralized by the ETs. By using blocking antibodies to MPO and α-defensin, it was possible to demonstrate that the viral neutralization was dependent on the presence of MPO and α-defensin in the NET structure. The production of NETs by neutrophils in this case was associated with TLR-7 and TLR-8 signaling as well as with ROS production (Saitoh et al., 2012). In addition, the investigators also showed that the anti-inflammatory cytokine IL-10 could reduce the release of extracellular DNA by neutrophils into the surrounding milieu. It is important to note, however, that these studies were carried out *in vitro* and, although this is an exciting new aspect of ET function in host immunity, it still remains to be demonstrated in the *in vivo* setting. Whether ETs produced by immune cells other than neutrophils are also capable of trapping and inactivating virus particles may be deserving of future investigation. The finding that different

Table 3 | Microorganisms able to trigger the release of ETs by specific cell types.

Microorganism	Cell type	Reference
Aspergillus fumigatus	Neutrophils	Bruns et al. (2010), McCormick et al. (2010)
Candida albicans	Neutrophils	Urban et al. (2006)
Cryptococcus gattii	Neutrophils	Springer et al. (2010)
Cryptococcus neoformans	Neutrophils	Urban et al. (2009)
Eimeria bovis	Neutrophils	Behrendt et al. (2010)
Enterococcus faecalis	Neutrophils	Lippolis et al. (2006)
Escherichia coli	Neutrophils, monocytes	Lippolis et al. (2006), Grinberg et al. (2008), Webster et al. (2010)
Haemophilus influenzae	Neutrophils	Hong et al. (2009), Hakkim et al. (2011)
Helicobacter pylori	Neutrophils	Hakkim et al. (2011)
Human Immunodeficiency Virus-1 (HIV-1)	Neutrophils	Saitoh et al. (2012)
Klebsiella penumoniae	Neutrophils	Papayannopoulos et al. (2010)
Leishmania amazonensis	Neutrophils	Guimaraes-Costa et al. (2009)
Listeria monocytogenes	Neutrophils	Ermert et al. (2009)
Mycobacterium canettii	Neutrophils	Ramos-Kichik et al. (2009)
Mycobacterium tuberculosis	Neutrophils	Ramos-Kichik et al. (2009)
Pseudomonas aeruginosa	Mast cells	von Kockritz-Blickwede et al. (2008)
Serratia marcescens	Neutrophils	Lippolis et al. (2006)
Shigella flexneri	Neutrophils	Brinkmann et al. (2004)
Staphylococcus aureus	Neutrophils, Mast cells	Brinkmann et al. (2004), von Kockritz-Blickwede et al. (2008)
Streptococcus dysgalactiae	Neutrophils	Lippolis et al. (2006)
Streptococcus pneumoniae	Neutrophils, Mast cells	Beiter et al. (2006), Crotty Alexander et al. (2010)
Streptococcus pyogenes	Neutrophils, Mast cells	Buchanan et al. (2006), von Kockritz-Blickwede et al. (2008)

pathogens are able to induce ETs in different innate immune cells argues for a general role of ETs in the innate immune response to pathogenic microorganisms and is supported by a number of *in vivo* studies revealing ET formation in necrotizing soft tissue infections caused by *S. pyogenes* (Buchanan et al., 2006), polymicrobial sepsis after cecal ligation and puncture (Yousefi et al., 2008) and *S. pneumoniae* infections in murine models (Buchanan et al., 2006).

The molecular mechanism/s responsible for the entrapment and killing of microorganisms within ETs is a matter of debate, though several hypotheses have been proposed. One such hypothesis is that entrapment is facilitated by the occurrence of electrostatic interactions arising from the cationically charged ET structure and the anionically charged bacterial surfaces (Brinkmann and Zychlinsky, 2007). The subsequent killing of the pathogen is postulated to arise from the ability of the ETs to increase the local concentration of certain antimicrobial peptides and therefore intensifying the contact between microorganisms and the antimicrobial agents (von Kockritz-Blickwede et al., 2008). Potential candidates being discussed to have antimicrobial properties within ETs are the histones. Several types of histones and histone-related peptides isolated from various organisms and cell types exhibit a broad spectrum of antimicrobial activities (Kawasaki and Iwamuro, 2008). In particular, the histone H2B displays antimicrobial properties against Gram-positive and Gram-negative bacteria and fungi (Li et al., 2007). An overview of the antimicrobial activities of histones is displayed in **Table 4**. In addition to histones, there are other cell specific components

Table 4 | Short overview of histones and their antimicrobial properties.

Histone	Origin	Antimicrobial spectrum	Reference
Histone H1	macrophages, epithelial cells, liver, intestine, skin	*S. aureus*, *L. moncytogenes*, *S. typhimurium*, *E. coli*, *C. neoformans*	Hiemstra et al. (1993), Rose et al. (1998)
Histone H2A	Placenta, skin, liver	*E. coli*, *S. aureus*, *B. subtilis*, *S. flexneri*, *S. typhimurium*, *S. pneumonia*, *C. albicans*	Kim et al. (2002), Cho et al. (2002), Fernandes et al. (2002), Li et al. (2007)
Histone H2B	Placenta, skin, liver	*S. aureus*, *L. moncytogenes*, *S. typhimurium*, *B. subtilis*	Li et al. (2007)

located within the ETs that may have antimicrobial effect. The two most important antimicrobial peptide families in mammals are the defensins (Lehrer and Ganz, 2002b) and a group of cationic molecules, classified as cathelicidins (Lehrer and Ganz, 2002a; Zaiou and Gallo, 2002). Cathelicidins belong to a family of antimicrobial peptides found in the lysosomes of several immune cells including neutrophils, mast cells, and macrophages (Nizet et al., 2001; Zanetti, 2004). The presence of these antimicrobial peptides has been demonstrated in ETs released by neutrophils as well as by mast cells (Brinkmann et al., 2004; von Kockritz-Blickwede et al., 2008). However, the question remains whether antimicrobial peptides bound to the DNA backbone of the ETs still retain their antimicrobial capacity.

Although the antimicrobial effect of ETs has been extensively demonstrated in many experimental settings, the extent to which these structures contribute to pathogen killing during productive infection remains a subject of debate. Furthermore, in certain circumstances, the production of ETs can be detrimental for the host. For example, the release of high quantities of DNA and histones can induce autoimmune reactions that may be involved in the development of autoimmune diseases like lupus erythematosus or rheumatoid arthritis (Mohan et al., 1993; Zhong et al., 2007). Preeclampsia, a severe disorder of late pregnancy characterized by an increasing level of cell free DNA in the maternal plasma, is another pathological disorder in which ET formation may also be involved (Clark et al., 1998). In this disorder, a massive release of DNA probably in response to high levels of inducing factors (e.g., IL-8 or microdebris of the placenta) has been observed (Gupta et al., 2005, 2007). Similarly, the release of ETs by platelet-activated neutrophils under blood flow conditions can result in reduced blood perfusion of the tissue and ischemia (Clark et al., 2007). The beneficial or detrimental effect of ETs can be determined by the extent of the response. Moderate release of ETs during infection can contribute to pathogen killing and control of the infection, thus conferring a beneficial effect. Conversely, massive release of ETs during pathological conditions can induce autoimmunity as well as organ damage and is thus highly deleterious for the host.

PATHOGEN EVASION OF EXTRACELLULAR TRAPS

Successful pathogens have evolved intricate countermeasures to subvert the mechanisms of host defense. Shortly after ETs were discovered, a number of studies reported the ability of certain pathogens to circumvent the antimicrobial activity of these structures. One of the main strategies used by pathogenic bacteria to escape the ETs is through the production of DNases that cleave DNA and therefore dismantle their DNA backbone. This mechanism has been described for *S. pyogenes*, which produces a very potent bacteriophage-encoded DNase designated Sda1. Strains of *S. pyogenes* producing Sda1 are more resistance to ET-dependent killing than strains lacking the *Sda1* gene (Sumby et al., 2005; Buchanan et al., 2006). A similar strategy has been reported for *S. pneumoniae* (Buchanan et al., 2006) and *S. aureus* (Udou and Ichikawa, 1979; Berends et al., 2010). Changes in the composition of the bacterial cell wall can also help to avoid the antimicrobial activity of ETs. Thus, *S. pneumoniae* mutant strains lacking positively charged D-alanyl residues on their lipoteichoic acid (LTA) have been shown to be more susceptible to ET killing than the corresponding wild-type strain (Wartha et al., 2007a,b). D-alanylation of LTA by bacterial species harboring a homolog of the *dlt* operon like *S. pyogenes* (Kristian et al., 2005) or *S. aureus* (Kraus et al., 2008) are known to be much more resistant against the antimicrobial activity of cathelicidins. An indirect strategy of microbes to avoid the antimicrobial effect of ETs is to reduce the recruitment of immune cells involved in the production of ETs. This is achieved by the blocking or cleaving of chemotactic mediators involved in the recruitment of immune cells to the site/s of infection. An example of this is provided by the IL-8 degrading protein SpyCEP of *S. pyogenes* (Gupta et al., 2005).

CONCLUDING REMARKS

Despite the large number of studies that have been conducted on ETs, they still remain enigmatic structures, and many aspects regarding their nature and significance is deserving of further investigation. The specific mechanism/s responsible for pathogen entrapment by ETs is still unsolved. Although some light has been shed regarding the killing mechanisms employed by ETs, the actual process is still largely unknown and requires detailed exploration. In particular, the role of antimicrobial agents like cathelicidins or histones is still under discussion. The extent to which the binding of these molecules to DNA may alter their biological functionality is also unknown. Another question that remains open is related to why only a small proportion of cells within the total population are primed to release ETs. This argues against a primary role of ETs in the functional biology of these cells. Perhaps, a major function of ETs is to contain the pathogen at the site of infection, thereby limiting its spread and dissemination. Indeed, this is a feature also ascribed in the late 1980s to fibrin networks where it was also demonstrated that they were able to interfere with the phagocytic function of neutrophils by blocking effective phagocytosis (Bruns et al., 2010). An additional problem in investigations of the release of ETs is the high variability of experimental settings employed by different laboratories. For example, the concentration of PMA used in different studies ranges from 20 to 200 μM. Furthermore, *in vitro* growth conditions such as nutrient and serum supplementation as well as the time frame for induction are heterogeneous in the literature. This variability may lead to incorrect assumptions and serious misinterpretations.

Future research should be directed to addressing the limitations of these investigations and detailing the signaling pathways leading to etosis. More importantly, further insights into the mechanism/s underlying the regulation of etosis are required. This is of particular importance given that the process of cell death releases many biologically active components that may be both beneficial but also detrimental to the host.

ACKNOWLEDGMENTS

The authors would like to thank Dr. M. Rhode for kindly providing the electron micrographs and Dr. A. Oxley for critically reading the manuscript. Financial support for this study was provided by the DFG-Deutsche Forschungsgemeinschaft, SPP 1394: "Mast cells-promoters of health and modulators of disease," project ME 1875/2-2.

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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.

Received: 31 October 2012; accepted: 20 December 2012; published online: 11 January 2013.

Citation: Goldmann O and Medina E (2013) The expanding world of extracellular traps: not only neutrophils but much more. Front. Immun. ***3****:420. doi: 10.3389/fimmu.2012.00420*

This article was submitted to Frontiers in Molecular Innate Immunity, a specialty of Frontiers in Immunology.

frontiers in IMMUNOLOGY

METHODS ARTICLE
published: 09 January 2013
doi: 10.3389/fimmu.2012.00413

Automatic quantification of *in vitro* NET formation

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Neutrophil Extracellular Traps (NETs) consist of decondensed chromatin studded with granular and some cytoplasmic proteins. They are formed by activated neutrophil granulocytes, also called polymorphonuclear leukocytes (PMN) as the result of an active cell death program, named NETosis. NETosis can be induced by a wide range of stimuli including coculture of neutrophils with pathogens (bacteria, fungi, parasites, virus particles), activated platelets, or pathogen components. The first step of the NETotic cascade is stimulation of one or several receptors followed by activation of the Raf/MEK/ERK pathway that culminates in the assembly of the multimeric NADPH oxidase complex and the production of reactive oxygen species (ROS). Later, intracellular membranes disintegrate, the granular protein Neutrophil Elastase enters the nucleus and processes core histones that also get hypercitrullinated. This leads to decondensation and mobilization of chromatin. The amount of NET formation varies with the degree of stimulation, and this is dependent on the type and concentration of the stimulus. NETs can be quantified using various methods including fluorescence microscopy or measuring DNA release. Each of these methods have specific drawbacks: analysis of fluorescence microscopy is prone to subjective variations, and DNA release does not differentiate between DNA that has been released by NETosis or by other forms of cell death. Here we present a protocol to semi-automatically quantify NET formation. It relies on the observation that anti-chromatin antibodies bind more readily to decondensed chromatin present in the nuclei of cells undergoing NETosis and in the NETs. Relating the fluorescence signals of the anti-chromatin antibody to the signals of a DNA-binding dye allows the automatic calculation of the percentage of netting neutrophils. This method does not require sophisticated microscopic equipment, and the images are quantified with a public-domain software package.

Keywords: NEtosis, chromatin, immunofluorescence, segmentation, quantification

INTRODUCTION

NETs are a fibrous structure consisting of a chromatin backbone with attached globular domains (Brinkmann et al., 2004; Urban et al., 2009). These domains contain granular proteins and peptides as well as some cytoplasmic components (Urban et al., 2009). The main components of NETs are the core histones (H2A, H2B, H3, H4) which together account for about 70% of the protein mass (Urban et al., 2009). Histones have long been recognized as potent antimicrobials (Miller et al., 1942; Hirsch, 1958), and together with the microbicidal granular enzymes and peptides, NETs have been shown to have activity against bacteria, fungi, parasites, and viruses (Brinkmann et al., 2004; Beiter et al., 2006; Urban et al., 2006; Bianchi et al., 2009; Aulik et al., 2010; Behrendt et al., 2010; Abdallah et al., 2012; Saitoh et al., 2012). The intact structure is indispensable for the microbicidal activity of NETs since it ensures a high local concentration of the active components in close vicinity to the pathogens. Treatment of NETs with DNase destroys their antimicrobial potency (Brinkmann et al., 2004). Interestingly, bacteria evolved extracellular nucleases that free them from NETs and allow spreading (Beiter et al., 2006; Buchanan et al., 2006). Besides their role in host defense, NETs have been shown to be involved in blood clotting (Fuchs et al., 2010; Massberg et al., 2010; von Brühl et al., 2012) and in the activation of dendritic cells (Lande et al., 2011).

Both the generation and the destruction of NETs has to be tightly regulated to provide prompt defense against invading pathogens as well as timely coagulation and to avoid negative effects that are associated with overshooting release or reduced clearance of NETs. Lack of NET formation has severe consequences as exemplified by patients suffering from Chronic Granulomatous Disease. These patients cannot make NETs due to an inactive NADPH oxidase complex (Fuchs et al., 2007; Bianchi et al., 2009) and suffer from severe recurrent infections. On the other hand, abundance of NETs due to imbalanced production and destruction can lead to endothelial damage (Saffarzadeh et al., 2012), uncontrolled thrombus formation or induction of autoantibodies against NET components (Hakkim et al., 2010) as well as other disorders (reviewed in Kaplan and Radic, 2012) and (Brinkmann and Zychlinsky, 2012). Thus, molecules that affect the balance of NET creation and destruction can be of therapeutical relevance.

The signaling pathways that lead to NET formation are just being unraveled, many aspects so far remain obscure. Numerous compounds that either promote or prevent NET formation have been tested *in vitro*, and several read out systems have been used to estimate the amount of NET release. The results obtained in different laboratories are hard to compare since different methodologies were used and in many reports the readout was prone to individual bias. Therefore, there is a need for a method that allows automatic quantification of NET formation, which is ideally also useful for high-content screening (HCS).

We present here a protocol that uses dual channel fluorescence staining and automatic image segmentation to determine the percentage of NETotic neutrophils. An antibody against a subnucleosomal complex (Losman et al., 1992) detects relaxed chromatin which is a hallmark of NETosis (Fuchs et al., 2007) and stains both neutrophils that undergo all phases of NETosis as well as NETs after their release from dying cells (Ermert et al., 2009; Brinkmann and Zychlinsky, 2012). The DNA-intercalating dye Hoechst 33342 is used to determine the number of cells per field of view. This method does not demand a sophisticated optical setup, image analysis is performed with the public domain software ImageJ (Schneider et al., 2012). The protocol could be useful to provide unbiased results that are adequately standardized to allow comparison of data sets that were generated in different laboratories. It can also be adapted to HCS.

MATERIALS AND METHODS

PMN ISOLATION

PMN were isolated from freshly drawn blood of healthy donors as described previously (Aga et al., 2002). Briefly, whole blood was separated by spinning on a Histopaque 1119 cushion. The resulting neutrophil-rich fraction was washed and layered on a discontinuous Percoll gradient (85 – 65% in PBS) for centrifugation. Bands on the 80, 75, and 70% layers were collected, pooled, and washed with PBS. The cells were counted and kept in PBS for the induction experiment.

NET INDUCTION

PMN were seeded on 13 mm glass coverslips in 24-well-plates in 490 µl of RPMI supplemented with glutamine, pyruvate, and 0.5% human serum albumin at a density of 10^5 cells per well. The plates were incubated for 15–30′ at 37 °C to allow adhesion of the cells. Prediluted aliquots of the test compounds were prepared at 50×, 5×, and 0.5× of the planned maximum concentrations. Proinflammatory cytokines were used at following concentrations: TNF-α, 1 ng/ml; G-CSF, 1 ng/ml; IL-1β, 10 ng/ml. As positive control, we used phorbol 12-myristate 13-acetate (PMA, 50 nM). Negative controls were DMSO (diluted to match the maximum dose to come with the test compounds) and seeding medium. To start the induction, 10 µl of the prepared aliquots were added to each well and the plates were kept at 37°C for 10′, 2 h (controls) or 6 h (controls and test compounds) before fixation by adding 167 µl of 8% PFA in PBS to reach a final PFA concentration of 2%. For initial analysis of the staining patterns, PMN samples prepared as described here were stimulated with PMA for various periods of time (240′, 130′, 80′, 10′ and 0′) before fixing all samples simultaneously.

For coculturing experiments, *Pseudomonas aeruginosa* strain PA14 at MOI 1, 10, and 100 was added to PMN on coverslips in 24-well plates and centrifuged (5 min at 300 × g). The plates were incubated for 5 h at 37°C.

SAMPLE STAINING

Coverslips with the fixed cells were removed from the plates and processed by floating on drops kept on hydrophobic laboratory film. After washing with PBS, the samples were permeabilized for 1′ with 0.5% Triton X100 in PBS, washed again with PBS and blocked for 20′ with blocking buffer (3% normal donkey serum, 3% cold water fish gelatin, 1% BSA, and 0.05% Tween 20 in PBS). Solutions of antibody (mouse mAB PL2-3) directed against the subnucleosomal complex of Histone 2A, Histone 2B, and chromatin (Losman et al., 1992) and against neutrophil elastase (rabbit pAB Calbiochem 481001) were applied in blocking buffer for 1–2 h. After washing with PBS, dye-conjugated secondary antibody solutions were applied for 30′–1 h (donkey anti mouse Cy3, donkey anti rabbit AlexaFluor 488, Jackson). The samples were washed with PBS and distilled water, stained with aqueous Hoechst 33342 (100 ng/ml, Sigma), washed again in distilled water and mounted with Mowiol.

IMAGE ACQUISITION AND BATCH QUANTIFICATION

Five images taken randomly from different regions of each coverslip in an experiment were taken with the 10× lens on a Leica DMR upright fluorescence microscope equipped with a Jenoptic B/W digital microscope camera. Exposure times of each channel were kept constant over the whole series in an experiment after calibrating on a bright representative sample to avoid saturated pixels. The image files were loaded as separate image stacks for each channel in ImageJ/FIJI software (Schindelin et al., 2012; Schneider et al., 2012). The parameters used for segmentation were controlled by scrolling through and checking the image stacks. To collect the data of total cell number, the Hoechst 33342 fluorescence image stack was binarized with Bernsen automatic local threshold function set to diameter 15 and threshold 35. Automatic particle analysis was set to 20 pixels minimum size and summarized result output. The resulting list of results was saved for further processing. We used the stack of images in the channel with the anti-chromatin immunolabeling to collect NET data. The threshold was set interactively to the minimum threshold that rendered no objects larger than 75 pixels on an image with 10′ PMA activated cells (except spontaneous NETs that are >75pixels). The result of particle analysis with a size-cut-off of 75 pixels was exported as before. Result tables of the Hoechst 33342 cell count and immunofluorescent NETs count were imported into a spreadsheet program and analyzed further. NET-rate was calculated as follows:

$$\text{NET-rate [\%]} = 100 \times \text{Objects counted (chromatin channel)/ Objects counted (Hoechst channel).}$$

The standard deviation was determined by comparing the results of the five individual images analyzed for each specimen.

RGB-merged stacks of the three channels DNA, (relaxed) chromatin, and Neutrophil Elastase with an overlay of the segmented

object outlines were generated and used solely as control images to visually assess and exclude the influence of non-neutrophil cells or imaging artifacts on the results. Immunostaining of Neutrophil Elastase was not further included in the analysis.

ANALYSIS OF STAINING PATTERNS

Images of the time course of PMA-activated neutrophils were taken with a 100× lens. The Hoechst 33342, Cy3, and AlexaFluor 488 images were merged into RGB with ImageJ/FIJI software, leaving the single channels open. Line selections of representative cells were made in the merged RBG image and profile plots of the same selection taken in the respective single channel images. The data lists of the profile plots were imported into a spreadsheet program and represented in combined line diagrams. For the time-course diagram, representative areas of the stained cells in the time-course were taken with the 40× lens and the images of the Hoechst DNA and the Chromatin IF channels loaded as stacks into ImageJ. Segmentation in the Cy3 (chromatin) channel with automatic threshold ("Moments"-algorithm) identified objects (nuclei . . . NETs) that were loaded into the ROI-manager. The intensities of the Hoechst and the Cy3 channel were measured for each ROI, the data lists were exported to a spreadsheet program and represented in a line diagram.

RESULTS

CHANGE OF DNA/CHROMATIN STAINING PATTERNS DURING NETosis

After induction of NETosis, neutrophils undergo a series of morphological modifications. The most prominent is the transformation of the nucleus which is characterized by a gradual decondensation (Fuchs et al., 2007). While unstimulated neutrophils retain a lobulated nucleus (**Figure 1A**), after treatment with PMA or other stimuli, neutrophils rapidly become adherent (**Figure 1B**). Later, their nuclei start to decondense and to lose their lobulation (**Figure 1C**). In the final phase of NETosis, the entire cell is filled with a homogenous mass of intermingled cytoplasm and karyoplasm (**Figure 1D** and Fuchs et al., 2007).

The relaxation of chromatin during NETosis results in the exposure of the epitopes of anti-chromatin antibodies and consequently a more intense fluorescence signal (Ermert et al., 2009; Brinkmann and Zychlinsky, 2012). In contrast, the staining intensity of DNA-intercalating dyes like Hoechst 33342 decreases during NETosis due to the drop in DNA concentration.

The relative staining intensity of Hoechst 33342 (blue) and the anti-chromatin antibody (red) is specified in line plots of individual cells after various periods of stimulation in **Figures 1E–H**. Unstimulated cells are characterized by a condensed lobulated nucleus which stains brightly with Hoechst 33342, but weakly with the chromatin antibodies (**Figure 1E**). This proportion of intensity values is inverted during later phases of NETosis when the nuclei decondense and allow better antibody penetration of the chromatin (**Figures 1F–H**). In late phases of NETosis, chromatin staining is more intense than staining with DNA-intercalating dyes resulting in a more reddish signal of the overlay of fluorescence channels (**Figure 1D**). In parallel, the average diameter of the DNA/chromatin staining increases from about 10 µm for the spherical unstimulated cells (**Figures 1A,E**) to more than 20 µm for neutrophils in late stages of NETosis (**Figures 1D,H**).

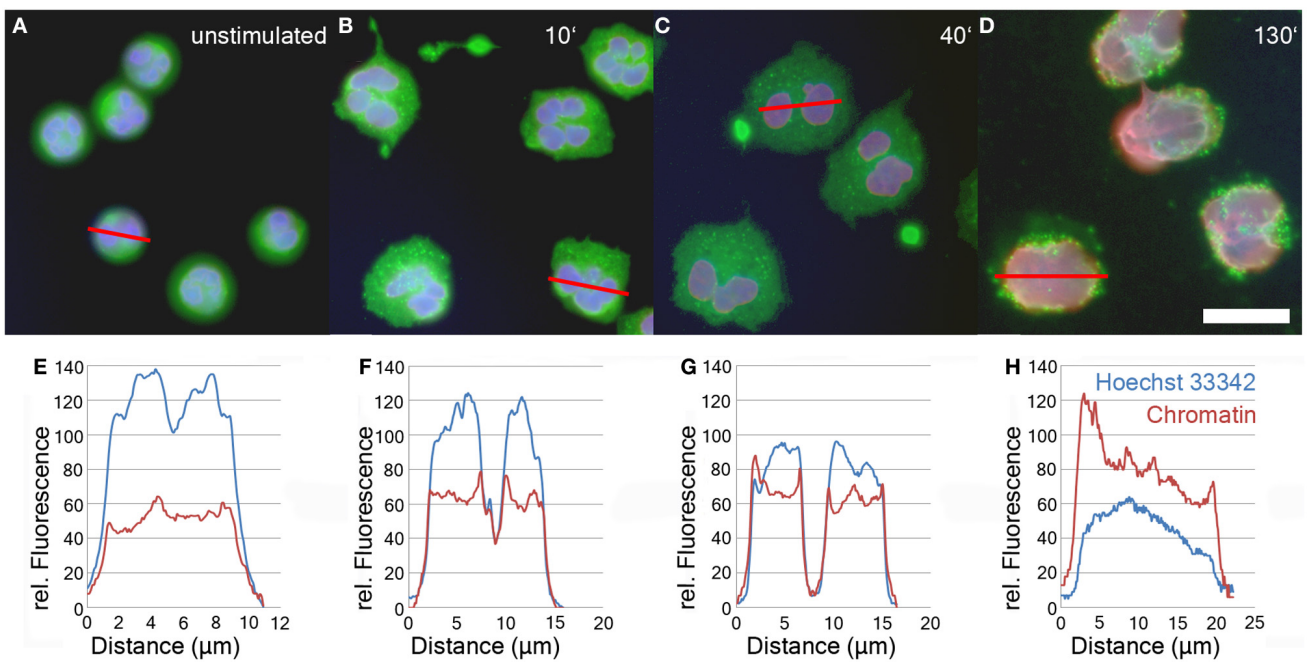

FIGURE 1 | Immunofluorescence analysis of unstimulated neutrophils (A) and neutrophils stimulated with 50 nM PMA for 10'(B), 40'(C), and 130' (D). Blue: DNA (Hoechst 33342), red: chromatin (antibody PL2-3), green: Neutrophil Elastase. Bar represents 20 µm. Panels **1E–H** show line plot quantifications of DNA and chromatin fluorescence intensity of individual cells depicted in panels **1A–D**.

TIME COURSE OF PMA STIMULATION

About 90 min after stimulation with PMA, neutrophil nuclei start to decondense. This leads to a significant increase in the area stained by the anti-chromatin antibody (**Figure 2**, green line). In parallel, already after 10 min of PMA stimulation, the intensity of Hoechst 33342 staining decreases (**Figure 2**, blue line), while the staining with anti-chromatin antibodies becomes brighter, reaching a plateau after about 120 min of stimulation (**Figure 2**, red line). Both Hoechst 33342 and chromatin staining intensities remain stable until most cells have reached late phases of NETosis or have released NETs after 240 min of stimulation.

SEGMENTATION OF HOECHST 33342 AND CHROMATIN IMMUNOFLUORESCENCE SIGNALS

Using 5 image sets taken with a 10× lens, about 5%, of the specimen area is recorded and at a seeding density of 10^5 cells per well, about 1000–5000 cells are analyzed. **Figure 3** depicts a sample of neutrophils stimulated with PMA for 130 min. The upper row (**Figures 3A–C**) displays the entire 10× micrograph, the boxed insert is magnified to better show the fluorescence signals (**Figures 3D–F**) and the segmentation (**Figures 3G–I**). The cell number per field of view is determined using the Hoechst 33342 signal (**Figures 3A,D**; blue in **Figures 3C,F**). After 130 min of PMA stimulation, a fraction of cells have decondensed nuclei and stain with the chromatin antibody (**Figures 3B,E**; red in **C,F**), indicating NETosis. The image stacks are automatically segmented by the ImageJ software as shown in **Figure 3G** (Hoechst 3342), **Figure 3H** (chromatin) and **Figure 3I** (overlay of segmentations and fluorescence signals). Cells that have not (yet) responded to the PMA stimulus are only detected in the Hoechst 33342 segmentation (blue arrowheads in **Figure 3I**), while NETotic neutrophils and NETs are identified in the chromatin segmentation (**Figure 3H**). While neutrophils even in late phases of NETosis are properly segmented, NETs that are produced by several cells are possibly counted as single particles (yellow arrowheads in **Figure 3I**). If this is the case, the NETosis percentage will be underrepresented.

MEASURING NET INDUCTION

To test whether the protocol is generally applicable for measuring NET induction, we tested neutrophils from different donors as well as various inducers of NET formation. These included PMA, ligands of Toll-like receptors (TLR), coculture with pathogens, (in-) organic particles as well as cytokines.

DONOR VARIATION

After 2 h of stimulation with 50 nM PMA, around 40% of the neutrophils isolated from four healthy donors were in the process of NETosis (**Figure 4**). PMN from two of those donors were stimulated for up to 6 h leading to NET induction in about 80% of neutrophils from one donor (**Figure 4**). Neutrophils that were cultured without stimulation showed NETosis rates below 5%. These data indicate that there is considerable variation in NETosis rates between donors, especially at later time points.

STIMULATION OF PATTERN RECOGNITION RECEPTORS

Neutrophils are activated by the presence of pathogens through the detection of pathogen-associated molecular patterns (PAMPs) by pattern-recognition receptors. We tested the ability of different PAMPs to induce NET formation, namely the synthetic lipopeptide (BLP, Pam3Cys-SKKK), a ligand of the TLR1/2-dimer (Aliprantis et al., 1999; Brightbill et al., 1999), a synthetic oligonucleotide rich in CpG-motifs that activates TLR9 (Bauer et al., 2001) and flagellin, a TLR5 ligand (Hayashi et al., 2001).

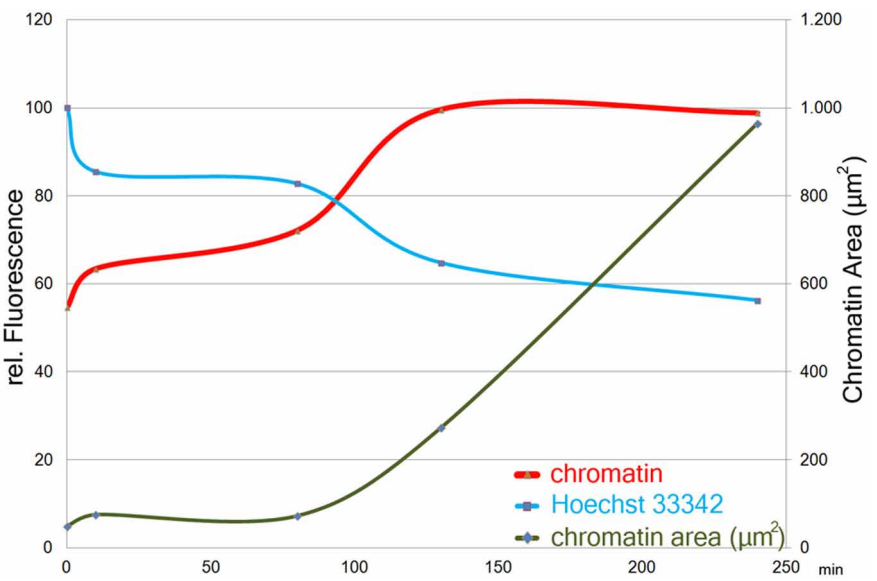

FIGURE 2 | Size (right y-axis) and staining intensities (left y-axis) of automatically segmented objects in images of chromatin- and Hoechst33342- stained cells from a time course of PMA induced NETosis. One image with 47 objects (avg.) per timepoint was measured. Staining intensity plotted as means per timepoint of the maximum intensity-value in each object.

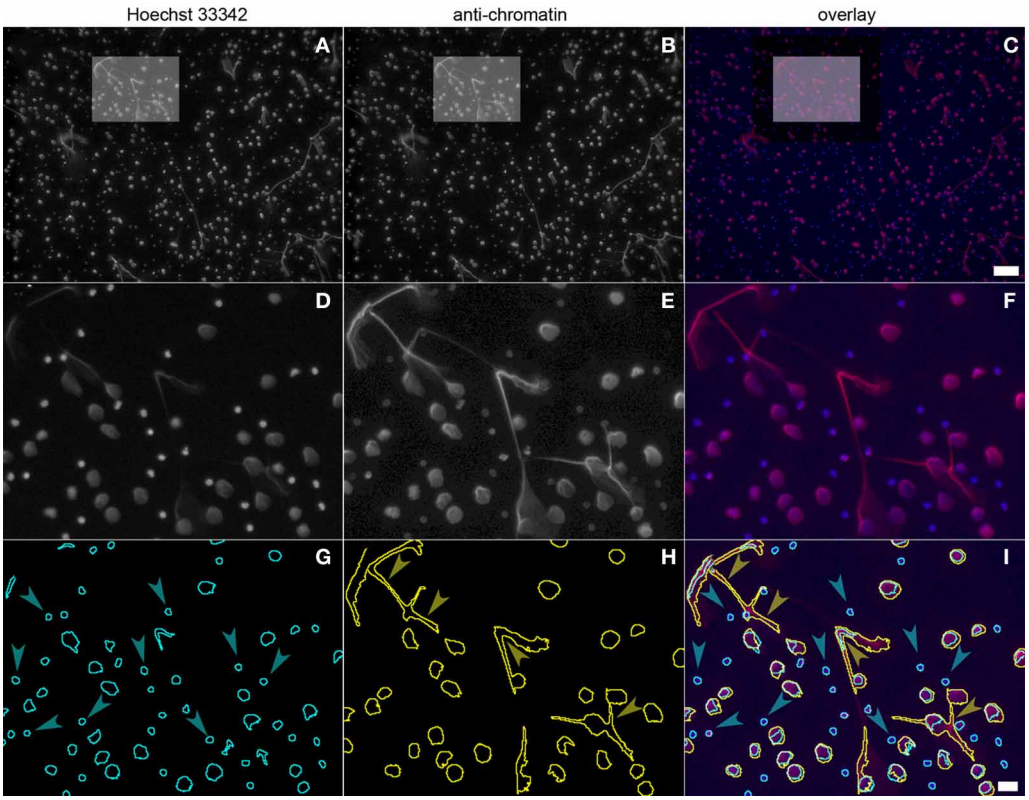

FIGURE 3 | Neutrophils stimulated with PMA for 130 min and stained with Hoechst 33342 (panel 3A, boxed area panel 3D) and PL2-3 (panel 3B, boxed area panel 3E). The overlap is shown in panel **3C** (blue, DNA; red, chromatin; boxed area in panel **3F**). Automatic segmentation of the fluorescence signals is shown in panel **3G** (blue, DNA) and panel **3H** (yellow, chromatin). The overlay of the segmentations and the fluorescence signals is depicted in panel **3I**. Bars represent 100 μm for **(A–C)** and 20 μm for **(D–I)**.

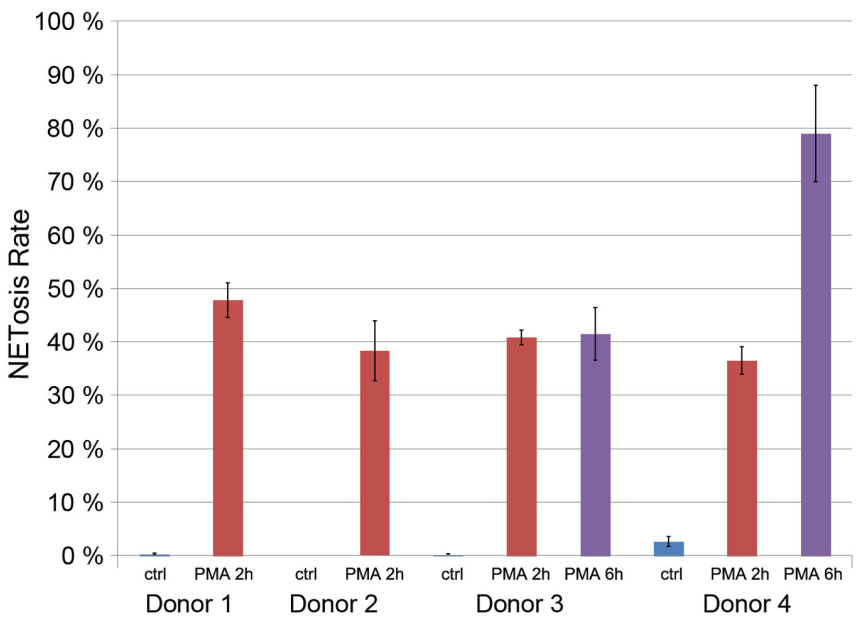

FIGURE 4 | Quantification of the NETosis rate of neutrophils from for donors unstimulated (blue) and stimulated with 50 nM PMA for 2 h (red) or 6 h (violet, only donors 3 and 4).

Each of these PAMPs induced a dose-dependent degree of NET formation. BLP was active at 1 µg/ml and 100 ng/ml (**Figure 5A**), while CpG and flagellin induced NETosis at 10 µg/ml and 1 mg/ml, respectively (**Figure 5B**). While the induction rate of flagellin was rather low (about 3%, **Figure 5B**), both BLP and CpG induction rates were between 5 and 10% (**Figures 5A,B**).

Notably, when we cultured PMN with *Pseudomonas aeruginosa* at different multiplicities of infection (MOI) for 5 h, we found a dose-dependent NETosis response. Even a MOI 1 led to clear NET activation of about 10%, while MOI 10 and 100 resulted in NETosis rates of 20 and 40%, respectively. The standard deviation for higher MOIs is high since the amount of bacterial DNA compromises the automatic quantification.

Notably, individual PAMPS are poor NET inducers when compared to bacteria (**Figure 5**), suggesting that several pattern recognition receptors need to be stimulated in parallel to efficiently drive NETosis.

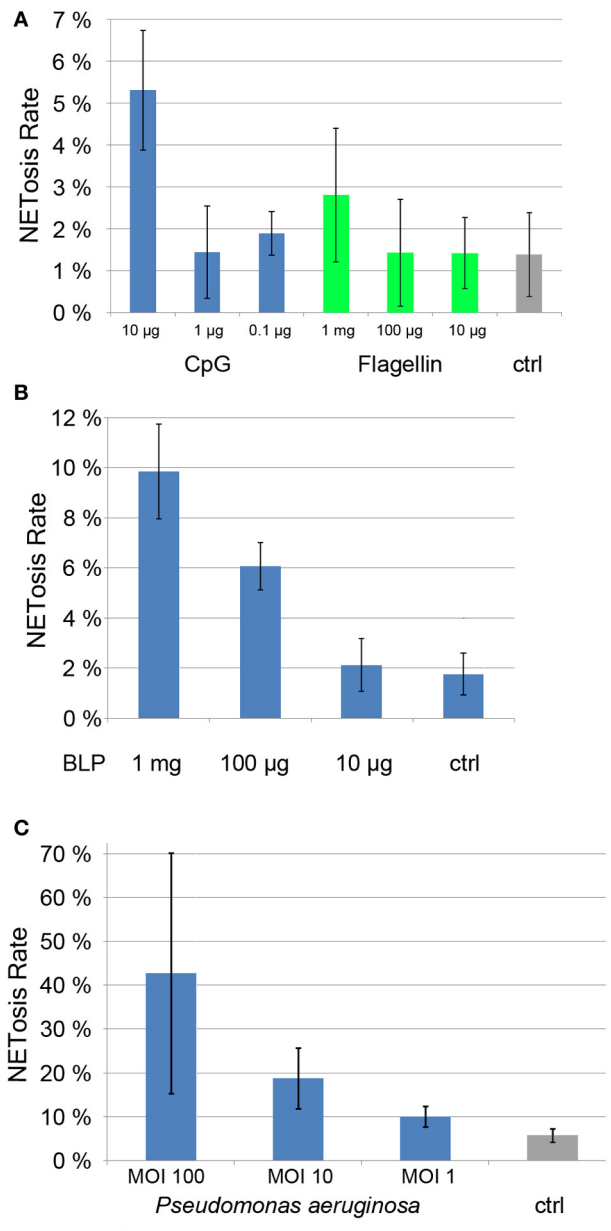

FIGURE 5 | NETosis rates of ligands of Toll-like receptors (CpG, Flagellin, panel 5A and BLP, panel 5B) as well as NETosis rate of neutrophils cocultured with *Pseudomonas aergunisosa* (panel 5C).

NET INDUCED BY PARTICLES

Monosodium urate crystals (MSU) are present in the joint fluid of patients suffering from gout and are involved in acute inflammatory arthritis. Recently it was found that synovial fluid of gout patients as well as MSU crystals induce NET formation (Mitroulis et al., 2011). Also inorganic particles are known to have inflammatory potential, especially silica particles that when inhaled can lead to severe pulmonary diseases such as chronic obstructive pulmonary disease (Hnizdo and Vallyathan, 2003). Treatment of mouse neutrophils with silica particles resulted in the production of ROS (van Berlo et al., 2010).

When we incubated human neutrophils with MSU and silica particles, we found a strong, dose dependent NET induction (**Figure 6**). At 1 mg/ml, NETosis rate was >20% with both types of particle.

NET INDUCTION BY CYTOKINE TREATMENT

Sepsis is a generalized inflammation potentially leading to multiorgan failure and death. It has been shown that during sepsis, activated platelets can induce neutrophils to produce NETs (Clark et al., 2007). The amount of circulating NETs has been proposed to serve as a prognostic marker with less NETs indicating a better chance of survival (Margraf et al., 2008). Both serum from septic patients and a mixture of inflammatory mediators (TNF-α, G-CSF, and IL-1β) induced NETosis in neutrophils from healthy donors (Kambas et al., 2012). When we incubated neutrophils with this mixture for 6 h, we found a NETosis rate of 6% compared to less than 2% in unstimulated controls (**Figure 7**). It remains to be determined if neutrophils cultured

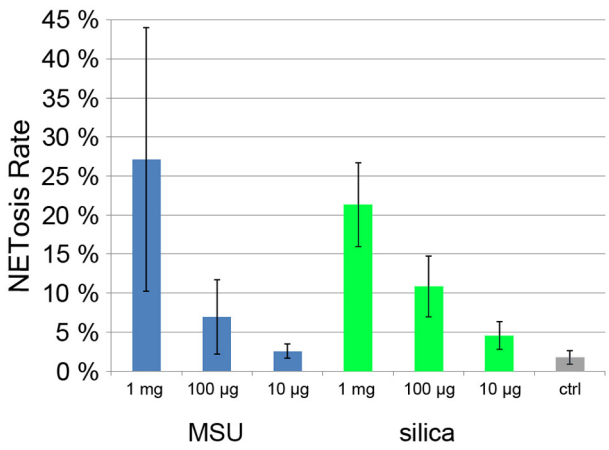

FIGURE 6 | Quantification of NET induction by monosodium urate (MSU) and silica particles.

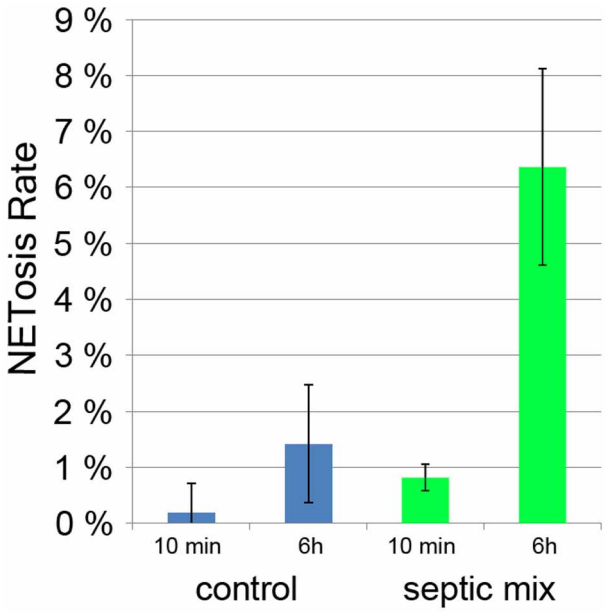

FIGURE 7 | NETosis rate of neutrophils treated for 10 min and 6 h for with proinflammatory cytokines (TNF-α, G-CSF, and IL-1β).

with inflammatory mediators will produce NETs more readily if treated with a second stimulus.

DISCUSSION

We present here a method for (semi-) automatic quantification of the induction of Neutrophil Extracellular Traps. The protocol can be used with simple microscopic equipment (fluorescence microscope with digital camera), but it is also suitable for high-throughput screening using micro titer plate format and hardware-based autofocussing. In this case, the chromatin antibody should be used directly labeled to minimize pipetting steps during preparation of the samples.

The method detects the decondensation of chromatin which is a prerequisite of NET formation. We measured a decrease of Hoechst 33342 fluorescence and an increase of chromatin staining already 10 min after PMA stimulation (**Figure 2**). After about 120 min, plateaus for both fluorescence intensities are reached and remain stable until most cells have reached the final phase of NETosis or have released NETs.

The increase in chromatin fluorescence intensity allows to detect neutrophils even in early phases of NETosis. Setting the threshold above the low chromatin staining of neutrophils which have not (yet) expanded their nuclei, cells with relaxed chromatin can reliably be segmented due to the increase of their fluorescence signal (**Figures 1G,H**; **Figure 3H**). Although the intensity of Hoechst 33342 staining decreases over time, both nuclei of resting and stimulated neutrophils can be segmented to allow identification and enumeration of all cells in the micrographs (**Figure 3G**).

If NETs are produced by several adjacent neutrophils and detected as one fluorescence event, they can possibly be segmented by the computer as one structure (yellow arrowheads in **Figure 3I**). The number of NETotic events will then be underestimated. To avoid this, a lower number of cells can be seeded, or the cells can be stimulated for a shorter period of time to avoid formation of confluent NETs. The optimal time point for quantification can be determined in a time course experiment.

This quantification method provides results with low standard deviation under conditions with low and intermediate NETosis rate (**Figures 5A,B**; **Figure 7**). Conditions that induce higher PMN mobility (MSU crystals, **Figure 6**) or a second source of DNA (i.e., cocultivation h pathogens, **Figure 5C**) result in a higher standard deviation since the number of cells per micrograph will have great variations, and the pathogen DNA will hamper proper Hoechst 33342 signal segmentation. Additionally, these conditions can result in a higher rate of cells that detach from the plate and will be lost during washing steps. In this case, lower standard deviation can be achieved by fine-tuning the cell number and the duration of the experiment.

The protocol presented here might prove useful for the detection of molecules which can regulate the signaling cascade that lead to NET formation and could thus be used as therapeuticals.

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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.

Received: 23 November 2012; accepted: 19 December 2012; published online: 09 January 2013.

Citation: Brinkmann V, Goosmann C, Kühn LI and Zychlinsky A (2013) Automatic quantification of in vitro NET formation. Front. Immun. ***3****:413. doi: 10.3389/fimmu.2012.00413*

This article was submitted to Frontiers in Molecular Innate Immunity, a specialty of Frontiers in Immunology.

frontiers in
IMMUNOLOGY

REVIEW ARTICLE
published: 29 November 2012
doi: 10.3389/fimmu.2012.00360

Activation of PAD4 in NET formation

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Peptidylarginine deiminases, or PADs, convert arginine residues to the non-ribosomally encoded amino acid citrulline in a variety of protein substrates. PAD4 is expressed in granulocytes and is essential for the formation of neutrophil extracellular traps (NETs) via PAD4-mediated histone citrullination. Citrullination of histones is thought to promote NET formation by inducing chromatin decondensation and facilitating the expulsion of chromosomal DNA that is coated with antimicrobial molecules. Numerous stimuli have been reported to lead to PAD4 activation and NET formation. However, how this signaling process proceeds and how PAD4 becomes activated in cells is largely unknown. Herein, we describe the various stimuli and signaling pathways that have been implicated in PAD4 activation and NET formation, including the role of reactive oxygen species generation. To provide a foundation for the above discussion, we first describe PAD4 structure and function, and how these studies led to the development of PAD-specific inhibitors. A comprehensive survey of the receptors and signaling pathways that regulate PAD4 activation will be important for our understanding of innate immunity, and the identification of signaling intermediates in PAD4 activation may also lead to the generation of pharmaceuticals to target NET-related pathogenesis.

Keywords: PAD4, citrullination, deimination, neutrophil, NET

THE PEPTIDYL ARGININE DEIMINASE FAMILY

The mammalian genome encodes 20 natural amino acids; however, this diversity is greatly increased by posttranslational modification of the original set to yield more than one hundred unique amino acids (Uy and Wold, 1977). Citrullination, or deimination, is the posttranslational modification of an arginine to a citrulline residue. Hydrolysis of the guanidino group of the arginine yields a ureido group and the loss of an ammonia (**Figure 1**). Citrullination is catalyzed by the peptidyl arginine deiminase family of enzymes, or PADs. This process results in the loss of positive charge and an approximately 1 Da increase in mass. While this modification seems quite modest, the loss of positive charge, and hydrogen bond acceptors, can have dramatic effects on cell signaling because these types of interactions are critical for stabilizing protein–protein, protein–DNA, and protein–RNA interactions. Additionally, this PTM may disrupt intra-molecular interactions, which could trigger major conformational changes in a protein, potentially altering intermolecular interactions and decreasing protein stability (Vossenaar et al., 2003).

Five PAD enzymes are expressed in humans and mice, and the major difference between these isozymes appears to be tissue localization. PADs 1, 3, and 6 are expressed in the skin and uterus, hair follicles, and egg, ovary and embryo, respectively (Vossenaar et al., 2003; Wang et al., 2012). PAD4 expression has been reported in granulocytes, as well as some cancerous cell lines and tumors (Vossenaar et al., 2003; Chang et al., 2009; Jones et al., 2009; Hemmers et al., 2011; Wang et al., 2012), and most recently in mammalian oocytes and the preimplantation embryo (Brahmajosyula and Miyake, 2011). PAD2 has a much broader tissue expression profile and can be found in the CNS, skeletal muscle, and cells of the immune system (Vossenaar et al., 2003). PADs 1, 2, and 4 are the only PADs expressed in the hematopoietic lineage, and, thus, are especially of immunological interest.

PADI genes, encoding the PAD enzymes, are located in a single gene cluster on chromosome 1p36.1 in humans and chromosome 4pE1 in mice (Vossenaar et al., 2003). The regions encoding the *PADI* locus in humans and mice span 334.7 and 230.4 kb, respectively (Vossenaar et al., 2003). All PAD enzymes are highly conserved, sharing at least 50% sequence homology among isozymes and 70% or greater homology of each vertebrate ortholog (Vossenaar et al., 2003). Eighteen per cent sequence identity is shared among all PADs (Vossenaar et al., 2003). Catalysis by all of these enzymes is calcium dependent, and, at least *in vitro*, requires calcium concentrations that are higher than that available in homeostatic cytoplasm, indicating calcium flux or a calcium-producing event is necessary to induce activity (Arita et al., 2004; Kearney et al., 2005; Raijmakers et al., 2007; Knuckley et al., 2010). Alternatively, a PTM or interacting protein may decrease the calcium concentration required for activation to physiologic levels.

PAD4

Of all of the PADs, PAD4 is of specific interest because of its importance in innate immunity and its putative role in a variety of pathogenic states, including autoimmune diseases, such as rheumatoid arthritis (RA), multiple sclerosis (MS), ulcerative colitis (UC), and systemic lupus erythematosus (SLE), and other inflammatory conditions, such as sepsis and thrombosis (Jones et al., 2009). Many autoantibodies in RA are directed against citrullinated proteins. In fact, the presence of anti-citrullinated protein

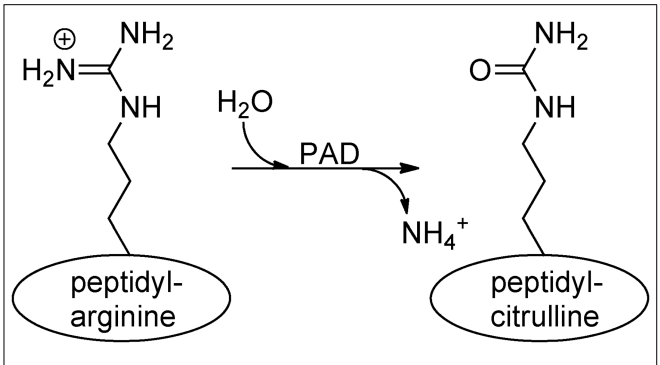

FIGURE 1 | Conversion of arginine into citrulline. Peptidylarginine deiminase (PAD) enzymes convert an arginine residue into citrulline.

antibodies (ACPA) is a better predictor of RA than rheumatoid factor (Vossenaar et al., 2003), and, in 2011, ACPA were included in the new classification criteria for RA (Willemze et al., 2012). A genome-wide association study identified a PAD4 haplotype that is associated with RA in a Japanese population, albeit with a low odds ratio (OR = 1.14; Suzuki et al., 2003). The mutations in the PAD4 gene appear to confer prolonged stability to the transcript, leading to a model where increased expression of PAD4 in these populations would favor the generation of citrullinated self-epitopes to prime the autoimmune response (Suzuki et al., 2003). Though this association has been confirmed in other Asian populations, it has not been replicated in studies using patients from all Western European populations, indicating that the RA-associated PAD4 haplotype found in Asian RA patients can not explain the presence of ACPA's in all ethnicities (Klareskog et al., 2008). Interestingly, the PAD4 RA-associated disease haplotype has also been found in some Japanese patients with UC (Chen et al., 2008). MS patients have increased levels of the citrullinated form of myelin basic protein (Wood et al., 1996; Moscarello et al., 2007), and both PAD2 and PAD4 are overexpressed in the brains of MS patients (Wood et al., 2008). Finally, as will be discussed later in this review, in response to microbes, neutrophils can extrude their nuclear contents to form antimicrobial neutrophil extracellular traps (NETs; Brinkmann and Zychlinsky, 2007). Since PAD4 is essential for the formation of NETs (Li et al., 2010; Hemmers et al., 2011), PAD4 has also been implicated in NET-related pathologies, such as SLE and thrombosis, where NETs presumably promote deleterious inflammatory responses (Kessenbrock et al., 2009; Logters et al., 2009; Fuchs et al., 2010; Hakkim et al., 2010; Garcia-Romo et al., 2011; Villanueva et al., 2011). Thus, PAD4 may be a relevant target for several disease indications.

PAD4 is a 74 kDa protein that exists as a head-to-tail dimer (Arita et al., 2004; Liu et al., 2011). Each monomer consists of two N-terminal immunoglobulin (Ig) domains, formed by Ig subdomain 1, which contains nine β-sheets, and Ig subdomain 2, which contains 10 β-sheets and four short α-helices. The C-terminal catalytic domain adopts the α/β propeller fold that is characteristic of the deiminase superfamily (Shirai et al., 2001; Arita et al., 2004). The C-terminal catalytic domain is the most highly conserved area of the molecule (Vossenaar et al., 2003), suggesting that the active sites are likely quite similar among PADs. While a high degree of conservation exists among PADs, PAD4 is the only family member to contain a classic nuclear localization sequence (56-PPAKKKST-63), found in Ig1 near the N-terminus, and, thus, is trafficked to the nucleus (Nakashima et al., 2002; Vossenaar et al., 2003; Arita et al., 2004). However, it is worth noting that recent data indicates that other PADs, most notably PAD2, are localized to the nucleus (Zhang et al., 2012).

PAD4 binds five calcium molecules, designated Ca1–Ca5, in a cooperative manner (Arita et al., 2004; Kearney et al., 2005; Liu et al., 2011). Ca1 and Ca2 bind in the C-terminal catalytic domain, and their binding induces major conformational changes that move several active site residues into positions that are competent for catalysis (Arita et al., 2004). This calcium-induced formation of the active site is unique to the PADs, and represents a novel mechanism of enzyme activation (Arita et al., 2004). Calcium binding also induces large structural changes in the N-terminus of the protein. For example, binding of Ca3, Ca4, and Ca5, along with two water molecules, induces the formation of the a1 α-helix, which is disordered in the apoenzyme (Arita et al., 2004). These conformational changes may provide, or remove, docking sites for other proteins, which may serve to further regulate PAD activity.

BIOCHEMICAL ACTIVATION OF PAD4

While it is unknown whether all PAD enzymes are capable of multimerizing, the dimer has been suggested to be required for PAD4 activity (Arita et al., 2004; Liu et al., 2011). However, the effects on enzyme activity and the calcium dependence of the enzyme are relatively minor (approximately twofold), and we routinely see robust enzyme activity at concentrations of protein that are an order of magnitude below the K_d of the dimer (Kearney et al., 2005). Nevertheless, dimer formation may represent a possible regulatory mechanism (Liu et al., 2011). Dimerization is mediated by both hydrophobic interactions and salt bridges between adjacent monomers (Arita et al., 2004; Liu et al., 2011).

The PADs display limited substrate specificity and citrullinate many proteins *in vitro*, preferring to modify arginine residues present in unstructured regions; the rate of substrate turnover is inversely proportional to the structural order of the substrate (Arita et al., 2006; Knuckley et al., 2010). Structurally, PAD4 interacts with the backbone atoms surrounding the site deimination, i.e., R-2, R-1, R0, and (R + 1), with few, if any, contacts with the side chains (Arita et al., 2006). The only requirement appears to be a small side chain at the R-2 position so as to avoid steric contacts with the active site (Arita et al., 2006). Upon binding to PAD4 the backbone of the substrate adopts a β-turn-like conformation within the substrate binding cleft (Arita et al., 2006), thereby explaining why the enzymes show such a high level of substrate promiscuity. In contrast to the situation *in vitro*, the PADs are believed to show greater substrate specificity *in vivo*. Presumably, interacting proteins modulate the substrate specificity of the enzyme or spatially target the enzyme to specific regions of the cell. For example, PAD4 is present in the nucleus and may be targeted to chromatin where it citrullinates a number of nuclear proteins, including the histones and protein arginine methyltransferase 1 (PRMT1; Vossenaar et al., 2003; Slack et al., 2011b). Although PAD4 was reported to convert monomethylated arginine residues to citrulline (Wang et al., 2004), this modification

occurs at rates that are 10^2- to 10^3-fold slower than an unmodified arginine, suggesting that the, so-called "demethylimination" reaction is not physiologically relevant (Hidaka et al., 2005; Kearney et al., 2005; Raijmakers et al., 2007; Thompson and Fast, 2006) and that citrullination simply antagonizes arginine methylation as originally suggested by Cuthbert et al. (2004).

In addition to the aforementioned protein substrates, PAD4, as well as the other PADs, autocitrullinate at several sites on the enzyme (Andrade et al., 2010; Mechin et al., 2010; Slack et al., 2011a). Although autocitrullination has been reported to directly modulate PAD4 activity (Andrade et al., 2010; Mechin et al., 2010), in our hands, this self-modification has no direct effect on enzyme activity, but it does appear to modulate protein–protein interactions (Slack et al., 2011b). For example, Slack et al. (2011b) demonstrated that citrullination of PAD4 reduces its ability to interact with PRMT1 and histone deacetylase (HDAC) 1, perhaps modulating its ability to alter gene transcription.

PAD MECHANISM AND INHIBITION

Given the substrate promiscuity of the PADs, it is unsurprising that the PADs also citrullinate a number of small molecule arginine mimics, including benzoyl arginine ethyl ester (BAEE) and benzoyl arginine amide (BAA). In fact, these compounds have served as important mechanistic probes of PAD4 catalysis and provided the molecular scaffold for the construction of the first highly potent PAD4 inhibitors. Below we highlight key mechanistic insights that guided the design of these inhibitors.

Briefly, there are four key catalytic residues, Asp350, His471, Asp473, and Cys645. Asp473 binds to both ω-nitrogens and Asp350 coordinates to one ω-nitrogen and the δ-nitrogen (**Figure 2A**). Cys645 and His471 lie on opposite sides of the guanidinium group and are appropriately positioned to promote catalysis via nucleophilic attack on the guanidinium carbon (Cys645) and protonation of the developing tetrahedral intermediate (His471; **Figure 2B**). Collapse of this intermediate leads to the loss of ammonia and the formation of the stable *S*-alkyl thiouronium intermediate that is subsequently hydrolyzed via a second tetrahedral intermediate to form citrulline; His471 likely activates the water molecule for nucleophilic attack (**Figure 2B**). Mechanistic studies (Knuckley et al., 2007, 2010), including mutagenesis, pH rate profile, solvent isotope effects, and solvent viscosity effects, as well as crystal structures of PAD4 bound to several substrates (i.e., BAA; Arita et al., 2006), and histone H3 and histone H4 tail analogs; Arita et al., 2004, 2006), and inhibitors (i.e., F-amidine, Cl-amidine, *o*-F-amidine, *o*-Cl-amidine, and TDFA; Luo et al., 2006a; Causey et al., 2011; Jones et al., 2012), provide strong support for the above mechanism and helped drive our thoughts about inhibitor design. For example, the presence of a reactive active site Cys prompted us to consider the synthesis of irreversible inhibitors (Knuckley et al., 2007, 2010). Additionally, the fact that mono-methylated arginine residues were exceptionally poor PAD substrates, as well as the steric restraints of the active site (Arita et al., 2004; Kearney et al., 2005; Raijmakers et al., 2007), told us that the reactive moiety would have to be relatively isosteric with respect to the substrate guanidinium. Furthermore, mutagenesis studies on the two active site aspartyl groups in PAD4, Asp350 and Asp473, showed that these residues are critical for catalysis (Knuckley et al., 2007), indicating that proper positioning, hydrogen bonding, and electrostatic interactions between these residues and the substrate guanidinium are critical determinants for efficient substrate turnover, and would have to be maintained for efficient enzyme inactivation. As such, we initially focused our efforts on synthesizing F-amidine and Cl-amidine (**Figure 2C**), two haloacetamidine-containing compounds that we

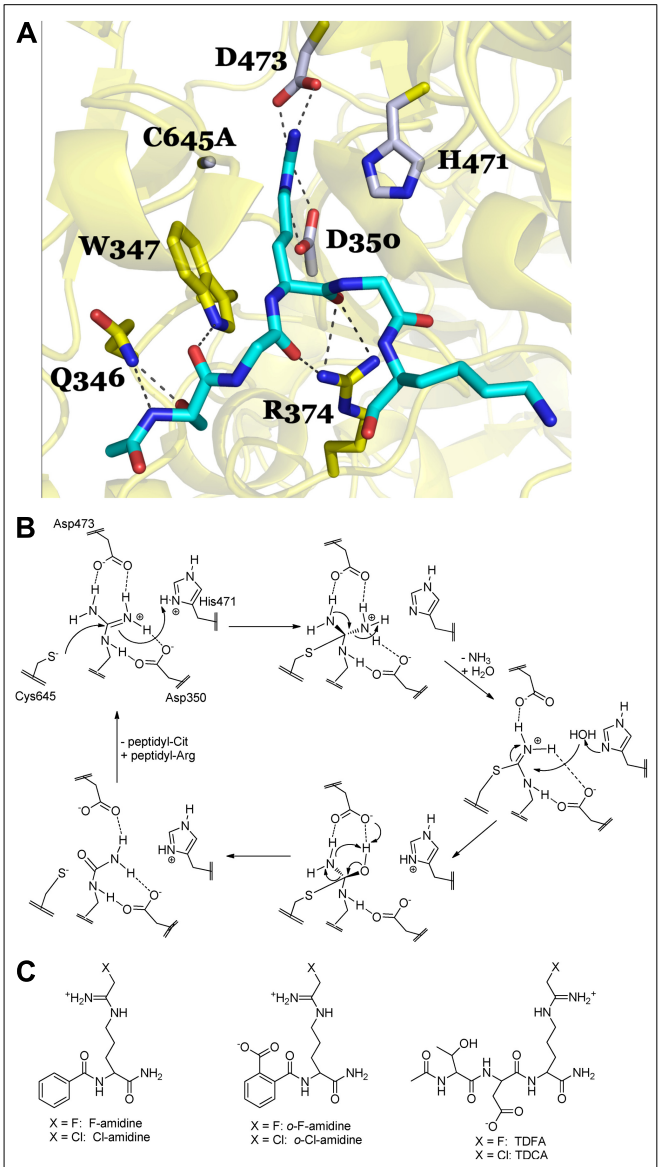

FIGURE 2 | (A) PAD4 in complex with Histone H4 1–5 (SGRGK). PAD4 active site residue side chains are colored gray (D350, H471, D473, C645A) and residues that are involved in binding the H4 1–5 backbone and S1 are colored yellow (Q346, W347, R347). N-terminally acetylated H4 1–5 is shown in cyan with Arginine 3 bound in the PAD4 active site. Polar residue interactions between PAD4 and H4 1–5 are indicated by dashed lines. The mutation of the active site cysteine residue (C645) to an alanine (C645A) was necessary to achieve substrate binding in the crystal structure as described in Arita et al. (2006). Figure was created from the structure filed under PDB code 2dey. **(B)** Proposed catalytic mechanism for PAD4. **(C)** Haloacetamidine-based inhibitors targeting the PAD isozymes.

hypothesized, and later confirmed, would inactivate the PADs by alkylating Cys645 (Luo et al., 2006a,b). These initial inhibitors, F-amidine and Cl-amidine, as well as our second generation compounds *o*-F-amidine, *o*-Cl-amidine, and TDFA (Knuckley et al., 2010; Causey et al., 2011), which show enhanced potency and selectivity, are bioavailable and have been used to show that the PADs play important roles in controlling gene transcription (Li et al., 2008; Yao et al., 2008; Jones et al., 2012; Zhang et al., 2011, 2012), fertility (Kan et al., 2012), differentiation (Slack et al., 2011c), and NET formation (Wang et al., 2009; Li et al., 2010). Additionally, Cl-amidine, or a Cl-amidine analog, decrease disease severity in animal models of RA, UC, nerve damage, and cancer (Chumanevich et al., 2011; Lange et al., 2011; Willis et al., 2011; Wang et al., 2012). Specifically, we were the first to show that the PAD inhibitor, Cl-amidine dose dependently decreased inflammation by up to 55% in the mouse collagen-induced arthritis (CIA) model of RA. Concomitant with the decreased severity there were significant decreases in the levels of citrullinated proteins, complement deposition, and epitope spreading (Willis et al., 2011). Similar dose-dependent effects were observed in the dextran sodium sulfate (DSS) model of UC where dosing of up to 75 mg/kg after the onset of disease led to significant reductions in weight loss, inflammation score, and colon lengthening (Chumanevich et al., 2011). The effects of Cl-amidine on nerve damage was examined in a chick embryo model of spinal cord injury where treatment with Cl-amidine reduced the abundance of deiminated histone 3, consistent with inhibition of PAD activity, and significantly reduced apoptosis and tissue loss following injury at embryonic day 15 (Lange et al., 2011). Finally, Wang et al. (2012) showed that a Cl-amidine analog, YW3-56, decreased tumor growth in a mouse sarcoma S-180 cell-derived solid tumor model and that additive effects on growth inhibition were observed when this compound was combined with the histone deacetylase inhibitor SAHA. We have observed similar effects with Cl-amidine in xenografts model of ductal carcinoma *in situ* (McElwee et al., 2012).

THE FUNCTION OF PAD4 IN NEUTROPHILS

Neutrophils are terminally differentiated granulocytes, which differentiate from hematopoietic stem cells in the bone marrow, and make up to 75% of white blood cells in the circulation (Ermert et al., 2009b). Mature neutrophils are released into circulation, where they have a very short life span of several hours to one day before undergoing apoptosis (Borregaard, 2010). Neutrophils are an important component of the innate immune system and form the first line of defense against invading pathogens, such as bacteria and fungi (Borregaard, 2010). Neutrophils contain an arsenal of antimicrobial proteins and peptides in primary (or azurophilic), secondary (or specific), and tertiary granules. Primary granules are mostly composed of proteases, such as myeloperoxidase and neutrophil elastase (NE), and antimicrobial peptides, such as defensins (Ermert et al., 2009b). Secondary and tertiary granules contain lactoferrin and gelatinase, respectively (Mantovani et al., 2011). Secretory granules also harbor stores of membrane proteins, such as the NADPH oxidase machinery (Nox; Borregaard, 2010), which can be trafficked to the surface of the cell quickly when necessary. In response to chemoattractants, neutrophils are guided to areas of infection, where they respond with several effector mechanisms to invading pathogens, including phagocytosis, release of bactericidal products, and ROS production (Borregaard, 2010). Neutropenia, or the state of having too few neutrophils, leads to extreme immunosuppression and susceptibility to bacterial infections, which can be fatal (Janeway, 2005).

In 2004, Brinkmann et al. (2004) recognized the formation of NET structures, which were extruded by neutrophils in response to bacteria. NETs are composed of nuclear DNA that are decorated with a variety of nuclear and granular proteins, actively thrown out into the extracellular space, and result in death of the NET-producing cell (Brinkmann et al., 2004). Cell death by this mechanism is unique from apoptosis and necrosis and has been termed "NETosis"(Gupta et al., 2010). NETs ensnare extracellular bacteria, which are killed by the inherent antimicrobial properties of NET-associated proteins, such as histones (Parseghian and Luhrs, 2006), NE (Papayannopoulos and Zychlinsky, 2009), and lactoferrin (Papayannopoulos and Zychlinsky, 2009). These structures represent a novel method for pathogen killing, independent of both degranulation and phagocytosis, and have been shown to effectively kill a variety of pathogens, including bacteria, fungi, and protozoa (Papayannopoulos and Zychlinsky, 2009; Remijsen et al., 2011a). NETs have also been reported to occur in response to viral infection; however, they not appear to show any observable anti-viral capabilities (Hemmers et al., 2011). NETs may represent a killing mechanism for pathogens too large for the neutrophil to phagocytose, such as fungal hyphae or helminthes (Urban et al., 2006). Interestingly, bacteria have adapted defense mechanisms to NET formation. For example, the Group A *Streptococcus* express a DNase enzyme that can degrade NETs (Buchanan et al., 2006), and *Pseudomonas aeruginosa* expresses surface molecules that can prevent neutrophil activation and NET formation (Khatua et al., 2012).

Histone citrullination is thought to promote NET formation by inducing chromatin decondensation and facilitating the expulsion of chromosomal DNA coated with antimicrobial molecules (Neeli et al., 2008; Wang et al., 2009; Li et al., 2010). In fact, chemical inhibition of PAD4 activity significantly reduces histone decondensation and NET formation in response to either the calcium ionophore ionomycin or the bacterium *Shigella flexneri* (Wang et al., 2009). Our group and the Wang group have independently created PAD4-deficient mice (Li et al., 2010; Hemmers et al., 2011). Neutrophils isolated from PAD4-deficient mice are unable to citrullinate histones, decondense chromatin, and generate NETs (Li et al., 2010; Hemmers et al., 2011). Because of their inability to form NETs, PAD4 KO mice were shown to be more susceptible to bacterial infection (Li et al., 2010), and, thus, PAD4 is an important mediator of innate immunity.

Neutrophil elastase, a neutrophil serine protease, resides in the azurophilic granules and is a component of NETs (Borregaard, 2010; Amulic et al., 2012). The cleavage of microbial virulence factors by NE is essential for the clearance of specific Gram-negative bacteria (Pham, 2006). NE also cleaves histones to drive chromatin decondensation during NET formation (Papayannopoulos et al., 2010). Indeed, NE is essential for the process of NETosis (Papayannopoulos et al., 2010), and it is interesting to speculate that histone citrullination, by PAD4, promotes a relaxing

of the chromatin structure, allowing NE to gain access. Thus, the activity of NE and PAD4 may converge upon the chromatin decondensation process and NET formation. Neutrophils isolated from PAD4-deficient mice will be useful to delineate the hierarchy between PAD4 and other molecules, like NE, that are required for NETosis.

STIMULATION OF PAD4 ACTIVITY

A number of stimuli, including live and heat-killed bacteria, fungi, protozoa, and chemokines have been reported to induce NET formation (Guimaraes-Costa et al., 2012). Because NET formation is PAD4-dependent (Li et al., 2010; Hemmers et al., 2011), these same stimuli likely also induce PAD4 activation. However, the activity of PAD4 in relation to each stimuli must be assessed by looking for citrullination of histones, which is both a hallmark of PAD activity (Neeli et al., 2008, 2009) and a component of NETs (Li et al., 2010; Hemmers et al., 2011). Only a handful these, including live bacteria, the Gram-negative bacterial cell wall component lipopolysaccharide (LPS), the Gram-positive bacterial cell wall component lipoteichoic acid (LTA), the fungal cell wall component zymosan, the proinflammatory cytokine TNFα, and H_2O_2 have been shown to induce PAD4 activity and histone citrullination (Neeli et al., 2008, 2009; Li et al., 2010; Hemmers et al., 2011). As discussed earlier, PAD4 is calcium-dependent, and it is thought that PAD4 requires calcium levels higher than are found in the homeostatic cell to be active (Vossenaar et al., 2004). Not surprisingly, the calcium ionophore ionomycin activates PAD4 to induce histone citrullination and elicit NET formation (Neeli et al., 2008, 2009; Wang et al., 2009; Li et al., 2010). **Table 1** catalogs the variety of stimuli reported to stimulate NET formation.

Although little is known about the downstream signaling pathways required for PAD4 activation in neutrophils, cytoskeletal activity may be involved in PAD4 activation. Pretreatment of cells with nocodazole or cytochalasin D, which inhibit microtubule polymerization, prior to LPS stimulation leads to a reduction of histone citrullination and NET formation (Neeli et al., 2009). Additionally, blockade of integrin signaling through Mac-1 and cytohesin-1 impeded PAD4 activity and NET formation (Neeli et al., 2009). How cytoskeletal signaling impacts PAD4 is unknown; however, it has been proposed that the same receptors establish whether a cell will undergo phagocytosis or NET formation (Neeli et al., 2009). Indeed, studies have indicated that neutrophils initiate NET formation when phagocytosis of a large particle fails (Urban et al., 2006). Perhaps cytoskeletal activity and PAD4-mediated citrullination are linked because the initiation of NET formation represents a back-up killing mechanism following unsuccessful phagocytosis.

PAD4 ACTIVITY AND ROS

The generation of reactive oxygen species (ROS) is initiated by a wide variety of neutrophil stimuli, including phagocytosis of pathogens and signaling by LPS and TNF (Kohchi et al., 2009), which are also NET-inducing stimuli. Indeed, ROS generation is required for NET formation, and, thus, it is likely that ROS generation is required for PAD4 activation as well. In neutrophils, superoxide ($O_2^{\bullet -}$) is generated by the NADPH complex (Nox) and by the electron transport chain in mitochondria (Kohchi et al., 2009). $O_2^{\bullet -}$ is then converted to H_2O_2 spontaneously or by the enzyme superoxide dismutase (SOD; Kohchi et al., 2009). H_2O_2 acts directly on target cells and is converted to additional effectors by enzymes such as myeloperoxidase (MPO). Interestingly, the addition of H_2O_2 to primary murine or human neutrophils induces PAD4-dependent histone citrullination (Neeli et al., 2008; Li et al., 2010). ROS molecules are highly cytotoxic and act as antimicrobial agents, but they can also play a dual role as reversible signal transduction mediators to regulate redox-sensitive target proteins (Amulic et al., 2012).

The link between ROS and NET formation was first recognized by the fact that patients with chronic granulomas disease (CGD), who are missing the Nox2 protein essential for NADPH assembly and, thus, cannot form ROS. Neutrophils isolated from CGD patients do not make NETs in response to *S. aureus* or phorbol myristate acetate (PMA; Fuchs et al., 2007). This phenotype is rescued by addition of H_2O_2 or exogenous glucose oxidase, which generates H_2O_2 (Fuchs et al., 2007), indicating that the ROS production facilitated by Nox2 is necessary for NETs. Catalase removes intracellular H_2O_2 by reduction to water and oxygen (Kohchi et al., 2009), and catalase inhibition increases intracellular H_2O_2, leading to increased NET production in healthy neutrophils (Fuchs et al., 2007). Subsequent studies have demonstrated that ROS generation is upstream of chromatin decondensation (Remijsen et al., 2011b), suggesting that NADPH oxidase activation may also be a prerequisite for PAD4 activation. Indeed, LPS-induced citrullination of histone H4 is decreased when cells are pre-incubated with the NADPH oxidase inhibitor apocynin (Neeli et al., 2009). Although, to our knowledge, the activity of PAD4 in CGD neutrophils has not yet been directly explored, since chromatin decondensation is not observed in CGD neutrophils, we would predict PAD4-mediated histone citrullination is also impaired. Since H_2O_2 treatment can activate PAD4-mediated histone deimination in primary murine and human neutrophils (Neeli et al., 2008; Li et al., 2010), and since NADPH activation seems to be upstream of NET formation (Neeli et al., 2009), we speculate that PAD4 activation may be downstream of NADPH (**Figure 3**).

The contribution of specific ROS molecules to NET formation has also been examined. NADPH oxidase or mitochondrial ROS selective inhibitors established a requirement for NADPH oxidase generated $O_2^{\bullet -}$ (Nishinaka et al., 2011), but excluded a need for mitochondrial ROS in PMA-induced NET generation (Kirchner et al., 2012). MPO catalyzes the oxidation of Cl^- ions to generate hypochlorous acid (HOCl) and other reactive products using H_2O_2 as a cosubstrate (Arnhold and Flemmig, 2010). In the absence of MPO activity, NET generation is absent (Papayannopoulos et al., 2010; Metzler et al., 2011; Kirchner et al., 2012), but this phenotype can be rescued by addition of HOCl (Palmer et al., 2012). In fact, the HOCl product of MPO has also been found to be both necessary and sufficient for NET formation, and in CGD neutrophils, the addition of HOCl is also sufficient to initiate NET formation (Palmer et al., 2012). Taurine is a cellular antioxidant capable of reducing HOCl and H_2O_2 to promote cell survival (Palmer et al., 2012). Accordingly, preincubation of neutrophils with taurine prior to PMA or HOCl stimulation reduces NET formation (Palmer et al., 2012). Additionally, while SOD

Table 1 | NET/PAD4 stimuli.

NET stimuli	Shown to activate PAD4	Reference
Activated endothelial cells	n.d.	Gupta et al. (2010)
Aspergillus fumigatus	n.d.	Bruns et al. (2010), McCormick et al. (2010)
Candida albicans	n.d.	Ermert et al. (2009a), Urban et al. (2009), Yost et al. (2009), Hakkim et al. (2010), Svobodova et al. (2012)
Opsonized *Candida albicans*	n.d.	Metzler et al. (2011)
Cryptococcus species	n.d.	Urban et al. (2009), Springer et al. (2010)
Escherichia coli	Yes	Grinberg et al. (2008)), Wang et al. (2009), Yost et al. (2009), Yan et al. (2012)
f-MLP	Yes	Neeli et al. (2008)
H_2O_2	Yes	Neeli et al. (2008), Li et al. (2010)
Haemophilus influenzae	n.d.	Juneau et al. (2011)
IL-8+ *Shigella flexneri*	Yes	Wang et al. (2009)
IL-8[1]	n.d.	Brinkmann et al. (2004), Gupta et al. (2005)
Ca^{2+} ionophore	Yes	Wang et al. (2009)
Klebsiella pneumoniae	n.d.	Papayannopoulos et al. (2010)
Leishmania species	n.d.	Guimaraes-Costa et al. (2009), Gabriel et al. (2010)
Listeria monocytogenes	n.d.	Ermert et al. (2009a), Munafo et al. (2009)
LPS	Yes	Neeli et al. (2008), Li et al. (2010), Munafo et al. (2009)
Lipoteichoic acid	Yes	Neeli et al. (2009)
Mycobacterium species	n.d.	Ramos-Kichik et al. (2009)
Nitric Oxide	n.d.	Patel et al. (2010)
Platelet activating factor	n.d.	Yost et al. (2009), Fuchs et al. (2010)
Platelet TLR-4	n.d.	Clark et al. (2007)
Phorbol-12-myristrate-13-acetate (PMA)	Yes	Brinkmann et al. (2004), Ermert et al. (2009a), Munafo et al. (2009), Neeli et al. (2009), Chow et al. (2010), Li et al. (2010), Papayannopoulos et al. (2010), Pilsczek et al. (2010), Lin et al. (2011), Metzler et al. (2011), Remijsen et al. (2011b), Villanueva et al. (2011), Menegazzi et al. (2012), Palmer et al. (2012), Parker et al. (2012), Yan et al. (2012)
Pseudomonas aeruginosa	n.d.	Pilsczek et al. (2010)
Salmonella typhimurium	n.d.	Brinkmann et al. (2004)
Shigella flexneri	Yes	Brinkmann et al. (2004), Li et al. (2010)
Staphylococcus aureus	n.d.	Brinkmann et al. (2004), Chow et al. (2010), Pilsczek et al. (2010)
Opsonized *Staphylococcus aureus*	n.d.	Palmer et al. (2012)
Staphylococcus epidermidis δ-toxin	n.d.	Cogen et al. (2010)
Streptococcus species	Yes[2]	Beiter et al. (2006), Buchanan et al. (2006), Lauth et al. (2009), Oehmcke et al. (2009), Crotty Alexander et al. (2010), Li et al. (2010), Pilsczek et al. (2010)
Streptococcus pneumoniae α-Enolase	n.d.	Mori et al. (2012)
TNFα	Yes	Neeli et al. (2009), Wang et al. (2009)
Toxoplasma gondii	n.d.	Abi Abdallah et al. (2012)
Yersinia enterocolitica	n.d.	Casutt-Meyer et al. (2010)
Zymosan	Yes	Neeli et al. (2009)

n.d., not determined.
[1] *Some investigators have reported that IL-8-induced NET formation may be sensitive to cell culture conditions (Marcos et al., 2011).*
[2] *M1 GAS deficient in an extracellular DNase (Sda1) could induce PAD4-dependent NETs (Li et al., 2010).*

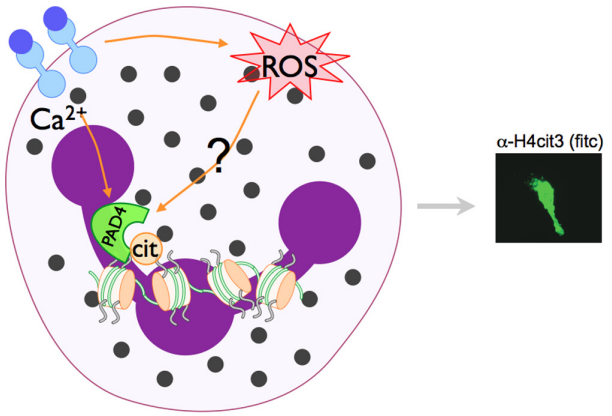

FIGURE 3 | Model of PAD4 activation in NET formation. Pathways that activate NET formation are less defined than phagocytic pathways, but are known to require NADPH oxidase activity and the activation of PAD4 and subsequent histone citrullination. PAD enzymes are Ca^{2+}-dependent. Since PAD4-mediated histone citrullination is abrogated by the NADPH inhibitor apocyanin (Neeli et al., 2009), we speculate that NADPH regulated ROS generation and increase Ca^{2+} levels may converge to activate PAD4 in neutrophils.

inhibition does not impede NET formation, addition of exogenous SOD does seem to increase NET production, perhaps owing to the increase in available H_2O_2 (Palmer et al., 2012). These studies indicate a model in which NADPH oxidase activity generates $O_2^{\bullet -}$, which then dismutates to H_2O_2 either spontaneously or with the help of SOD, and is then used by MPO to generate HOCl, which is necessary and sufficient to induce NET formation. It will be interesting to determine whether HOCl can also directly regulate PAD4 activation.

PAD4 AND AUTOPHAGY

Like ROS, autophagy has been shown to be required for chromatin decondensation during NET generation (Remijsen et al., 2011b); however, these two processes seem to be independent of each other (Remijsen et al., 2011b). Blockade of PI3K with wortmannin inhibits autophagy, and pretreatment of PMA stimulated neutrophils with wortmannin prevented chromatin decondensation (Remijsen et al., 2011b). However, no direct role between autophagy, PI3K and citrullination has been shown. Recently, newly developed PAD4 inhibitors were found to reduce autophagy processes in an osteosarcoma cell line (Wang et al., 2012), further providing evidence of a link between PAD4 activity and autophagy.

CONCLUSION

Neutrophil extracellular traps have been reported in several pathological scenarios, including SLE, sepsis, thrombosis, and infectious disease, and may induce or exacerbate inflammation through prolonged inflammatory response, tissue damage, and presentation of neo-antigens. Indeed, our group recently described the PAD4-dependent formation of NETs in a murine model of the effector phase of RA; however, PAD4 was dispensable for disease in this model (Rohrbach et al., 2012). Pathology caused or exacerbated by NETs is an expanding field of research. Because PAD4 is required for NET formation, inhibition of PAD4 activity may improve clinical outcomes in patients experiencing these inflammatory diseases. In fact, PAD inhibitors have demonstrated efficacy in a variety of immune pathologies (Chumanevich et al., 2011; Lange et al., 2011; Slack et al., 2011c; Wang et al., 2012; Willis et al., 2011). Of course, because PAD4 is required for microbial-induced NET formation (Li et al., 2010; Hemmers et al., 2011), the side effects of PAD4-targeted therapeutics may also include increased susceptibility to infectious diseases.

PAD4 is an important component in the innate immune system, however its activity has been linked to a wide variety of disease states, including cancer, autoimmunity, and other inflammatory conditions. Despite its significance, little is understood about how the PAD4 enzyme becomes active in order to impart its helpful and harmful effects. At the protein level, calcium binding, dimerization, and autocitrullination may help regulate its activity. ROS may also play a role in regulating PAD4 activation (see model in **Figure 3**), and recently associations between PAD4 activity and autophagy have been proposed. Despite these efforts, much is left to understand about PAD4 enzyme regulation. Anti-PAD4 therapies have been proposed for inflammatory conditions and cancer, thus a more comprehensive understanding of the pathways that activate PAD4 in neutrophils will be important.

ACKNOWLEDGMENTS

The work in Dr. Mowen's laboratory was supported grants GM085117 and AI067460 from the National Institutes of Health (Kerri A. Mowen). The work in Dr. Thompson's laboratory was supported by grants GM079357 and CA151304 from the National Institutes of Health as well as an American College of Rheumatology Research Foundation Within Our Reach Grant. We apologize to investigators whose important contributions were not included due to space limitations. This is manuscript #21941 from The Scripps Research Institute.

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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.

Received: 17 September 2012; paper pending published: 04 October 2012; accepted: 11 November 2012; published online: 29 November 2012.

*Citation: Rohrbach AS, Slade DJ, Thompson PR and Mowen KA (2012) Activation of PAD4 in NET formation. Front. Immun. **3**:360. doi: 10.3389/fimmu.2012.00360*

This article was submitted to Frontiers in Molecular Innate Immunity, a specialty of Frontiers in Immunology.

frontiers in IMMUNOLOGY

ORIGINAL RESEARCH ARTICLE
published: 04 October 2012
doi: 10.3389/fimmu.2012.00307

PAD4 mediated histone hypercitrullination induces heterochromatin decondensation and chromatin unfolding to form neutrophil extracellular trap-like structures

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NETosis, the process wherein neutrophils release highly decondensed chromatin called neutrophil extracellular traps (NETs), has gained much attention as an alternative means of killing bacteria. *In vivo*, NETs are induced by bacteria and pro-inflammatory cytokines. We have reported that peptidylarginine deiminase 4 (PAD4), an enzyme that converts Arg or monomethyl-Arg to citrulline in histones, is essential for NET formation. The areas of extensive chromatin decondensation along the NETs were rich in histone citrullination. Here, upon investigating the effect of global citrullination in cultured cells, we discovered that PAD4 overexpression in osteosarcoma U2OS cells induces extensive chromatin decondensation independent of apoptosis. The highly decondensed chromatin is released to the extracellular space and stained strongly by a histone citrulline-specific antibody. The structure of the decondensed chromatin is reminiscent of NETs but is unique in that it occurs without stimulation of cells with pro-inflammatory cytokines and bacteria. Furthermore, histone citrullination during chromatin decondensation can dissociate heterochromatin protein 1 beta (HP1β) thereby offering a new molecular mechanism for understanding how citrullination regulates chromatin function. Taken together, our study suggests that PAD4 mediated citrullination induces chromatin decondensation, implicating its essential role in NET formation under physiological conditions in neutrophils.

Keywords: pad4, hypercitrullination, neutrophil extracellular traps, chromatin decondensation, heterochromatin protein 1, histone modifications

INTRODUCTION

Neutrophils serve as an integral part of the body's innate immune system as they are the first line of defense against invading microbes (Kanthack and Hardy, 1894; Nathan, 2006). Upon release from circulation, a chemotactic gradient guides neutrophils to specific sites of infection. Equipped with an arsenal of antimicrobial proteins, neutrophils are then able to rapidly attack and destroy the pathogens they encounter (Wang et al., 2012). Once a neutrophil recognizes its target, the process of phagocytosis commences. First, actin reorganization under the neutrophil membrane brings the pathogen into a phagosome, where the subunits of the membrane associated nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system would assemble to facilitate formation of reactive oxygen species (ROS). These cytotoxic ROS then attack the pathogen following the formation of the phagolysosome—an intracellular structure where the phagosome fuses with numerous antimicrobial peptide-containing granules and lysosomes. Together, ROS and the antimicrobial peptides effectively destroy the microbe (Klebanoff, 1999). Until recently, phagocytosis has been the most widely accepted method by which neutrophils destroy pathogens.

A novel antimicrobial mechanism, by which neutrophil extracellular traps (NETs) mediate bacterial killing, is now being widely accepted (Brinkmann et al., 2004). Neutrophils are observed to generate extracellular chromatin fibers upon activation with interleukin-8 (IL-8), phorbol myristate acetate (PMA), lipopolysaccharide (LPS) and bacteria (Brinkmann et al., 2004). Following activation, neutrophils undergo distinct morphological alterations leading to ultimate NET formation. First the lobular shape of the neutrophil nucleus is lost followed by nuclear envelope disintegration. Then nuclear, cytoplasmic and granular components mix together followed by a rupture of the cell membrane and the release of intact chromatin into the extracellular space (Fuchs et al., 2007). This extracellular chromatin—NETs—is composed of proteases (i.e., elastase and myeloperoxidase) and histones, which render NETs the antimicrobial ability to restrict pathogens at the site of infection and to ultimately destroy the bacteria they come in contact with (Brinkmann et al., 2004). The significance of NETs as an essential host defense mechanism cannot be underestimated. In chronic granulomatous disease (CGD) patients with impaired NADPH oxidase activity and ROS production, neutrophils have poor antimicrobial activity, which is in part due to the inability to produce NETs (Fuchs et al., 2007; Bianchi et al., 2009). Reversely, gene therapy with the NADPH gene in CGD patients restores NET formation and is a viable treatment for this disease (Bianchi et al., 2009).

The discovery of NETs was a landmark discovery in the field of immunology because it established a novel way by which the body

can fight off infections. "Netting" neutrophils comprise a significant division of the host innate defense mechanism as evidenced with the finding of extensive extracellular DNA structures at sites of infection. However, too much NETs are implicated in several diseases, such as deep vein thrombosis (Reayi and Arya, 2005) and multiple sclerosis (Mastronardi et al., 2006). Furthermore, genetic studies linked PAD4 with rheumatoid arthritis (Suzuki et al., 2003) and PAD4 in the synovial fluid of RA patients likely produces citrullinated autoimmune antigens during disease progression (Kinloch et al., 2008). Overall, NETs represent a doubled-edged sword in that these extracellular chromatin structures aid the body to eliminate infections but can also cause diseases. By studying the molecular processes underlying NET formation, there is a strong clinical potential to better understand and find means of regulating the pathological conditions caused by NETs.

NETs are comprised of extensively decondensed chromatin, suggesting that higher-order chromatin is involved in NET formation. In the eukaryotic nucleus, 147 bp of DNA tightly associates with the histone octamer (two of each histones H3, H2B, H2A, and H4) to form a nucleosome—the basic structural unit of chromatin (Richmond and Davey, 2003). The subsequent binding of linker histone H1 further compacts the DNA to form 30 nm chromatin fibers (Horn and Peterson, 2002). It is well recognized that the formation of higher-order chromatin and its structural changes are integral for regulating gene expression (Schalch et al., 2005). A prominent hallmark of NETosis is the rapid decondensation of nuclear chromatin into 15–25 nm chromatin fibers (Brinkmann et al., 2004), which suggests that there is a regulatory mechanism leading to NET formation at the level of chromatin structure regulation. Although the exact mechanisms that control chromatin structure during specific nuclear events remain to be tested, posttranslational histone modifications are known to play a significant role. Acetylation, methylation and phosphorylation of histone proteins regulate chromatin functions, such as transcription as well as chromatin condensation and decondensation (Shilatifard, 2006; Kouzarides, 2007; Li et al., 2007). Heterochromatin binding protein 1 (HP1) is a well-established non-histone protein that binds to modified histones in particular histone H3 Lys9 methylation and regulates chromatin structure. Maintenance of a heterochromatin state relies on the recruitment of HP1 to methylated histone H3 (Verschure et al., 2005). Thus via HP1 binding to specific sites on chromatin, a cell is able to tightly regulate a gene active or inactive state depending on the need for specific cellular functions.

During the search for how neutrophils regulate higher-order chromatin, our lab discovered that peptidylarginine deiminase 4 (also called PAD4 or PADI4) catalyzed histone hypercitrullination mediates chromatin decondensation and is essential for NET formation (Wang et al., 2009; Li et al., 2010). PAD4 is a neutrophil enriched nuclear enzyme that targets histone arginine and monomethylarginine residues for citrullination in a calcium dependent reaction (Nakashima et al., 2002; Wang et al., 2004). Significantly, extensive citrullination is correlated with chromatin decondensation (Neeli et al., 2008; Wang et al., 2009). Research on PAD4 demonstrates that histone modifications play a substantial role in the change of higher-order chromatin from a condensed to a highly decondensed state during NET formation. Additionally, we have shown that PAD4 is an essential factor for NET-mediated innate immune functions (Li et al., 2010).

In the past decade, ample research has been done to try to dissect the mechanism of NET-mediated bacterial killing to better understand the immune system and how neutrophils respond to infection. Although many mechanisms have been proposed, we hypothesize that the molecular events underlying NET formation involve a global reorganization of chromatin. We postulate that gene regulation plays a significant role in NET formation and that the histone-modifying enzyme PAD4 is one of the main mediators of this immunological process. In the process of preliminary research for PAD4, we unexpectedly observed that mere PAD4 overexpression in osteosarcoma U2OS cells yielded extracellular fibers similar to those observed in NETs. The released chromatin stains strongly by a histone citrulline-specific antibody analogous to what has been observed in the literature upon stimulation of neutrophils with IL-8, PMA, LPS, etc. (Neeli et al., 2008; Wang et al., 2009; Li et al., 2010). Moreover, histone hypercitrullination after PAD4 overexpression excludes HP1β from binding to chromatin. This offers a new mechanism by which PAD4 is able to regulate higher-order chromatin structures to induce NET formation. Our findings reinforce the notion that the regulation of chromatin by histone modifications is essential for the process of NET formation.

RESULTS

PAD4 INDUCES EXTENSIVE CHROMATIN DECONDENSATION IN NON-GRANULOCYTIC CELLS

To elucidate the role of PAD4 in chromatin decondensation, we overexpressed PAD4 in osteosarcoma U2OS cells via transient transfection of a plasmid containing the full-length PAD4 gene. Strikingly, transient transfection of PAD4 for 36 h induced U2OS cells to rupture and release extensive web-like chromatin fibers into the extracellular space (**Figure 1**). *In vitro*, neutrophils undergo NETosis in the presence of pro-inflammatory cytokines, bacteria, or after calcium ionophore treatment (Brinkmann et al., 2004; Fuchs et al., 2007; Li et al., 2010). However, we observed NET-like structures devoid of any of these stimulants in a non-granulocytic cell line, suggesting that increased PAD4 activity induces the formation of these structures. To assess the similarity between these extracellular fibers and NETs induced *in vitro*, U2OS cells were stained with α-H3Cit, α-HA to localize HA-PAD4 and Hoechst following PAD4 overexpression. We found that H3Cit staining was greatly increased at areas of highly decondensed chromatin (**Figures 1A,B**). However, PAD4 was not strongly detected there (**Figure 1A**), suggesting that PAD4 dissociates from the NET-like structure upon rupture of the nuclear and cell membranes. Moreover, the H3Cit antibody stained chromatin was only weakly labeled with the Hoechst reagent, suggesting that the underlying chromatin is highly decondensed. Taken together, these results support that PAD4 overexpression and hypercitrullination of chromatin led to the formation of highly decondensed chromatin structures reminiscent of NETs.

A previous study hinted that PAD4 overexpression induces apoptosis due to release of cytochrome *c* and activation of caspases (Liu et al., 2006). To further analyze the nature of the

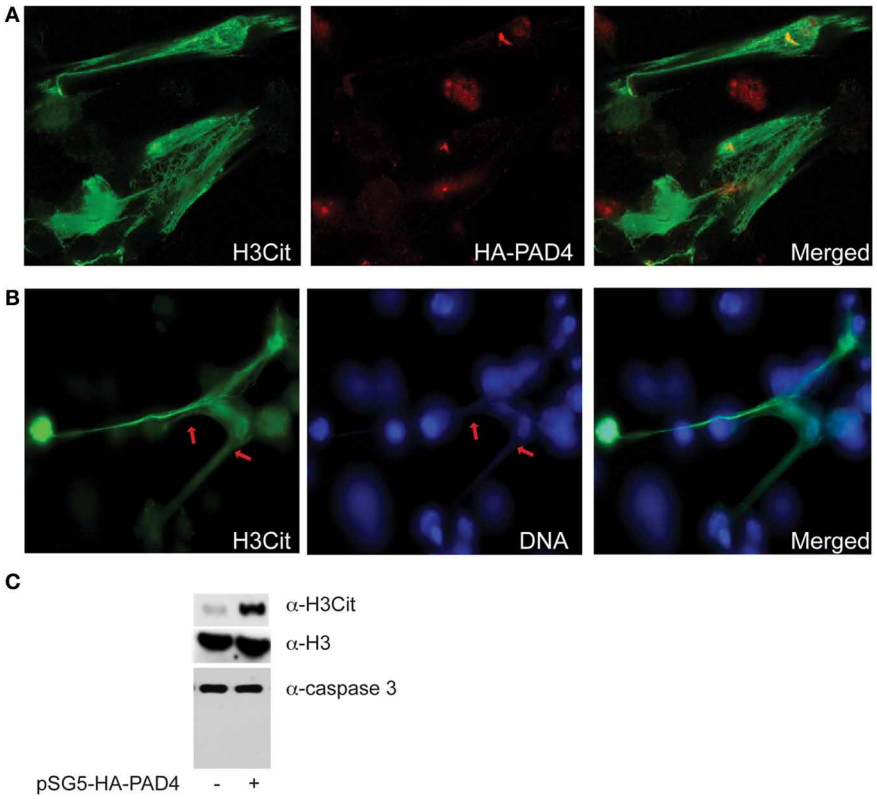

FIGURE 1 | Dramatic chromatin decondensation and formation of NET-like structures upon forced PAD4 expression. (A) Immunostaining of U2OS cells with the H3Cit and the HA antibodies after forced HA-PAD4 expression by transient transfection. Note the dramatic global histone H3 hypercitrullination. **(B)** Immunostaining of H3Cit and DNA staining showing the enrichment of H3Cit with the highly decondensed chromatin denoted by red arrows. **(C)** Western blot analyses of the H3Cit levels, and the caspase-3 cleavage in U2OS cells with or without forced HA-PAD4 expression. Histone H3 was probed to ensure equal protein loading.

cell death elicited by PAD4 overexpression, Western blot analyses were performed using U2OS cell lysate with or without PAD4 overexpression. An increase in H3Cit but no cleavage of caspase-3 was detected in cells over-expressing PAD4 as compared to the control cells (**Figure 1C**). Given that caspase-3 activation was not detected and the released chromatin remained intact after PAD4 overexpression, we favor that PAD4 overexpression induced the NETosis type of cell death instead of apoptosis under above experimental conditions in the U2OS cells.

CHROMATIN RELEASED FROM U2OS CELLS IS SIMILAR TO NETs

It was apparent that the "NET-like" structures following transient transfection of PAD4 in U2OS cells correlated, at least from an immunohistochemical standpoint, with previous studies examining NETs derived from neutrophils. However, it is important to note that neutrophil released chromatin assumes a distinct morphology and is not surrounded by membrane (Brinkmann et al., 2004). To further evaluate the chromatin structures formed by PAD4 overexpressing U2OS cells, scanning electron microscopy (SEM) was performed. U2OS cells that did not overexpress PAD4 were flat, attached firmly to the cover slip with visible nuclei and no extracellular fibers (**Figure 2A**). However, after PAD4 overexpression, many cells became round and lost attachment to the substratum (**Figures 2B,C**). Upon closer examination, transfected cells made prominent extracellular fiber structures analogous to NETs (**Figure 2, denoted by red arrows**). These structures assumed thin and thick stretches that literally extruded from the cells and in some instances this fibril network encompassed the entire cell it originated from (**Figure 2D**). Furthermore, high magnification images showed vesicular membrane structures interspersed within the larger chromatin stretches (**Figure 2E**). These results offer supporting evidence that PAD4 overexpressing cells release "NET-like" structures into the extracellular space.

PAD4 MEDIATED CHROMATIN DECONDENSATION IS DEPENDENT ON THE ACTIVITY OF PAD4

If mere PAD4 protein elevation and histone citrullination can induce chromatin decondensation, it is expected that the relative enzymatic activity of PAD4 is essential for this process. To test this idea, we analyzed the NET-induction ability of a plasmid expressing an enzymatically inactive PAD4 mutant—HA-PAD4^{C645S}—in U2OS cells. As controls, we found that the pSG5 plasmid vector alone did not induce NET-like structures

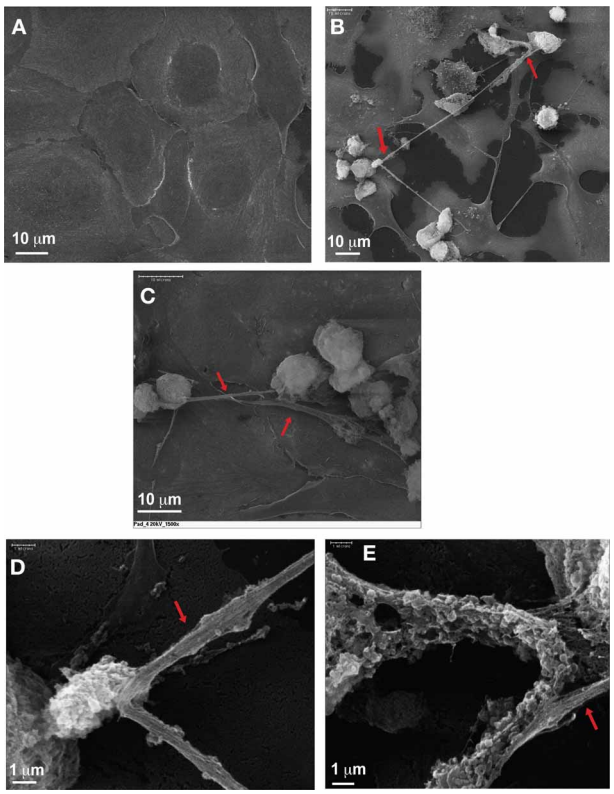

FIGURE 2 | Scanning electron microscope analyses of extracellular chromatin fibers. (A) U2OS cells without forced HA-PAD4 expression. **(B–E)** U2OS cells with forced HA-PAD4 expression, showing the decondensed chromatin fibers (denoted by red arrows). Also noticeable is the membrane vesicles attached to the chromatin fibers in **(E)**.

(**Figure 3A**), while pSG5-HA-PAD4 plasmid did (**Figure 3B**). In contrast, after transient transfection of the pSG5-HA-PAD4^{C645S} plasmid, NET-like structures were not detected, suggesting that the activity of PAD4 is required for the NET-like structure induction (**Figure 3C**). The equal amount expression of the HA-PAD4 protein or the HA-PAD4^{C645S} mutant protein was detected by Western blot (**Figure 3D**). Consistent with the immunostaining experiments, histone H3 citrullination was detected by the H3Cit antibody only in cells with the forced expression of the HA-PAD4 protein (**Figure 3D**). The amount of histone H3 and actin was also monitored to ensure equal protein loading (**Figure 3D**, two bottom panels). The number of cells that are positive for H3 hypercitrullination staining or double positive for both histone H3 hypercitrullination and chromatin decondensation from independent fields in immunostaining experiments was tabulated as percentage of total H3Cit positive cells and displayed in a bar graph (**Figure 3E**). This quantification indicated that cells positive for only citrullination or double positive for both citrullination and chromatin decondensation were detected after the expression of HA-PAD4 but not after the expression of HA-PAD4^{C645S} (**Figure 3E**). Taken together, these results support the notion that the PAD4 activity is crucial for extensive chromatin decondensation.

PAD4 MEDIATED CHROMATIN DECONDENSATION IS CALCIUM DEPENDENT

Using the U2OS cell system for analyzing extensive chromatin decondensation makes it possible to dissect the molecular processes leading to this event. Literature has shown that *in vivo* citrullination was induced when cells were treated with calcium ionophore (Takahara and Sugawara, 1986; Vossenaar et al., 2003). Interestingly, after PAD4 overexpression in U2OS cells, we observed abundant citrullination without calcium ionophore treatment, raising a question if calcium elevation is required for histone citrullination under the condition of PAD4 overexpression. To test this idea, we employed an intracellular calcium chelator 1,2-Bis(2-aminopheoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis (acetoxymethyl ester), better known as BAPTA-AM (Takahashi et al., 1999), at non-toxic concentrations. HA-PAD4 was first expressed in U2OS by transient transfection. Cells were then treated with 0, 5, and 10 μM BAPTA-AM, followed by fixation and immunostaining with α-HA-PAD4 and α-H3Cit antibodies as well as Hoechst. A similar experiment was performed in parallel to prepare protein samples for Western blot analyses. After treatment with an increasing amount of BAPTA-AM, an apparent decrease in the extent of cells undergoing histone citrullination and chromatin decondensation was detected (**Figure 4A**). Moreover, western blot analyses detected equal amount of PAD4 expression but reduced levels of H3Cit after treatment with an increasing amount of the calcium chelator (**Figure 4B**), suggesting that calcium is important for histone citrullination. The number of cells that are positive staining of H3 citrullination or that are double positive for both H3 citrullination and chromatin decondensation in three independent experiments was tabulated as a percentage of H3Cit positive cells and displayed in a bar graph (**Figure 4C**). We found that at increasing concentrations of BAPTA-AM, the percentage of cells that were solely H3Cit positive increased while the percentage of cells that were H3Cit and decondensation double positive decreased (**Figure 4C**). These results reveal that U2OS cells must achieve sufficient intracellular calcium concentrations to induce PAD4 mediated histone citrullination and chromatin decondensation.

HISTONE CITRULLINATION INDUCES HP1β DISSOCIATION AND HETEROCHROMATIN DECONDENSATION

That PAD4 overexpression induces chromatin decondensation underscores the significance of chromatin modifiers and post-translational modifications in NET formation. However, the exact role of citrullination at histone arginine residues to induce chromatin decondensation is still unclear. Several possible mechanisms can be envisioned. For example, global hypercitrullination of histones neutralizes the net positive charge of chromatin and as a result there is a loss of electrostatic interaction between DNA and histones leading to chromatin decondensation. Alternatively, citrullination at arginine residues serve as an epigenetic mark to recruit additional chromatin modifiers that "unravel" chromatin. Moreover, it is possible that histone citrullination prevents the binding of known chromatin condensation factors. An understanding of how the higher-order chromatin state is altered leading to chromatin decondensation will provide a molecular mechanism by which NET formation occurs.

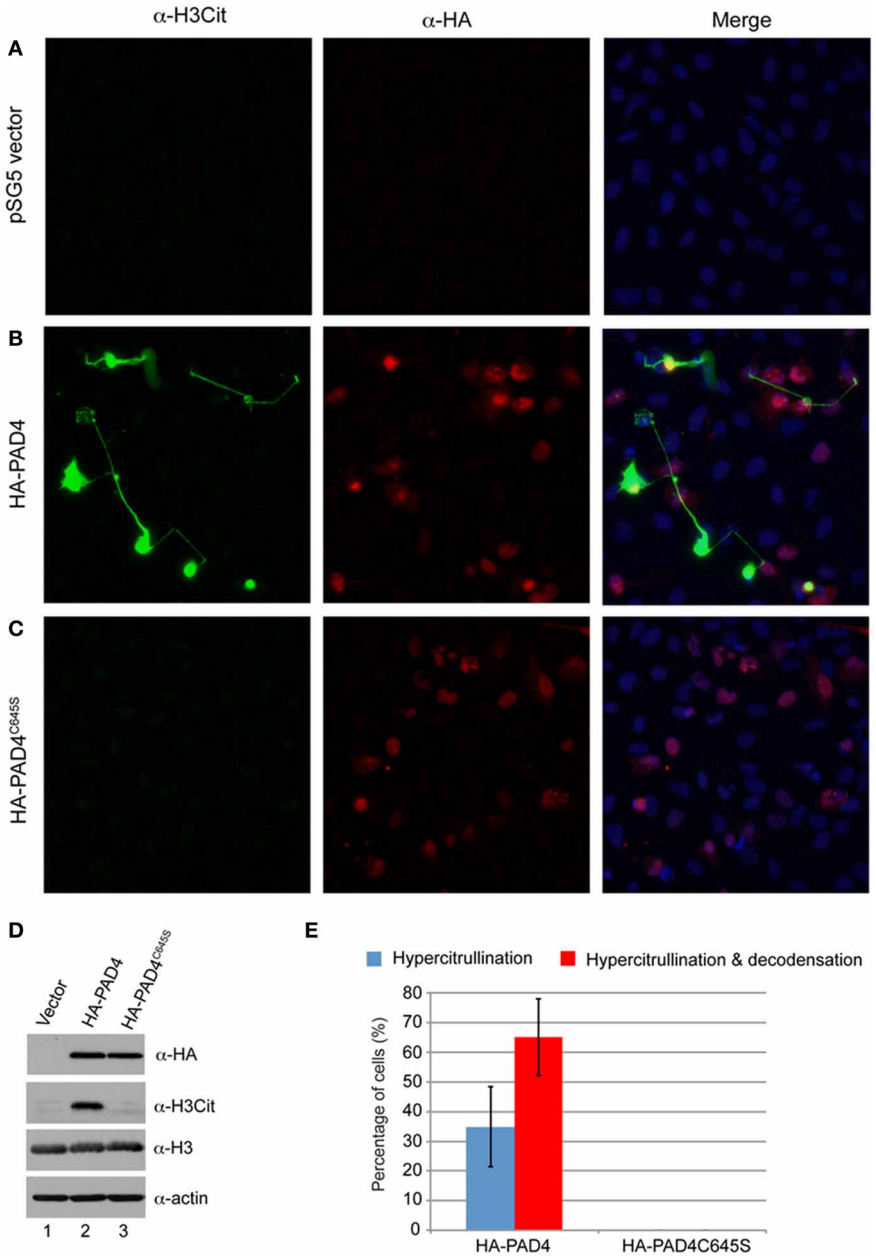

FIGURE 3 | PAD4 activity is important for the induction of NET-like structures. (A–C) Fluorescent microscope analyses of histone H3Cit and chromatin morphology in U2OS cells transfected with the pSG5 vector, the pSG5-HA-PAD4 plasmid, or the pSG5-HA-PAD4^{C645S} plasmid. **(D)** Western blot analyses of HA-fusion protein expression, histone H3Cit levels in U2OS cells after transient transfection. Histone H3 and actin were probed to ensure equal protein loading. **(E)** The number of H3Cit positive cells without obvious chromatin decondensation (hypercitrullination) or H3Cit positive cells with obvious chromatin decondensation (hypercitrullination and decondensation) were numerated as a percentages of cells that are H3Cit positive in U2OS cells transfected with the pSG5-HA-PAD4 plasmid or the pSG5-HA-PAD4^{C645S} plasmid.

The heterochromatin protein 1 (HP1) family members are chromatin regulators that bind to H3 Lys9 methylated residues to regulate heterochromatin formation and function. Overexpression of HP1β is sufficient to induce local chromatin condensation. More significantly, the binding of HP1β to chromatin is regulated by posttranslational histone modifications such as methylation and phosphorylation (Fischle et al., 2005; Verschure et al., 2005). Since a preferred target of PAD4, the H3 Arg8 residue, is adjacent to H3 Lys9, we hypothesized that citrullination of Arg8 affects the binding of HP1β to methylated histone H3 and thereby perturbing the function of heterochromatin during NET formation. To test this hypothesis, we employed NIH 3T3 cells, a mouse fibroblast cell line well known for the formation of discrete regions of heterochromatin within the nucleus,

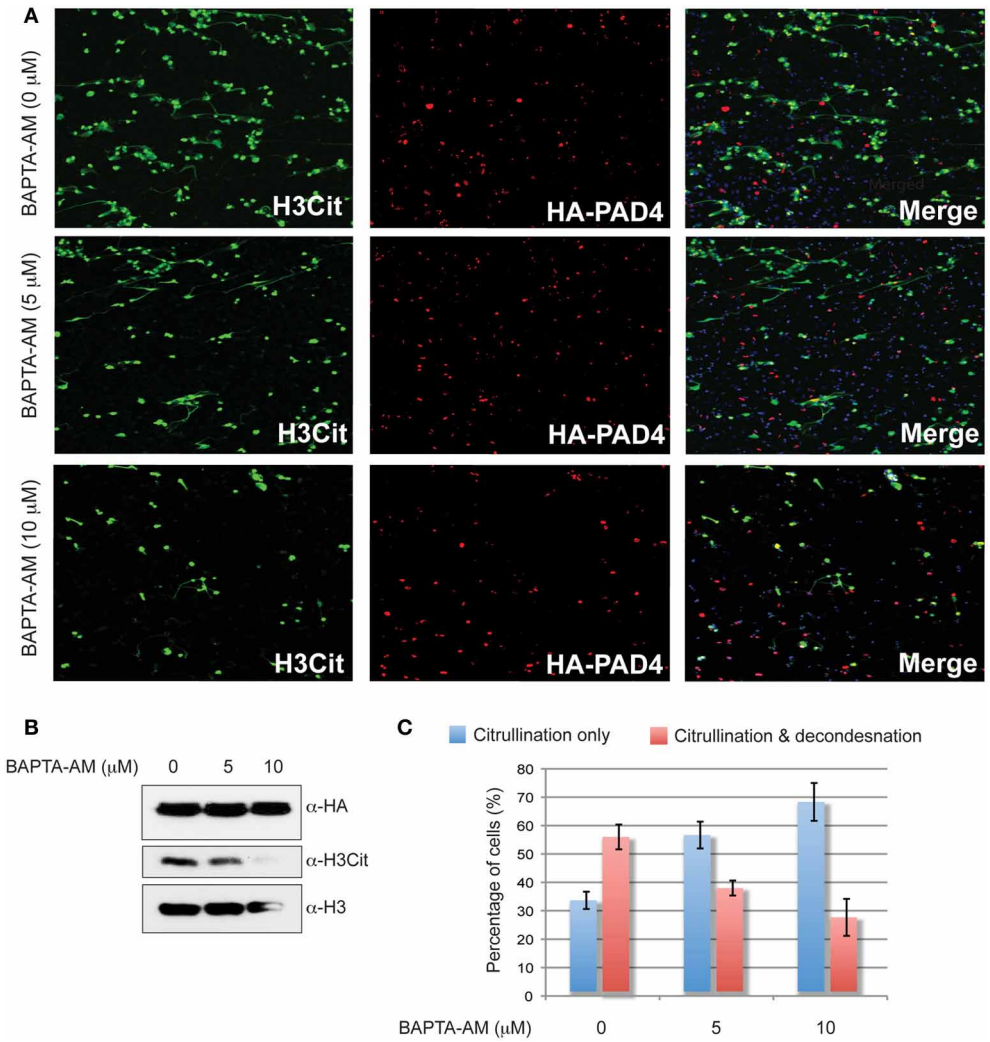

FIGURE 4 | The calcium chelator BAPTA-AM attenuates histone hypercitrullination and chromatin decondensation induced by forced HA-PAD4 expression. (A) Fluorescent microscope analyses of histone H3Cit levels in U2OS cells transfected with the pSG5-HA-PAD4 plasmid and then treated with BAPTA-AM at 0, 5, and 10 μM concentrations. **(B)** Western blot analyses of histone H3Cit levels in U2OS cells transfected with the pSG5-HA-PAD4 plasmid then treated with BAPTA-AM. Histone H3 blot was performed to show the amount of histone H3 in each sample. HA western blot was performed to monitor the HA-PAD4 expression. **(C)** The number of H3Cit positive cells without obvious chromatin decondensation (citrullination only) or H3Cit positive cells with obvious chromatin decondensation (citrullination and decondensation) were numerated as a percentages of cells that are H3Cit positive in U2OS cells transfected with the pSG5-HA-PAD4 plasmid and then treated with BAPTA-AM at different concentrations.

with foci of condensed chromatin stained by the HP1β mouse monoclonal antibody (**Figure 5A**). We found that overexpression of HA-PAD4 in NIH 3T3 cells induced histone H3 citrullination detected by the H3Cit antibody staining (**Figure 5A**) as well as extensive chromatin decondensation showing strong H3Cit antibody staining (**Figure 5B**). This experiment was crucial for illustrating that PAD4 mediated chromatin decondensation after PAD4 overexpression can occur in other cells lineages besides U2OS cells, i.e., PAD4 mediated NET-like structure formation is not cell type specific. Interestingly, cells positive for H3Cit or double positive for H3Cit and chromatin decondensation did not stain with the HP1β antibody (**Figures 5A,B**). Furthermore, cells stained strongly with the H3Cit antibody lost the foci of dense heterochromatin (**Figure 5A**, denoted by arrows). These results suggest that mere PAD4 overexpression can decondense euchromatin and heterochromatin. It has recently been reported that a synthetic H3Arg8Cit peptide can prevent the binding of H3K9me2/3 to HP1β (Bock et al., 2011). The fact that HP1β is lost in H3Cit positive cells promoted us to assess whether H3 Arg8 citrullination adjacent to the methylated Lys9 residue inhibits the binding of HP1β. Toward this end, three H3 N-terminal peptides were synthesized (illustrated in **Figure 5C**). Peptide 1 (P1, H3 residues 1–18) was unmodified with C-terminal biotin conjugation. Peptide 2 (P2) is the same as P1 but contains K9me3 modification, while peptide 3 (P3) contains Cit8 and K9me3 dual modification. The amount

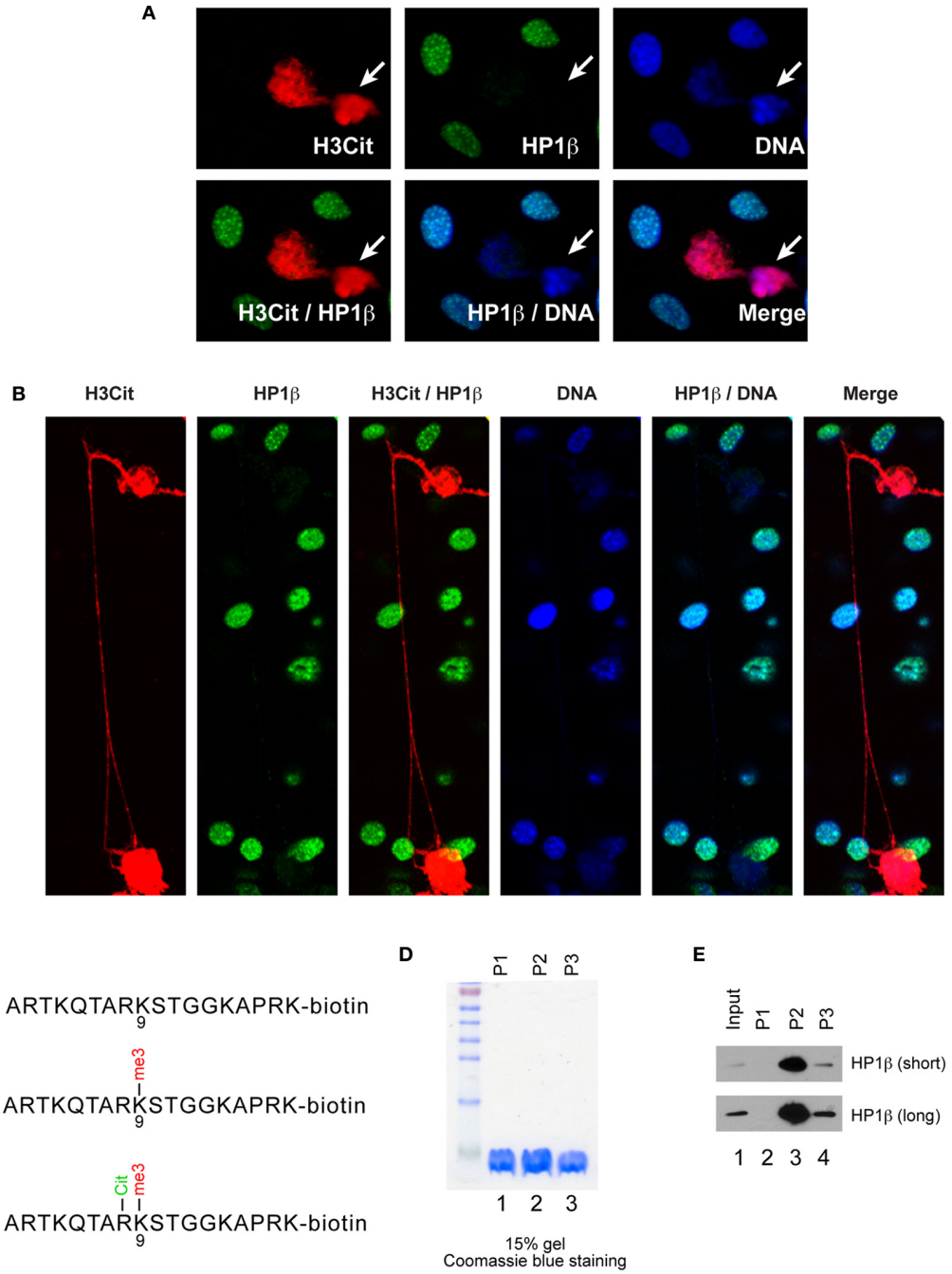

FIGURE 5 | Forced HA-PAD4 expression induced heterochromatin decondensation and HP1β dissociation in NIH 3T3 cells. (A) Immunostaining assays of H3Cit and HP1β in NIH 3T3 cells after forced HA-PAD4 expression. Arrows denote a cell with an increase in H3Cit, a loss of HP1β and the organization of distinct heterochromatic loci, a distinct feature of these cells. **(B)** Immunostaining with H3Cit and HP1 β in NIH 3T3 cells with forced HA-PAD4 expression. Note the extreme chromatin decondensation in the extracellular space. **(C)** The sequence of the biotin conjugated H3 N-terminal peptides with no modification, K9me3, or Cit8K9me3 modifications. **(D)** Coomassie blue staining to show the amount of each peptide. **(E)** Peptide pull-down experiments to analyze the binding of HP1β to H3 unmod, K9me3, and Cit8K9me3 peptides. Top panel shows a short exposure and bottom panel shows a long exposure time in Western blot assays.

of the three peptides was analyzed in a 15% SDS-PAGE gel followed by Coomassie blue staining (**Figure 5D**). In peptide pull down experiments, H3K9me3 peptide but not the H3 unmodified peptide was able to retain HP1β (**Figure 5E**). In contrast, the efficacy of HP1β interaction with H3K9me3 was significantly decreased by the Cit8 modification at the neighboring Arg8 residue (**Figure 5E**), suggesting that histone H3 citrullination in particular H3Cit8 modification can regulate the binding of HP1β

to chromatin thereby the organization of high order chromatin structure.

This observation is consistent with the idea that PAD4 is released from dying and NET-forming neutrophils in the joint of RA patients during disease progression to produce citrullinated autoimmune antigens.

DISCUSSION

In this study, we showed that (1) PAD4 overexpression causes extensive chromatin decondensation in non-granulocytic cells in a manner similar to NETs, (2) the process of extensive chromatin decondensation is dependent upon the enzymatic activity of PAD4 and sufficient intracellular calcium concentrations, and (3) citrullination prevents the binding of HP1β to chromatin. This study supports the essential role of PAD4 in inducing NET formation and provides a potential model system for assessing the biological role of PAD4 in this process.

PAD4 is responsible for histone "hypercitrullination" found along NETs as well as required for bacterial killing mediated by these chromatin "webs" (Neeli et al., 2008; Wang et al., 2009; Li et al., 2010). These findings suggest a role for histones and their epigenetic modifications in a physiological process that was not fully recognized. Here we show that PAD4 overexpression in non-granulocytic cells devoid of pro-inflammatory cytokines or calcium ionophore treatment triggers extensive chromatin decondensation. The decondensed chromatin stained positive for histone citrullination, similar to NETs and upon further analysis occurred without caspase-3 cleavage, a prominent mark of cell death via apoptosis. Other work has also shown that NET formation results in neutrophil death in a manner independent from apoptosis because NET-forming cells do not display "eat-me signals" such as phosphatidyl-serine and no caspase activity is detectable (Remijsen et al., 2011). Our results therefore illustrate that PAD4 overexpression triggers extensive chromatin decondensation biochemically and morphologically analogous to NETs in a manner independent of apoptosis in U2OS cells.

As previously stated, immunofluorescence studies following transfections experiments validated that PAD4 is crucial for mediating chromatin decondensation. However, to further solidify our findings, it was necessary to closely examine the "NET-like" structures formed by PAD4 overexpressing cells. Some of the primary literature examining NETs provides electron microscopy studies illustrating that neutrophils undergoing NETosis form extensive membrane protrusions and the released DNA forms a meshwork of fibers (Brinkmann et al., 2004). Similarly, our SEM analysis revealed that PAD4 overexpressing cells emanated both long and dense stretches of chromatin. The denser areas of fibril matrix were also marked by vesicular structures probably the result of the chromatin interaction with the cell membrane before extracellular release. Taken together, the fluorescent and EM microscopic analyses showed the great similarity between U2OS derived extracellular chromatin fibers and NETs.

Although PAD4 is a crucial mediator of NETosis (Li et al., 2010), how PAD4 fits into the molecular mechanism for inducing NET formation is poorly understood. NETosis induction requires pre-treated with calcium ionophore to promote global activation of PAD4 in primary neutrophils or HL-60 granulocytes (Takahara and Sugawara, 1986; Wang et al., 2009). Inhibition of PAD4 with Cl-amidine followed by calcium ionophore treatment of differentiated HL-60 cells results in a significant reduction of histone citrulline positive decondensed chromatin (Wang et al., 2009). Intriguingly, our experiments showed that chromatin decondensation could be induced in cells without treatment with calcium ionophore. Moreover, chelating intracellular calcium with BAPTA-AM in PAD4 overexpressing cells results in a marked decrease in decondensed chromatin with double positive H3Cit staining and chromatin decondensation. Our results thus highlight a possible calcium regulated mechanism leading to PAD4 mediated chromatin decondensation. Under physiological conditions, calcium is sequestered in subcellular organelles to prevent aberrant signaling cascades. From the perspective of PAD4, the intracellular calcium concentration of 10^{-8}–10^{-6} molar is required for robust PAD4 activation (Takahara and Sugawara, 1986). Our work shows blocking calcium signaling by a chelator can inhibit PAD4 overexpression-mediated NET formation. As calcium triggers multiple signaling events, the cellular pathways leading to a full activation of PAD4 remains unknown. The notion that the activity of PAD4 is needed for chromatin decondensation is further supported from our study employing the inactive mutant $PAD4^{C645S}$.

To date, the mechanism of NETosis has been tackled at the macroscopic level via visualization of live neutrophils stimulated for NET formation. From these studies, we know that prior to chromatin release there is a loss of nuclear integrity, mixing of chromatin with cellular granules and finally, disintegration of the nuclear membrane (Medina, 2009). Additionally, from a signaling standpoint, CXCR2 and Src family kinases seem to mediate NET formation (Marcos et al., 2010). However, there seems to be a lack of emphasis on gene regulation and alteration of higher order chromatin structure as an underlying mechanism leading to NET formation. Overall our work has demonstrated that PAD4 is an chromatin modifier that alters the structure of chromatin via catalyzing citrullination at Arg residues on histones (Wang et al., 2009), and is required for NET-mediated bacterial killing (Li et al., 2010) and PAD4 triggers chromatin decondensation in non-granulocytic cells (this study). Therefore the mechanism of NET formation, i.e., the extensive "unraveling of nuclear chromatin," is tightly linked to PAD4 and histone hypercitrullination.

One of the best-characterized proteins associated with chromatin condensation is HP1 (Eissenberg and Elgin, 2000). HP1 preferentially binds to histone H3K9me3 residues allowing for nucleation of chromatin into a highly condensed state (Verschure et al., 2005). Our studies support that citrullination may antagonize the binding of HP1β to chromatin allowing the cell to decondense heterochromatin during NET formation. It has recently been shown in peptide array assays that H3Cit8 will inhibit the binding of HP1β to the H3K9me3 (Bock et al., 2011). HP1β is negatively regulated by other post-translation modification, such as by the phosphorylation of serine 10 adjacent to methyl-lysine 9 (Fischle et al., 2005). The fact that citrullination affects the binding of HP1β to chromatin suggests

another mechanism for PAD4 mediated chromatin decondensation during the process of NET formation. Based upon our findings we can derive a model for PAD4 mediated chromatin decondensation as occurs in neutrophils undergoing NETosis (**Figure 6**). In non-stimulated neutrophils, nuclear heterochromatin is regulated by HP1β bound to H3K9me2/3. Upon stimulation, PAD4 is activated and globally citrullinates Arg8 residues adjacent to the K9me2 or K9me3 residues. The shift in equilibrium to chromatin in a more citrullinated state would prevent HP1 from binding to chromatin thus promoting chromatin decondensation.

NET formation is a truly unique but also effective immune response that illustrates the importance of histone modifications at the level of higher-order chromatin in inducing a physiological process. PAD4 plays a prominent role in inducing the extensive chromatin decondensation that occurs in NET producing cells. With a better understanding of how NETs form and the role PAD4 plays in the process, it is possible to find ways of controlling NET-associated diseases such as systematic lupus erythematousus (SLE), deep venous thrombosis (DVT), preeclampsia, etc. As we have recently shown that the newly developed PAD4 inhibitors could serve as putative cancer therapeutics (Wang et al., 2012), inhibiting PAD4 could offer a new strategy for treatment of NET associated ailments.

METHODS

CELL CULTURE AND TRANSIENT TRANSFECTION

U2OS and NIH 3T3 cells were cultured in DMEM medium supplemented with 10% FBS and 1% Penicillin-Streptomycin in a 5% CO_2 incubator at 37°C. To start the transient transfection, 2–3 × 10^5 U2OS or NIH 3T3 cells were plated in a 6-well plate. Replace the medium to fresh medium without antibiotics early in the morning on the day of transfection. When cells reached ∼70–90% confluence, 4 μg of DNA (pSG5-HA-PAD4 or pSG5-HA-PAD4^{C645S}) was diluted with 250 μl OPTI-MEM and 10 μl of Lipofectamine 2000 (Invitrogen) was combined with 240 μl OPTI-MEM and incubated for 5 min at RT. The DNA/OPTI-MEM and Lipofectamine 2000/OPTI-MEM was combined and incubated for 20 min at RT. Five-hundred microliters of plasmid/lipofectamine complex was added to the six-well plate and then placed in a 5% CO_2, 37°C incubator. After 12 h, the transfection medium was replaced with fresh complete medium.

IMMUNOSTAINING AND FLUORESCENT MICROSCOPY

Immunostaining was performed using a previously established protocol. After fixation of samples with 3.7% paraformaldehyde in PBS supplemented with 1% Triton X-100 and 2% NP-40, cells were washed with PBST three times 10 min each. Following the third wash, cells were blocked in 2% BSA in PBST for at least 2 h at RT. Primary antibodies were diluted in PBST supplemented with 2% BSA and 5% normal goat serum as follows: α-HA (Sigma, H9658, mouse mAb, 1:200 dilution), α-H3Cit (Abcam, Ab5103, rabbit pAb, 1:200 dilution), and α-HP1β (Active motif, 39979, 1:200, dilution). Cellular staining was performed in a humid chamber overnight at 4°C. After application of the primary antibodies, the cells were washed with PBST three times 10 min each. Cells were then stained with the appropriate secondary antibodies conjugated with Cy3 or Alexa488 at a 1:500 dilution in a humid chamber for at least 2 h at RT. After washing three times 10 min each with PBST, cells were stained with 1 μg/ml Hoechst (Sigma, 94403) in PBS for 15 s followed by a final wash with H_2O. Slides were then mounted and imaged with a fluorescent microscope (Axioscope 40; Carl Zeisss, Inc.). Fluorescent images were captured via an AxioCam MRM camera (Carl Zeiss, Inc.) using the Axiovision AC software (Carl Zeiss, Inc.). Confocal fluorescent images were also captured at the Center for Quantitative Cell Analysis at the Pennsylvania State University. Images were later processed and manipulated using the Adobe Photoshop program or the Image J program as appropriate.

PROTEIN EXTRACTION AND WESTERN BLOT

For Western blot analyses, cells were lysed in an appropriate volume of cold IP buffer (10 mM Tris-HCl pH 8.0, 2 mM EDTA, 150 mM NaCl, 0.2% Triton X-100, and 0.2% NP-40) supplemented with protease inhibitors (1 mM PMSF, 1 μg/mL leupeptin, 1 μg/mL aprotinin, and 1 μg/mL pepstatin). Crude extract was then sonicated for ∼5 min at 4°C followed by SDS denaturation. The appropriate volume of denatured protein was separated in a 14% SDS-PAGE gel. Proteins were then transferred to nitrocellulose membrane, using a Semi-Dry Transferring system for 1 h. Following Ponceau S staining, the membrane was blocked in 5% fat free milk in TBST for ∼30 min at RT to which

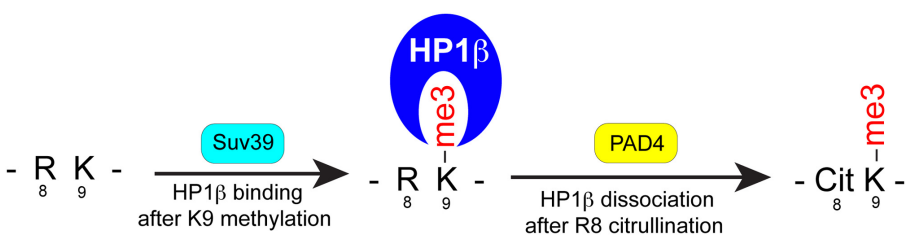

FIGURE 6 | Working model for the effects of H3Arg8 (R8) citrullination on the binding of HP1β to H3K9me3. (1) Upon methylation of H3K9 by the methyltransferase (e.g., Suv39), HP1β recognizes this modification and is recruited to a chromatin region to regulate heterochromatin structure and gene repression. (2) Reversely, PAD4-catalyzed citrullination of H3R8 produce a dual histone H3 modification—H3Cit8K9me3—to dissociate HP1 and mediate heterochromatin decondensation during NET formation.

the following primary antibodies were added: α-H3Cit (AbCam, Ab5103, 1:2000 dilution), α-HA (Sigma, H9658, 1:1000 dilution), α-histone H3 (AbCam, Ab1791, 1:3000 dilution), and α-HP1β (Active motif, 39979, 1:200, dilution). Following overnight incubation at 4°C, membranes were washed 3 times 10 min each in TBST and were then incubated for a minimum of 2 h at 4°C with the proper horseradish peroxidase-conjugated secondary antibody. Signals were detected using the Lumi-Light PLUS Western blotting substrate (Roche Inc.).

CELLULAR CHROMATIN DECONDENSATION ASSAY AND DECONDENSATION ASSAY WITH BAPTA-AM

To assess cellular chromatin decondensation as a result of PAD4 overexpression, U2OS cells were transiently transfected with 4 μg of PSG5-HA-PAD4, a construct that allows detection of HA-PAD4 protein because of the HA epitope. After 12 h of transfection, the medium was removed and replaced with fresh DMEM with antibiotics. At this point, BAPTA-AM (Sigma) was added to 5 and 10 μM final concentrations for treatment. After additional 24 h, the medium was removed and the cells were immunostained with α-HA and α-H3Cit and viewed with fluorescence microscopy to observe increased PAD4 expression and chromatin decondensation.

SCANNING ELECTRON MICROSCOPY (SEM)

For SEM imaging, U2OS cells after pSG5-HA-PAD4 transfection to induce chromatin decondensation were fixed in 2% glutaraldehyde in 0.1 M pH 7.4 phosphate buffer. Dehydrate is performed by gradual wash of ethanol followed by the CO_2 critical time point dry. SEM analyses of the morphology of treated or control cells were performed at the Penn State Electron Microscopy Facility.

PEPTIDE PULL-DOWN ASSAYS

C-terminal Biotin conjugated H3 peptides (residues 1–18) were synthesized by Peptide 2.0 Inc. About 10 μg of each peptide was incubated with the streptavidin beads (Pierce, 20349) then washed with IP buffer (10 mM Tris-HCl pH 8.0, 2 mM EDTA, 150 mM NaCl, 0.2% Triton X-100, and 0.2% NP-40) freshly supplemented with protease inhibitors. Nuclear extracts were prepared from NIH 3T3 following a previously described protocol (Li et al., 2008). After incubation with the nuclear extracts, peptide-streptavidin beads were washed with IP buffer three times 10 min each. The retained proteins were analyzed by Western blot analyses with the HP1β antibody.

ACKNOWLEDGMENTS

We thank members of the Wang group and the Center for Eukaryotic Gene Regulation for helpful discussions and comments. We thank the Penn State University Microscopy and Cytometry Facility for technical help. This work is supported by a National Cancer Institute of NIH grant R01 CA136856 to Yanming Wang. Marc Leshner is a recipient of a Grier Summer Research Award from PSU.

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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.

Received: 27 July 2012; accepted: 17 September 2012; published online: 04 October 2012.

*Citation: Leshner M, Wang S, Lewis C, Zheng H, Chen XA, Santy L and Wang Y (2012) PAD4 mediated histone hypercitrullination induces heterochromatin decondensation and chromatin unfolding to form neutrophil extracellular trap-like structures. Front. Immun. **3**:307. doi: 10.3389/fimmu.2012.00307*

This article was submitted to Frontiers in Molecular Innate Immunity, a specialty of Frontiers in Immunology.

frontiers in
IMMUNOLOGY

ORIGINAL RESEARCH ARTICLE
published: 20 February 2013
doi: 10.3389/fimmu.2013.00038

Opposition between PKC isoforms regulates histone deimination and neutrophil extracellular chromatin release

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In response to inflammation, neutrophils deiminate histones and externalize chromatin. Neutrophil extracellular traps (NETs) are an innate immune defense mechanism, yet NETs also may aggravate chronic inflammatory and autoimmune disorders. Activation of peptidylarginine deiminase 4 (PAD4) is associated with NET release (NETosis) but the precise mechanisms of PAD4 regulation are unknown. We observed that, in human neutrophils, calcium ionophore induced histone deimination, whereas phorbol myristate acetate (PMA), an activator of protein kinase C (PKC), suppressed ionophore-induced deimination. Conversely, low doses of chelerythrine and sanguinarine, two inhibitors of PKC, reversed PMA inhibition and enhanced ionophore-stimulated deimination. In addition, a peptide inhibitor of PKCα superinduced ionophore activation of PAD4, thus identifying PKCα as the PMA-induced inhibitor of PAD4. At higher doses, chelerythrine, sanguinarine, and structurally unrelated PKC inhibitors blocked histone deimination, suggesting that a different PKC isoform activates histone deimination. We identify PKCζ as activator of PAD4 because a specific peptide inhibitor of this PKC isoform suppressed histone deimination. Confocal microscopy confirmed that, in the presence of PMA, NETosis proceeds without detectable histone deimination, and that ionophore cooperates with PMA to induce more extensive NET release. Broad inhibition of PKC by chelerythrine or specific inhibition of PKCζ suppressed NETosis. Our observations thus reveal an intricate antagonism between PKC isoforms in the regulation of histone deimination, identify a dominant role for PKCα in the repression of histone deimination, and assign essential functions to PKCζ in the activation of PAD4 and the execution of NETosis. The precise balance between opposing PKC isoforms in the regulation of NETosis affirms the idea that NET release underlies specific and vitally important evolutionary selection pressures.

Keywords: NETosis, PAD4, protein kinase C, deimination, inflammation

INTRODUCTION

Neutrophils are the first responders of the innate immune system (Nathan, 2006). By their rapid and effective response to infections, neutrophils perform an essential role in the defense of the host from various pathogens. Neutrophils circulate in the blood which carries them to the site of an infection. Within seconds, they adhere to the activated endothelium and pass through or between endothelial cells into tissues (Phillipson et al., 2006). There, neutrophils migrate along gradients of inflammatory mediators toward the ongoing infection. Upon arrival, they engage bacteria, viruses, or fungi through alternative and complementary mechanisms, including phagocytosis, production of reactive oxygen, and the secretion of bactericidal substances (Kennedy and DeLeo, 2009). Despite the vital role of neutrophil responses to infections, we have only an incomplete understanding of neutrophil functions. The study of neutrophils *ex vivo* is complicated by their short lifetime and the difficulty of recreating characteristics of an infection in a culture dish. Pharmacological stimuli that are capable of eliciting neutrophil activation *in vitro* are therefore widely used in the analysis of neutrophil responses to infections.

Phorbol-12-myristate-13-acetate (PMA) is useful for its ability to activate neutrophils and elicit responses that include enhanced adhesion, production of reactive oxygen, and degranulation (Tauber, 1987). PMA elicits these responses because it can penetrate the cell membrane and mimic diacylglycerol (DAG), a cellular signal that activates two of the three families of protein kinase C (PKC). The PMA-responsive PKC belong to the classical (α, β, γ) and novel (δ, υ, τ) PKC families, whereas the alternative PKC isoforms (ζ, λ/ι) function independent of DAG/PMA (Steinberg, 2008). The classical PKC are distinguished from all other PKC because they also require elevated calcium for maximal activity. The compounds A23187 and ionomycin serve as ionophores that form channels in the plasma membrane and allow influx of calcium ions (Erdahl et al., 1994). In many studies, ionophores are used in combination with PMA to elicit maximal activation of cellular responses that require elevated intracellular calcium and PKC activation.

Phorbol myristate acetate and ionophore are strong stimuli for multiple pathways in neutrophils. Thus, a plausible prediction was that PMA or ionophore would affect the manner in which neutrophils die. PMA induces a unique cell death that differs from apoptosis and necrosis (Takei et al., 1996; Suzuki and Namiki, 1998). The discovery that this novel form of neutrophil cell death involves the release of nuclear chromatin and serves in

the innate neutrophil response to pathogens (Brinkmann et al., 2004) led to a fundamental paradigm shift in our thinking about innate immune responses to infections. Indeed, the cell death that is induced by PMA is in many ways analogous to the response of neutrophils to bacterial, fungal, protozoan, and viral pathogens (Brinkmann and Zychlinsky, 2012). Thus, PMA found a new use in studies on the mechanisms of neutrophil innate responses to pathogens.

When exposed to pathogens, or to particulates in the presence of inflammatory stimuli, neutrophils decondense chromatin, and rupture the nuclear envelope which allows the nuclear chromatin to expand into the cytoplasm (Fuchs et al., 2007). In parallel, cytoplasmic granules burst, releasing bactericidal contents into the cytoplasm where they associate in a tight complex with the unraveling chromatin. The last and most dramatic step in this programed cell death pathway is the release of chromatin to the extracellular space. The externalized chromatin can immobilize pathogens and is therefore referred to as a neutrophil extracellular trap (NET). The cell death resulting in NET chromatin release is called NETosis (Steinberg and Grinstein, 2007). A variant form of NETs is composed of mitochondrial DNA and their release may not lead to cell death (Yousefi et al., 2009). Indeed, *in vivo* observations in mice infected by *S. aureus* indicate that release of nuclear chromatin NETs may not induce the immediate loss of cellular functions (Yipp et al., 2012), although functions that depend on gene expression are presumably compromised in these neutrophils.

The release of chromatin follows the activation of peptidylarginine deiminase 4 (PAD4), an enzyme that converts arginine residues to citrullines (Neeli et al., 2008; Wang et al., 2009). PAD4 is activated by inflammatory stimuli and bacterial breakdown products (Neeli et al., 2008). Its activation depends on signals from the cell surface and on an intact cytoskeleton (Neeli et al., 2009). Of the five mammalian deiminases, PAD4 is the enzyme that localizes to the nucleus and modifies histones (Nakashima et al., 2002). Because mutant mouse neutrophils that lack PAD4 cannot release NETs (Li et al., 2010; Hemmers et al., 2011), it has been argued that NET release depends on the activity of PAD4. Just how PAD4 mediates NET release is not known. One possibility is that deimination serves a structural role in the global relaxation of chromatin that precedes NET release: deimination reduces the positive charge of histones which may unravel the tightly packed nuclear chromatin and promote NETosis (Wang et al., 2009). Alternatively, deimination could reprogram gene expression in analogy with other post-translational modification (PTM) and thus set the stage for the execution of NETosis in response to inflammation (Cuthbert et al., 2004; Wang et al., 2004). To distinguish between these possibilities, we must learn more about the regulation of PAD4 in neutrophils. Here, we report consequences of PKC isoform activation and repression on histone deimination and NETosis.

MATERIALS AND METHODS

ANTIBODIES AND CHEMICALS

We obtained the rabbit antibody (Ab) to the carboxy terminus of histone H3 and the Ab to modified citrulline from Millipore (Temecula, CA, USA). The rabbit Ab to deiminated histone H3 (dH3) was from Abcam (ab 5103). The horseradish peroxidase (HRP)-conjugated secondary Abs to rabbit immunoglobulin G (IgG; A0545), goat anti-rabbit conjugated to AF488, calcium ionophore (A23187), ionomycin (I0634), PMA (P8139), and protease inhibitor cocktail (P8340) were purchased from Sigma-Aldrich. Sytox orange was obtained from Invitrogen Life Technologies. All enzyme inhibitors used in this paper were purchased from Calbiochem (Millipore).

HUMAN NEUTROPHIL ISOLATION

Neutrophils were isolated from buffy coats obtained from healthy donors (Lifeblood Biological Services, Memphis, TN, USA) in accord with protocols approved by the University of Tennessee Institutional Review Board and isolated following published methods (Neeli et al., 2008). Briefly, neutrophils were purified at room temperature (RT), enriched in the supernatant of an Isolymph sedimentation and recovered in the pellet of an Isolymph density gradient (Gallard Schlesinger) under endotoxin-free conditions. The contaminating erythrocytes were lysed in ice-cold hypotonic (0.2%) sodium chloride solution for 30 s, at which point the solution was rendered physiologic saline by addition of hypertonic (1.6%) sodium chloride. The neutrophils were rinsed once in Hanks' balanced salt solution (HBSS; without calcium or magnesium) and re-suspended at 2×10^6 cells/ml. At that point, neutrophil viability was assessed by trypan blue dye exclusion and exceeded 98%. Neutrophils were kept at RT (for up to 30 min) before use in experiments.

TREATMENTS OF NEUTROPHILS AND DETERMINATION OF HISTONE DEIMINATION

One million cells in 500 μl of HBSS were incubated in polypropylene tubes with or without different inhibitors for 30 min at 37°C. Then, 2× stimuli were added in 500 μl of HBSS containing 200 μM calcium. For lipopolysaccharide (LPS) stimulation, we added 1% human serum as a source of LPS-binding protein to phosphate buffered saline (PBS) containing 100 μM calcium and 100 ng/ml LPS. Following 2 h of incubation, the cells were centrifuged at 1200 rpm for 5 min, the buffer was discarded and the cell pellet was lysed by adding 50 μl of sodium dodecyl sulfate (SDS) lysis buffer. Samples were boiled for 5 min, cooled and stored at −20°C until use.

PREPARATION AND ANALYSIS OF NETs

To prepare NETs, 1×10^6 purified neutrophils were plated in 500 μl of serum-free HBSS containing 100 μM $CaCl_2$ per well in a 24-well tissue culture plate. Ionophore, PMA or a combination of the two compounds were added as stimuli to induce NETosis. At the end of 1, 2, or 4 h incubations, 0.5 U of micrococcal nuclease (Worthington) was added to the wells and the plate was incubated for another 10 min at 37°C. Supernatants were collected and adjusted to 2 mM ethylenediaminetetraacetic acid (EDTA), clarified by centrifugation at 5,000 *g* for 5 min, and saved for analysis of DNA and myeloperoxidase (MPO). DNA concentrations were determined by Pico-Green fluorescence ("Quant-It," Life Technologies). MPO activity was measured in parallel aliquots by oxidation of tetramethylbenzidine (TMB, 2 mM) in the presence of H_2O_2, followed by spectrophotometry.

WESTERN BLOTTING

Cell lysates were resolved by 12% SDS-PAGE (polyacrylamide gel electrophoresis), and blotted to nitrocellulose membranes. Membranes 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 in TBST. Subsequently, membranes were washed and incubated for 1 h with goat anti-rabbit Ab conjugated to HRP, washed three times with TBST and twice with TBS alone. The HRP activity was detected by using chemiluminescence reagent plus (PerkinElmer Life Sciences) and exposure to autoradiographic film. For detection of citrullines, membranes were blocked with 1% ovalbumin and incubated with chemical reagents that modify citrulline by formation of an ureido group, as suggested by the manufacturer.

CONFOCAL MICROSCOPY

Neutrophils were allowed to settle for 30 min at 37°C onto glass coverslips that were precoated with poly-L-lysine. The cells were treated with stimuli in the presence or absence of various inhibitors (or left untreated) and incubated for 2 h at 37°C. The coverslips were washed with ice-cold HBSS, the cells were fixed with 4% paraformaldehyde in HBSS and blocked 1 hr at RT with blocking solution (HBSS with 1% BSA, 0.05% Tween 20, and 2 mM EDTA). The coverslips were washed with wash buffer (HBSS with 1% BSA), incubated with rabbit anti-citrullinated histone H3 Abs (diluted 1/100 in wash buffer), washed again, incubated with goat anti-rabbit IgG coupled with AF488 together with Sytox orange for 30 min at RT, and analyzed by confocal microscopy, as previously described. Viability of neutrophils exposed to various inhibitors was assessed by trypan blue exclusion following a 2-h incubation. Cell viability ranged between 90 and 95% for any of the inhibitors at the highest concentrations used in our assays. The exception was calphostin C that at 100 μM reduced viability of treated cells to 80%.

RESULTS

DISTINCT EFFECTS OF NEUTROPHIL ACTIVATORS ON HISTONE DEIMINATION AND NET RELEASE

A wide variety of inflammatory stimuli induce histone deimination and NET release (Rohrbach et al., 2012). Quite commonly, PMA or the A23187 ionophore have been used as stimuli for NETosis. Hence, we wished to determine the extent of histone deimination following administration of these compounds to purified human neutrophils. We also set out to determine whether the two stimuli can be used in combination. In buffers that contained 100 μM calcium, ionophore strongly induced histone deimination (**Figure 1A**). In contrast, PMA did not increase histone deimination above background. Instead, PMA diminished the low level of histone deimination that was induced by calcium alone. Strikingly, PMA reduced ionophore-induced deimination to background levels when the two were added simultaneously to neutrophils. To exclude the possibility that PMA induces a citrulline distribution in histone H3 that escapes detection by the anti-citrullinated H3 reagent, we probed a duplicate blot with an Ab that recognizes modified citrullines (**Figure 1B**). This reagent confirmed that PMA treatment does not induce deimination of any proteins in the cell and that ionophore treatment results in the deimination of histones H3 and H4 as the predominant PAD4 substrates. At 2 h after PMA addition, one additional band was detected at approximately 50 kD (**Figure 1B**). This band may correspond to the previously identified PAD2 substrate actin (Darrah et al., 2012). We must insert a cautionary note here: certain modifications may remain below the detection limit of the reagents we used to detect modified citrullines. However, it is valid to point out that the reagents clearly detect citrulline in lysates from ionophore treated neutrophils. Thus, PMA does not induce histone deimination that is detected by histone-specific or by modified citrulline-specific antibodies.

Suppression of deimination by PMA was observed in different physiological buffers and with different calcium ionophores. In PBS or HBSS (each supplemented to 100 μM calcium), the A23187 ionophore induced histone deimination that was inhibited to background levels by PMA (**Figure 1C**). Substitution of ionomycin for A23187 (in HBSS buffer) induced deimination that was greatly diminished by addition of PMA (**Figure 1C**). These results indicate that an increase in cytoplasmic calcium stimulates histone deimination by PAD4 but that a target of PMA, presumably a PMA-dependent PKC isoform, acts in a dominant fashion to suppress deimination.

Because most stimuli for histone deimination also induce NETosis (Neeli et al., 2009), we decided to test whether PMA and ionophore, separately or combined, induce NETosis. Untreated, freshly isolated neutrophils displayed multilobed nuclei of uniform size (**Figure 1D**). Upon incubation with PMA, nuclei swelled and constrictions between the nuclear lobes widened. Some nuclei released their chromatin into the cytoplasm and other cells dispersed NETs (**Figure 1E**). In agreement with western blot results, NETs induced by PMA contained very little or no dH3. The NETs shown here (indicated by arrow heads in **Figures 1E,G**) resemble the diffuse and cloud-like structures seen by live-cell microscopy (Fuchs et al., 2007) or following minimal fixation of cells (Hakkim et al., 2011; Brinkmann and Zychlinsky, 2012), leading us to conclude that NETs acquire the extended, fibrous structure after buffer exchange and cover slip manipulations that are common steps in cell fixation protocols.

A23187 ionophore induced deimination that, in many neutrophils, coincided with nuclear swelling and chromatin release (**Figure 1F**). In contrast, ionophore in combination with PMA readily induced NETosis, yet histone deimination was below our ability to detect (**Figure 1G**). Microscopy thus indicated that A23187 ionophore or PMA, as well as the combination of the two compounds, efficiently induce NETosis, which, in the presence of PMA, proceeds with no detectable histone deimination. The microscopy experiments thus suggested that histone deimination may be dispensable for PMA-induced NETosis.

To quantify NET release induced by different treatments, we solubilized NETs with micrococcal nuclease to measure DNA by fluorescence (**Figure 1H**) and released MPO by enzymatic reaction (**Figure 1I**). Externalized MPO correlates with NET release as most of the MPO is NET-associated (Parker et al., 2012a). In our assays, DNA and MPO measurements concordantly showed that ionophore or PMA induce NETosis but that a combination of the

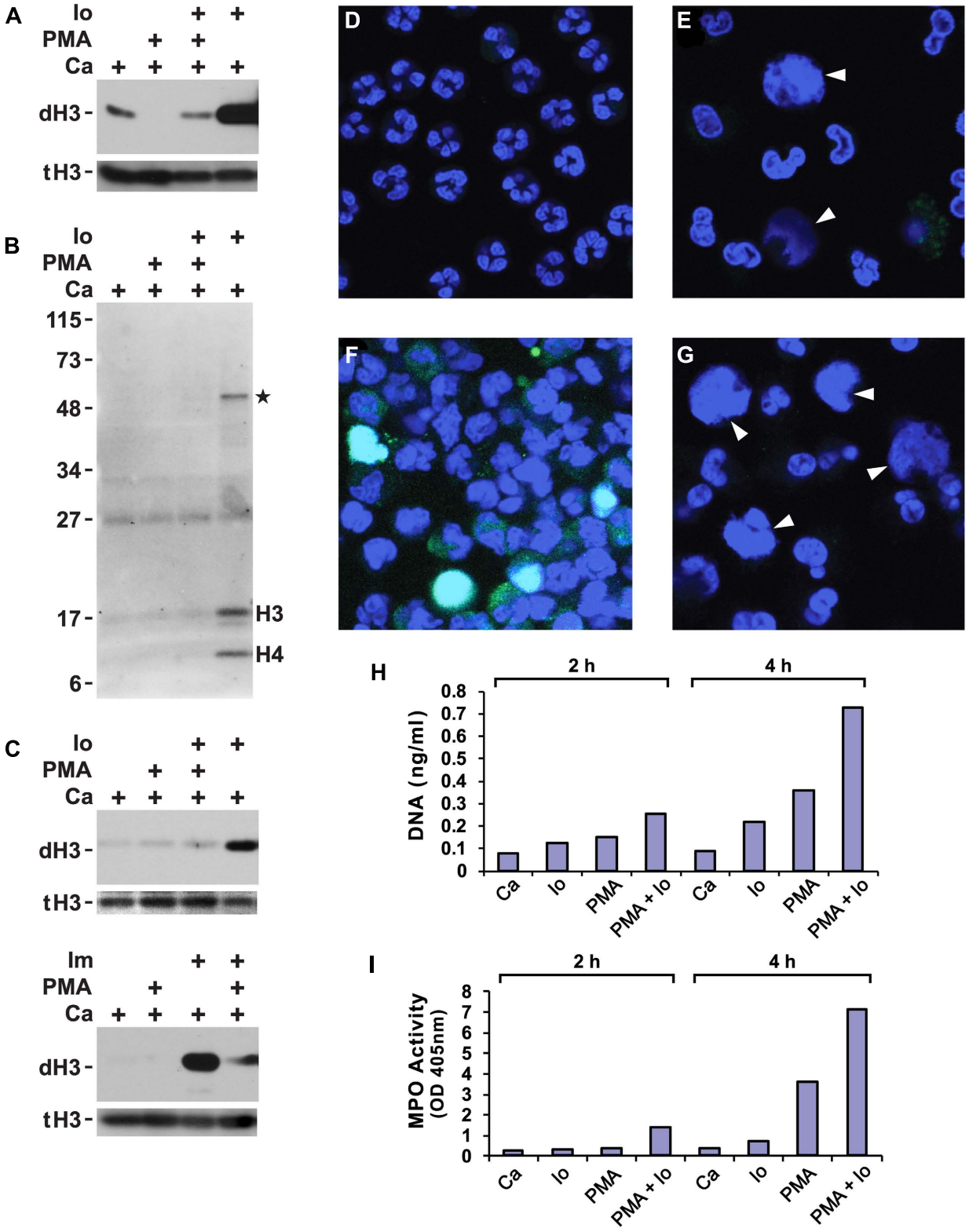

FIGURE 1 | Phorbol myristate acetate (PMA) suppresses histone deimination, whereas ionophore or ionomycin stimulate deimination. Purified neutrophils were incubated in buffers containing calcium (Ca), A23187 ionophore (Io) or ionomycin (Im), and/or PMA for 2 h. Cells were lysed and analyzed by western blotting to detect deiminated histone H3 (dH3) vs. total H3 (tH3). The presence of stimuli is indicated by a plus sign.

(Continued)

FIGURE 1 | Continued
Panels **(A,B)** show blots of lysates from cells incubated with the indicated stimuli or calcium alone. Panel **(A)** shows results of a blot detecting dH3, whereas panel **(B)** shows antibody binding to modified citrullines. Migration of H3 and H4 histones and position of molecular weight markers are indicated. Asterisk indicates a protein of approximately 50 kD that is deiminated in response to ionophore treatment. Substitution of PBS for HBSS did not qualitatively alter the results of the treatments (**C**, top), nor did substitution of ionomycin for ionophore (**C**, bottom). The highest levels of dH3 were observed after ionophore or ionomycin treatment. Inclusion of PMA reduced histone deimination and PMA alone failed to stimulate deimination. Microscopy of unstimulated neutrophils showed cells with multilobed nuclei **(D)**, whereas PMA treatment induced nuclear swelling, rupture, and NET release **(E)**. In these pictures, DNA is displayed in blue and binding of antibodies to deiminated histone H3 in green. Diffuse NETs are indicated by arrow heads here and in **(G)**. Ionophore induced histone deimination in cytoplasm and decondensed nuclei **(F)**. A combination of PMA and ionophore led to enhanced NETosis with little to no detectable histone deimination **(G)**. Purified NET DNA was solubilized by micrococcal nuclease digestion and quantified by fluorescence **(H)**. MPO released by nuclease treatment was measured in solution by the TMB method **(I)**. All experiments were performed at least five times with consistent results.

two stimuli is even more effective at eliciting NETosis. These assays confirmed that the two stimuli cooperate in inducing NETosis, whereas PMA counteracts ionophore in the induction of histone deimination.

PMA INDUCES A PKC ISOFORM THAT SUPPRESSES HISTONE DEIMINATION

Phorbol myristate acetate inhibition of histone deimination may be mediated by a PMA-induced PKC isoform. To test whether PAD4 inhibition could be relieved by repression of the inhibitory PKC, we measured histone deimination in cells treated with PKC inhibitors prior to stimulation with PMA. Indeed, we identified a relatively narrow concentration range of chelerythrine, a plant alkaloid (Powell and Chen, 1955), that could relieve the repression of histone deimination by PMA. To confirm this possibility, we used the related compound sanguinarine (Schenck and Hannse, 1954) and observed that, in the presence of ionophore, 5 μM chelerythrine or 1 μM sanguinarine optimally reversed PMA inhibition (**Figure 2A**). Higher concentrations of the PKC inhibitors (20 μM chelerythrine or 10 μM sanguinarine), however, suppressed deimination. These results suggested that PMA induces, and low concentrations of these PKC inhibitors suppress, a PKC isoform that inhibits histone deimination. Once this isoform is repressed, histone deimination again becomes inducible by calcium ionophore.

To test whether ionophore activates the inhibitory PKC isoform, we added low concentrations of chelerythrine and sanguinarine prior to initiating ionophore treatment of neutrophils. We used this experiment to fine-tune the dose at which chelerythrine and sanguinarine were most effective. Inhibitor concentrations falling between 2.5 and 5.0 μM chelerythrine and near 2.5 μM sanguinarine further enhanced ionophore-induced histone deimination (**Figure 2B**). These results indicated that an elevation of intracellular calcium activates the inhibitory PKC. Because classical PKC isoforms respond to calcium and PMA (Steinberg, 2008), we deduced that the inhibitory PKC isoform is likely a member of the classical group of PKC enzymes. As above, higher levels of the

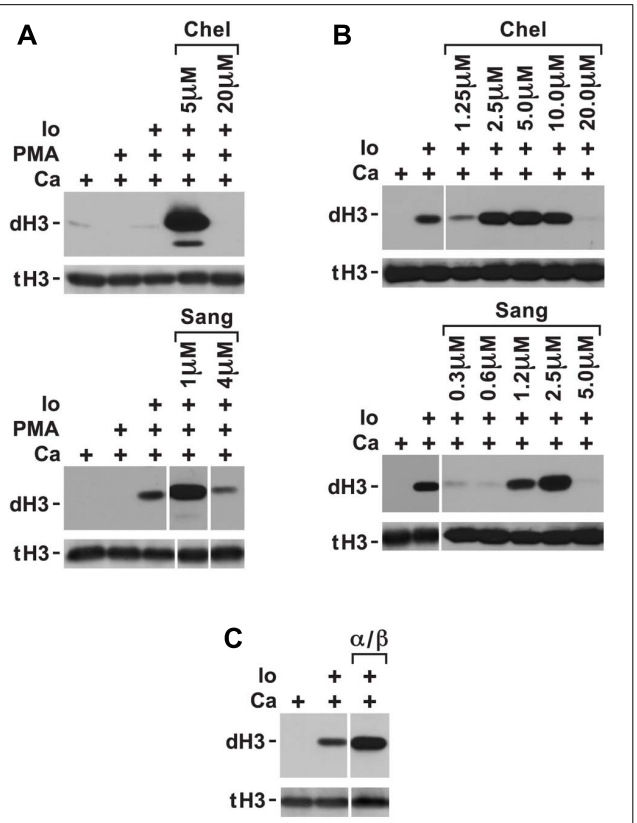

FIGURE 2 | Conditions in which PKC inhibitors enhance histone deimination. (A) Histone deimination that is repressed by PMA can be derepressed by 5 μM chelerythrine (Chel) or 1 μM sanguinarine (Sang) but higher concentrations of these inhibitors lead to renewed repression. Levels of deiminated histone H3 (dH3) vs. total H3 (tH3) were estimated by western blotting. **(B)** A narrow range of inhibitor concentrations also superinduced histone deimination in response to ionophore (Io). **(C)** Pseudosubstrate peptide inhibitor for PKCα/β enhanced histone deimination that was stimulated by ionophore. The vertical white lines separate lanes from the same gel that were taken with identical autoradiographic exposure but were arranged differently in the original gel. The presence of stimuli is indicated by a plus sign.

PKC inhibitors, or EDTA added to the medium (data not shown), suppressed deimination.

Because neutrophils express PKCα (Nixon and McPhail, 1999), we repeated the ionophore stimulation in the presence of a specific PKCα/β inhibitor peptide. The myristoylated inhibitor peptide passes the plasma membrane and interacts with the catalytic domain of the classical PKC isoform. The peptide inhibits substrate binding, thus acting as a competitive inhibitor. Addition of the PKCα/β inhibitory peptide increased the extent of histone deimination induced by ionophore (**Figure 2C**), suggesting that PKCα inhibits histone deimination.

PKCζ IS REQUIRED FOR INDUCTION OF HISTONE DEIMINATION

The inhibition of histone deimination by high levels of chelerythrine and sanguinarine (**Figures 2A,B**) suggested that histone deimination is contingent on the activation of a second PKC isoform. This possibility was consistent with our previous observation that treatment of neutrophils with staurosporine, a potent

PKC inhibitor, blocks histone deimination (Neeli et al., 2008). To confirm the contribution of a PKC to deimination, we treated neutrophils with calcium ionophore in the presence or absence of additional, broadly effective PKC inhibitors. Pretreatment of neutrophils with calphostin C (**Figure 3A**) or bisindolylmaleimide 1 (BIM1; **Figure 3B**) diminished but failed to completely inhibit the induction of histone deimination by calcium ionophore. Calphostin C and BIM1 are considered relatively ineffective for atypical PKC isoforms (Martiny-Baron et al., 1993; Xu and Clark, 1997).

To pinpoint the PKC isoform that promotes histone deimination, we used peptide inhibitors of PKCτ and PKCζ, two PKC isoforms that have been implicated in neutrophil responses to external stimuli (Laudanna et al., 1998; Bertram et al., 2012). The PKCζ inhibitor completely blocked histone deimination, whereas the PKCτ peptide was ineffective (**Figure 3C**). These results indicated that activation of histone deimination by calcium ionophore depends on the activation of PKCζ.

To test whether PKC isoforms regulate histone deimination induced by stimuli encountered *in vivo*, we incubated neutrophils with specific PKC isoform inhibitors prior to stimulation with LPS (**Figure 3D**). LPS treatment induced histone deimination that could be suppressed by a peptide inhibitor of PKCζ, whereas peptides against PKCα/β or PKCτ were ineffective at suppressing LPS-induced PAD4 activation. This observation suggested that PAD4 activation by PKCζ is a shared signal among diverse neutrophil stimuli.

EFFECT OF PKC INHIBITORS ON NETosis

To examine the effect of enhanced vs. inhibited histone deimination on the morphology of NETosis, we incubated neutrophils in the presence of various inhibitors prior to stimulation with calcium ionophore. With chelerythine, we observed two different results, depending on the concentration of inhibitor that was used. At 5 μM chelerythrine, neutrophils generated increased levels of deiminated histones and progressed through various stages of NETosis (**Figure 4A**). Interestingly, we detected the highest levels of dH3 in the cytoplasm of neutrophils with decondensing nuclei and the levels remained high in cells with mixed nuclear and cytoplasmic contents. In contrast, neutrophils incubated with 20 μM chelerythrine exhibited drastically reduced histone deimination, and cell morphologies associated with NETosis were largely

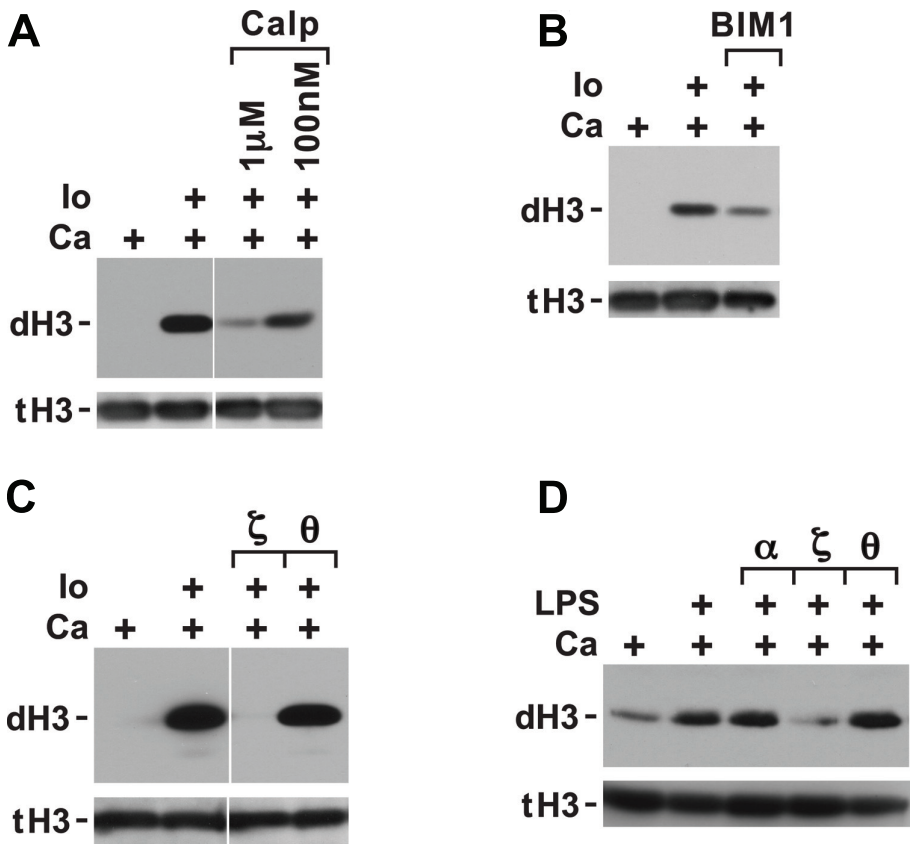

FIGURE 3 | Histone deimination induced by ionophore is only partially suppressed by calphostin C or bisindolylmaleimide 1 (BIM1) but is fully suppressed by a specific peptide inhibitor of PKCζ. Calphostin C (Calp) partially suppressed histone deimination **(A)**, as did addition of 100 nM BIM1 **(B)**. These two compounds do not effectively block atypical PKC isoforms. In contrast, ionophore-induced histone deimination was completely suppressed by a peptide inhibitor of PKCζ but not by an inhibitor of PKCτ **(C)**. The vertical white lines separate lanes from the same gel that were taken with identical autoradiographic exposure but were arranged differently in the original gel. The presence of stimuli is indicated by a plus sign. Addition of LPS in PBS (100 ng/ml) enhanced histone deimination, and a peptide inhibitor of PKCζ but not of PKCτ or α/β suppressed the elevated level of deimination **(D)**.

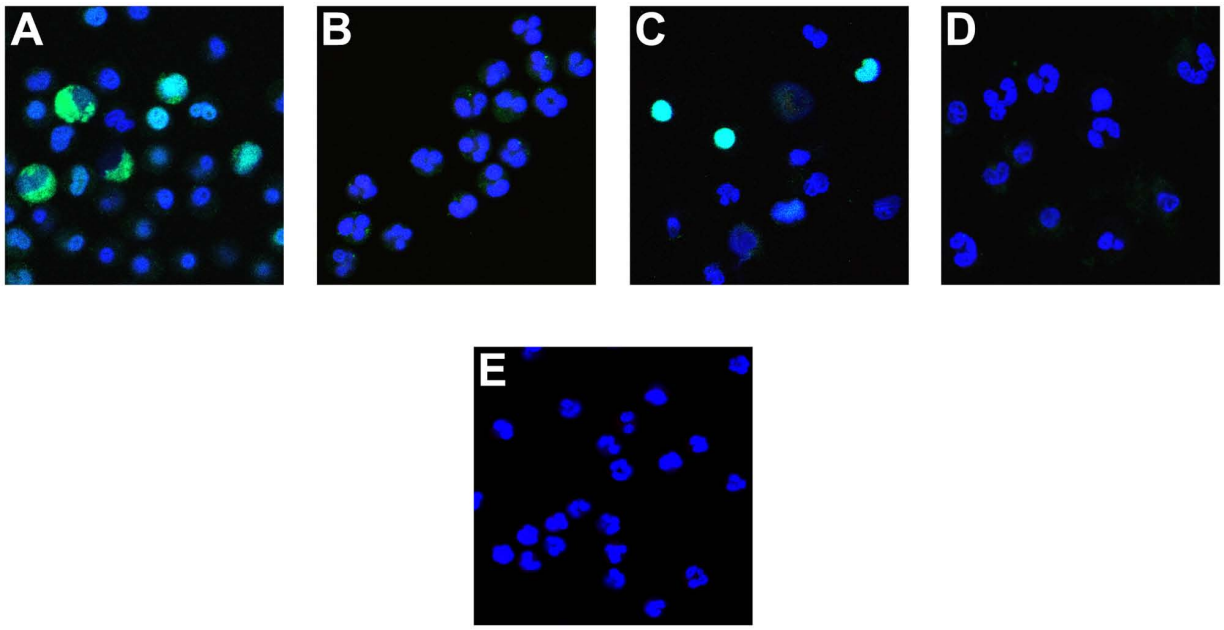

FIGURE 4 | Effect of PKC inhibitors on NETosis. Neutrophils were incubated with A23187 ionophore in the presence of 5 μM chelerythrine **(A)**, or 20 μM chelerythrine **(B)**. Antibody binding to deiminated histone H3 (green) and the DNA contained in cells or released as NETs (blue) was observed in 5 μM chelerythrine, whereas 20 μM inhibitor precluded deimination and most other morphologic features of NETosis. Cells incubated with PKCα/β pseudosubstrate peptide inhibitor deiminated histone H3 and proceeded to NET release **(C)**. The PKCζ inhibitor peptide suppressed histone deimination and NET release but earlier stages of NETosis, such as a partial heterochromatin decondensation and nuclear de lobulation, were observed **(D)**. As in **Figure 1E**, untreated neutrophils showed typical multilobed nuclei without apparent evidence of NETosis **(E)**.

absent (**Figure 4B**). Therefore, at the lower concentration of chelerythrine, histone deimination and NETosis execution were strongly induced, whereas, at the higher concentration of this PKC inhibitor, both histone deimination and NETosis were largely repressed.

The peptide inhibitors had dramatic effects on histone deimination and NET release. Exposure to the PKCα/β pseudosubstrate inhibitor prior to stimulation by ionophore induced strong histone deimination that was most notable in cells with decondensed and expanded nuclei (**Figure 4C**). Importantly, neutrophils stimulated with ionophore in the presence of PKCα inhibitor, readily completed NETosis. In contrast, neutrophils treated with PKCζ inhibitor showed dramatically decreased deimination and impaired NETosis (**Figure 4D**). We observed early nuclear changes of NETosis, such as nuclei with widened interlobe constrictions, yet the release of NETs was largely repressed and cells resembled neutrophils that were left untreated (**Figure 4E**). Collectively, these results indicate that PKC inhibitors provide versatile tools for the analysis of enzymatic steps that regulate the transitions in nuclear structure during NETosis.

DISCUSSION

NETosis brings high risks and high rewards for the host (Brinkmann and Zychlinsky, 2012; Kaplan and Radic, 2012). NETs represent an efficient mechanism to immobilize a broad range of pathogens and expose them to a high local concentration of damaging and potentially lethal compounds. Thus, NETs may be a life-saving antimicrobial response. NETs also interface with other granulocyte responses and thus establish an integrated set of defenses against infections. However, the release of NETs along with tissue degrading compounds from granulocytes at the site of an infection has long been recognized as a risky strategy that can damage host tissues (Nathan, 2006). An added risk to the host arises from the contribution of NETs to blood clotting and the formation of thrombi (Fuchs et al., 2010). Therefore, it is vital that the release of NETs be carefully regulated.

One way to regulate NETosis is through regulation of PAD4. PAD4 is intensely induced by inflammation, such that up to one-fifth of all histones in the cell acquire citrullines (Nakashima et al., 2002). Most PAD4 substrate arginines are localized to the flexible histone amino termini that are important for nucleosome stacking and histone DNA interactions. Loss of the positive charges along the histone termini reduces electrostatic interactions with DNA and thus allows chromatin to assume an extended conformation (Leshner et al., 2012). The activation of PAD4 hence may provide the mechanical lever whose mass action drives the major morphologic transformation seen in NETosis. This view is consistent with the severely impaired ability of PAD4-deficient neutrophils to initiate NETosis (Li et al., 2010; Hemmers et al., 2011). Although PAD4 has numerous substrates in the cell, including PAD4 itself (Andrade et al., 2010), modification of core histones may be the major reason for chromatin decondensation and NET release. However, our study indicates that PMA-induced NETosis unfolds in the absence of detectable histone deimination. This finding was unexpected because inflammatory and infection-related stimuli for NETosis that invariably induce both the deimination

of histones and the release of NETs. It seems likely that the PMA signal for NETosis is qualitatively different from more physiologic stimuli (Parker et al., 2012b). Nevertheless, it is remarkable that a NET stimulus as potent as PMA induces NETs without apparent activation of PAD4. Thus, it is important to more clearly understand the regulation and consequences of PAD4 activation in the induction of NETosis.

Many authors have proposed that PAD4 activation results from an increase in intracellular calcium concentration. This passive model of PAD4 regulation implies that any spike in intracellular calcium, such as may result from a break in the membrane barrier of neutrophils, would activate PAD4. The model is consistent with the strong activation of PAD4 by ionophore and ionomycin (**Figure 1C**). However, our results demonstrate that PAD4 activation is not a direct outcome of elevated levels of intracellular calcium. Even in the presence of elevated cytoplasmic calcium, PMA was capable of repressing deimination. This suggests that neutrophils possess a PMA-responsive mechanism that inhibits PAD4 activation. Under physiologic conditions, this PKC-dependent pathway may prevent the premature activation of PAD4. A PKC isoform that blocks deimination is induced by PMA and calcium and is suppressed by a narrow concentration range of chelerythrine and sanguinarine. Because a specific peptide inhibitor of PKCα/β could overcome PMA repression of histone deimination, we conclude that PKCα functions as a repressor of histone deimination. PKCα, by its association with adhesion receptors (Rucci et al., 2005), may inhibit PAD4 activation early during the attachment of neutrophils to the endothelium and may release PAD4 from repression only during later stages of chemotactic migration.

The most striking finding of our study is that repression by a PKC isoform is not the only safeguard preventing histone deimination by PAD4. In addition, a second PKC isoform is required to induce histone deimination in response to PMA or LPS. Because the induction of deimination was prevented by a specific peptide inhibitor of PKCζ, we conclude that histone deimination requires activation through PKCζ. PKCζ is a conserved, atypical isoform of PKC that contributes to various functions in metazoans, ranging from cell polarity and migration to innate responses to infection (Xu and Clark, 1997; Laudanna et al., 1998; Feng and Longmore, 2005; Nishimura et al., 2005). The remarkable range of PKCζ regulated functions reflects its multiple cellular locations and interacting partners. In infections, PKCζ mediates functions of Toll-like receptors (TLR) at the cell surface (Yang et al., 2011), the assembly of a functional nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex (Dang et al., 2001; Raad et al., 2009), the activation of latent cytoplasmic nuclear factor-kappaB (NF-κB) subunits (Duran et al., 2003), and their association with active chromatin domains (Levy et al., 2011). From these known PKCζ functions, it follows that PKCζ may have diverse opportunities to regulate histone deimination.

The PKCζ isoform could activate PAD4 as soon as a suitable stimulus binds a cell surface receptor. The ζ isoform participates in the response to *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), adhesion, and chemotaxis (Laudanna et al., 1998). Alternatively, PAD4 could be activated indirectly, following PKCζ-assisted assembly of an active NADPH oxidase complex (Raad et al., 2009). This possibility may link PAD4 activation to the generation of reactive oxygen. A third possibility is that PKCζ regulates the translocation of PAD4 into the nucleus, by analogy to the regulation of LKB1 nuclear localization by PKCζ (Xie et al., 2009). Even though cytoplasmic and nuclear pools of PAD4 may coexist, it is unknown whether PAD4 redistributes following stimulation. A fourth possibility is that PKCζ regulates PAD4 activity by altering the structure of chromatin or its interactions with enzymes that establish the histone epigenetic code (Wang et al., 2010). These alternatives are now amenable to experimentation.

It is remarkable that histone deimination is regulated by two PKC isoforms. This indicates that granulocytes precisely control PAD4 activation and that NETosis depends on a specific set of circumstances. Our results further emphasize that evolutionary pressure ensures histone deimination is closely regulated by an ancient family of kinases that coordinate numerous other cellular responses to environmental conditions. Thus, it is no longer conceivable that NETosis is a random or accidental event. Rather, NETosis is a fundamental response to a challenge to the survival of the organism.

ACKNOWLEDGMENTS

The authors acknowledge the research support of the Lupus Research Institute of New York and the expert assistance of Tim Higgins, senior illustrator.

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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.

Received: 09 November 2012; accepted: 31 January 2013; published online: 20 February 2013.

*Citation: Neeli I and Radic M (2013) Opposition between PKC isoforms regulates histone deimination and neutrophil extracellular chromatin release. Front. Immun. **4**:38. doi: 10.3389/fimmu.2013.00038*

This article was submitted to Frontiers in Molecular Innate Immunity, a specialty of Frontiers in Immunology.

frontiers in
IMMUNOLOGY

HYPOTHESIS AND THEORY ARTICLE
published: 17 January 2013
doi: 10.3389/fimmu.2012.00428

NETs: the missing link between cell death and systemic autoimmune diseases?

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For almost 20 years, apoptosis and secondary necrosis have been considered the major source of autoantigens and endogenous adjuvants in the pathogenic model of systemic autoimmune diseases. This focus is justified in part because initial evidence in systemic lupus erythematosus (SLE) guided investigators toward the study of apoptosis, but also because other forms of cell death were unknown. To date, it is known that many other forms of cell death occur, and that they vary in their capacity to stimulate as well as inhibit the immune system. Among these, NETosis (an antimicrobial form of death in neutrophils in which nuclear material is extruded from the cell forming extracellular traps), is gaining major interest as a process that may trigger some of the immune features found in SLE, granulomatosis with polyangiitis (formerly Wegener's granulomatosis) and Felty's syndrome. Although there have been volumes of very compelling studies published on the role of cell death in autoimmunity, no unifying theory has been adopted nor have any successful therapeutics been developed based on this important pathway. The recent inclusion of NETosis into the pathogenic model of autoimmune diseases certainly adds novel insights into this paradigm, but also reveals a previously unappreciated level of complexity and raises many new questions. This review discusses the role of cell death in systemic autoimmune diseases with a focus on apoptosis and NETosis, highlights the current short comings in our understanding of the vast complexity of cell death, and considers the potential shift in the cell death paradigm in autoimmunity. Understanding this complexity is critical in order to develop tools to clearly define the death pathways that are active in systemic autoimmune diseases, identify drivers of disease propagation, and develop novel therapeutics.

Keywords: cell death, apoptosis, NETs, NETosis, necrosis, autoimmune disease

INTRODUCTION

A hallmark feature of systemic autoimmune diseases is the circulation of autoantibodies that recognize intracellular antigens thought to be expressed by all cells, yet are strikingly associated with specific disease phenotypes and outcomes. These diseases include systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), Sjögren's syndrome (SS), autoimmune myopathies, systemic vasculitis, and scleroderma. The ubiquitous expression of many autoantigens and their diverse intracellular function and distribution has long posed a problem in understanding the mechanisms by which these proteins become targets of the autoimmune response. It stands to reason that several requirements must be met in order for immune tolerance to be broken to a self-protein: (i) accessibility of the antigen to the immune system; (ii) presence of proinflammatory factors or motifs; and (iii) non-homeostatic state of the protein/altered self (i.e., increased levels of expression or modified protein sequence/structure). Cell death has long been implicated in this process, and for historical reasons, apoptosis has been the most widely studied. However, the recent finding that NETosis can reproduce critical immune features initially ascribed to apoptosis in systemic autoimmune diseases has exposed a previously unrecognized level of complexity in the role of cell death in autoimmunity. Indeed, virtually every form of physiologic cell death that has been described has the potential to meet these requirements, especially in the setting of clearance defects impairing the efficient and rapid removal of dead cells.

Before we lay a framework implicating cell death pathways in the initiation and propagation of systemic autoimmune diseases, it is critical to point out that cell death and damage is a normal and necessary process in a multi-cellular organism. From an evolutionary perspective, no physiologic form of death should have deleterious effects to the host. Any form of cell death, be it organized and planned (i.e., apoptosis) or spectacular and frightening (i.e., NETosis), is physiologic. Redundant mechanisms have evolved to ensure rapid and efficient clearance of dead cell corpses and debris, and development of immune tolerance to self-proteins. Therefore, no form of cell death should have an autoimmune advantage over any other mode of death, unless specific host abnormalities related to unique forms of death exist. The development of autoimmune diseases is known to be highly complex and likely involves the contribution of several genetic and environmental components. Due to this unlikely confluence of predisposing factors, autoimmune diseases are uncommon in the general population with a prevalence of 7.6–9.4% (Cooper et al., 2009). With these caveats in mind, this review will build a framework to better understand the role of cell death in the current pathogenic

model of systemic autoimmune diseases with an emphasis on the neutrophil and suggest four theories which integrate NETosis into the existing paradigm.

THE EXISTING PATHOGENIC MODEL OF CELL DEATH AS A SOURCE OF AUTOANTIGENS IN SYSTEMIC AUTOIMMUNE DISEASES

A HISTORICAL PERSPECTIVE

The first evidence that suggested a role for cell death in autoimmune disease pathogenesis came from studies by Schwentker and Rivers (1934) in the early 1930s. In these studies, immunization of rabbits with homologous brain suffering autolysis (i.e., spontaneous necrosis) induced anti-brain antibodies and in some cases paralysis, while immunization with freshly isolated brain did not. Although no direct association was made between cell death and the development anti-brain autoantibodies, this observation supported the growing idea that allergic encephalomyelitis and other antibody-mediated diseases may result from autoimmunization against altered tissue generated by the action of pathogens, toxins, chemicals, and/or physical agents (Zoutendyk and Gear, 1951). Later, Burnet (1959) proposed that immune cells reactive with antigenic determinants not readily accessible to the immune system (only in inflamed or traumatized organs) may not be eliminated and be responsible for inducing a vicious cycle of inflammation, most readily when the organism is under stress. This was an important step in recognizing that normally sequestered or altered self-proteins may drive autoimmunity by stimulating autoreactive cells that were not eliminated or rendered tolerogenic during development.

The seminal description of apoptosis as a programmed form of cell death, distinct from necrosis, in 1972, influenced subsequent studies of cell death and ultimately shaped our current pathogenic model of autoimmune diseases (Kerr et al., 1972). It is likely that if another form of programmed cell death had been identified at that time (e.g., necroptosis or NETosis), the current paradigm of cell death and autoimmune disease pathogenesis, may very well center around a different form of death. A central role for apoptosis in autoimmune disease pathogenesis began to emerge in 1990 when two studies provided evidence that apoptosis was a driver of inflammation in autoimmunity. Bell and colleagues observed that nucleosomes released from cells dying by apoptosis could stimulate the production of anti-DNA antibodies *in vitro*, and suggested that this process may occur *in vivo* in SLE (Bell et al., 1990; Bell and Morrison, 1991). Rumore and Steinman (1990) identified that patients with SLE have circulating DNA "closely resembling the characteristic 200 bp ladder found with oligonucleosomal DNA," and suggested that this DNA may be produced by apoptotic cells. They also suggested the possibility that oligonucleosomal DNA generated during apoptosis may escape phagocytosis, and thus gain access to the extracellular fluid. Later, soluble nucleosomal DNA was found in circulation in other autoimmune diseases including SS, scleroderma, and anti-neutrophil cytoplasm antibodies (ANCA)-associated vasculitis (Holdenrieder et al., 2006), as well as in synovial fluid in RA (Yu et al., 1997).

Although the model linking apoptosis to the pathogenesis of autoimmune diseases was gaining momentum, there was no direct evidence that dead or dying cells were active participants in the process. This culminated in 1994 when two papers were published that placed the apoptotic cell in the spotlight as an important factor in SLE pathogenesis. The first paper, by Emlen et al. (1994), described that patients with SLE have accelerated lymphocyte apoptosis *in vitro* and suggested that "abnormal apoptosis of lymphocytes in SLE may provide a source of extracellular nuclear antigen to drive the immune response and to allow the formation of immune complexes (IC)." Shortly thereafter, a paper by Casciola-Rosen et al. (1994) revealed that SLE autoantigens clustered at the surface of apoptotic blebs (membrane protrusions that form on cells dying by apoptosis). The novelty of this paper was that it showed a model in which not only DNA, but other autoantigens (i.e., ribonucleoproteins, RNP) are potentially exposed to the immune system during apoptosis. Moreover, it proposed that during this process, autoantigens can suffer changes in immunogenicity as result of clustering and potentially through posttranslational modifications.

Based on these studies as well as several others, apoptosis has become a critical part of the pathogenic model of autoimmune diseases, and has been widely considered to be the source of autoantigens (e.g., DNA and RNP) and adjuvants (e.g., HMGB-1) that can initiate and propagate the autoimmune process (Lovgren et al., 2004; Vollmer et al., 2005; Marshak-Rothstein and Rifkin, 2007; Urbonaviciute et al., 2008). However, since apoptotic cells are largely considered anti-inflammatory, secondary necrosis of apoptotic cells is a further step that is necessary in this model to expose the cellular contents of dying cells to the immune system. The extracellular exposure of intracellular antigens and endogenous adjuvants, together with the abnormal clearance and/or response to these molecules is the most widely-accepted hypothesis in the paradigm of autoantibody production and systemic autoimmunity (Suber et al., 2008; Munoz et al., 2010; Mahoney et al., 2011; Wickman et al., 2012).

THE COMPLEXITY OF CELL DEATH

THREE MAIN CATEGORIES OF CELL DEATH

Given the wide acceptance of the hypothesis that apoptosis plays a central role in autoimmune disease pathogenesis, it is surprising that no successful therapeutics have been developed which target this pathway in human autoimmune diseases (e.g., to improve the clearance of dying cells). If we accept that cell death is playing an important role, this likely reflects a lack of understanding of the vast complexity of cell death mechanisms, something that is only beginning to be appreciated. According to the last recommendations of the "Nomenclature Committee on Cell Death," cell death can be divided into three major groups: programmed, regulated, and accidental (Galluzzi et al., 2012). "Programmed" applies to those physiological instances of cell death that occur in the context of embryonic/post-embryonic development and tissue homeostasis (e.g., apoptosis and necroptosis). "Regulated" is used to indicate cases of cell death – be they programmed or not – whose initiation and/or execution is mediated by a dedicated molecular machinery, implying that they can be inhibited by targeted pharmacological and/or genetic interventions (e.g., autophagic death and NETosis). "Accidental" indicates cell death triggered by extremely harsh physical conditions (e.g., freeze–thawing cycles or high concentrations of pro-oxidants), which cannot be inhibited by pharmacological

and/or genetic manipulation and usually exhibits morphological features of necrosis.

A form of "accidental death" is secondary necrosis, which is defined as an autolytic process of cell disintegration with release of cell components that occurs when there is no intervention of scavengers after cell death is completed (Silva, 2010). In this regard, it is important to distinguish accidental necrosis from programmed necrosis or necroptosis, a tightly controlled programmed form of death with morphological resemblance of necrosis, but that is orchestrated by the serine-threonine kinases RIP1 and RIP3, and can be specifically inhibited by necrostatins (Wu et al., 2012). Necroptosis may play a regulatory role in the development of the immune system and in the response to viral infection by serving as a backup mechanism of cell death if apoptosis is impaired (Kaiser et al., 2011; Oberst et al., 2011; Zhang et al., 2011; Mocarski et al., 2012). In principle, every form of programmed and regulated cell death may be at risk of suffering secondary necrosis if efficient clearance is delayed. Therefore, although secondary necrosis has been long considered a consequence of inappropriate clearance of apoptotic cells (Silva, 2010), it is possible that secondary necrosis may result from any form of death in which the corps is inadequately cleared.

PROGRAMMED AND REGULATED CELL DEATH

To date, 13 modes of programmed and regulated cell death have been proposed based on biochemical pathways, the effect of specific death inhibitors, and morphological changes occurring in the dying cell and its organelles (reviewed in Galluzzi et al., 2012). The induction of each form of death depends on different factors including the cell type, the death stimuli, the cytokine environment, the presence of pathogens, and the presence of death inhibitors, among others. Thus, death by pyroptosis (a caspase-1-dependent process activated by intracellular bacteria) is restricted to macrophages and dendritic cells (DCs), ETosis (also referred as NETosis when occurs in neutrophils) appears to be specific for granulocytes, and cornification (a program of terminal differentiation that is dependent on caspase-14) is limited to keratinocytes (Galluzzi et al., 2012). Moreover, death signals can be modulated to trigger different forms of death. For example, although signals from members of the tumor necrosis factor receptor (TNFR) family activate the caspase cascade to induce death by apoptosis, inhibition of caspase-8 (e.g., by virus infection) changes the death pathway toward programmed necrosis (i.e., necroptosis; Han et al., 2011; Mocarski et al., 2012). Interestingly, lupus T cells exhibit persistent mitochondrial hyperpolarization as well as depletion of ATP and glutathione, which results in the induction of necrosis (likely necroptosis), instead of apoptosis, in response to activation-induced death (Gergely et al., 2002; Fernandez and Perl, 2009; Fernandez and Perl, 2010).

Cytokines add an additional level of complexity by modulating pathways that promote cell survival, cell death, and the mode of death. For example, in the case of neutrophils which are programmed to undergo spontaneous apoptosis as a mechanism to maintain immune system homeostasis, granulocyte-macrophage colony-stimulating factor (GM-CSF) can delay this process and prolong neutrophil survival (Geering and Simon, 2011). However, when neutrophils are exposed to GM-CSF upon ligation of CD44, the cells die by an autophagy-related form of neutrophil necroptosis (Mihalache et al., 2011). Moreover, if GM-CSF primed neutrophils are exposed to LPS or complement factor 5a (C5a), the cells generate a unique form of neutrophil extracellular traps (NETs) made of mitochondrial DNA (Yousefi et al., 2009).

In cells that may contain more than one death program, there is the potential to select specific modes of death depending on stimuli in the environment. However, once the cell is committed to die by one specific form of death, this program appears to be irreversible and in some cases incompatible with other forms of death (at least for *in vitro* studies). Thus, there are types of death that seem to be antagonistic (i.e., they cannot co-exist in the same cell) because the activation of one death pathway inhibits the others, for example, apoptosis and NETosis or apoptosis and necroptosis (Galluzzi et al., 2012). The capacity to activate diverse forms of death appears to provide an advantage to the host by switching modes of death under conditions in which specific death pathways may be inhibited, for example, in the clearance of pathogens by necroptosis when apoptosis is inhibited (Han et al., 2011; Mocarski et al., 2012). The development of highly specific probes and ability to study cell death *in vivo* will be critical to determine what form of cell death predominates in a given target tissue and may provide valuable insights into the pathogenesis of autoimmune diseases. This is particularly relevant in the study of cells that contain multiple death programs and are known to be present in areas of inflammation, such as neutrophils.

PROGRAMMED AND REGULATED CELL DEATH IN NEUTROPHIL FUNCTION AND HOMEOSTASIS

Among the different modes of programmed and regulated cell death, at least four types have been described in neutrophils. Apoptosis and NETosis, which are known to occur *in vivo* (Yipp et al., 2012), and autophagic-like cell death and an autophagy-related form of necroptosis, which have been induced *in vitro* (von and Simon, 2007; Mihalache et al., 2011). Although autophagic cell death and programmed necrosis have been implicated in controlling both innate and adaptive immune functions (Lu and Walsh, 2012), the role of these forms of death in neutrophil function and their potential consequences in autoimmunity still need to be defined.

NEUTROPHIL APOPTOSIS AND NETOSIS: PARTNERS IN THE FIGHT AGAINST PATHOGENS

Neutrophils are unique cells that use death as a mechanism to modulate inflammation and to ensure the efficient clearance of microorganisms during infections. Two forms of neutrophil death have been implicated in these processes, apoptosis and NETosis. Apoptosis is the mechanism by which aged neutrophils constitutively die in order to maintain homeostatic cell numbers (Cartwright et al., 1964; Geering and Simon, 2011). In addition, apoptosis plays a critical role in the innate response against bacterial, fungal, and protozoal infections (reviewed in Kennedy and DeLeo, 2009; Geering and Simon, 2011). During infection, neutrophils phagocytose bacteria, fungi and protozoa, and the ingested microorganisms are destroyed by the combination of reactive oxygen species (ROS) and antimicrobial granule components. Neutrophils contain or produce many cytotoxic molecules

that can cause significant damage to surrounding tissues if the inflammatory response is not tightly regulated. The engulfment of microorganisms by neutrophils typically accelerates neutrophil apoptosis, which ultimately promotes the resolution of infection. Thus, neutrophil apoptosis guaranties the safe disposal of engulfed bacteria, leads to a loss of functional properties in neutrophils, and drives the production of anti-inflammatory cytokines through clearance of the apoptotic cells by resident or infiltrating macrophages.

Neutrophils also die by NETosis in response to pathogens. During this process, neutrophils extrude extracellular fibrillary networks composed of DNA associated with histones and granular antimicrobial proteins such as proteinase 3 (PR3), myeloperoxidase (MPO), and α-defensins, among others (Urban et al., 2009). NETs act as a mesh that traps microorganisms and facilitates their interaction with neutrophil-derived effector molecules, limiting the spread of rapidly disseminating pathogens (Brinkmann et al., 2004). In addition, NETs can induce the production of antimicrobial cytokines, such as interferon-α (IFN-α; Garcia-Romo et al., 2011; Lande et al., 2011; Villanueva et al., 2011), a relevant cytokine in the control of viral, bacterial, and protozoal infections (Bogdan et al., 2004). Thus, although neutrophil apoptosis and NETosis have been studied as separate processes that occur during infection, it is more likely that these death pathways coexist and work cooperatively for the safe and efficient clearance of pathogens by limiting pathogen spreading, activating inflammatory antimicrobial pathways, and promoting the resolution of inflammation.

Interestingly, although neutrophils are theoretically exposed to the same environmental stimuli during infection, it is intriguing that some neutrophils become phagocytic and die by apoptosis, while others become NETotic. Indeed, in mouse neutrophils in which NETs were induced by IL-8 priming and exposure to *S. flexneri*, only a fraction (13.9 ± 1.8%) formed NETs (Li et al., 2010). Thus, it is possible that this differential response may result from distinct populations of neutrophils which are specifically programmed to activate apoptosis or NETosis in response to pathogens or cytokines. Alternatively, cytokines and/or growth factors that alter the default death program of neutrophils to prolong their survival during infection (e.g., GM-CSF) may allow neutrophils not yet committed to die by apoptosis to release NETs.

NETs vs NETosis

Since the description of NETs, it has been controversial whether neutrophils die while extruding their intracellular material and if this phenomenon occurs *in vivo*. Initial *in vitro* studies using the non-physiological stimulus, phorbol-12-myristate-13-acetate (PMA), demonstrated that formation of NETs required rupture of the cell membrane in a process marked by increased cell permeability and exposure of inner membrane phospholipids (Fuchs et al., 2007). Although this process was clearly distinguishable from apoptosis and necrosis, the striking damage suffered by the cell supported the notion that the production of NETs was associated with neutrophil death (i.e., NETosis). In this model, NET formation required cell death, resulting in the terms NETs and NETosis being used interchangeably.

However, further studies demonstrated that when using more physiological stimuli (i.e., GM-CSF priming and subsequent short-term TLR4 ligation or C5a receptor stimulation), NETs are generated by viable cells (Yousefi et al., 2009). A recent study by Yipp et al. (2012) elegantly suggests a novel paradigm in NET formation. By directly visualizing neutrophil behavior during Gram-positive skin infections in mice and humans, it was demonstrated that viable neutrophils form NETs while crawling, resulting in widespread NET deposition in tissue. As result of this process, neutrophils were rendered anuclear, but did not lyse or exhibit features of programmed cell death. In fact, anuclear neutrophils contained bacteria, suggesting that phagolysosome maturation and NET release can be separately compartmentalized, such that bacteria cannot escape from inside the cell during NET formation. Whether the anuclear neutrophil should or should not be considered dead is questionable, and it remains unclear if these cells retain the capacity to activate other death programs. In this regard, it is important to note that similar to erythrocytes and platelets (Mason et al., 2007; Lang and Qadri, 2012), cytoplasts (anuclear neutrophils generated *in vitro*) retain full capacity to die by apoptosis (Maianski et al., 2004). In this scenario, death by apoptosis of post-NET anuclear neutrophils may guaranty the safe disposal of engulfed bacteria, their efficient clearance to avoid secondary necrosis, and the induction of anti-inflammatory cytokines by phagocytes. For practical reasons we will continue using the term NETosis as a form of death, although it may be somewhat inaccurate. Although further study is needed to understand the interplay between death programs in the neutrophil, it is clear that distinct cell death mechanisms may be active in systemic autoimmune diseases and contribute differentially to the initiation and propagation of disease.

CLEARANCE OF DEAD CELLS AND AUTOIMMUNITY

MECHANISMS OF DEAD CELL CLEARANCE ARE NOT UNIQUE TO APOPTOTIC CELLS

Programmed and regulated forms of cell death are physiologic processes that play critical roles in many different aspects of host development and homeostasis, including tissue turnover, proper development, and the elimination of transformed and infected cells. The rapid and efficient clearance of dead cells and debris is therefore critical to prevent the accumulation of aged, damaged, infected, or dangerous cells. Although the study of apoptosis has brought about an important understanding of pathways activated by dying cells that modulate inflammation, immune tolerance, and the efficient clearance of cell debris (reviewed in Ravichandran, 2011; Wickman et al., 2012), similar mechanisms must exist for any form of programmed or regulated cell death in order to maintain host homeostasis.

During apoptosis, dying cells advertise their presence to phagocytes through the release of soluble "find-me" signals (e.g., the lipid lysophosphatidylcholine, sphingosine 1-phosphate, CX3CL1, and the nucleotides ATP and UTP), which induce migration of phagocytes toward the dying cells. Apoptotic cells also expose "eat-me" signals on their surface (e.g., phosphatidylserine, PS) that are recognized by phagocytes through specific engulfment receptors. Interestingly, other forms of death including anoikis (death induced by detachment of anchorage-dependent cells), autophagic

cell death, caspase-independent apoptosis, and necroptosis also expose PS as a mechanism for efficient non-inflammatory clearance by phagocytes (Hirt and Leist, 2003; Brouckaert et al., 2004; Petrovski et al., 2007a, 2011). In addition, cells dying by anoikis and necroptosis are efficiently engulfed by PS-independent process (Hirt et al., 2000; Petrovski et al., 2007a, 2011).

Efficient mechanisms of clearance are not limited to the recognition and engulfment of intact dead cells, but include pathways that recognize and remove necrotic cell fragments and cell debris such as DNA, histones, and RNP. These pathways have the potential to be universal in the clearance of cell debris generated independently of the mode of cell death. For example, necrotic lymphocyte debris is efficiently removed by macrophages via PS, αvβ3, CD14, CD36, and complement C1q (Bottcher et al., 2006), and debris from neutrophils dying by secondary necrosis is cleared by pathways involving thrombospondin-1 and αvβ3 (Ren et al., 2001). In both cases, cell debris is removed without eliciting inflammatory cytokine secretion. Pentraxins such as C-reactive protein (CRP), serum amyloid P component (SAP), and pentraxin 3 (PTX3) are also involved in the clearance of damaged cells and their soluble constituents. CRP binds to the membranes of damaged cells (both apoptotic and necrotic) likely via phosphatidylcholine, contributing to clearance by phagocytes (Volanakis and Wirtz, 1979; Gershov et al., 2000; Hart et al., 2005; Krysko et al., 2006). In addition, CRP binds to small nuclear RNP particles and chromatin (via histones) and is believe to be involved in the clearance of potentially autoantigenic nuclear material released from dying cells (Du Clos et al., 1988; Du Clos, 1989; Jewell et al., 1993). The avid binding of SAP to chromatin displaces H1-type histones, solubilizing chromatin fragments otherwise quite insoluble at the physiological ionic strength of extracellular fluids (Butler et al., 1990). Since SAP binds to chromatin exposed by necrotic and apoptotic cells *in vivo* (Hintner et al., 1988; Breathnach et al., 1989), it may participate in the disposal of chromatin exposed during cell death, potentially including DNA found in NETs.

Components of the complement pathway are also important in the clearance of dead cells. Early components of the component complement classical pathway bind to cells undergoing secondary necrosis, promoting their engulfment by phagocytes (Gullstrand et al., 2009). In addition, C1q binds DNA and, together with DNase I, promotes degradation of necrotic cell-derived chromatin (Gaipl et al., 2004). The complement inhibitor C4b-binding protein (C4BP) binds strongly to necrotic cells, limiting DNA release from these permeable cells and inhibits the complement cascade at the level of C3 (Trouw et al., 2005).

Chromatin released from dying cells is normally degraded by serum endonucleases, such as DNase I (Napirei et al., 2004; Nathan, 2006). In this regard, although antimicrobial peptides and C1q appear to protect NET DNA from DNase I degradation (Lande et al., 2011; Leffler et al., 2012; a process likely required to enhance NET antimicrobial activity), serum from healthy controls efficiently degrades NETs (Hakkim et al., 2010; Leffler et al., 2012), suggesting the existence of additional mechanisms of NET clearance. Certainly, further studies are necessary to define the role of known and novel clearance pathways in the removal of cells dying by newly discovered forms of cell death and determine how these processes modulate inflammation in infection and autoimmunity.

IMPAIRED CLEARANCE OF DEAD CELLS AND AUTOIMMUNITY: INSIGHTS FROM *IN VIVO* SYSTEMS

The existence of clearance defects is critical to support the current model of cell death in autoimmunity, yet there are still important gaps in the knowledge of relevant pathways that can be modulated by therapy and the forms of death responsible for driving autoimmune diseases in humans. Complete genetic deficiencies in components involved in the clearance of dead cells are very uncommon in humans and limited to a few pathways (e.g., early components of complement and DNases; Yasutomo et al., 2001; Manderson et al., 2004; Al-Mayouf et al., 2011) but are strikingly associated with SLE, suggesting that these pathways are relevant in the protection against autoimmunity. However, while these findings have been considered strong evidence to support the role of apoptosis in autoimmunity, it is important to highlight that these pathways are also involved in the clearance of dead cells generated by other mechanisms (e.g., NETosis and necroptosis). In this regard, clearance defects cannot be used as direct evidence that apoptosis alone is responsible for triggering autoimmunity.

The study of mice with targeted disruption of specific genes associated with the clearance of dying cells (assuming that this is the only function of these genes) have been used to support the role of apoptosis in autoimmunity, but some of these models have important caveats. Interestingly, although some of these models develop lupus-like features and abnormal accumulation of apoptotic cells (e.g., C1q-, C4-, SAP-, c-Mer-, DNase I-, and MFG-E8-deficient mice; Botto et al., 1998; Bickerstaff et al., 1999; Chen et al., 2000; Napirei et al., 2000; Cohen et al., 2002; Hanayama et al., 2004), disease expression is frequently dependent on background genes (e.g., C1q-, C4-, SAP-deficient mice; Botto et al., 1998; Bickerstaff et al., 1999; Chen et al., 2000; Mitchell et al., 2002; Paul et al., 2002; Gillmore et al., 2004). In some cases, an increase in the number of apoptotic bodies, although related to the targeted gene, is not associated with disease expression (Mitchell et al., 1999). Moreover, not all clearance defects in mice lead to autoimmunity (e.g., CD14- and MBL-deficient mice) despite an abnormal *in vivo* accumulation of apoptotic material (Devitt et al., 2004; Stuart et al., 2005).

These discrepancies suggest that the development of autoimmunity is not solely dependent on the accumulation of apoptotic cells and highlight the influence of background genes on disease expression. Strikingly, it was demonstrated that the autoimmune phenotype described in some gene-targeted knock-out mice (i.e., SAP-deficient mice) might be primarily due to combinations of background genes originated from the parental 129 and C57BL/6 mouse strains (Bygrave et al., 2004; Heidari et al., 2006; Carlucci et al., 2007; widely used in the generation of gene-targeted mice, including the C1q-, C4, SAP-, DNase I-, and MFG-E8-deficient mice), which may or may not interact with the target gene. In this scenario, although a targeted gene may predispose to the accumulation of apoptotic cells (e.g., C1q-deficient mice); this may not necessarily be important or sufficient for development of the autoimmune phenotype (Mitchell et al., 2002). These nuances are important to consider when translating findings from these models to human autoimmunity and reveal important limitations in interpreting

the mechanistic link between the genetic predisposition to accumulate apoptotic cells and development of disease. Further studies are necessary to exclude the possibility that other forms of death may also play a role or that other functions of these pathways not related to clearance of dead cells are not responsible for the development of autoimmunity (Carroll, 2004; Carroll and Isenman, 2012).

DO NO HARM: A UNIVERSAL REQUIREMENT FOR PHYSIOLOGIC CELL DEATH

The end point of any physiologic form of death is to carry out a required function and be appropriately cleared, without deleterious effects to the host. However, although this process requires an anti-inflammatory ending, each form of death has distinct interactions and consequences on the immune system with pro- or anti-inflammatory effects depending on the mode of death and the circumstances associated with the induction of cell death (Petrovski et al., 2007b; Miao et al., 2010; Johansson et al., 2011; Rock et al., 2011; Han et al., 2011; Fesus et al., 2011; Remijsen et al., 2011; Schiller et al., 2012). Thus, for example, while apoptotic cell death occurring in steady-state conditions (e.g., during development and cell turnover) must be tolerogenic (Voll et al., 1997), induction of tolerance could have harmful consequences in circumstances in which apoptotic cell death is increased, but a pro-inflammatory response is required (e.g., during infections and cancer). In this scenario, there are factors (e.g., cytokines, pathogens and tumor antigens) which modulate the response to apoptotic cells, allowing the induction of adaptive immune responses if immunogenic antigens are present (Le et al., 2003; Lin et al., 2008; Alfaro et al., 2011; Rock et al., 2011; Johansson et al., 2011). Collectively, these responses help to protect the host and limit the injurious process, but can themselves cause tissue damage and disease. The precise balance between pro- and anti-inflammatory effector functions driven by dying cells and the surrounding immune players is therefore critical to determine the net effect of dying cells. When this balance is shifted, the consequences may drive an autoimmune process or allow the uncontrolled growth of transformed cells.

ANTI-INFLAMMATORY PATHWAYS INDUCED BY DEAD CELLS ARE NOT LIMITED TO APOPTOSIS

The study of apoptosis provided initial clues that dying cells are important players in limiting inflammation. However, since no physiologic form of death should harm the host under normal circumstances, it is likely that in addition to apoptosis, other modes of death may have the capacity to provide signals to resolve inflammation and/or promote healing. After ingestion of apoptotic cells, phagocytes produce "tolerate me" cytokines (e.g., transforming growth factor β, TGF-β and IL-10), and decrease secretion of pro-inflammatory cytokines (e.g., TNFα, IL-1, and IL-12), which actively creates an anti-inflammatory milieu at sites of apoptotic cell death (Voll et al., 1997; Munoz et al., 2010). Interestingly, the efficient engulfment of necroptotic cells also inhibits the production of pro-inflammatory cytokines in macrophages (Hirt and Leist, 2003; Bottcher et al., 2006). Intriguingly, despite the view that necrotic cells are pro-inflammatory, some reports suggest that the efficient phagocytosis of these cells can also trigger anti-inflammatory and tissue repair pathways (Li et al., 2001; Ren et al., 2001; Bottcher et al., 2006). Moreover, engulfment of necrotic neutrophils by immature DCs down-regulates CD80, CD86, and CD40, rendering them unable to induce allogeneic T cell responses (Clayton et al., 2003).

Although NETosis may be viewed as a dangerous form of death based on recent associations with SLE, Wegener's granulomatosis, and Felty's syndrome, several antimicrobial proteins that are enriched in NETs (e.g., α-defensins and the cathelicidin LL37; Brinkmann et al., 2004; Lai and Gallo, 2009; Urban et al., 2009), also have anti-inflammatory activities which favor resolution of infection and repair of damaged tissues (reviewed in Lai and Gallo, 2009). In macrophages, α-defensins inhibit the secretion of TNFα and nitric oxide, and protect mice from a murine model of peritonitis (Miles et al., 2009). LL37 can inhibit LPS-induced cytokine release from monocytes and protects mice against endotoxin shock (Nagaoka et al., 2001). In addition, cathelicidins inhibit TLR4-mediated DC maturation and cytokine release (Di et al., 2007). LL37 also appears to play a role in skin wound healing by promoting keratinocyte migration that is required for re-epithelialization of the wound (Heilborn et al., 2003; Carretero et al., 2008). Interestingly, patients with SLE have antibodies against α-defensins (also known as human neutrophil peptides or HNPs) and LL37 (Lande et al., 2011) suggesting that autoantibodies may affect the function of these antimicrobial peptides in the context of inflammation and autoimmunity. Thus, by attenuating exacerbated inflammatory responses and stimulating certain beneficial aspects of inflammation, antimicrobial peptides in NETs may have an important role in regulating and balancing inflammatory responses. Indeed, recent evidence has shown that lupus-prone mice which are unable to generate NETs due to a deficiency in Nox-2 (phagocyte NADPH oxidase) have markedly exacerbated lupus (Campbell et al., 2012), supporting the importance of NETs in immune homeostasis.

AUTOANTIBODIES: POTENT ADJUVANTS THAT CHANGE THE INFLAMMATORY FATE OF DEAD CELLS

Different circumstances can change the inflammatory outcome of dying cells, including pathogens, cytokines, cellular transformation, and cell damage by toxic agents, among others (Casares et al., 2005; Petrovski et al., 2007b; Miao et al., 2010; Fesus et al., 2011; Han et al., 2011; Johansson et al., 2011; Remijsen et al., 2011; Rock et al., 2011; Garg et al., 2012; Schiller et al., 2012). In 2002, Leadbetter et al. (2002) revealed an unexpected mechanism that explained how immune cells might perceive autoantigens (generated under sterile and/or non-toxic conditions) as noxious structures or DAMPs (damage-associated molecular patterns), provoking the production of pro-inflammatory cytokines. By studying mechanisms that activated transgenic rheumatoid factor (RF)-B cells, they demonstrated that IC consisting of IgG bound to mammalian chromatin effectively activated RF-B cell through a dual process involving B cell antigen receptor (BCR) recognition and delivery of the DNA to TLR9 sequestered in endosomal compartments. A few years later, the same group demonstrated that the "two-receptor" paradigm can be extended to IC containing RNA-associated autoantigens by dual BCR and TLR7 engagement (Lau et al., 2005).

Based on this model, further studies identified that although apoptotic and necrotic material has minimal inflammatory activity, autoantibodies could convert cell debris into a potent inducer of IFN-α by plasmocytoid dendritic cells (pDCs) via TLRs and FcγRIIa ligation (Bave et al., 2000, 2001, 2003; Lovgren et al., 2004). Similarly, although NETs can induce production of IFN-α by pDCs (likely a physiologic response to aid in pathogen clearance), this effect is strikingly enhanced (as much as 10-fold) by the presence of IC containing NET components and antibodies against DNA, HNP, or LL37, with anti-DNA antibodies as the most prominent amplifier of this response (Lande et al., 2011). A similar effect is likely responsible for the induction of IFN-α by pDCs in the presence of NETs and anti-RNP antibodies (Garcia-Romo et al., 2011).

The "two-receptor" paradigm has also been extended to specific autoantigens found in RA (Sokolove et al., 2011). Here, it has been demonstrated that IC containing citrullinated fibrinogen co-stimulate macrophages via the TLR4 and Fcγ receptor to induce production of TNF-α by macrophages. Although fibrinogen is not an intracellular antigen, it is possible that IC containing citrullinated autoantigens released from dying cells may have a similar inflammatory effect. Taken together, these data suggest that under normal conditions, dying cells have the capacity to modulate pro- and anti-inflammatory activities to avoid host damage. However, the presence of autoantibodies against components released by dying cells shifts this balance toward an abnormal pro-inflammatory response. Although parallel studies are necessary to determine if autoantigens released by different forms of cell death have distinct inflammatory properties, the available data suggests that autoantigens released from any source have the same capacity to form IC with autoantibodies and activate the immune system.

NEUTROPHILS IN SYSTEMIC AUTOIMMUNE DISEASES

Since the discovery of NETs, there has been renewed interest in the neutrophil as a potential driver of autoimmune disease. The neutrophil has long been implicated in playing a variety of roles in systemic autoimmune diseases from immune effector to autoantigenic target. This stems from the ability of the neutrophil to wear many different hats during the course of an immune response from phagocyte, to secretor of cytokines, producer of anti-bacterial agents and NETs, and stimulator of adaptive immune cells. The primary role of the short-lived neutrophil in host defense is to rapidly accumulate at sights of tissue injury, in the presence or absence of infection, to protect against invasion by bacteria or fungi and then die by the mechanisms described above (Nathan, 2006).

While neutrophils are present in high numbers at the sites of autoimmune damage and are thought to play active role in disease pathogenesis, their mechanistic role in autoimmunity remains unclear. Neutrophils and leukocytoclasia (i.e., neutrophil debris) are the dominant infiltrate in vasculitis affecting small vessels in systemic autoimmune diseases (Carlson and Chen, 2006), and neutrophils are the second most abundant infiltrating cell type in dermatomyositis (DM) skin lesions (Caproni et al., 2004). In SLE, increased levels of apoptotic (Courtney et al., 1999), activated (Molad et al., 1994), and immature neutrophils (Bennett et al., 2003) are found circulating in the blood of patients, and the percentage of apoptotic and activated neutrophils positively correlates with disease activity (Courtney et al., 1999). Furthermore, neutrophils are the most abundant cell type present in RA synovial fluid and are enriched at the pannus/cartilage interface, where most tissue damage occurs (Mohr et al., 1981).

AUTOANTIGEN EXPRESSION BY NEUTROPHILS

Interestingly, the neutrophil is also thought to be a major source of autoantigens in systemic autoimmune diseases. Neutrophil-specific autoimmunity, which is strikingly associated with the small-vessel vasculitides (i.e., microscopic polyangiitis, Wegener's granulomatosis, Churg–Strauss syndrome, and polyarteritis nodosa), was first reported in 1982 with the identification of ANCAs in a few patients with necrotizing glomerulonephritis (Davies et al., 1982). MPO and PR3 were subsequently identified as the predominant autoantigens in 1988 and 1990, respectively (Falk and Jennette, 1988; Ludemann et al., 1990). Anti-neutrophil autoantibodies (not necessarily targeting MPO and PR3) have also been described in SS and SLE (Lamour et al., 1995; Manolova et al., 2001), with recent evidence that neutrophil antimicrobial peptides are among the antigens targeted in SLE (Lande et al., 2011). It is also important to note that neutrophils express high levels of peptidylarginine deiminase enzymes (PAD2 and PAD4; Darrah et al., 2012), which are responsible for generating citrullinated proteins, currently the most specific targets of the immune response defined in RA (Wegner et al., 2010). Thus, it appears that patients with different forms of systemic autoimmunity target ubiquitously expressed proteins as well as those uniquely expressed by neutrophils. The reason that several neutrophil-specific proteins are targeted remains unclear, but may be related to their propensity to die at sites of inflammation resulting in exposure of normally sequestered antigens to the immune system.

MODIFIED DURING DEATH: A UNIFYING PROPERTY OF AUTOANTIGENS

Clustering and structural modification of autoantigens during apoptosis meets two key requirements for breaking tolerance to self-proteins: accessibility of the antigen to the immune system (i.e., through clustering in apoptotic blebs) and non-homeostatic state of the protein/altered self (i.e., through non-tolerized post-translational modifications, PTMs). These features initially made apoptosis an attractive central component of the autoimmune disease paradigm (Casciola-Rosen et al., 1994; Hall et al., 2004). However, although these requirements might be highly relevant to autoimmune disease pathogenesis, they have the capacity to be met by other forms of cell death.

Unbiased proteomic analysis of NETs by two different groups have identified a total of 23 proteins present in these structures (Urban et al., 2009; Saffarzadeh et al., 2012), and two additional components have been described in other studies, including LL37 (Lande et al., 2011) and DNA/chromatin (Brinkmann et al., 2004; **Table 1**). These components have diverse functions and subcellular distributions in live cells, but are redistributed and extruded from neutrophils during NETosis. A literature search revealed that 84% of NET components have been identified as autoantigens in patients with autoimmunity, cancer, or both, in independent studies (**Table 1**; **Figure 1A**). In fact, 74% have

Table 1 | Autoantigens are enriched in NETs.

NET-protein	Autoantigen	Disease family	Disease	Reference
Actin, cytoplamsmic 1 and 2 (β/γ)*,ψ	Yes	Autoimmunity	RA	Darrah et al. (2012)
α-Actinin 1 and/or 4*	Yes	Autoimmunity	SLE nephritis	Renaudineau et al. (2007)
Annexin AIψ	Yes	Autoimmunity	SLE	Iaccarino et al. (2011)
		Cancer	Lung cancer	Brichory et al. (2001)
Azurocidin*	Yes	Autoimmunity	Vasculitis	Zhao et al. (1995)
Catalase*	Yes	Autoimmunity	SLE	Mansour et al. (2008)
			RA	
Cathepsin G*	yes	Autoimmunity	Vasculitis	Schultz and Tozman (1995)
Cytokeratin-10*	Yes	Infection-induced autoimmunity	Chronic Lyme arthritis	Ghosh et al. (2006)
Neutrophil defensins (HNP)*,£	yes	Autoimmunity	SLE	Lande et al. (2011)
dsDNA/chromatin$	Yes	Autoimmunity	SLE	Shen et al. (1998)
Leukocyte elastase*	Yes	Autoimmunity	Vasculitis	Schultz and Tozman (1995)
Leukocyte elastase inhibitorψ	No			
α-Enolase*,ψ	Yes	Autoimmunity	RA	Wegner et al. (2010)
Glyceraldehyde 3-phosphate dehydrogenaseψ	Yes	Autoimmunity	Multiple sclerosis	Kolln et al. (2006)
			Ovarian autoimmunity	Edassery et al. (2010)
			SLE	Takasaki et al. (2004)
			Dilated cardiomyopathy	Buse et al. (2008)
Histones*	Yes	Autoimmunity	SLE	Shen et al. (1998)
			Drug-induced lupus	Shen et al. (1998)
			Felty's syndrome	Dwivedi et al. (2012)
			RA	Sokolove et al. (2012)
Lactoferrin*,ψ	Yes	Autoimmunity	Autoimmune pancreatitis	Hayakawa et al. (2009)
			RA	Mulder et al. (1993)
			Vasculitis	Schultz and Tozman (1995)
LL37£	Yes	Autoimmunity	SLE	Lande et al. (2011)
Lysozyme C*	Yes	Autoimmunity	Vasculitis	Schultz and Tozman (1995)
Myeloid cell nuclear differentiation antigen*	No	–	–	–
Myeloperoxidase*,ψ	Yes	Autoimmunity	Vasculitis	Schultz and Tozman (1995)
Myosin-9*	Yes	Genetic autoimmunity	APS-1	Lindh et al. (2012)
Plastin-2*,ψ	Yes	Cancer	Non-Hodgkin's lymphoma	Ueda et al. (2008)
Profilin-1ψ	No	–	–	–
Protein S100*,ψ	No	–	–	–
Proteinase 3*	Yes	Autoimmunity	Vasculitis	Schultz and Tozman (1995)
Transketolase*	Yes	Autoimmunity	Multiple sclerosis	Lovato et al. (2008)

**Saffarzadeh et al. (2012);* ψ *Urban et al. (2009);* $ *Brinkmann et al. (2004);* £ *Lande et al. (2011).*
APS-1, autoimmune polyendocrine syndrome type I.

been reported to be autoantigens in systemic autoimmune diseases, most dominantly in SLE, RA, and vasculitis (**Figures 1A,B**). This observation suggests that redistribution into NETs may be a previously unappreciated unifying property of several autoantigens targeted across the spectrum of autoimmune diseases and cancer.

Interestingly, although the exposure of autoantigens is a common feature shared by NETosis and apoptosis, which may similarly balance their potential relevance in autoimmunity, it is noteworthy that these processes are clearly distinguishable by the exposed autoantigens and the way that these molecules are structurally modified. Thus, in contrast to apoptotic blebs,

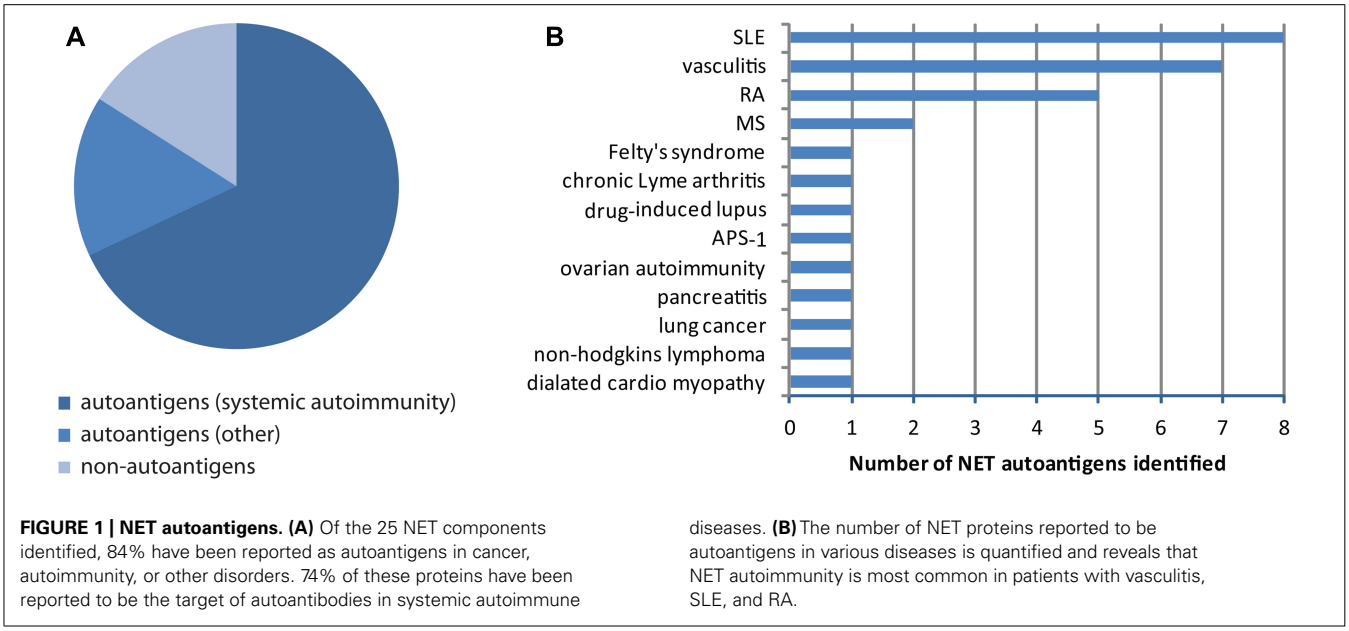

FIGURE 1 | NET autoantigens. (A) Of the 25 NET components identified, 84% have been reported as autoantigens in cancer, autoimmunity, or other disorders. 74% of these proteins have been reported to be the target of autoantibodies in systemic autoimmune diseases. **(B)** The number of NET proteins reported to be autoantigens in various diseases is quantified and reveals that NET autoimmunity is most common in patients with vasculitis, SLE, and RA.

major autoantigens in systemic autoimmune disease such as RNPs (e.g., Ro, La, Sm, and U1-70K) are not found in NETs (Urban et al., 2009; Villanueva et al., 2011; Saffarzadeh et al., 2012). Similarly, it is unknown whether neutrophils dying by NETosis expose phospholipids targeted by anti-phospholipid antibodies.

Apoptotic and NETotic cells also generate different PTMs. Depending on the stimuli, apoptotic cells can contain autoantigens modified by proteolysis (induced by caspases and/or granzymes), phosphorylation/dephosphorylation, photo-induced damage, methylation, ADP-ribosilation, and transglutamination (Casciola-Rosen et al., 1999; Rutjes et al., 1999; Hall et al., 2004; Andrade et al., 2005; Dieker et al., 2007; Utz et al., 1997, 2000; Darrah and Rosen, 2010; van Bavel et al., 2011; Bernard et al., 2012). In some cases, these modifications can be recognized by autoantibodies (e.g., phosphorylation, acetylation, and methylation) and have been suggested to play a role in the loss of tolerance to self-proteins. During NETosis, however, the induction of PTMs appears to be more limited. In this regard, methylation, citrullination, and acetylation have been detected in histones (Liu et al., 2012), but is unknown whether other autoantigens may be modified during NETosis. Moreover, although histone citrullination is a hallmark in NETs formation and a target for autoantibodies in Felty's syndrome (Li et al., 2010; Dwivedi et al., 2012), citrullination of major autoantigens targeted in RA (e.g., enolase and vimentin; Wegner et al., 2010) have not reported to occur during NETosis. Although citrullination appears not to occur in apoptotic cells dying by camptothecin or staurosporine (Neeli et al., 2008), whether other apoptotic stimuli can activate citrullination is unknown.

Apoptosis and NETosis may therefore have distinct features (neither of which are mutually exclusive) that may offer different advantages in the disease-specific paradigms of autoimmunity. Thus, while the induction of protein citrullination, the exposure of PR3 and MPO, and the extrusion of chromatin during NETosis are attractive elements (but not exclusive for NETosis) for diseases like RA, ANCA-associated vasculitis, and SLE, respectively, features like the targeting of Ro and La and their association with photosensitivity in SLE can be more easily explained by ultraviolet-B (UVB)-induced keratinocyte apoptosis (Casciola-Rosen et al., 1994) than by NETosis. Finally, it is unlikely that apoptosis and NETosis are the only modes of death with the capacity to modify protein immunogenicity, which may add layers of complexity to the autoimmune paradigm and may support the development of disease-specific models.

THEORICAL MODELS TO INTEGRATE NETOSIS AND APOPTOSIS INTO THE PARADIGM OF SYSTEMIC AUTOIMMUNE DISEASES

The finding that apoptotic cells and nucleosomal DNA are abnormally increased in the circulation and target tissues of patients with systemic autoimmune diseases strongly supports the notion that abnormal production and/or clearance of apoptotic cells is ongoing in this group of diseases (Yu et al., 1997; Courtney et al., 1999; Andrade et al., 2000; Makino et al., 2003; van Rossum et al., 2005; Holdenrieder et al., 2006; Lin et al., 2007; Midgley et al., 2009). In addition, apoptotic material in complex with autoantibodies can induce production of IFN-α by pDCs (Bave et al., 2000, 2001, 2003; Lovgren et al., 2004; Vollmer et al., 2005), a critical cytokine in the current paradigm of SLE (Ronnblom et al., 2011) that is gaining interest in the pathogenic model of SS, autoimmune myositis, scleroderma, and RA (Greenberg et al., 2005; Hjelmervik et al., 2005; Gottenberg et al., 2006; Walsh et al., 2007; Higgs et al., 2011, 2012; van et al., 2011). However, while it is widely accepted that apoptosis is occurring in the setting of systemic autoimmunity, it remains unclear if this process is an initiator of disease, a propagator of the feed-forward cycle of immune-mediated tissue damage, or a byproduct of unchecked inflammation.

These unanswered questions, together with the growth in our understanding of the complexities of cell death, have sparked

interest in other cell death programs that may be equally or more important than apoptosis in driving autoimmunity. Recent studies on NETotic cell death have demonstrated that similar to apoptotic material, NETs (especially in complex with anti-NET antibodies) can activate IFN-α production by pDCs (Garcia-Romo et al., 2011; Lande et al., 2011; Villanueva et al., 2011), adding a novel mechanistic player into SLE pathogenesis and potentially other systemic autoimmune disease in which IFN-α may play a pathogenic role (Knight and Kaplan, 2012). Moreover, NETs have been implicated in the generation and release of autoantigens targeted in small-vessel vasculitis and Felty's syndrome (Kessenbrock et al., 2009; Dwivedi et al., 2012). However, how NETosis may influence the role of apoptosis in the model of systemic autoimmune diseases is still unclear.

Based on the general concepts discussed in the initial part of this review and recent experimental data, we will provide four hypothetical models in an attempt to integrate apoptosis and NETosis into a pathogenic paradigm of systemic autoimmunity. It is not our intention to suggest that dying neutrophils are the only source of autoantigens or the primary target during disease initiation. Depending on the target tissue, many other cells can be the primary source of autoantigens (e.g., keratinocytes, synoviocytes, lymphocytes, monocytes, platelets, myocytes, etc.) in which case, the neutrophil may still play a role, but not as the origin of autoantigens toward which tolerance is initially broken. In addition, considering that the growing literature in regard to NETs and autoimmune diseases is biased toward SLE, these models will be largely based upon this prototypic systemic autoimmune disease. Model I will discuss the possibility that defects in common clearance pathways are the driver of systemic autoimmunity, while models II–IV suggest that apoptosis and NETosis play independent roles in systemic autoimmune disease pathogenesis. These three additional models are largely based on three independent studies in 2011, which suggested that NETosis may play a pathogenic role in SLE. While they agreed that NETs can induce IFN-α production by pDCs (with some differences about the mechanism), they differed dramatically in their conclusions about the mode by which NETs are generated (Garcia-Romo et al., 2011; Lande et al., 2011; Villanueva et al., 2011). These differences have major effects on the development of a unified pathogenic model of systemic autoimmunity.

THEORY I: THE SHARED MECHANISM MODEL

If systemic autoimmune diseases are the result of genetic or acquired defects in universal clearance pathways, it is possible that any form of cell death may provide the signals required to activate an autoimmune response (**Figure 2A**). In this regard, it is interesting that the same clearance defects that have been used to support the role of apoptosis in autoimmune diseases are now being translated into the NETotic model of autoimmunity (e.g., impairment of DNase1 function; Martinez et al., 2008; Hakkim et al., 2010). This suggests that diseases in which abnormal accumulation or clearance of apoptotic and NETotic cells has been implicated, may result from a common defect (e.g., SLE). In this scenario, although some forms of death may dominate at distinct disease stages depending on the environment (e.g., apoptosis, necroptosis, and/or autophagic cell death during viral infections, but NETosis in bacterial or fungal infections); any form of death may have the same potential to initiate and/or to propagate the disease. Certainly, it is possible that unique autoantigen modifications (e.g., cleavage by death proteases, phosphorylation, and citrullination, among others; Hall et al., 2004; Darrah and Rosen, 2010), generated by specific forms of death, may influence autoantigen selection and disease phenotype. It is important to determine if common clearance defects are playing a dominant role in these diseases before attributing disease development to a particular death pathway since therapies targeting unique forms of death (e.g., NETosis or apoptosis) would have little effect in this setting. Instead, therapies should be focused on enhancing universal pathways involved in the anti-inflammatory clearance of dead cells.

Attempts to improve the clearance of cell debris as therapy in SLE have not been deeply explored. In 1959–1960, bovine DNase I was first used in a small number of patients with encouraging results (Lachmann, 2003). However, following clinical improvement, patients developed antibodies to the bovine DNase which precluded further treatment. Forty years later, human recombinant DNase I was used in patients with SLE in a Phase 1b trial (Davis et al., 1999). Although this study did not identify clinical benefit, it was limited by the small number of patients and by failure to achieve sufficient bioactive serum concentrations. No further studies have followed in the use of DNase or any other component involved in the clearance of dead cells for the treatment of SLE.

THEORY II: THE "APOPTOCENTRIC" MODEL

The study by Garcia-Romo et al. (2011), demonstrated that SLE neutrophils undergo accelerated death *in vitro* (defined by trypan blue staining which indicates secondary necrosis) and identified that these cells are dying by apoptosis (determined by TUNEL assay). This data is consistent with previous observations both in pediatric and adult SLE (Courtney et al., 1999; Midgley et al., 2009), and supports the notion that in SLE, neutrophils are actively dying by apoptosis and undergoing secondary necrosis. Interestingly, the presence of anti-RNP immunoglobulin (Ig) induced a prominent reduction in the apoptosis rate of SLE neutrophils (*in vitro*) by changing the cell death program to NETosis. Moreover, although they detected a prominent type-I IFN signature in SLE neutrophils, they found that neutrophils exposed to IFN-α *in vitro* did not die by apoptosis and concluded that IFN is not responsible for the accelerated apoptosis observed in this cell type. Instead, they found that IFN-α increased the expression of TLR7 in neutrophils, which made them more susceptible to die by RNP-Ig-induced NETosis via FcγRIIa/TLR7 ligation.

Although there are some aspects here that need further mechanistic analysis (like the switch from apoptosis to NETosis), this data suggests a model in which apoptosis and NETosis co-exist in the lupus paradigm, but have different roles in the disease process (**Figure 2B**). Here, IFN-α activated neutrophils appear to be a bystander target for autoantibodies, and NETosis is a consequence of this process. In this model, the requirement of TLR-ligation suggests that cells suffering from secondary necrosis (likely from the large pool of apoptotic neutrophils in SLE)

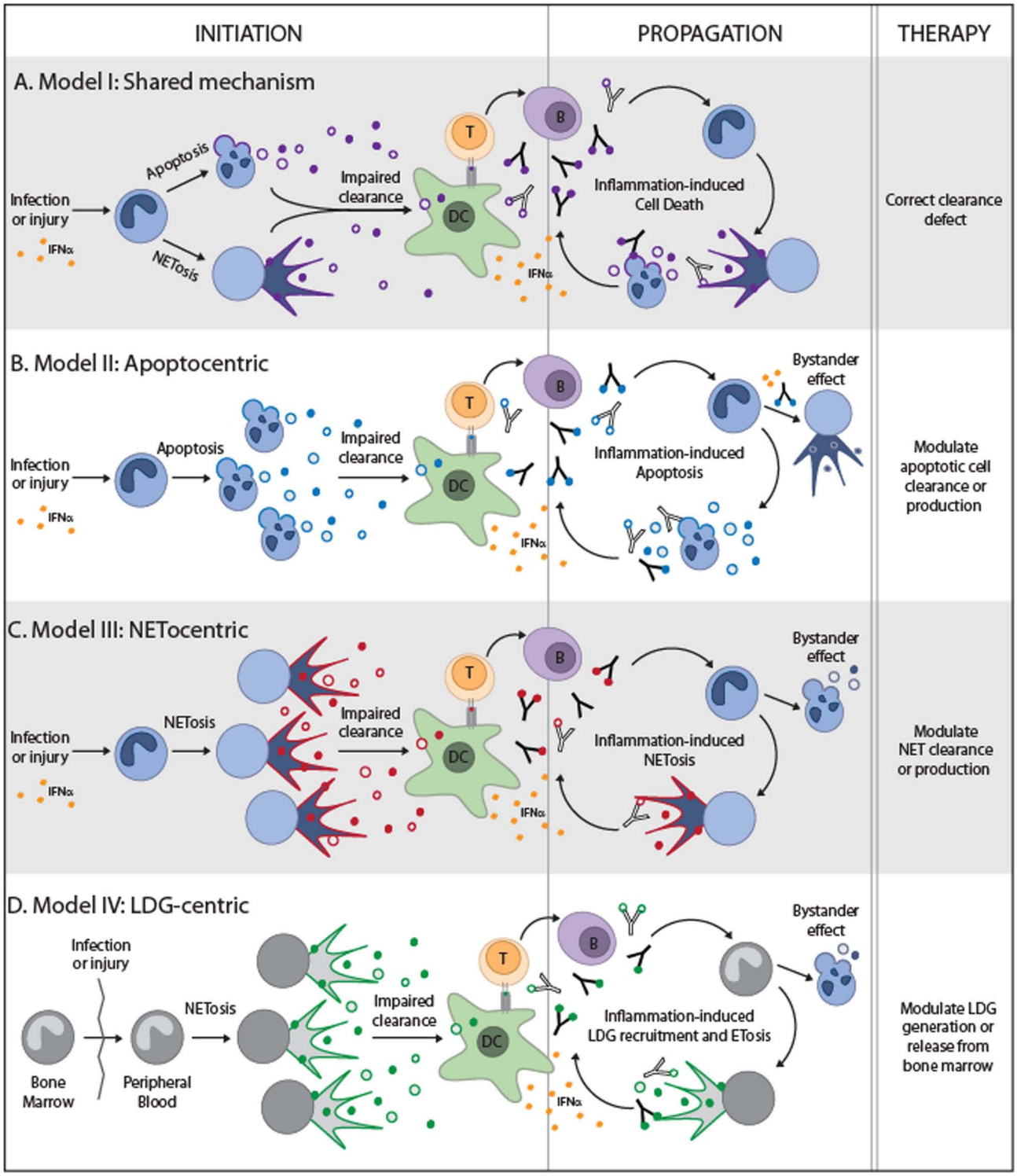

FIGURE 2 | Theoretical models to integrate NETosis and apoptosis into the paradigm of systemic autoimmune diseases. The four models are depicted and illustrate the form of cell death that is predicted to play a role during disease initiation and propagation. For simplicity, the neutrophil is shown in all models as the initiating source of autoantigens, but other tissue sources of cell death may be the primary driver of autoimmunity in models **(A,B)**. The neutrophil and large density granulocyte (LDG) are the primary initiating sources of autoantigens in models **(C)** and **(D)**, respectively. All models require the impaired clearance of cell debris, activation of dendritic cells and production of IFNα, and presentation of autoantigens to T helper cells. T helper cells would then stimulate autoantibody production which may lead to pro-inflammatory clearance of apoptotic cells and may combine with IFNα to induce NET formation. The nuances of each model are depicted and suggested therapeutic targets.

play a role in disease initiation by releasing antigens (i.e., RNP) to form IC which induce NETs. Together, this data suggest that in accordance with the apoptosis theory in SLE, accelerated apoptosis (and/or clearance defects) are likely involved in disease initiation, autoantibody production, and the early burst of IFN-α. Importantly, this model is not limited to apoptotic neutrophils, but can be applied to apoptotic material generated from any tissue in SLE. Induction of NETs by the effect of IFN-α and "two-receptor" ligation of RNP-IC may be part of an amplifying process of disease propagation that enhances the exposure of autoantigens (i.e., DNA) and endogenous adjuvants (e.g., LL37) and stimulates further IFN-α production in SLE. In this study, NETs induced by RNP-IC can activate IFN-α production by pDCs independently of FcγRIIa.

The recent finding that NETs are not necessary, but apparently protective in lupus-prone mice further support the model that NETs may be an epiphenomenon (at least in the lupus model), and open the possibility that NETs may have important effects in immunoregulation (Campbell et al., 2012). Although this finding cannot be directly translated into the human model, it certainly challenges investigators to better understand the role of NETs in inflammation in humans, before considering it as a potential target for therapy. Instead, therapies that correct the primary defect in the apoptotic pathway or enhance apoptotic cell clearance may have therapeutic benefit.

THEORY III: THE "NETOCENTRIC" MODEL

The study by Lande et al. (2011) has novel features of interest which support a primary role for NETosis in the initiation and propagation of systemic autoimmunity (**Figure 2C**). First, they discover that patients with SLE have circulating IC containing DNA and neutrophil antimicrobial peptides (LL37 and HNPs), and that a large proportion of patients have autoantibodies against LL37 and HNP. Second, LL37 and HNP are expressed on the surface of SLE neutrophils and this process is likely induced by IFN-α. Third, mouse monoclonals against LL37 and HNP induced NETs in SLE neutrophils and in IFN-α primed neutrophils. Finally, the study showed that freshly isolated SLE neutrophils release DNA in culture and suggested that this process reflects the spontaneous production of NETs by SLE patient neutrophils. Although this study neither addressed whether circulating IC containing DNA-LL37/HNPs are indeed generated from NETs (but not other mode of neutrophil death), whether human anti-LL37 and anti-HNP antibodies can trigger NET formation, nor confirmed that the DNA released spontaneously by SLE neutrophils was from NETs, the data suggests a model in which NET production in SLE results from a vicious cycle whereby autoantibodies target NET components and induce further NET formation by IFN-α activated neutrophils. In this study, although NETs can induce IFN-α production by pDCs, this process is strongly increased by IC formation with anti-DNA, anti-LL37, or anti-HNP antibodies.

This data supports a more "NETocentric" model that the previous one (Theory II) because the critical autoantigens that may be sufficient to initiate disease (i.e., DNA, LL37, and HNP) can be directly exposed in NETs. Thus, in the context of an adequate genetic predisposition, the abnormal clearance of NETs generated during infection may predispose individuals to the production of anti-DNA, anti-LL37, and anti-HNP antibodies. The presence of these autoantibodies in combination with subsequent infections may amplify an interferogenic response until compensatory mechanisms are surpassed, leading to disease propagation and development of clinical symptoms. In this model, apoptosis may occur as result of chronic inflammation and tissue damage induced by NETs (likely providing additional autoantigens not expressed in neutrophils), and suggests that the finding of accelerated apoptosis in SLE may correspond to an epiphenomenon not necessarily associated with the induction of disease. In contrast to the previous models, therapies to improve NET clearance or decrease NET production may be useful to ameliorate disease.

THEORY IV: THE LOW-DENSITY GRANULOCYTE-CENTRIC MODEL

The last model of NETs in systemic autoimmunity is based on the study by Villanueva et al. (2011); **Figure 2D**. The major finding in this study is strikingly different from the others (Garcia-Romo et al., 2011; Lande et al., 2011); the authors conclude that that low-density granulocytes (LDGs), but not mature neutrophils, are responsible for generating NETs in SLE. LDGs appear to represent an immature form of granulocyte that is prematurely released from the bone marrow in patients with SLE and RA (Hacbarth and Kajdacsy-Balla, 1986; Denny et al., 2010). Because their morphological features are quite different from mature neutrophils, LDGs are co-purified with mononuclear cells during peripheral blood cell gradient separation using Ficoll-Hypaque. Contamination with LDGs appears to be responsible for the prominent granulocytic signature found in peripheral mononuclear cells (PBMCs) from patients with SLE (Bennett et al., 2003; Denny et al., 2010). Strikingly, Villanueva et al. (2011) found that LDGs spontaneously generate NETs immediately after purification. However, in contrast to the data by Lande et al. (2011), mature SLE neutrophils showed no differences in spontaneous NET formation compared to controls.

There are some noteworthy features about LDGs that make them unique as the source of NETs in SLE. Immature neutrophils do not respond to type I IFN (e.g., IFN-α) because they fail to phosphorylate STAT1 (signal transducer and activator of transcription 1) in response to receptor binding (Martinelli et al., 2004), a feature likely shared by LDGs. This finding is consistent with gene expression analysis of lupus LDGs in which among 302 differentially expressed genes (compared to control neutrophils) there is no evidence of IFN-induced gene activation (Villanueva et al., 2011). Thus, this may indirectly support the hypothesis that IFN-α plays no role in the activation or generation of LDGs. Moreover, in contrast to other studies in which the pre-existence of IFN-α is required to prime neutrophils to generate NETs (Garcia-Romo et al., 2011; Lande et al., 2011), this study suggests that IFN-α may have no role in inducing NETs by LDGs, providing a model in which LDGs may precede IFN-α production in SLE. Instead, this study shows that IL-17 is exposed in LDG-NETs, suggesting that this cytokine may be involved in tissue damage and immune dysregulation induced by LDG-NETs. An intriguing part of this study is that although SLE neutrophils do not generate NETs spontaneously; these cells in culture can release DNA (the mechanism is undefined) that can induce IFN-α mRNA expression by a pDC

cell line in a similar proportion to LDG-NETs. In this study, the effect of anti-NET antibodies on IFN-α production by pDCs was not addressed.

It is still unknown whether circulating LDGs are found before clinical disease initiation in SLE and the mechanism(s) by which these cells are activated and abnormally released from bone marrow. However, it is possible that the abnormal release and NETosis of LDGs (a process potentially boosted during infection) may be the initial trigger for the aberrant production of IFN-α in SLE. Moreover, abnormally cleared NETotic LDGs may serve as source of autoantigens for autoantibody production in SLE. In this model, the abnormal apoptotic cell death found in SLE would be a consequence of the autoimmune process and may contribute to disease propagation, but is not the primary cause of disease. Importantly, this model offers a unique and specific target (LDGs) of therapy for the treatment of SLE.

CONCLUSION

The discovery of NETs has brought renewed interest in the neutrophil as a dominant player in the pathogenesis of systemic autoimmune diseases. It is clear that NET formation by neutrophils is a biological phenomenon with pathogenic potential, but how this relates to apoptosis in the pathogenic model of systemic autoimmune diseases remains undefined. This review puts forth several models to integrate the growing body of data on NETosis with the historically appreciated role of apoptosis in these diseases. The study of NETs is still in its infancy and in order to adopt a new pathogenic model, further studies are necessary to determine how these structures and neutrophil remains (i.e., post-NET anuclear neutrophils) modulate the immune response. Moreover, the pathogenic mechanisms and pathways by which NETs are generated need to be identified and will require the development of highly specific probes to study these structures *in vivo*. Although NETs can be distinguished morphologically when induced *in vitro*, the study of NETs in tissues requires specific markers to clearly distinguish NETosis from other types of granulocyte damage that may release DNA and cytoplasmic contents (e.g., secondary necrosis). In this regard, measuring soluble dsDNA (e.g., in plasma or in supernatants; Margraf et al., 2008; Lande et al., 2011) to quantify NETosis is questionable, since other forms of death (e.g., apoptosis and necrosis) may also be responsible for this effect (van der Vaart and Pretorius, 2008), especially in diseases with a high rate of apoptosis and secondary necrosis, such as SLE (Garcia-Romo et al., 2011).

Undoubtedly, the early findings about the potential pro-inflammatory effect of NETs are stimulating and have rejuvenated the study of cell death in systemic autoimmune diseases. As more forms of cell death are discovered and the growing complexity of cell death is understood, it is important to reexamine the role of different forms of cell death in the generation and modification of autoantigens. This will require the discovery of useful markers to distinguish unique forms of death in blood and tissues and may have important implications in the development of novel therapies that target cell death pathways in the treatment of systemic autoimmune diseases.

ACKNOWLEDGMENTS

Erika Darrah was supported by NIH grant T32 AR048522 and Sibley Memorial Hospital Fund. Felipe Andrade was supported by The Dana Foundation Scholars Program in Human Immunology, The Donald B. and Dorothy L. Stabler Foundation and NIH grant P30 AR053503.

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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.

Received: 22 October 2012; accepted: 26 December 2012; published online: 17 January 2013.

Citation: Darrah E and Andrade F (2013) NETs: the missing link between cell death and systemic autoimmune diseases? Front. Immun. ***3****:428. doi: 10.3389/fimmu.2012.00428*

This article was submitted to Frontiers in Molecular Innate Immunity, a specialty of Frontiers in Immunology.

frontiers in
IMMUNOLOGY

REVIEW ARTICLE
published: 14 December 2012
doi: 10.3389/fimmu.2012.00380

Proteins derived from neutrophil extracellular traps may serve as self-antigens and mediate organ damage in autoimmune diseases

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Neutrophils are the most abundant leukocytes in circulation and represent one of the first lines of defense against invading pathogens. Neutrophils possess a vast arsenal of antimicrobial proteins, which can be released from the cell by a death program termed NETosis. Neutrophil extracellular traps (NETs) are web-like structures consisting of decondensed chromatin decorated with granular and cytosolic proteins. Both exuberant NETosis and impaired clearance of NETs have been implicated in the organ damage of autoimmune diseases, such as systemic lupus erythematosus (SLE), small vessel vasculitis (SVV), and psoriasis. NETs may also represent an important source of modified autoantigens in SLE and SVV. Here, we review the autoimmune diseases linked to NETosis, with a focus on how modified proteins externalized on NETs may trigger loss of immune tolerance and promote organ damage.

Keywords: neutrophil, NETs, autoimmunity, posttranslational modifications, systemic lupus erythematosus (SLE), psoriasis, vasculitis, citrullination

INTRODUCTION

Neutrophils are the most abundant leukocyte population in peripheral blood and have a lifespan of as little as 4 h in the circulation; this short half-life is balanced by continuous and tightly regulated release from the bone marrow. Neutrophils are among the first line of defense against invading microbes (Kobayashi and Deleo, 2009), targeting pathogens through diverse mechanisms including phagocytosis, reactive oxygen species (ROS) generation, the release of microbicidal molecules from cytoplasmic granules, and the recently described extrusion of an extracellular chromatin meshwork—so-called NETosis (Brinkmann et al., 2004).

In 2004, Brinkmann et al. described a distinct mechanism of neutrophil cell death, resulting in the programmed externalization of a meshwork of chromatin fibers decorated with granule-derived antimicrobial proteins (neutrophil extracellular traps or NETs) (Brinkmann et al., 2004). NETosis has subsequently been shown to be an important strategy by which neutrophils trap and kill invading microorganisms (Brinkmann and Zychlinsky, 2012; Kaplan and Radic, 2012). NETs can also damage the vasculature and have the potential to trigger thrombosis (Fuchs et al., 2010; Gupta et al., 2010; Brill et al., 2012; Saffarzadeh et al., 2012).

Although there is still much to learn regarding the triggers and signaling pathways that facilitate NETosis, important roles have been suggested for the NADPH oxidase machinery (Fuchs et al., 2007; Ermert et al., 2009; Bianchi et al., 2011; Remijsen et al., 2011), ROS (Nishinaka et al., 2011; Palmer et al., 2012), the Raf/mitogen-activated protein kinase/extracellular signal-regulated kinase pathway (Hakkim et al., 2011), histone citrullination (Neeli et al., 2008; Wang et al., 2009; Li et al., 2010; Hemmers et al., 2011), MPO/neutrophil elastase (NE) (Papayannopoulos et al., 2010; Metzler et al., 2011), autophagy (Mitroulis et al., 2011; Remijsen et al., 2011), and microtubule polymerization (Neeli et al., 2009). The characterization of pathways implicated in the development of NETs has potential implications for pharmacologic strategies to block NETosis, which is particularly appealing in the context of the "sterile" NETosis that will be described below. The protein fraction of NETs classically contains histones, MPO, and various serine proteases, although the specific composition continues to be defined (Urban et al., 2009; Liu et al., 2012). Here, we will first review recent discoveries pertaining to how NETs may play a role in the pathogenesis of systemic autoimmune diseases, and will then consider the protein composition of NETs in more detail.

SMALL VESSEL VACULITIS

The first compelling link between NETs and autoimmunity was in 2009 with the characterization of NETosis in small vessel vasculitis (SVV) (Kessenbrock et al., 2009). SVV is a systemic autoimmune disease of unknown etiology, with disease flares that result in necrotizing inflammation of small blood vessels—especially targeting the kidneys, lungs, skin, and peripheral nerves. The majority of SVV patients have detectable anti-neutrophil cytoplasmic antibodies (ANCA) with specific reactivity against either proteinase 3 (PR3) or MPO. In addition to their important role in diagnosis, ANCA activate neutrophils *in vitro* (Chen and Kallenberg, 2009), and are sufficient to induce vasculitic disease in animal models (Xiao et al., 2002; Pfister et al., 2004). Kessenbrock

and colleagues showed that NETs externalize PR3 and MPO, and, reciprocally, that ANCA (and specifically anti-PR3 antibodies) induce NETosis (Kessenbrock et al., 2009). Furthermore, MPO-DNA complexes, presumably derived from NETs, can be detected in the circulation, the levels of which track with SVV disease activity. In addition, extracellular DNA (co-localizing with histones, MPO, and PR3) was detected in kidney biopsies from the majority of SVV patients (Kessenbrock et al., 2009).

While the Kessenbock study, as well as one additional case report (Abreu-Velez et al., 2009), have hinted at an important role for NETs in the organ damage of SVV, more recent studies have begun to mechanistically explore the specialized role of NET proteins as autoantigens in SVV. To this end, Nakazawa and colleagues studied the drug propylthiouracil (PTU) which is a known inducer of anti-MPO autoantibodies and SVV in humans (Wada et al., 2002; Nakazawa et al., 2012). In the presence of PTU, phorbol 12-myristate 13-acetate (PMA)-induced NETs had an abnormal, globular conformation, which was relatively resistant to DNase I digestion (Nakazawa et al., 2012). When these PMA/PTU NETs were injected into rats, the animals not only developed anti-MPO autoantibodies, but also pulmonary capillaritis reminiscent of human vasculitic disease (Nakazawa et al., 2012). Whether the driving feature of autoimmunity was enhanced NET stability, differences in the tertiary structure of PTU NETs or modification of NET proteins such as MPO remains to be determined.

Another recent study provided a tantalizing link between NETs and adaptive immunity, demonstrating that NET proteins were preferentially uploaded into myeloid dendritic cells (mDCs) *in vitro*, an affect that was lost when the NET structure was dismantled with DNase (Sangaletti et al., 2012). Injection of the NET-loaded mDCs into mice resulted in anti-MPO autoantibodies and an autoimmune phenotype including glomerulitis, although the kidney histopathology was in some ways more reminiscent of lupus lesions than the typical pauci-immune disease of SVV. This study hints that NETs provide a unique, stimulatory microenvironment that can break normal immune tolerance, and thereby predispose to autoimmunity.

PSORIASIS

Psoriasis is a common inflammatory disease of the skin and is typically thought of as an autoimmune disease given the recognized importance of autoreactive T-cells. In psoriasis, local production of type I IFNs, such as IFNα, by plasmacytoid dendritic cells (pDCs) is an important upstream event in the activation of autoimmune T-cells (Nestle et al., 2005). pDCs are a specialized type of dendritic cell with unique, high-level expression of toll-like receptors (TLRs) 7 and 9, which recognize nucleic acids from viruses and other microbes—the result being robust expression of type I IFNs (Kadowaki et al., 2001). In 2007, Lande et al. identified the cationic NET protein cathelicidin/LL37 as a factor that binds and converts inert self DNA into a complex capable of activating pDCs (Lande et al., 2007); this leads to robust production of IFNα in psoriasis skin, with implications for driving autoimmunity. More recently, it has also been suggested that the combination of secretory leukocyte proteinase inhibitor (SLPI) and NE, both derived from NETs, can bind DNA and serve a similar role in converting self DNA into an activator of pDCs in psoriasis lesions (Skrzeczynska-Moncznik et al., 2012).

Interleukin-17 (IL-17) is a proinflammatory cytokine, linked to autoimmune diseases such as rheumatoid arthritis (RA), inflammatory bowel disease, and psoriasis (Wilson et al., 2007). Historically, production of IL-17 has been attributed to Th17-cells, and, indeed, both IL-17 mRNA and increased numbers of Th17-cells have been identified in psoriasis lesions (Kryczek et al., 2008; Lowes et al., 2008). A novel take on this story was the recent identification of extracellular traps from both mast cells and neutrophils as an important, and perhaps predominant, source of IL-17 in psoriasis lesions (Lin et al., 2011). Further, IL-23, a known activator of Th17 differentiation, can also stimulate mast cells to release extracellular traps decorated with IL-17 (Lin et al., 2011).

GOUT

Acute gout is a common, inflammatory arthritis driven by the deposition of monosodium urate (MSU) crystals in appendicular joints; a critical impetus for MSU deposition is elevated serum uric acid, which correlates with obesity, hypertension, diabetes, and other metabolic risk factors. Although gout is not a typical autoimmune disease, it shares the characteristic of acute, sterile inflammation; and, in recent years, the recognition of the potency by which anti-IL-1 agents can ameliorate gout flares has opened the door to what will surely be additional cytokine manipulation in the future.

Given the now well-recognized role of MSU crystals as activators of the NLRP3 inflammasome with resultant production of the pro-inflammatory cytokine IL-1β (Martinon et al., 2006), as well as the consistent documentation of neutrophilia in acute gout synovial fluid (Popa-Nita and Naccache, 2010), investigators have begun to address the extent to which NETs factor into gout pathogenesis. Indeed, MSU crystals, IL-1β, and both synovial fluid and serum from patients with acute gout, all stimulate neutrophils to release NETs (Mitroulis et al., 2011). These "gout NETs" contain DNA, MPO, and the alarmin, high mobility group box chromosomal protein 1 (HMGB1), and may propagate the inflammatory response. Furthermore, the IL-1 inhibitor anakinra blocks NET release when control neutrophils are exposed to gout serum or synovial fluid (Mitroulis et al., 2011).

More recently, basophils and eosinophils (along with neutrophils) were been shown to release extracellular traps in response to MSU crystals. In contrast, monocyte-lineage cells, despite phagocytizing the crystals, did not release extracellular DNA (Schorn et al., 2012). The authors argued that MSU-induced NETs were qualitatively different from those induced by bacteria or PMA in that MSU NETs extended more "widely" in the culture plate, and were relatively resistant to inhibition (and perhaps degradation) by high concentrations of plasma in the culture medium (Schorn et al., 2012). The protein content of MSU-induced NETs was not, however, further explored.

FELTY'S SYNDROME

Patients with RA—the prototypical chronic, inflammatory polyarthritis—form autoantibodies to citrullinated (deiminated) proteins, the detection of which has emerged as the most

compelling serologic test for RA (Wegner et al., 2010). A small subset of patients with RA develop so-called Felty's syndrome, which manifests clinically as marked neutropenia and splenomegaly; and, which appears to be closely related to a syndrome of oligoclonal T-cell expansion, large granular lymphocyte leukemia (Balint and Balint, 2004; Liu and Loughran, 2011). Given the classical recognition of anti-histone antibodies in patients with systemic lupus erythematosus (SLE) (Suzuki et al., 1994), and to a lesser extent in RA and Felty's syndrome (Cohen and Webb, 1989; Tuaillon et al., 1990)—as well as the well-recognized deimination of histones in NETs (Neeli et al., 2008; Wang et al., 2009; Li et al., 2010; Hemmers et al., 2011)—a logical question is whether autoantibodies from SLE, RA, and Felty's syndrome patients specifically target deiminated histones.

In a 2012 study, autoantibodies from all three diseases showed reactivity with NETs, with a preference for deiminated histones in Felty's syndrome that was not readily apparent in either SLE or RA serum (Dwivedi et al., 2012). Further linking deimination to autoimmunity, deiminated histones were detected in circulating neutrophils of patients with RA, while serum from patients with SLE and Felty's syndrome stimulated the *ex vivo* deimination of neutrophil histones (Dwivedi et al., 2012).

SYSTEMIC LUPUS ERYTHEMATOSUS

SLE is an autoimmune syndrome characterized by autoantibody formation against nuclear antigens, with resultant immune complex deposition, inflammation, and organ damage (Tsokos, 2011). While intensive study has shown that both T- and B-cells are required for the lupus phenotype (Crispin et al., 2010; Dorner et al., 2011), neutrophils and other mediators of the innate immune response have, by comparison, received considerably less attention (Knight and Kaplan, 2012).

Various abnormalities in neutrophil phenotype and function have been described over the years, including abnormalities in phagocytic activity, aggregation, and intravascular activation (Brandt and Hedberg, 1969; Hashimoto et al., 1982; Abramson et al., 1983; Jonsson and Sturfelt, 1990; Molad et al., 1994; Courtney et al., 1999). Further, a subset of neutrophils in the peripheral blood of lupus patients have lower density and consequently co-purify with peripheral blood mononuclear cells (PBMCs) during sedimentation of whole blood (Hacbarth and Kajdacsy-Balla, 1986; Bennett et al., 2003; Denny et al., 2010). This population may represent the accelerated release of immature granulocytes from the bone marrow, although the origin, function, and pathogenic significance of these cells remain to be fully determined (Denny et al., 2010; Villanueva et al., 2011).

Evidence of a role for neutrophils in SLE pathogenesis is emphasized by the observation that various bactericidal proteins released by activated neutrophils are present at higher-than-expected levels in lupus blood (Sthoeger et al., 2009; Vordenbaumen et al., 2010; Ma et al., 2012). Neutrophils, and in particular low-density granulocytes (LDGs), have been associated with endothelial damage as well as promotion of abnormal endothelial differentiation, and have been posited to play a critical role in the well-recognized accelerated atherosclerosis of SLE (Denny et al., 2010; Kaplan, 2011). Neutrophilic infiltrates are a recognized feature of diffuse proliferative lupus nephritis (Austin et al., 1984), while proteins released from neutrophilic granules are toxic to glomerular structures (Henson, 1972; Johnson et al., 1988; Hotta et al., 1996).

A particularly exciting development of the past 2–3 years has been the description of aberrant NETosis in SLE, which might explain, at least in part, the longstanding recognition of increased circulating DNA in lupus patients (Tan et al., 1966). Indeed, mutations in DNase I have been reported among SLE patients, and seem to promote autoantibody formation (Yasutomo et al., 2001; Shin et al., 2004). In addition, two groups have recently described a DNase I-inhibititory activity in SLE serum that prevents degradation of NETs, and is associated with more active disease (Hakkim et al., 2010; Leffler et al., 2012). Specifically, experiments by Hakkim and colleagues demonstrate that 36.1% of SLE sera degrade NETs poorly, with inhibitors of DNase I detectable in some patients, while others coat the NETs with autoantibodies to mechanically protect against degradation (Hakkim et al., 2010). SLE patients with poor NET degradation have higher anti-double-stranded DNA antibody titers, display more complement activation, and are more likely to carry a diagnosis of lupus nephritis (Hakkim et al., 2010; Leffler et al., 2012).

Further, in 2011, three papers described *ex vivo* models of enhanced NETosis in SLE patients (Garcia-Romo et al., 2011; Lande et al., 2011; Villanueva et al., 2011), with the aforementioned lupus LDGs particularly capable of releasing spontaneous NETs (**Figure 1**). All three papers also demonstrated that NETs stimulate pDCs to release type I IFNs (Garcia-Romo et al., 2011; Lande et al., 2011; Villanueva et al., 2011), and, indeed, most current models of lupus pathogenesis include a role for activation of the type I IFN pathway, which lowers the threshold for autoreactivity of both antigen-presenting and antibody-producing cells (Banchereau and Pascual, 2006; Elkon and Stone, 2011).

Continuing the theme discussed above for SVV and Felty's syndrome, NETs may provide novel antigens for autoantibody formation in SLE (Liu et al., 2012). There are also hints that NETs may be a source of vascular and organ damage in SLE (Villanueva et al., 2011), which would not be surprising if parallels were drawn to other inflammatory diseases where NET toxicity has been documented such as SVV, cystic fibrosis, transfusion-related acute lung injury (TRALI), and sepsis (Clark et al., 2007; Kessenbrock et al., 2009; Caudrillier et al., 2012; Dubois et al., 2012; Thomas et al., 2012).

NET PROTEINS AND SLE

There are at least two general frameworks by which NET proteins might impact on SLE pathogenesis, both of which have already been touched upon in this review. The first posits a role for NETs in organ damage, which is supported by both the potential toxicity of NET proteins and the recognition that many of these proteins have been detected at increased levels in SLE patients. Proof of this principle will depend on animal models where specific proteins can be targeted by genetic or pharmacologic approaches.

The second concept is that NET proteins may be uniquely modified and positioned to break tolerance and thereby trigger or exacerbate autoimmunity. Certainly, the idea of modified proteins serving as autoantigens in SLE is not new (Casciola-Rosen

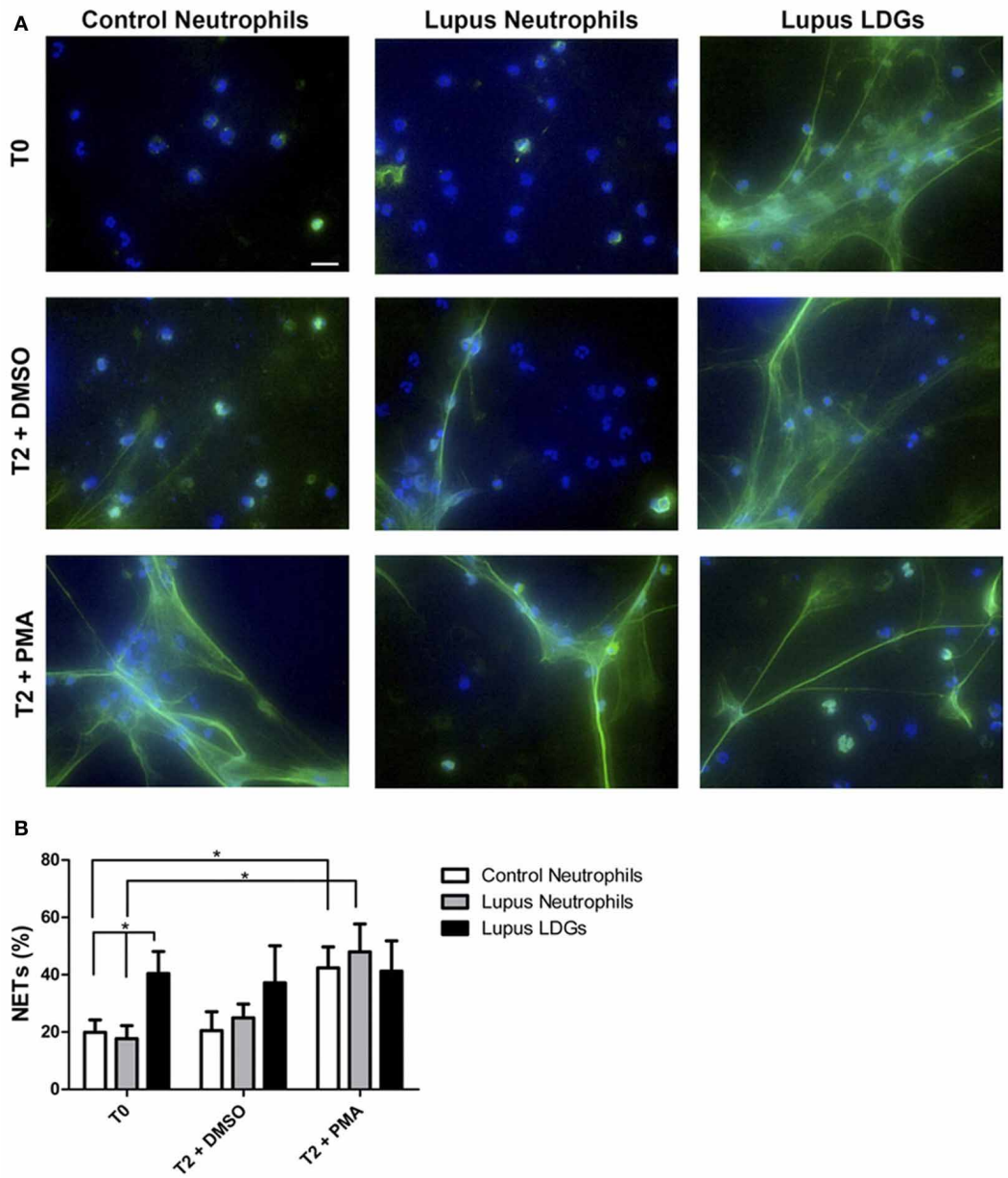

FIGURE 1 | Circulating lupus LDGs undergo increased NETosis. **(A)** Representative images of control neutrophils, lupus neutrophils, and lupus LDGs isolated from peripheral blood and analyzed at baseline (T0) or after stimulation for 2 h with DMSO or PMA. Panels show merged images of neutrophil extracellular traps (NETs) in which neutrophil elastase is stained green by immunofluorescence and DNA is stained blue by Hoechst 33342; 40× images, scale bar: 20 μm. **(B)** Quantification of the percentage of NETs (elastase-labeled cells over total number of cells) are plotted as mean ± SEM ($n = 6$ patients/group; $^*p = 0.05$). [Obtained with permission from Villanueva et al. (2011) and The Journal of Immunology. Copyright 2011. The American Association of Immunologists, Inc.].

et al., 1999; Utz et al., 2000; Graham and Utz, 2005; Dieker and Muller, 2010), and the milieu of NETs may represent a novel environment—replete with pathogens and immunostimulatory host molecules—where this can take place.

At this point, relatively few proteins have been definitively detected in lupus NETs, with the definition of "lupus NETs" being somewhat arbitrary and based on the *ex vivo* study of lupus neutrophils. Of the lupus-associated NET proteins, the most in-depth work has involved LL37/cathelicidin, with the demonstration that this small cationic protein circulates in complex with chromatin fragments and anti-DNA autoantibodies in lupus serum, thereby enhancing stimulation of pDCs and protecting against DNase-mediated destruction (Lande et al., 2011). Although not directly linked to SLE, LL37/cathelicidin can also complex with RNA to activate dendritic cells through TLR7 and TLR8 (Ganguly et al., 2009); this work is particularly interesting given the suggestion by

Garcia-Romo and colleagues that anti-RNP antibodies and TLR7 may play a role not only in dendritic cell activation, but also in the activation of lupus neutrophils to release NETs (Garcia-Romo et al., 2011).

Other proteins identified by immunofluorescence (no lupus-NET proteomics have been undertaken outside of the histone studies described below) include C1q (Leffler et al., 2012), NE (Garcia-Romo et al., 2011; Villanueva et al., 2011), histones (Villanueva et al., 2011; Liu et al., 2012), HMGB1 (Garcia-Romo et al., 2011), HNP (Lande et al., 2011), IL-17 (Villanueva et al., 2011), LL37/cathelicidin (Lande et al., 2011; Villanueva et al., 2011), and MPO (Garcia-Romo et al., 2011; Lande et al., 2011; Villanueva et al., 2011). These proteins, along with those identified in the aforementioned autoimmune/inflammatory diseases, are summarized in **Table 1**. In the absence of a definitive proteomics approach, this list is certainly not exhaustive, and one might therefore extrapolate from other studies (Urban et al., 2009), with the caveat that the NETs heretofore characterized by proteomics were isolated from neutrophils treated with PMA, and therefore have unknown *in vivo* relevance from the perspective of SLE. We will now consider some of these individual lupus-NET proteins.

Both the peroxidase MPO and serine protease PR3 have compelling roles as autoantigens in SVV, as discussed above (Kessenbrock et al., 2009). And, given the common availability of commercial assays, anti-MPO and anti-PR3 titers have been frequently assessed in SLE patients (Nassberger et al., 1990; Cambridge et al., 1994; Manolova et al., 2001; Pan et al., 2008). The available data is heterogeneous and no clear trend has emerged, although one can be relatively confident in saying that—at least for the assays that are commercially available—anti-MPO/PR3 autoantibodies do not specifically identify SLE patients, nor do they track with specific disease manifestations. In contrast to the assessment of autoantibodies, studies that examine the role of the MPO protein in SLE are relatively limited, although at least one study has shown increased MPO plasma levels in lupus patients as compared to healthy controls (Telles et al., 2010), albeit without a clear correlation to disease activity. NE is also a recognized trigger of autoantibodies in SLE (Nassberger et al., 1989, 1990), but the clinical significance remains to be determined. In terms of the NE protein, one study has suggested higher plasma levels in SLE patients (Zhang et al., 1989).

Both the iron-chelator lactoferrin and the serine protease cathepsin G have been objectively identified in PMA-induced NETs (Urban et al., 2009), and both appear to function as autoantigens in SLE (Lee et al., 1992; Galeazzi et al., 1998; Zhao et al., 1998; Manolova et al., 2001; Caccavo et al., 2005); although, again, no clear clinical correlation has emerged. In terms of circulating protein, there is no correlation between plasma lactoferrin and either active or inactive SLE (Adeyemi et al., 1990; Tsai et al., 1991), while cathepsin G protein levels have not been considered.

Alarmins are endogenous mediators capable of enhancing innate and adaptive immune response through recruitment and activation of antigen-presenting cells. From the perspective of NET proteins, both the α-defensins (sometimes called neutrophil defensins or human neutrophil peptides/HNPs) and HMGB1 would be classified as alarmins. α-defensins 1 and 3 were identified in the proteomic analysis of PMA-induced NETs (Urban et al., 2009), while both HMGB1 (Garcia-Romo et al., 2011) and α-defensins/HNP (Lande et al., 2011) have been described in the context of lupus NETs.

α-defensins activate monocyte-lineage cells to release pro-inflammatory cytokines such as TNF-α and IL-1β; they also serve as chemokines for recruitment of diverse cell types

Table 1 | NET proteins with potential roles in autoimmunity.

Protein	Present in disease-specific NETs (by IF)	Present in PMA-induced NETs (by proteomics*)	AutoAbs	Role(s) in auto-immunity
Histones	All (by definition)	Yes	SLE, Felty's	AutoAg in SLE and Felty's; pro-thrombotic
MPO	SLE, psoriasis, SVV, gout	Yes	SVV, SLE	AutoAg in SVV; oxidative stress?
Proteinase 3	SVV	Yes	SVV, SLE	AutoAg in SVV
LL37	SLE	No	SLE	Binds ICs to activate pDCs
HNP/α-defensins	SLE	Yes	SLE	Binds ICs; predisposes to CVD?
HMGB1	SLE, gout	No	Unknown	Binds ICs; pro-inflammatory
IL-17	SLE, psoriasis	No	SLE, psoriasis	Pro-inflammatory
C1q	SLE	No	SLE	Activates complement; protects from degradation?
Elastase	SLE, psoriasis	Yes	SLE	Unknown
Lactoferrin	Unknown	Yes	SLE	Unknown
Cathespin G	Unknown	Yes	SLE	Unknown
Calprotectin	Unknown	Yes	Unknown	Unknown
α-enolase	Unknown	Yes	SLE	Unknown
Catalase	Unknown	Yes	SLE	Oxidative stress?

(Urban et al., 2009); autoAb, autoantibody; autoAg, autoantigen; IC, immune complex.

NET, neutrophil extracellular trap; IF, immunofluorescence; SLE, systemic lupus erythematosus; SVV, small vessel vasculitis; CVD, cardiovascular disease.

including T-lymphocytes and dendritic cells; and can regulate activation of the complement cascade (Lehrer et al., 1993; Lehrer and Ganz, 2002). α-defensins have been linked to SLE both from the perspective of defensin-specific autoantibodies that correlate with disease activity (Tamiya et al., 2006), as well as circulating protein levels that seem to be higher in lupus patients (Sthoeger et al., 2009; Vordenbaumen et al., 2010); in fact, high α- and β-defensin levels were recently shown to correlate with cardiovascular disease in lupus patients (Vordenbaumen et al., 2012). One can certainly imagine a role for defensins in the induction of lupus inflammation and autoimmunity, and indeed this concept has been reviewed elsewhere (Froy and Sthoeger, 2009).

From a lupus perspective, HMGB1, a DNA-binding protein with alarmin potential when released into the extracellular space, has received considerable interest in recent years, as evidenced by the number of review articles written on this topic (Abdulahad et al., 2010; Pan et al., 2010; Urbonaviciute and Voll, 2011; Pisetsky, 2012). Initial reports described extracellular HMGB1 in cutaneous lesions (Popovic et al., 2005; Barkauskaite et al., 2007), but, more recently, this DNA-binding protein has been linked to lupus nephritis (Zickert et al., 2012)—where HMGB1 has been suggested as a novel urine biomarker for nephritis activity (Abdulahad et al., 2012). Similar to cathelicidin/LL37, HMGB1 associates with extracellular nucleosomes and potentiates their inflammatory potential through receptors such as TLR9 (Tian et al., 2007; Urbonaviciute et al., 2008). A recent review, however, points out that caution is necessary as HMGB1 function is critically-dependent on its redox state, and therefore detection may not always equate with pathologic potential (Pisetsky, 2012).

With the exception of the nuclear protein HMGB1, all of the aforementioned proteins are primarily derived from neutrophil granules. Cytoplasmic proteins such as the antimicrobial heterodimer calprotectin have been identified in NETs (Urban et al., 2009); the links between calprotectin and lupus are tenuous, although one study reported elevated circulating levels which, in a population of 100 patients, correlated with disease activity and anti-DNA autoantibodies (Haga et al., 1993). These findings have not been replicated in another study (Wahren et al., 1995). Autoantibodies to another NET protein, α-enolase (Urban et al., 2009)—which have gained notoriety for their possible association with Hashimoto's encephalopathy (Yoneda et al., 2007)—can also be detected in patients with SLE (Mosca et al., 2006). Similarly, autoantibodies to the NET protein catalase have been described in lupus (Mansour et al., 2008), with suggestion that these antibodies may be linked to oxidative stress.

To summarize, studies reporting autoantibodies to NET proteins are common in SLE, although with tenuous clinical correlations that have yet to be reproduced across studies; certainly, none of these autoantibodies are presently useful to the rheumatologist in clinic (with the possible exception of anti-histone antibodies). In contrast, some of the most clinically relevant autoantigens in SLE such as Ro, La, Smith, and RNP have yet to be identified in NETs (Villanueva et al., 2011). When circulating protein levels are considered, there is a trend toward NET proteins being increased in lupus plasma. HMGB1 probably has the most momentum presently for use as a biomarker in the clinical care of lupus patients—especially in the context of nephritis—but confirmation in additional patient populations is needed.

There are still several gaps in our understanding of how NETs may potentially trigger autoimmunity. First, there still does not seem to be an answer to the question of whether all NETs are created equal. Replicating the proteomics data for PMA-induced NETs (Urban et al., 2009) in other systems, such as NETs spontaneously released by lupus neutrophils (Villanueva et al., 2011), or NETs triggered by MSU crystals, seems desirable and would surely generate new hypotheses regarding the potential for organ damage, and the interplay between innate and adaptive immunity.

Along these same lines, there is also still much work to be done to understand potential triggers of sterile NETosis in the rheumatologic diseases. In SVV, anti-PR3 and anti-MPO autoantibodies have been suggested as possible triggers (Kessenbrock et al., 2009), while type I interferons as well as anti-LL37, anti-RNP, and anti-double-stranded DNA autoantibodies may play a role in SLE (Garcia-Romo et al., 2011; Lande et al., 2011; Villanueva et al., 2011). These concepts await confirmation by other investigators and in animal models.

Next, there is still no validated biomarker for enhanced NETosis *in vivo*. Quantifying a circulating protein may be inferior to the strategies described for MPO (Kessenbrock et al., 2009; Caudrillier et al., 2012) and LL37/cathelicidin (Lande et al., 2011) that identify and quantify that protein in complex with DNA.

Finally, the clinical studies described above typically rely on commercial assays for the detection of autoantibodies. As will be discussed in more detail below, NETs are an attractive milieu for post-translational modifications (Liu et al., 2012), and it may take a more refined look at autoantibodies (and their specificities) to prove relevant clinical correlations, should they exist.

MODIFIED NET PROTEINS AS AUTOANTIGENS

POSTTRANSLATIONAL MODIFICATIONS (PTMs)

PTMs are chemical alterations of a protein by the addition of biochemical functional groups (such as acetate, methyl, phosphate, lipids, and carbohydrate moieties; see **Table 2**), that change the chemical nature of an aminoacid (e.g., arginine > citruline) or by altering the secondary structure of the

Table 2 | Posttranslational modifications.

Modification	Residues modified	Function/notes
Acetylation	Lys	Protein stability, DNA regulation
Deimination	Arg	Transcription
Disulfide bond formation	Cys	Protein stability, inter- intra-molecular crosslink
Glycosilation (N-, O-linked)	Asn (N-linked) Ser (O-linked)	Cell–cell recognition, signaling
Methylation	Lys, Arg	Gene regulation
Nitration	Tyr	Oxidative damage during inflammation
Phosphorylation	Tyr, Ser, Thr	Activation/inactivation, signaling
Ubiquitination	Lys	Signaling, degradation

polypeptide (e.g., di-sulfide bonds). Such modifications orchestrate a variety of specific functions such as unraveling of chromatin, signaling, cell–cell recognition/communication, and enzyme activation/inactivation. Therefore, it is important to examine whether proteins externalized on the NETs undergo specific PTMs, and whether exposition of modified proteins can circumvent tolerance and promote the development of autoimmune syndromes in predisposed individuals. In this section, we will review the PTMs already reported in NET proteins.

The scaffold and most abundant proteins in the NETs are histones. They comprise about 70% of the proteins associated to chromatin fibers released during NETosis to the extracellular space (Urban et al., 2009). Nucleosome is the fundamental unit of the chromatin and it is composed of two copies of each of the core histones (H2A, H2B, H3, and H4) (Luger et al., 1997). The unstructured N-terminal tail of this proteins undergoes a series of modifications, important for their role during transcription, condensation, and decondensation of the DNA. Although detection of PTMs can be a challenge, today's armamentarium includes mass spectrometry with or without proteomic analysis, and immunoblot against most common modifications (e.g., methylation, acetylation, and ubiquitination). Liu and collegues reported a series of PTMs in NETs' histones isolated from H_2O_2-stimulated human neutrophils and from two neutrophil-like cell lines stimulated with H_2O_2, TNF, LPS, Ionomycin, or PMA (Liu et al., 2012) (**Table 3**). Methylation of histone H4K20 (mono-, di-, and tri-methyl), acetylation of histone H4K5 and H4K16 and citrullination of histone H3 and H4 increased upon stimulation with H_2O_2, when compared with unstimulated conditions. The same study reported that SLE sera reacted preferentially to unmodified histone H2B and acetylated H2BK12 and K20 peptides, although a subset reacted to citrullinated histone H3 (Liu et al., 2012). In addition, autoantibodies against acetylated histone H2B tail, histone H4, histone H3K27Me3, citrullinated H3 and H4 and ubiquitination of H2A have been reported in SLE patients (Suzuki et al., 1994; Dieker et al., 2007; Van Bavel et al., 2009, 2011; Liu et al., 2012). Histone epitopes are proposed as clinically important autoantigens in SLE, RA and other autoimmune diseases (Monestier et al., 2000; Robinson et al., 2002; Van Bavel et al., 2011). Indeed, Liu and colleagues found that many of the relevant SLE autoantigens were contained in NETs (Liu et al., 2012). As mentioned above, autoantibodies generated in Felty's syndrome bind preferentially to deiminated histones, in particular to histone H3 (Dwivedi et al., 2012) and sera from these patients binds to LPS-generated NETs (Dwivedi et al., 2012). These observations further support that NETs can be a source of modified autoantigens associated with autoimmunity. Future research directions will need to focus on whether "sterile" stimuli specific for certain autoimmune diseases can induce specific PTMs in proteins externalized in the NETs, and whether these specific modifications can preferentially trigger certain chronic inflammatory processes. PTMs of various cellular proteins may trigger formation of neoantigens with the capacity to induce adaptive immune responses (Rosen and Casciola-Rosen, 1999). Despite that purified NETs failed to exacerbate autoimmune phenotypes in certain strains of mice (Liu et al., 2012), it is possible that priming factors or second signals are needed to break tolerance in the presence of aberrant NETosis. These factors may vary from disease to disease and could include, in the case of SLE, type I IFNs (Baechler et al., 2003; Bennett et al., 2003; Banchereau and Pascual, 2006), other cytokines or specific environmental insults. Indeed, a high "interferon signature" in SLE is associated with high titers of autoantibodies against histones and other nuclear proteins that may be externalized during NETosis (Baechler et al., 2003; Bennett et al., 2003). It has also been reported that type I IFNs can potentiate production of NETs (Martinelli et al., 2004).

While most of the PTMs are often associated with reversible events involved in signal transduction, deimination (arginine to citrulline conversion)—catalyzed by a family of enzymes named peptidylarginine deiminases (PADs)—is not reversible.

Table 3 | Posttranslational modifications in NETs.

Source of NETs	Stimulation	Enriched PTMs found in NETs			Affected PTMs in NETs		
		Acetylation	Citrullination	Methylation	Acetylation	Citrullination	Methylation
Human peripheral neutrophils	Hydrogen peroxide	H4K5Ac H4K16Ac	H3Cit(2,18,17) H4Cit3	H4K20Me1/2/3	H3K9Ac		
ATRA/GM-CSF differentiated murine EPRO cells	Hydrogen peroxide Ionomycin LPS PMA TNF		H3Cit(2,8,17) H3Cit26 H4Cit3	H3K9Me2 H3K27Me1/2/3 H4K20Me1/2/3	H2BK12Ac H3K9Ac H3K27Ac H4K16Ac H3R17Me2(a) H4R3Me2(s)		H3K36Me2 H4K20Me2
ATRA differentiated human leukemia HL-60 cells	Hydrogen peroxide LPS TNF			H3K27Me1/2/3	H2BK12Ac H3K9Ac H3K27Ac	H3Cit(2,8,17) H3Cit26	H3K36Me2 H4K20Me2 H3R2Me2(a) H3R17Me2(a) H4R3Me2(s)

Abbreviations: K, lysine; R, arginine; Me, methyl; Ac, acetyl; Cit, citrulline; (a), asymmetric; (s), symmetric; TNF, tumor necrosis factor; EPRO, early promyelocyticcell line; ATRA, all-trans retinoic acid; PMA, phorbol 12-myristate 13-acetate; LPS, lipopolysaccharide.

The presence of this atypical aminoacid (citrulline), not encoded by the genome, plays an important role during NET formation since PAD4-deficient mice suffer of impairment in NETs formation (Li et al., 2010). Studies have suggested that changes in the polarity of the aminoacid (positive to neutral) can play putative roles in the generation of autoimmune responses (Vossenaar et al., 2003; Neeli et al., 2008). High levels of PADs have been described in the central nervous system of multiple sclerosis (MS) patients and animal models of inflammatory demyelinating diseases (Mastronardi et al., 2006). Strong evidence supports a pathogenic role for citrullinated autoantigens and the immunological response to them, in RA (Schellekens et al., 2000; Suzuki et al., 2003; Lundberg et al., 2005; Foulquier et al., 2007; Duskin and Eisenberg, 2010). As histone citrullination appears to be an important phenomenon in NET formation, it remains to be established if and how this modification may promote loss of tolerance or the development of deleterious immune responses (Hirsch, 1958; Neeli et al., 2008; Li et al., 2010). Further, citrullination of other peptides that have been described present in the NETs may alter their functionality. This is the case of LL37, present in the NETs and recently found to be substrate of citrullination *in vitro* by both PAD2 and PAD4 (Lande et al., 2007, 2011; Kilsgard et al., 2012). Indeed, LL37 can be citrullinated in 3 or 5 sites and the degree of modification dictates the activity and stability of the peptide (Kilsgard et al., 2012). Indeed, LL37 (5Cit) is more chemotactic to PBMCs and more pro-inflammatory compared to LL37 (3Cit) or unmodified LL37. Thus, immunoregulation can be induced by specific PTMs that occur during NETosis. Considering that citrullination by PAD4 is essential for the generation of NETs, allowing chromatin to decondense and be released during NETosis (Wang et al., 2009; Li et al., 2010), it will be important to determine whether other proteins are citrullinated or otherwise modified in the NETs, besides histones, and in the role of these additional modifications in the regulation of inflammation and adaptive immune responses.

AUTOANTIGENS GENERATED BY PROTEOLYTIC CLEAVAGE

Another process to be considered in the generation of neoantigens is proteolytic cleavage, the process of breaking the peptide bond between two residues in a protein. Enzymes such as peptidases and proteases carry out this process and generate fragments involved in cell signaling or activation of a zymogen, the inactive form of an enzyme. Proteins can be cleaved as a result of intracellular processing. As mentioned above, NE and MPO are important during NET formation (Papayannopoulos et al., 2010). Indeed, NE can translocate into the nucleus and partially and specifically degrade histones to promote nuclear decondensation (Papayannopoulos et al., 2010). Modified or unmodified fragments of histones generated by partial cleavage could potentially be recognized as neoantigens by B- and T-cells, thereby generating autoantibodies against them. Indeed, SLE autoantibodies can recognize peptides of five aminoacids (Pro-Glu-Pro-Ala-Lys) or more and other peptides containing modifications such as methylation or acetylation, in the case of antibodies against histone H2B, using an elegant silico-based peptide array that contain every possible overlapping peptide sequence in a linear fashion against H2B (Price et al., 2012). Although, the work focused mainly on histone H2B, it shows an innovative and powerful tool to define minimum epitopes for recognition by the adaptive immune system. It remains to be fully characterized and tested whether histone fragments generated by NE during NETosis can serve as autoantigens or resemble epitopes that can be recognized by the immune system.

Some important questions remain to be answered. First, if NETs are a main source of autoantigens, how can we account for the variability in autoantibody responses among the various autoimmune diseases and among individuals with the same autoimmune condition? We may consider that not all proteins in the NETs are equally exposed. Some epitopes can be uncovered by the help of chemical agents or other molecules. This is the case of the study mentioned above regarding PTU-induced vasculitis and the development of insoluble NETs by this drug (Nakazawa et al., 2012). In this case, the high similarity of PTU to a nitrogenous base and the presence of thione group ($\geq$S) in its structure may contribute to the high affinity to create inter and intra-molecular bonds with other sulfhydryl groups (-SH) in the NETs. Those reactions can be catalyzed by free radicals and oxidative species during NETosis, creating a compact conformation of the NETs that is insoluble. Indeed, conformational changes in the structure of the NETs may expose epitopes, such as MPO, that were not exposed in NETs in the absence of drug exposure, thereby triggering the generation of autoantibodies. Whether induction of aberrant NET structure may be one of the key mechanisms implicated in drug-induced lupus remains to be established. It will also be important to examine whether NETs triggered by specific conditions present in certain autoimmune diseases (ANCAs, anti-RNP antibodies, IFNs, etc.) induce different rearrangement of the chromatin and/or other modifications that promote specific protein content of the NETs and/or changes in their structure.

Taken together, exposure of altered proteins on the NETs, either by PTMs, proteolytic cleavage or specific environmental stimuli (e.g., drugs) in the context of an underlying pro-inflammatory milieu could promote deleterious consequences for the host. In addition, patients with deficiency in the clearance of NETs, such as that described in patients with SLE (Hakkim et al., 2010), may confer enhanced, persistent exposition of NETs and associated proteins that may promote generation and perpetuation of autoimmune responses. In this scenario, autoantibodies against specifically modified antigens could serve as prospective biomarkers for autoimmune diseases beyond RA.

CONCLUSIONS

NETs may represent an important source of neoantigens, where PTMs and proteolytic cleavage of proteins externalized in the NETs could promote the generation of autoantibodies in predisposed individuals. Indeed, the generation of autoantibodies to modified autoantigens has been described, suggesting a link between PTMs and autoimmunity. While NETs are unlikely to be the only source of autoantigens in SLE and other autoimmune/inflammatory diseases, the combination of PTMs derived from NETs and inflammatory molecules that may act as priming factors, represent an attractive milieu for the loss of tolerance and/or the activation of deleterious innate and adaptative immune responses.

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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.

Received: 19 October 2012; accepted: 28 November 2012; published online: 14 December 2012.

*Citation: Knight JS, Carmona-Rivera C and Kaplan MJ (2012) Proteins derived from neutrophil extracellular traps may serve as self-antigens and mediate organ damage in autoimmune diseases. Front. Immun. **3**:380. doi: 10.3389/fimmu.2012.00380*

This article was submitted to Frontiers in Molecular Innate Immunity, a specialty of Frontiers in Immunology.

frontiers in
IMMUNOLOGY

ORIGINAL RESEARCH ARTICLE
published: 10 December 2012
doi: 10.3389/fimmu.2012.00376

Bonding the foe – NETting neutrophils immobilize the pro-inflammatory monosodium urate crystals

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In the presence of sodium, uric acid from purine metabolism precipitates as monosodium urate (MSU) needles and forms renal calculi or causes gouty arthritis in kidneys and joints, respectively. The latter is characterized by red, hot, and swollen arthritic joints. Here we report the *in vitro* effect of MSU crystals on blood granulocytes and analyze their contribution to granuloma formation and neutrophil extracellular traps (NETs) formation (NETosis) in synovial fluid of patients with gouty arthritis *in vivo*. We observed that MSU crystals induce NETosis *in vitro* in a reactive oxygen species (ROS)-dependent manner. Indeed, blocking ROS (e.g., the oxidative burst) by various anti-oxidants partially inhibited NETosis induced by MSU crystals. Analyses of synovial fluids and of tissue sections of patients suffering from gout revealed that NETs are also formed *in vivo*, especially during acute gouty flares and/or granuloma formation. Since prolonged exposure to NETs carries the risk for the development of chronic inflammation we also studied the opsonization of NETs, as a prerequisite for their clearance. The established dead cells' opsonins C3b, galectin-9, and CRP decorated the residual dead cells' corpses and opsonized these for disposal. Surprisingly, all three soluble pattern recognizing molecules spared the spread NET structures. We conclude that (i) MSU crystals are strong inducers of ROS-dependent NETosis and (ii) that the prolonged presence of NET-pathogen or NET-crystal aggregates observed in patients with systemic autoimmunity, especially in those with low serum DNase-1 activity, cannot be compensated by CRP, complement, and galectin-mediated phagocytic clearance.

Keywords: NETosis, NETs, MSU, opsonins, inflammation, ROS, gout

INTRODUCTION

Uric acid is the final product of the purine catabolism in humans. Most uric acid dissolves in blood and is excreted in urine via the kidneys. Reduced removal of uric acid or increased purine breakdown (caused by purine rich food or massive tissue damage and cell death) leads to elevated levels of circulating uric acid. If the latter exceeds its solubility limit (approximately 6.8 mg/dl), it precipitates in sodium containing fluids as monosodium urate (MSU) crystals in the extracellular milieu (Schorn et al., 2009). The accumulation of MSU crystals in joints is the etiological reason for the painful inflammatory attacks in patients suffering from gout.

Once formed, MSU crystals act as endogenous danger signal stimulating the innate immune system to elicit an inflammatory response. Depositions of MSU crystals are often accompanied by massive leukocyte infiltrations (Popa-Nita and Naccache, 2010). After phagocytosis by monocytes, MSU crystals activate the NALP3 inflammasome with concomitant IL-1β release by monocytes (Schorn et al., 2010, 2011). Along with chemotactic factors, IL-1β attracts and activates neutrophils and other immune cells, which leave the blood vessels and migrate along the chemotactic gradient toward the MSU depots. In response to chemical stimuli, microbial pathogens or MSU crystals, neutrophils, eosinophils, basophils, and mast cells have been shown to release DNA into the extracellular milieu (Brinkmann et al., 2004; von Kockritz-Blickwede et al., 2008; Schorn et al., 2012). If these structures are released from neutrophils they are usually referred to as neutrophil extracellular traps (NETs). In analogy, for extranuclear DNA released from eosinophils, basophils, and mast cells the terms EETs, BETs (Schorn et al., 2012), and MCETs (von Kockritz-Blickwede et al., 2008) may be used, respectively.

Neutrophil extracellular traps are composed of DNA, histones, granular enzymes, and anti-microbial proteins, ejected in response to strong phagocytic stimuli (Brinkmann et al., 2004; Urban et al., 2006). Thus, NETs provide a powerful arsenal of anti-microbial factors in high local concentrations. Beside MSU crystals PMA, bacteria or fungi are potent NETs inducers (Brinkmann and Zychlinsky, 2012). The sticky DNA fibers of the NETs bind and immobilize pathogens and thus inhibit their further spreading.

Abbreviations: BETs, basophil extracellular traps; BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; CRP, C-reactive protein; DAPI, 4′,6-diamidino-2-phenylindole; DCFH-DA, dichlorofluorescein-diacetate; EETs, eosinophil extracellular traps; FSc, forward scatter; Gal, galectin; IL, interleukin; MCETs, mast cell extracellular traps; MSU, monosodium urate; NADPH, nicotinamide adenine dinucleotide phosphate; NET, neutrophil extracellular trap; PAD4, protein arginine deiminase 4; PFA, paraformaldehyde; PI, propidium iodide; PMN, polymorphonuclear neutrophils; ROS, reactive oxygen species; snRNP, small nuclear ribonucleoprotein; SSc, side scatter.

Therefore, beside phagocytosis, intraphagosomal killing and secretion of anti-microbials, NET formation is a crucial mechanism to control and fight various classes of pathogens. It has been shown that neutrophils exert protective effects in endotoxemia and sepsis by releasing NETs and thus capturing circulating bacteria from the blood stream (McDonald et al., 2012).

When cells release NETs they undergo a cell death pathway called "NETosis," morphologically distinct from all other cell death pathways like apoptosis and necroptosis and others (Fuchs et al., 2007). However, some features of other cell death pathways may also operate during NETosis. The following steps are required for NETosis: (i) the generation of reactive oxygen species (ROS) by the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Fuchs et al., 2007), (ii) the translocation of neutrophil elastase and myeloperoxidase from the granules to the nuclei, (iii) the conversion of arginine residues to citrulline in core histones by protein arginine deiminase 4 (PAD4), being necessary for proper decondensation of chromatin before release as NETs (Neeli et al., 2008), and (iv) the rupture of the plasma membrane (Brinkmann and Zychlinsky, 2012).

In this study, we analyzed the role of ROS in MSU-induced NET formation. We observed in accordance with published data for bacterial-induced NETosis, that anti-oxidants also inhibit the formation of NETs after stimulation of neutrophils with MSU. Extracellular chromatin containing structures with the appearance of NETs can also be detected in tissue sections and synovial fluids of patients suffering from gout. Since NETs are not bound by opsonins we consider NETs as problematic prey for the opsonin-mediated clearance and a potential antigenic structure for etiology and pathogenesis of systemic autoimmunity.

MATERIALS AND METHODS

HUMAN MATERIAL

All analyses of human material were performed in full agreement with institutional guidelines. We prepared paraffin sections of gouty granulomas from patients suffering from gouty arthritis and compared them with sections of synovial controls ($n = 5$). We analyzed synovial fluids (anti-coagulated with 20 U/ml heparin) from patients suffering from gout and patients with MSU-free synovitis. Heparinized (20 U/ml) venous blood was obtained from normal human blood donors. Autologous plasma was extracted from heparinized venous blood by centrifugation at 3400 *g* for 10 min (Rotina 46, Hettich). CRP was isolated from human sera of patients with bacterial infections by affinity chromatography using phosphocholine sepharose (Thermo Fisher). We labeled CRP with FITC according to the instructions of the manufacturer (Sigma-Aldrich).

ISOLATION OF PMN FROM HUMAN BLOOD

Polymorphonuclear neutrophils (PMN) were isolated from heparinized blood (20 U/ml) by ficoll density gradient centrifugation using standard protocols. Shortly, PMN were collected from the buffy coats. Residual erythrocytes were eliminated by hypotonic lysis. Viable cells were counted by trypan blue exclusion in a Neubauer chamber. The cell count was adjusted to 2 to 5×10^6 PMN/ml. PMN were cultured in autologous active plasma containing functional complement.

OPSONIN BINDING TO NETs

Isolated PMN were incubated with 200 μg/ml MSU crystals for 5 h at 37°C and then fixed with 1% paraformaldehyde. Cytospins were prepared and treated for 30 min at 37°C with fresh human plasma to allow complement binding. NETs were visualized by propidium iodide (PI) staining employing fluorescence microscopy. The binding of opsonins was analyzed by CRP-FITC, anti-C3b-FITC (Dako), and biotinylated Gal-9 plus streptavidin-FITC (Sigma-Aldrich). As control we used an anti-dsDNA antibody plus anti-human IgG-FITC (Southern Biotech).

HISTOLOGY

DNA was stained for 30 min with 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen GmbH) or with PI (Sigma-Aldrich) at 1 or 4 μg/ml, respectively. After washing, the samples were analyzed by fluorescence microscopy using standard filter sets.

INTRACELLULAR ROS PRODUCTION

Dichlorofluorescein-diacetate (DCFH-DA) is freely permeable across cell membranes. Inside the cells, the acetate moiety is cleaved off by esterases to yield the membrane-impermeable non-fluorescent DCFH. In the presence of ROS, DCFH is oxidized and forms the fluorescent DCF. Anti-coagulated blood was incubated with 10 μM DCFH-DA (Sigma-Aldrich) at 37°C. After 30 min, 1 mg/ml MSU crystals were added, and the samples were incubated at 37°C for up to 8 h. After erythrocyte lysis, the intracellular DCF fluorescence of the leukocytes was recorded by flow cytometry.

NET FORMATION IN PRESENCE OF ANTI-OXIDANTS

Whole blood was incubated with 1 mg/ml MSU or co-incubated with 250 μM butylated hydroxytoluene (BHT), 200 μM butylated hydroxyanisole (BHA), or 300 μM ascorbic acid (all from Sigma-Aldrich) for 5 h at 37°C. After the lysis of erythrocytes and solubilization of MSU crystals, cytospins were prepared and stained with DAPI.

CYTOSPINS

We centrifuged 2×10^5 cells at 850 *g* for 10 min (Rotina 46, Hettich) with a cytospin cuvette on glass slides (Thermo Fisher). After draining the supernatants, the cells were centrifuged for 2 min at 2000 *g*. The fixed cells were then analyzed by light and fluorescent microscopy.

PREPARATION OF MSU CRYSTALS

For the synthesis of MSU crystals, a solution of 10 mM uric acid and 154 mM NaCl (both from Merck KGaA) was adjusted to pH 7.2 and agitated for 3 days. The resulting crystals were pelleted, washed with ethanol, and dried under sterile conditions. The crystals were deprived of lipopolysaccharides by heating to 180°C for 2 h and can be stored in PBS (pH 7.0) at ambient temperature for at least 1 month.

LYSIS OF ERYTHROCYTES, SOLUBILIZATION OF CRYSTALS, AND FLOW CYTOMETRY

In whole blood assays, the erythrocytes were automatically lysed using a TQprep Workstation (Beckman Coulter) before measurement with a Gallios cytofluorometer (Beckman Coulter). The data

were analyzed with the Kaluza software 1.2 (Beckman Coulter). Electronic compensation was used to eliminate bleed-through fluorescence. The erythrocyte lysis conditions also solubilized non-ingested MSU crystals.

STATISTICAL ANALYSIS

We performed statistical analyses with SPSS PASW statistics 18. The results are represented as mean ± SD of at least three and up to five independent experiments. Student's *t*-test or an analysis of variance for repeated measurements was used. The data were considered significant and highly significant for *P*-values <0.05 and <0.01, respectively.

RESULTS

MSU CRYSTAL-INDUCED NETosis DEPENDS ON ROS

It was previously shown that NET formation induced by chemicals, various pathogens, or cytokines is strongly augmented by the presence of ROS generated by activated NADPH oxidase (von Kockritz-Blickwede et al., 2008). Since we observed that MSU crystals are potent inducers of NETs, we analyzed if MSU-initiated NETosis is accompanied by and dependent on the formation of ROS in human whole blood *ex vivo* cultures. The ROS productions of individual cell populations were determined by DCF fluorescence. DCFH-DA is commonly used to quantify ROS on a single cell level in flow cytometry. DCFH-DA passively penetrates individual cells and is trapped as DCFH in the cytoplasm after deacylation by intracellular esterases. In the presence of ROS the latter forms the highly fluorescent DCF, which can be detected in cytofluorometry. Human anti-coagulated whole blood was incubated with 10 μM DCFH-DA at 37°C in the presence and absence of MSU crystals. Already 30 min after the addition of the crystals ROS was to be detected. The DCF fluorescence reached its maximum after 4.5 h. In the absence of MSU the DCF fluorescence was virtually stable for up to 8 h (**Figure 1A**).

The incubation of human whole blood *ex vivo* cultures with 1 mg/ml MSU for 5 h at 37°C resulted in the formation of extended supercellular structures containing extranuclear DNA as detected by DAPI staining (**Figure 1B**). The extracellular DNA was confirmed as NETs employing stainings against neutrophil elastase, myeloperoxidase, and histones (not shown). To analyze if the formation of NETs requires ROS, the experiment was also performed in the presence of the potent anti-oxidants BHA, BHT, and ascorbic acid. The treatment with the various anti-oxidants did not influence the cell viability as verified by Annexin V/PI stainings (not shown). The *ex vivo* culture of human whole blood with BHT or BHA clearly reduced the sizes of the NETs formed by blood granulocytes in the presence of >90% plasma (**Figures 1C,D**). Ascorbic acid was even more potent and abolished NET formation under these conditions completely (**Figure 1E**).

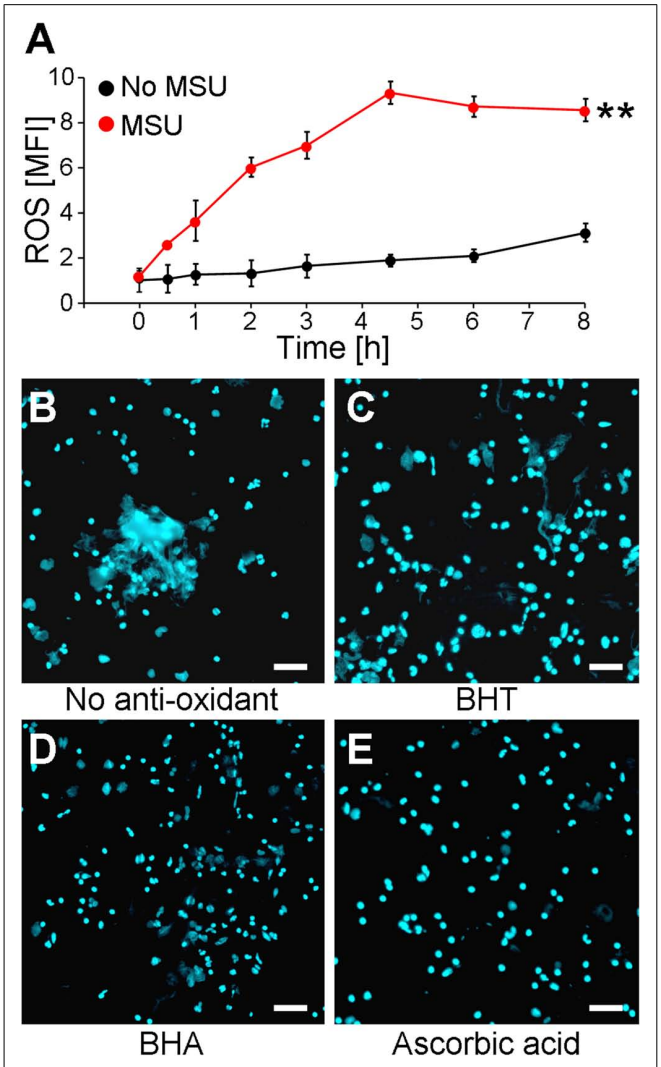

FIGURE 1 | MSU-induced NET formation depends on oxidative stress (ROS). (A) Whole blood was treated with or without MSU ($n = 3$ per group) and analyzed for ROS production by cytofluorometry. MSU crystals induce oxidative stress (ROS) in neutrophils. $^{**}P < 0.01$. **(B–E)** Whole blood was incubated with MSU in the absence **(B)** or the presence of BHT **(C),** BHA **(D)**, or ascorbic acid **(E)**. Cytospins were stained for extranuclear DNA. NET formation by neutrophils after incubation with MSU was inhibited by the anti-oxidants BHT **(C)**, BHA **(D)**, and ascorbic acid **(E)**. All experiments were performed at least three times. Scale bars, 100 μm.

MSU INDUCE NET FORMATION IN HUMAN TISSUE SECTIONS

The *in vitro* generation of NETs is well characterized, but the relevance for diseases requires *in vivo* data. Therefore, we analyzed whether MSU-associated NETs can also be found in human tissue sections. We analyzed gouty granulomas and surrounding non-granulomatous areas for the presence of extranuclear DNA structures, with the appearance of NETs generated in our *ex vivo* blood cultures. Extracellular DNA was detected by stainings with PI. As shown in **Figure 2A** extranuclear DNA is abundant in the granuloma (**Figure 2A**, lower part) whereas the chromatin is confined to the nuclei in the surrounding tissue (**Figure 2A**, upper part). Next we analyzed cytospins from synovial fluid from patients with gouty synovitis. Synovial sections and cytospins of MSU-negative arthritides served as controls. Again, extracellular thready chromatin was to be detected by DNA staining only in samples from patients suffering from gout (**Figures 2B,C**). In the controls the DNA was confined in the nuclei (**Figures 2D,E**). Due to the washing steps during the

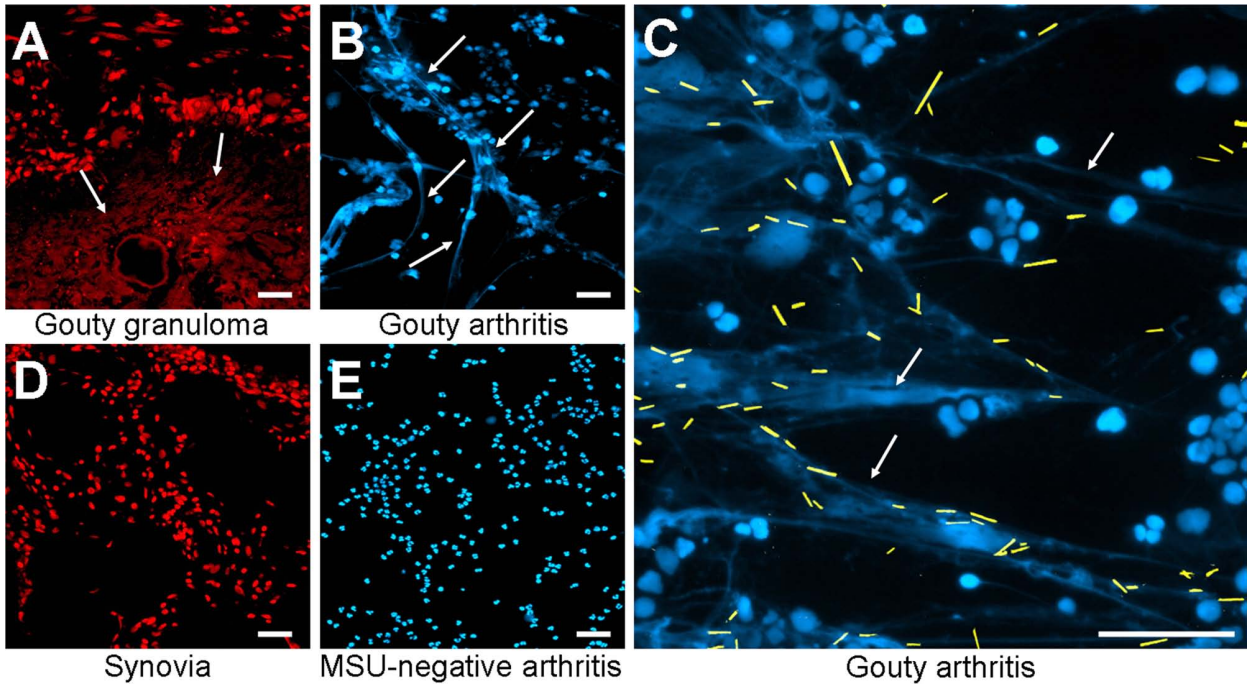

FIGURE 2 | Patients with MSU-induced inflammation display extranuclear DNA in tissue sections. Revealed by DNA staining with propidium iodide, sections of gouty granulomas **(A)** display extranuclear DNA (arrows). Synovial fluids of patients suffering from gouty arthritis display extranuclear DNA (arrows) visualized by staining with DAPI **(B,C)**. All MSU crystals (artificially colored in yellow in **C**) were associated with NET structures **(C)**. Sections of human synovial control **(D)** and cytospins of synovial fluids from MSU-negative arthritis **(E)** exclusively showed nuclear DNA distribution. All experiments were performed at least five times. Scale bars, 100 μm.

routine preparation of the tissue sections MSU crystals are usually dissolved and cannot be detected (**Figure 2A**). In contrast, the mild conditions used in the preparation of the cytospins from synovial fluids preserve the MSU. Interestingly, in the cytospins of the synovial fluids of patients with gouty arthritis the crystals are trapped within the extranuclear chromatin fibers (**Figure 2C**; MSU crystals artificially highlighted in yellow). Thus, in concordance with their role in the anti-bacterial responses, NETs reduce the uncontrolled spreading of pathogenic MSU crystals.

MSU-INDUCED NETs DO NOT BIND TO OPSONINS

The fast and efficient disposal of apoptotic and necrotic cell material is essential to avoid autoimmune reactions (Korns et al., 2011). NETs expose several nuclear autoantigens that are prototypical for the autoantigens targeted in systemic autoimmunity, e.g., SLE. NETs contain nucleic acids which are potent inducers of interferon-alpha, if they are recognized by endosomal TLR-3, -7, -8, and -9. Therefore, NETs may be considered hazardous waste that has to be disposed carefully. To clear toxic waste and pro-inflammatory cell remnants the phagocytes employ opsonizing immunoglobulins or complement proteins as well as other molecules bridging the phagocytes to their prey. If these molecules also opsonize/bind NETs structures was investigated by direct and indirect immune fluorescence analyses employing CRP-FITC/galectin-9-FITC/anti-dsDNA-FITC and anti-C3b-FITC, respectively. CRP is known to bind lysophosphatidylcholine on disturbed cellular membranes as well as nuclear components as histone H1 and small nuclear ribonucleoprotein (snRNP) particles (Jewell et al., 1993). Galectin-9 binds beta-galactosides exposed during the change of the glycomic profile during cell death and C3b is deposited during classical and alternative activation of the complement cascade. We therefore investigated the binding of CRP, galectin-9, and complement C3b to NET structures formed by isolated PMN cultured with 5×10^6 cells/ml in response to MSU crystals (**Figure 3**). Surprisingly, C3b (**Figure 3A**), CRP (**Figure 3B**), and galectin-9 (**Figure 3C**) indeed bound to the cellular corpses of PMN but not to the NETs (see arrows). In contrast, antibodies against dsDNA bound both cell remnants and NETs (**Figure 3D**).

DISCUSSION

MSU crystals are amongst the most potent NETosis inducing agents. Indeed, they represent the only stimuli that induce NETosis in whole blood *ex vivo* cultures or in neutrophils cultures the presence of pure plasma (this paper and Schorn et al., 2012). Here we showed that NET formation induced by MSU crystals is accompanied by ROS formation in neutrophils. This is in line with Fuchs et al. (2007) who reported that NETosis is dependent on the activation of NADPH oxidase and ROS production. Interestingly, diphenyleneiodonium, an inhibitor of the NADPH oxidase prevented NET formation. In our hands, the reduction of oxidative stress by the anti-oxidants BHT, BHA, and ascorbic acid abolished NETs formation as well. An ROS-dependent

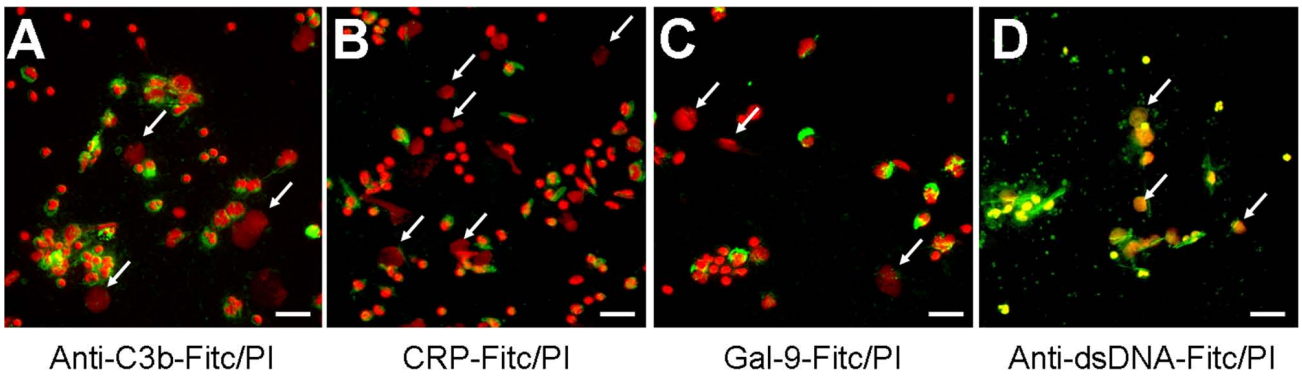

FIGURE 3 | The dead cells' ligands C3b, CRP, and galectin-9 do not opsonize NETs. (A–D) Isolated PMN were incubated with MSU for 5 h at 37°C and then fixed with 1% PFA. Cytospins were prepared and NETs visualized by PI staining (red). Anti-C3b **(A)**, CRP **(B)**, and galectin-9 **(C)** bind to PMN corpses but not to NETs (arrows). Antibodies against dsDNA as positive control **(D)** bind cell remnants and NETs (arrows). Scale bars, 50 μm.

NET formation has also been shown for mast cells in response to PMA, H_2O_2, or bacterial pathogens (von Kockritz-Blickwede et al., 2008). Patients suffering from chronic granulomatous disease (CGD) carry a mutation in the genes coding for the NADPH oxidase and are, therefore, deficient in NETosis. The deficiency in the NET formation of patients with CGD may contribute to their immune deficient phenotype (Fuchs et al., 2007). Consequently, the restoration of NADPH function by gene therapy resulted in the reconstitution of NETs formation and neutrophil elimination of *Aspergillus nidulans* (Bianchi et al., 2009, 2011). As shown during sepsis intravascular NETs capture bacteria from the vasculature thus preventing dissemination. Consequently, blocking NETs formation results in increased spreading of bacteria to distant organs (McDonald et al., 2012). These findings underline the fundamental role of NETs in anti-microbial activity *in vivo*.

Analyses of synovial fluids and of tissue sections of patients suffering from gout revealed that NETs are also formed *in vivo*, especially during acute gouty flares and/or granuloma formation. Mitroulis et al. (2011) showed that the formation of NETs can be induced by the transfer of synovial fluid from inflamed tissues to control PMN. The occurrence of NETs has also been described in SLE, where it has been identified as a potential antigen for autoimmunity that trigger B cell activation (Lande et al., 2011). NETs have been shown to activate TLR-9 in dendritic cells and to prime T cells. Interestingly, autoantibodies found in SLE are often directed against both self-DNA and anti-microbial peptides located in NET structures (Nassberger et al., 1989; Fauzi et al., 2004). NETs are covered with HMGB1, which represents a highly active danger signal endowed with pro-inflammatory potential. Therefore, it has been speculated that after inflammatory responses the timely removal of NETs is essential to avoid exposure of intracellular self-antigens and, consequently, the challenge of immune tolerance (Hakkim et al., 2010). The DNA backbone of the NETs has been described to be performed by DNase-1 and patients with low serum DNase-1 activity have an increased risk to develop nephritis (Hakkim et al., 2010).

We found that the established dead cells' opsonins C3b, galectin-9, and CRP decorated the residual dead cells' corpses and opsonized these for disposal. Surprisingly, all three soluble pattern recognizing molecules spared the spread NET structures. However, the chromatin of the NETs is accessible to a monoclonal polyreactive DNA autoantibody. These results are in concordance with the observation of Fuchs et al. (2007) who showed that AxV only binds to phosphatidylserine restricted to the necrotic cell remnant but not to the dispersed chromatin containing NETs structures. Besides phosphocholine from disturbed mammalian membranes nuclear components as snRNP or histone H1 are common target structures for CRP binding (Janko et al., 2009). Surprisingly, CRP did not bind to the dispersed extracellular chromatin in the NETs. However, if the nuclear CRP targets are not included into the NETs, lost from the NETs structure due to low binding affinity or are modified during NETosis requires further investigations. In this context histone modifications prior to NETting might also play a role (Liu et al., 2012).

The binding of nuclear structures by opsonins as CRP has been discussed previously to efficiently mask self-antigens to preclude immune activation and to foster anti-inflammatory clearance (Marnell et al., 2005). Usually, the binding of CRP results in the recruitment of complement C1q and in complement activation. Previously it has been shown that complement C1q promotes the degradation of necrotic cell-derived chromatin (Gaipl et al., 2004). Remarkably, in a previous study C1q binding to NETs was detected, which inhibited the degradation of NETs by a still unknown mechanism, whereas anti-C1q antibodies reversed this effect (Leffler et al., 2012). In our hands, anti-dsDNA antibodies bound to the NETs and formed nucleic acid containing immune complexes similar to those generated in SLE by secondary necrotic cells and lupus typical autoantibodies (Munoz et al., 2010). In this context pro-inflammatory activities are exerted by antibodies that bind secondary necrotic cells either directly or via a bridging molecule like CRP (Janko et al., 2011). The fact that immune complexes can trigger NET formation (Kessenbrock et al., 2009) and NETs can form immune complexes may be involved in the vicious circle operating in patients with clearance deficiency or low DNase-1 activities (Munoz et al., 2009). If NET-IgG immune complexes harbor a similar pro-inflammatory potency like IgG-opsonized secondary necrotic cells

(Meesmann et al., 2009) is currently investigated in our lab. Taking together, we consider NETs as a problematic prey especially in the presence of NET-binding autoantibodies.

ACKNOWLEDGMENTS

This project was supported by the Interdisciplinary Center for Clinical Research (IZKF) at the University Hospital of the University of Erlangen-Nuremberg, project A41 (to Christine Schorn and Martin Herrmann), by the Masterswitch project of the European Union (to Georg Schett and Martin Herrmann), by Deutsche Forschungsgemeinschaft (SFB643-TPB5; to Christina Janko), by the training Grant GK SFB 643 from the DFG (to Christine Schorn and Christina Janko), and the K. und R. Wucherpfennigstiftung (to Martin Herrmann).

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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.

Received: 28 September 2012; paper pending published: 12 October 2012; accepted: 23 November 2012; published online: 10 December 2012.
*Citation: Schorn C, Janko C, Krenn V, Zhao Y, Munoz LE, Schett G and Herrmann M (2012) Bonding the foe – NETting neutrophils immobilize the pro-inflammatory monosodium urate crystals. Front. Immun. **3**:376. doi: 10.3389/fimmu.2012.00376*
This article was submitted to Frontiers in Molecular Innate Immunity, a specialty of Frontiers in Immunology.

frontiers in IMMUNOLOGY

REVIEW ARTICLE
published: 18 December 2012
doi: 10.3389/fimmu.2012.00385

The emerging role of neutrophils in thrombosis—the journey of TF through NETs

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The production of TF by neutrophils and their contribution in thrombosis was until recently a matter of scientific debate. Experimental data suggested the *de novo* TF production by neutrophils under inflammatory stimuli, while others proposed that these cells acquired microparticle-derived TF. Recent experimental evidence revealed the critical role of neutrophils in thrombotic events. Neutrophil derived TF has been implicated in this process in several human and animal models. Additionally, neutrophil extracellular trap (NET) release has emerged as a major contributor in neutrophil-driven thrombogenicity in disease models including sepsis, deep venous thrombosis, and malignancy. It is suggested that NETs provide the scaffold for fibrin deposition and platelet entrapment and subsequent activation. The recently reported autophagy-dependent extracellular delivery of TF in NETs further supports the involvement of neutrophils in thrombosis. Herein, we seek to review novel data regarding the role of neutrophils in thrombosis, emphasizing the implication of TF and NETs.

Keywords: neutrophil extracellular traps, thrombosis, tissue factor, neutrophil, coagulation cascade

HISTORICAL INTRODUCTION

The role of neutrophils in the interface between inflammation and thrombosis remains a controversial scientific issue. Even though there is significant evidence that suggests a critical role for neutrophils in the thrombotic process (Lerner et al., 1971), their impact on thrombogenesis was until recently questioned. The ability of neutrophils to enhance or trigger *in vivo* thrombotic events via production and expression tissue factor (TF), the major *in vivo* initiator of coagulation (Rapaport and Rao, 1995; Bächli, 2000), is a matter of debate. During the past decade, experimental evidence using not physiologic inflammatory conditions reinforced the view that neutrophils acquire rather than produce TF (Osterud et al., 2000; Egorina et al., 2008). As a result, the significance of this cell population in thrombosis was considered minor and the study of neutrophils was not included in research models of thrombosis.

During the last few years several studies elucidated the crucial role of neutrophils in thrombosis (Looney et al., 2006; Zernecke et al., 2008; Darbousset et al., 2012; von Brühl et al., 2012). Neutrophil depletion was shown to be able to reverse *in vivo* experimental thrombosis, thus supporting the significance of this population (von Brühl et al., 2012). Several studies support the *in vivo* and *ex vivo* TF production by neutrophils (Maugeri et al., 2006; Ritis et al., 2006; Redecha et al., 2007, 2008; Kambas et al., 2008; Rafail et al., 2008; Kourtzelis et al., 2010).

The description of neutrophil extracellular trap (NET) release by neutrophils revealed a novel inflammatory role of these cells (Brinkmann et al., 2004; Clark et al., 2007; Fuchs et al., 2007; Medina, 2009; Mitroulis et al., 2011). Moreover, recent experimental data regarding the bridging of autophagy with neutrophils and immunity (Mitroulis et al., 2010), added a novel view on their functions. The implication of autophagy in NET release and TF delivery to NETs and the linkage between NETs and thrombosis suggest a critical role for neutrophils in the interaction between inflammatory and thrombotic pathways. Moreover, the attenuation of thrombotic manifestations in thrombotic animal models by neutrophil depletion demonstrates the contribution of these cells in thrombosis (von Brühl et al., 2012). The expression of produced and/or acquired TF by neutrophils is an attractive and realistic scenario for the pathogenesis of thrombotic events that characterize several inflammatory disorders, including sepsis (Aras et al., 2004), ANCA-associated vasculitis and Behçet's disease (Tomasson et al., 2009), or inflammatory bowel disease (Miehsler et al., 2004).

TF: THE ORCHESTRATOR OF COAGULATION

TF is a 47 kDa transmembrane glycoprotein that shares high homology in secondary and tertiary structure with interferon γ receptors and is a member of the human class II cytokine receptor family (Bazan, 1990). Currently, TF is considered as the main *in vivo* initiator of coagulation (Rapaport and Rao, 1995; Bächli, 2000; Manly et al., 2011). The presence of a multitude of binding sites in the gene's promoter region indicates multi-potency of expression in a large variety of cells and under a vast array of stimuli. Under normal conditions TF is not expressed in endothelial cells (Wilcox et al., 1989), but only in sub-endothelial tissue, thus creating a protecting envelope between blood and sites of expression (Drake et al., 1989; Fleck et al., 1990). However, under specific inflammatory conditions TF is expressed in endothelial cells and myeloid leukocytes (Parry and Mackman, 1995; Armesilla et al., 1999; Maugeri et al., 2006; Ritis et al., 2006; Kambas et al., 2008; Rafail et al., 2008; Kourtzelis et al., 2010).

There is emerging evidence indicating the presence of circulating TF in blood (blood-borne TF). There are three potential

sources of blood-borne TF—peripheral blood cells (Drake et al., 1989; Ritis et al., 2006; Kambas et al., 2008; Kourtzelis et al., 2010), microparticles (MPs) (Mallat et al., 2000) and the soluble alternative spliced variant of TF (Bogdanov et al., 2003). Although monocytes have been reported to constitutively express TF and while this cell population is considered the main source of TF-bearing MPs (Aleman et al., 2011), there are emerging evidence indicating the possible implication of other cell populations in the generation of blood-borne TF.

Intraluminal exposure of TF located on a serine-rich phospholipid membrane activates FVII, forming TF/FVIIa complex (Bach, 1988). This complex is able to activate FX, which in turn results in thrombin, which is responsible for thrombus stabilization (Monroe et al., 2002). Moreover, membrane-embedded TF is usually in inactive coagulant state (cryptic) while it requires activation to reach its full potency (decryption) (Rao et al., 2012). However, the mechanism behind the activation of circulating TF is not yet elucidated and such information would provide a significant breakthrough in the understanding of *in vivo* thrombosis.

Nevertheless, apart from the role of the extrinsic coagulation system (TF—thrombin axis) in thrombosis, this system has been implicated in several non-thrombotic models such as angiogenesis, tumor growth and metastasis, inflammation, and fibrosis. The serine proteases of this pathway, namely TF/VIIa, Xa, and thrombin, are able to signal through the protease activated receptor (PAR) receptor family to produce intracellular signals (Coughlin, 2005) *via* phosphoinositide 3-kinase (PI3K), Src tyrosine kinase, extracellular signal-regulated kinase (ERK), and mitogen-activated protein kinase (MAPK) pathways (Ramachandran and Hollenberg, 2008). The activation of these pathways results in the secretion of cytokines and chemokines implicated in several biological functions (Coughlin, 2005).

INFLAMMATION AND THROMBOSIS: A RECIPROCAL PROCESS

Increased prevalence of venous thrombotic events is a long standing observation in patients suffering from infectious and sterile inflammatory disorders. Venous thrombosis constitutes a major morbidity and mortality factor in inflammatory diseases, including sepsis, systemic lupus erythematosus (SLE), inflammatory bowel disease, or vasculitis (Zöller et al., 2012). Additionally, recent clinical data derived from patients with rheumatoid arthritis and SLE support the critical role of inflammation in accelerated atherothrombosis (Santos et al., 2012). Experimental evidence links the observed thrombogenicity with TF-dependent activation of extrinsic coagulation cascade. Increased TF expression by endothelial and blood cells exposed to inflammatory mediators is proposed as an essential part of the pathogenic mechanism for arterial and venous thromboembolism that characterizes inflammatory disorders (Mackman, 2009). These observations indicate a potential triggering role of inflammation in thrombosis. However, the relationship between inflammation and thrombosis is bidirectional since thrombosis can reignite inflammation creating a persistent or recurrent inflammatory environment. TF-thrombin axis enhances the inflammatory response in several clinical models such as arthritis (Busso et al., 2003), antiphospholipid syndrome (APS) (Ritis et al., 2006; Redecha et al., 2007, 2008), ischemia/reperfusion injury (Loubele et al., 2009), and sepsis (Osterud and Bjorklid, 2001; Aras et al., 2004; Wang et al., 2009). Signaling through PARs plays a critical role for this reciprocal process. TF:FVIIa complex has been implicated in the induction of inflammation in the aforementioned clinical models. In an endotoxemic animal model, both TF deficiency and combined inhibition of thrombin and deficiency in PAR2 reduced inflammation (Pawlinski et al., 2004). Further studies in animal models of sepsis demonstrated that extrinsic coagulation cascade inhibition with a varying range of anticoagulants [natural anticoagulants, Tissue Factor Pathway Inhibitor (TFPI), Protein C, and Antithrombin III] attenuated the persisting inflammation (Taylor et al., 1987, 1998a,b; Ramachandran and Hollenberg, 2008). Moreover, it has been recently shown that thrombin is able to generate biologically active C5a from C5 in the absence of C3, indicating a significant role in the reignition of inflammation (Huber-Lang et al., 2006; Krisinger et al., 2012). However, the physiological contribution of this pathway has to be further investigated. This data establish the reciprocal and close relationship between the two systems.

TF PRODUCTION BY NEUTROPHILS: A CONTROVERSIAL ISSUE

The expression of TF by neutrophils was initially reported almost 40 years ago (Lerner et al., 1971), although those studies did not include solid proof on their ability to produce TF protein. However, it is less than 10 years since evidence on the ability of neutrophils to produce functional TF arose. A turning point in the investigation of TF expression by blood cells was the observation that the promoter of TF gene is strictly regulated by methylation. TF promoter exists in its unmethylated form in both monocytes and neutrophils, allowing stimuli-driven TF mRNA transcription.

TF production by neutrophils was opposed by other reports (Osterud, 2004, 2012). It was reported that isolated neutrophils failed to produce TF protein when stimulated with LPS alone or in conjunction with phorbol myristate acetate (PMA) or TNF-α (Osterud et al., 2000). TF activity in culture supernatants was attributed to platelet activation by contaminating monocytes (Osterud et al., 2000). A study in an *in vivo* murine sepsis model demonstrated that the cluster of TF positive cells infiltrating the spleen were granulocytes. However, even though these cells expressed TF protein, they did not possess TF mRNA (de Waard et al., 2006). The authors attributed this finding to the uptake by neutrophils of TF produced by other cell types in the form of MPs. This was supported by a study suggesting that neutrophils acquire monocyte-derived TF rather than synthesize it by themselves (Egorina et al., 2008). Using a blood reconstitution model and cells transfected with si-RNA for TF, the authors were able to demonstrate that only monocytes contributed in LPS-induced TF expression. Interestingly, this TF was not localized on neutrophil membrane but intracellularly. Even though there is clear evidence for the acquisition of TF by granulocytes in this article, the authors used a limited number of mediators and conditions for neutrophil activation.

Even though these findings indicate a mechanism for TF transfer from monocytes to neutrophils, they do not exclude

the possibility for TF production by neutrophils under *in vivo* inflammatory conditions.

On the opposite side of the debate, it has been found that neutrophils produce functional TF protein after stimulation with P-selecting or N-formyl-methionyl-leucyl-phenylalanine (fMLP) but not PMA. Interestingly, cells expressed TF intracellularly and after stimulation with fMLP the protein was translocated to cell membrane in a small percentage of cells (Maugeri et al., 2006). At the same time, the ability of neutrophils to produce TF was reported in an *ex vivo* human model of APS (Ritis et al., 2006). It was shown that IgG immunoglobulin from patients with APS triggers the activation of complement and subsequently generation of C5a. The produced anaphylatoxin was able to induce *TF* gene transcription and production of active TF by human neutrophils. These findings were verified by another study in a murine *in vivo* APS model. It was demonstrated that TF responsible for fetal miscarriages was derived from myeloid cells and particularly neutrophils in a C5a-dependent manner (Redecha et al., 2007). Blocking of TF attenuated trophoblast damage and reduced miscarriages. The same group in a following study in the same murine model demonstrated that TF:FVIIa complex exhibits signaling through PAR2 receptor on activated neutrophils causing trophoblast damage and fetal death via reactive oxygen species release (Redecha et al., 2008). Mice with deletion of the cytoplasmic domain of TF, responsible for interaction with FVIIa and consequently PAR2 receptor, or PAR2 knockout exhibited lower neutrophil activation levels and normal pregnancies. Moreover, another study demonstrated the expression of TF by neutrophils isolated from the bronchoalveolar fluid (BALF) from patients with Acute Respiratory Distress Syndrome (ARDS) (Kambas et al., 2008). The ability of BALF from such patients to induce TF expression was attributed to the synergistic effect of C5a and TNF-α. C5a-dependent TF production by neutrophils was also observed in an extracorporeal circulation model (Kourtzelis et al., 2010). It was demonstrated that serum from End Stage Renal Disease (ESRD) patients induces functional TF production in both neutrophils and monocytes from healthy individuals in a C5a-dependent manner. Of interest, in the same study, granulocyte colony stimulating factor (G-CSF) levels were significantly correlated with TF expression. The significance of neutrophil priming in their activation by cytokines has been recently described (Yousefi et al., 2009; Hattar et al., 2010). Moreover, *in vitro* data supported the production of TF by neutrophils stimulated with leptin. Inhibition studies indicated that TF induction was partially mediated by TNF-α and JAK2/PI3K (Rafail et al., 2008).

Even though these data provide significant evidence for the ability of neutrophils to produce TF, they do not answer the question on how neutrophils can externalize TF in a functional manner. New breakthroughs in neutrophil biology and particularly the description of NETs reveal possible solutions to unwinding Ariadne's thread regarding this debatable issue (**Table 1**).

A CRITICAL ROLE OF NEUTROPHILS IN THROMBOSIS

Despite the debate whether neutrophils are able to produce TF or not, there are several articles demonstrating their active role in *in vivo* experimental thrombosis and inflammation-driven thrombotic diseases. In animal models of acute lung injury caused by differential etiology, including acid aspiration (Folkesson et al., 1995), ischemia/reperfusion (Eppinger et al., 1997), and transfusion related acute lung injury (TRALI) (Looney et al., 2006), neutrophil depletion before the initiation of inflammation attenuates lung injury. Furthermore, in a model of diet-induced atherosclerosis, circulating neutrophils were required for plaque formation in atherosclerotic lesions (Zernecke et al., 2008). An additional contribution of neutrophils in the activation of extrinsic coagulation cascade is the degradation of TFPI *via* elastase release (Massberg et al., 2010). TFPI is the main inhibitor of TF. Additionally, protein disulfide isomerise, a key protein for the activation of cytoplasmic TF (Reinhardt et al., 2008) was found to be expressed in neutrophils (von Brühl et al., 2012). Thus, neutrophils play an important role in the activation of extrinsic coagulation system, either by regulating its breaks or by activating its initiator.

Recently, Darbousset et al. (2012) clearly demonstrated that neutrophil binding to the injured endothelium was the initial step in the continuum of events that resulted in thrombus formation in a model of laser-induced endothelial injury. The critical role of neutrophils was reinforced by the observation that these cells were the main source of TF, which was required for thrombus formation. Additionally, in the same model, factor XII deficiency did not attenuate thrombus generation. Another recent study in a mouse model of deep vein thrombosis (DVT) provided evidence for the indispensable role of neutrophils in venous thrombosis, as shown by neutrophil depletion (von Brühl et al., 2012). Neutrophils were shown to promote thrombogenesis by binding and activating factor XII, which is in contrast to the study by Darbousset et al. using conditional mutants and bone marrow chimeras, the authors also demonstrated that TF derived from myeloid cells and not endothelial cells was responsible for the activation of coagulation system. However, monocyte-derived TF was not sufficient for the formation of thrombus. These well-organized studies provide convincing evidence for the significance of neutrophils in thrombosis and mark the restoration of neutrophils in the forefront of the investigation of thrombosis.

NETs AND THROMBOSIS

Neutrophils, as a critical part of innate immunity, have evolved mainly around their ability to fight bacterial infections. Novel insight on neutrophil biology demonstrated a new mechanism of neutrophils to defend against pathogens through the release of NETs. NETs are chromatin filaments that form a network of DNA, histones and several cytoplasmic and granule proteins with antibacterial or immune-modulating role (Jaillon et al., 2007; Sangaletti et al., 2012). NETs are released as a last measure of defence from neutrophils (Brinkmann and Zychlinsky, 2007).

Recent studies demonstrated the critical implication of NETs in animal thrombotic models. Using a murine model of endotoxemia and an *in vitro* model of blood flow, Clark et al. (2007) reported that the formation of NETs in the vasculature resulted in the entrapment of platelets. Subsequent platelet activation induced endothelial injury leading in the impairment of blood flow. A few years later, the contribution of NETs in thrombus formation was shown in a baboon DVT model (Fuchs et al., 2010). This study demonstrated that NETs entrapped both platelets and

Table 1 | Neutrophils, TF, and thrombosis—the historical overview.

Year	1971	2000	2006	2007	2008	2010	2012
For	"First evidence on neutrophils producing TF"—Lerner et al. *Proc. Soc. Exp. Biol. Med.*		"Neutrophils produce TF when stimulated with fMLP or P-selectin"—Maugeri et al. *J. Thromb. Haemost.* "C5a-dependent TF production by neutrophils in APS"—Ritis et al. *J. Immunol.*	"Neutrophils produce TF in *in vivo* APS murine model in a C5a dependent manner"—Redecha et al. *Blood* "Endotoxemic mice entrap and activate platelets in NETs"—Clark et al. *Nat. Med.*	"Neutrophils produce TF in ARDS in a C5a and TNF-α dependent manner"—Kambas et al. *J. Immunol.* "TF produced by neutrophils exert autocrine signaling through TF/FVIIa complex in APS"—Reinhardt et al. *J. Clin. Invest.*	"NETs entrap platelets and act as a scaffold for fibrin formation in baboon DVT"—Fuchs et al. *Proc. Natl. Acad. Sci. U.S.A.* "TF production by neutrophils in ESRD patients"—Kourtzelis et al. *Blood*	"Neutrophil depletion attenuates thrombosis in mouse DVT. Key role of NETs in DVT"—von Brühl et al. *J. Clin. Invest.* "Neutrophil TF is critical in laser induced endothelial injury"—Darbousset et al. *Blood* "TF delivery in NETs through autophagy in human sepsis"—Kambas et al. *PLoS ONE*
Against		"Neutrophils cannot produce TF when stimulated with LPS and PMA or TNF-α"—Osterud et al. *Thromb. Haemost.*	"In murine sepsis, granulocytes express TF protein but no TF mRNA transcripts"—de Waard et al. *Thromb. Haemost.*		"Neutrophils do not produce TF but acquire it from monocytes when stimulated with LPS and TNF-α or PMA"—Egorina et al. *Blood*		

erythrocytes, while NETs served as three dimensional scaffolds for fibrin deposition and the subsequent stabilization of thrombus. Moreover, histones were identified as the culprit for platelet activation. Another study demonstrated that neutrophils contribute in tissue injury through NET release in a TRALI mouse model (Caudrillier et al., 2012). More specifically, activated platelets were able to stimulate neutrophils for NET release which increased endothelial permeability. Moreover, inhibition of platelet activation reduced NET release and tissue injury. Pretreatment with either histone blocking or DNase I reduced endothelial damage in TRALI. In a murine DVT model, neutrophils were demonstrated as a major factor of thrombosis, as shown by the effect of neutrophil depletion. Neutrophils were found to contribute to DVT through NET release since treatment with DNase I suppressed DVT growth (von Brühl et al., 2012). Citrullinated H3 histone interaction with von Willebrand factor was proposed as a possible mechanism for the formation of erythrocyte rich thrombus (red thrombus). NET formation was also observed by neutrophils in the context of chronic myelogenous leukemia and in solid tumor models (Demers et al., 2012). The authors correlated NET release with cancer related thrombosis, through presence of citrullinated H3 histone and high plasma DNA concentrations. Finally, as already mentioned, NET release was associated with venous thrombosis through factor XII activation in a murine DVT model induced by partial vessel occlusion.

EXTRACELLULAR TF DELIVERY THROUGH NETs

The contribution of neutrophils in thrombosis was overshadowed by the debate on their ability to produce functional TF. Additionally, the intracellular localization of TF in neutrophils

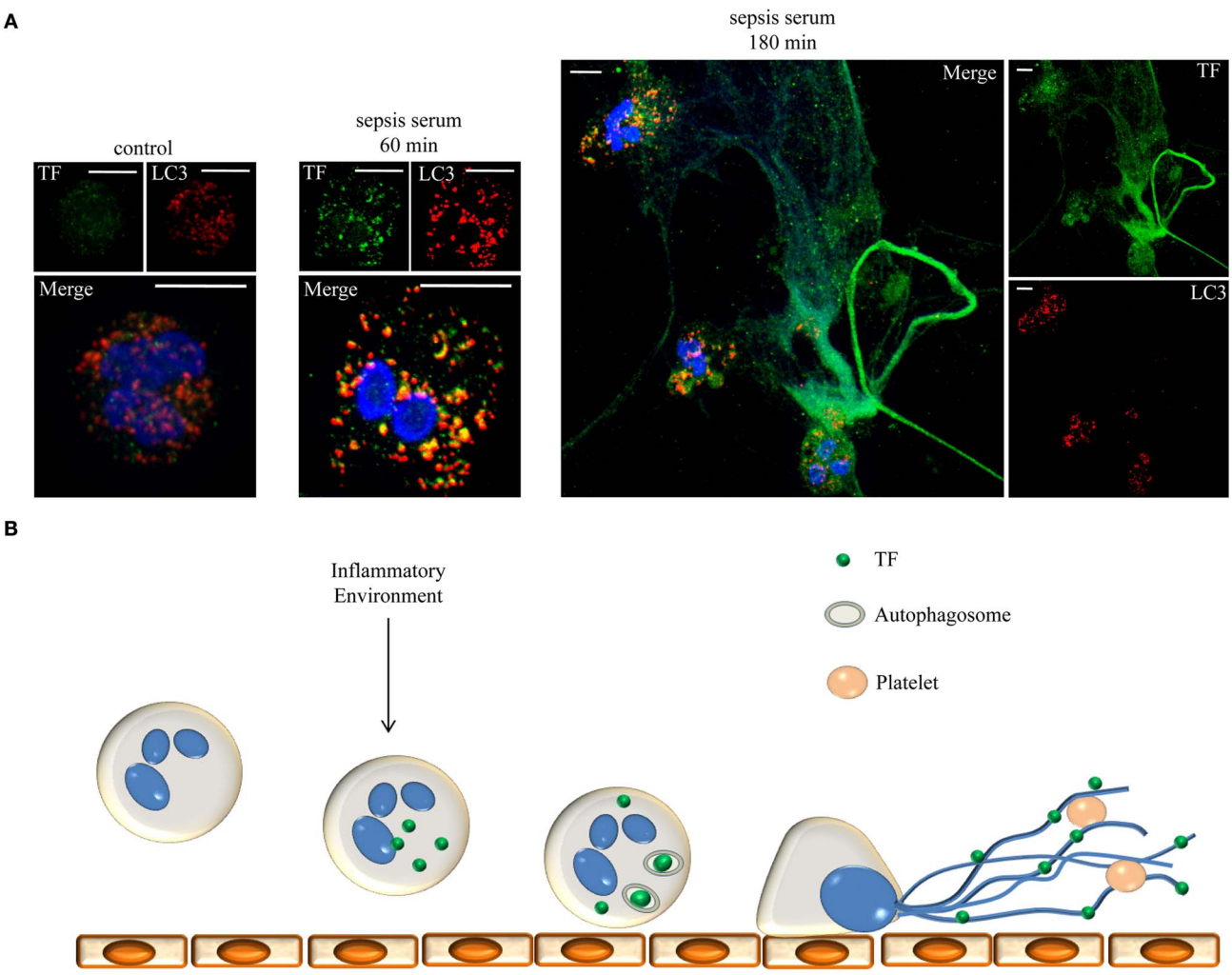

FIGURE 1 | Neutrophils release NETs decorated with TF under sepsis conditions. (A) Neutrophils were incubated for 60 or 180 min with serum from patients with sepsis to release NETs. TF (green staining) is localized in autophagosomes (LC3B: red staining) and in NETs, respectively. DNA stained with DAPI (blue). Z-stack analysis. Scale bars represent 5 μM. Original magnification 1000×. **(B)** Schematic representation of the proposed mechanism. Inflammatory conditions prime and stimulate neutrophils to produce TF which is engulfed in autophagosomes and translocated on NETs. TF coated NETs can further entrap circulating platelets to form thrombus and trigger cell signaling through PARs.

raised concerns regarding its ability to activate coagulation system, since only minimal TF amounts were detected on cell membrane.

An explanation for the extracellular delivery of neutrophil-borne TF was the identification of TF on NETs. The first description of TF expression in NETs was by the study of von Brühl et al. (2012). The authors proposed that activation of factor XII rather than TF on NETs was not essential for thrombus formation even though they did not investigate the role of TF present on NETs. Recently, we demonstrated that neutrophils from patients with sepsis release large amounts of TF in the form of NETs (Kambas et al., 2012). NET-borne TF was able to generate thrombin, which subsequently resulted in platelet activation. Microparticle depletion from sepsis serum suggested the *de novo* TF production and ensured that neutrophils did not acquire TF from TF-bearing MPs of unknown origin. The observed inclusion of TF in autophagosomes prior to its extracellular delivery in NETs suggested the involvement of autophagy in this process. This autophagy-dependent pathway was also shown for high-mobility group protein B1 (HMGB-1), proposing a role for autophagy as a secretory mechanism for the externalization of membrane bound or cytosolic proteins to NETs. Using *in vitro* stimulation studies, it was shown that neutrophil priming with pro-inflammatory cytokines is required for TF mRNA translation after bacterial phagocytosis. These findings further demonstrate the critical role of inflammatory mediators in the post-transcriptional regulation of TF. Even though there are speculations that *in vivo* NET-bound TF could be trapped MPs of different origin (neutrophil, platelet, monocyte, etc.), it does not alter the crucial role of NETs in the interplay between inflammation and thrombosis.

Based on the above findings, NETs act as mechanism for the localized extracellular expression of intracellular epitopes and anti-microbial proteins. These networks function as a scaffold for thrombus formation by exposing active TF to the other serine proteases of the extrinsic coagulation cascade. Moreover, entrapment and activation of circulating platelets further contributes in the obstruction of blood flow, while entrapped platelets prevent the degradation of this scaffold by DNase. This maelstrom of events, from neutrophil attachment to endothelium to NET release and the subsequent thrombi formation in the microvasculature could be critical in the pathophysiology of sepsis (**Figure 1**). Autophagy could function as a selective transport mechanism for the delivery of proteins to NETs. Moreover, an additional role for autophagy in the regulation of protein levels through degradation or even post-translation protein modification is implied.

CONCLUSION

Despite the well-established correlation between inflammation and thrombosis in clinical practice (Aras et al., 2004; Miehsler et al., 2004; Tomasson et al., 2009; Zöller et al., 2012), the role of neutrophils in thrombogenicity has only recently emerged. Growing evidence support the critical involvement of NET formation in this process. Neutrophil recruitment and activation at the site of endothelial damage is considered as the initial event in thrombus formation. The local intralluminal exposure of high levels of thrombogenic TF in NETs could be essential for the initiation and propagation of both venous and arterial thrombosis. Additionally, neutrophil-driven activation of the extrinsic coagulation cascade could exert through PAR signaling a significant contribution in non-thrombotic processes, including inflammation, cancer biology, or fibrosis.

ACKNOWLEDGMENTS

This review was supported by the Hellenic Ministry of Education and General Secretariat for Research and Technology (ESPA project/No 898).

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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.

Received: 28 September 2012; accepted: 30 November 2012; published online: 18 December 2012.

*Citation: Kambas K, Mitroulis I and Ritis K (2012) The emerging role of neutrophils in thrombosis—the journey of TF through NETs. Front. Immun. **3**:385. doi: 10.3389/fimmu.2012.00385*

This article was submitted to Frontiers in Molecular Innate Immunity, a specialty of Frontiers in Immunology.

frontiers in IMMUNOLOGY

REVIEW ARTICLE
published: 27 November 2012
doi: 10.3389/fimmu.2012.00362

Neutrophil NETs in reproduction: from infertility to preeclampsia and the possibility of fetal loss

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The intention of this review is to provide an overview of the potential role of neutrophil extracellular traps (NETs) in mammalian reproduction. Neutrophil NETs appear to be involved in various stages of the reproductive cycle, starting with fertility and possibly ending with fetal loss. The first suggestion that NETs may play a role in pregnancy-related disorders was in preeclampsia, where vast numbers were detected in the intervillous space of affected placentae. The induction of NETosis involved an auto-inflammatory component, mediated by the increased release of placental micro-debris in preeclampsia. This report was the first indicating that NETs may be associated with a human pathology not involving infection. Subsequently, NETs have since then been implicated in bovine or equine infertility, in that semen may become entrapped in the female reproductive tract during their passage to the oocyte. In this instance interesting species-specific differences are apparent, in that equine sperm evade entrapment via expression of a DNAse-like molecule, whereas highly motile bovine sperm, once free from seminal plasma (SP) that promotes interaction with neutrophils, appear impervious to NETs entrapment. Although still in the realm of speculation it is plausible that NETs may be involved in recurrent fetal loss mediated by anti-phospholipid antibodies, or perhaps even in fetal abortion triggered by infections with microorganisms such as *L. monocytogenes* or *B. abortus*.

Keywords: pregnancy, preeclampsia, infertility, recurrent fetal loss, neutrophil extracellular traps (NETs)

INTRODUCTION

Polymorphonuclear neutrophils (**PMNs**) are the most prevalent leucocyte cell type in the peripheral circulation. They are characterized by a uniquely lobulated nuclear structure and a highly granulated cytoplasm. PMNs play a leading role in combatting infection either by phagocytosis or by the release of antibacterial granules (Nathan, 2006). A more recent attribute of these cells is their ability to extrude their DNA into the extracellular environment, as so-called neutrophil extracellular traps (NETs), which serve to ensnare and kill bacteria (Brinkmann et al., 2004; Brinkmann and Zychlinsky, 2007). This unique form of cell death termed NETosis has been shown to be highly reliant on the action of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, the generation of reactive oxygen species (ROS) (Fuchs et al., 2007), and the combined actions of myeloperoxidase (MPO), neutrophil elastase (NE) (Papayannopoulos et al., 2010), and histone deamination by human peptidylarginine deiminase 4 (PAD4) (Neeli et al., 2008; Wang et al., 2009). It is this facet which forms the basis of this review.

PMNs are involved in several stages in the reproductive cycle. In many mammalian species, PMNs have been implicated in tissue remodeling during the oestrous cycle when the endometrium adapts to be receptive for oocyte implantation (Strzemienski, 1989; Wood et al., 2007).

In murine systems, the influx of PMNs into the vaginal vault appears to play a crucial role in the continued progression of the oestrous cycle, as their depletion via application of anti-Gr-1 monoclonal antibodies leads to a blocking of the cycle in diestrus (Sasaki et al., 2009). This blockage was reversible, and could be reverted by the re-introduction of circulatory PMNs into affected mice. This neutropenic abrogation of the oestrous cycle was accompanied by reduction in the levels of the sex hormones oestradiol and progesterone, which are necessary for its maintenance, implying some form of feedback mediated by normal endometrial presence of infiltrating PMNs. The feedback loop was facilitated by opioid peptides released by activated PMNs, which promote steroidogenesis by granulosa cells (Sasaki et al., 2011).

During the human menstrual cycle (MC), large numbers of PMNs have been noted in areas of tissue degradation (day 28) prior to onset of menstruation (Salamonsen and Lathbury, 2000). It appears that endometrial PMNs may have different phenotypes, as not all stained positive for metalloproteinase-9 (MMP-9) (Salamonsen et al., 2000). Endometrial PMNs have also been determined to express ELAFIN, a serine protease inhibitor and member of the whey acidic protein (WAP) family (King et al., 2003a,b). Elafin is a potent inhibitor of NE

Abbreviations: FRT, female reproductive tract; PMN, polymorphonuclear neutrophil; MC, menstrual cycle; SP, seminal plasma; AI, artificial insemination; IUGR, intra-uterine fetal growth restriction; NE, neutrophil elastase; aPL, antiphospholipid antibodies; β2GPI, beta-2-glycoprotein I; PAR-2, protease-activated receptor 2; ROS, reactive oxygen species.

and proteinase 3, and has been suggested to protect tissue from degradation by these enzymes (King et al., 2003a,b). Since Elafin has been demonstrated to possess anti-microbial activity, its presence could thereby contribute to innate immune defence in the female reproductive tract (FRT) (King et al., 2003a,b).

Hormonal changes occurring during the human MC have also been shown to alter the physiology of circulating PMNs. During the luteal phase of the MC, in which progesterone peaks, a notable increase in circulatory PMNs numbers was noted (Smith et al., 2007). Circulatory PMNs numbers remained high until the onset of menstruation at day 28, at which time they were still higher than those of comparable male donors. During the luteal phase circulatory PMNs exhibited reduced levels of MMP-9 and TNF-α expression (Smith et al., 2007). During periods of oestrogen surges (week 2 and 3 of the MC), circulatory PMNs in women expressed lower levels of CD89 (**FcaR for IgA**), CD11b, and CD18, which returned to normal by week 4. In general, circulatory PMNs in women expressed higher levels of CD89 than those from men (Smith et al., 2007).

The reduction in expression of the integrins CD11b and CD18 could explain the diminution of neutrophil adhesion by oestrogen, and partially explain the anti-inflammatory action of this sex hormone. The generally elevated expression of CD89 on peripheral PMNs by women indicates that they are more responsive to activation via an IgA-based mechanism. Women mostly exhibit a more robust humoral response than men.

In animal systems it is unclear if the oestrus cycle affects circulatory and FRT-associated PMN pro-inflammatory activity. In hormone primed mares no change in the bactericidal activity of circulatory PMNs was noted (Strzemienski et al., 1987).

INTERACTION BETWEEN NEUTROPHILS AND SEMEN IN THE FEMALE REPRODUCTIVE TRACT

During coitus billions of sperm are deposited into the FRT. During the sexual act, microorganisms originating from either the penis or the vagina are transported with the ejaculate either into the vagina or directly into the uterus. In order to maintain an environment favorable to implantation, these potential pathogens need to be successfully eliminated. A further task is the removal of the vast majority of sperm, as these can be antigenic to the recipient female, and immunization against sperm can result in infertility. A number of observations have shown that neutrophils are recruited to the FRT following insemination in a manner akin to inflammation, and that they play a major role in the removal of excess sperm, largely via phagocytosis (Strzemienski, 1989; Alghamdi and Foster, 2005; Alghamdi et al., 2009; Katila, 2012).

Even though human reproductive tract leucocytosis has been implicated in human infertility, interactions between neutrophils and sperm have been best studied in large domesticated animals such as cows and horses, due to the frequent use of artificial insemination (AI) for optimal breeding (Katila, 2012). In these instances it has been observed that the repeated deposition of spermatozoa in the presence of neutrophils can lead to diminished fertility (Alghamdi and Foster, 2005; Alghamdi et al., 2009).

In early studies it was noted that bovine seminal plasma (SP) reduced the ability of isolated PMNs to phagocytize bull spermatozoa (Strzemienski, 1989). In further exploratory studies examining the action of SP it was evident that equine SP contained factors that reduced neutrophil binding to spermatozoa *in vitro* (Alghamdi et al., 2004), thereby perhaps permitting a greater number of healthy mobile spermatozoa to reach the oviduct.

In these studies aggregates were noted between large numbers of PMNs and spermatozoa, which could be antagonized by SP. The issue of these PMN-spermatozoa aggregates was subsequently addressed in more detail once it emerged that PMNs were capable of producing extracellular traps (Brinkmann et al., 2004).

Since bovine SP was found to contain a fertility-promoting factor with homology to DNAse I, the question was raised whether such a factor would permit spermatozoa to evade the presence of any PMN NETs in the FRT. In one of the first publications recording the presence of NETs in another system than infection, Alghamdi and colleagues observed that the incubation of isolated peripheral PMNs with equine spermatozoa lead to the vigorous generation of NETs, with kinetics close to those mediated by *E. coli* (Alghamdi and Foster, 2005). They furthermore observed that the protein fraction of equine SP did indeed contain a molecule with DNAse activity, as it was capable of digesting plasmid DNA, in a manner very similar to that performed by DNAse I (Alghamdi and Foster, 2005).

The addition of this equine SP protein fraction to spermatozoa-PMN mixtures led to the digestion of PMN NETs, an aspect that could be partially mimicked by the addition of extraneous DNAse I. It was, however, clear that equine SP contains other factors that modulate PMNs response to spermatozoa, as it reduced the number of NETs generated by accessory PMNs in such cultures (Alghamdi and Foster, 2005) (**Figure 1**).

Of great interest is that equine SP protein fraction did not prevent NETs induction by *E. coli*, nor did it prevent their killing by activated PMNs. Of further note is that this equine DNAse-like protein was virtually absent in the SP of a stallion with a record of poor fertility. This strongly suggests that this factor assists in mediating optimal interaction between spermatozoa and PMN in the equine FRT, without compromising PMN bacteriocidal activity (Alghamdi and Foster, 2005).

A major difference between bovine and equine insemination is that in the former the spermatozoa are deposited in the vagina, whilst in the latter they are ejaculated directly into the uterus (Alghamdi et al., 2009; Katila, 2012). In bovines the spermatozoa need to migrate through the cervix into the uterus, in the process leaving most of the seminal fluid behind. Therefore, the interaction between SP proteins modulating PMN activity will be diminished in this case. Alternatively, in equines, SP proteins could have a different function, because spermatozoa are discharged adjacent to the site of fertilization.

This indeed turned out to be the case in that bovine SP mediates a different modality on PMN activity than equine SP, by promoting the binding of bovine spermatozoa to PMNs (Alghamdi et al., 2009). On the other hand, in contrast to the situation in

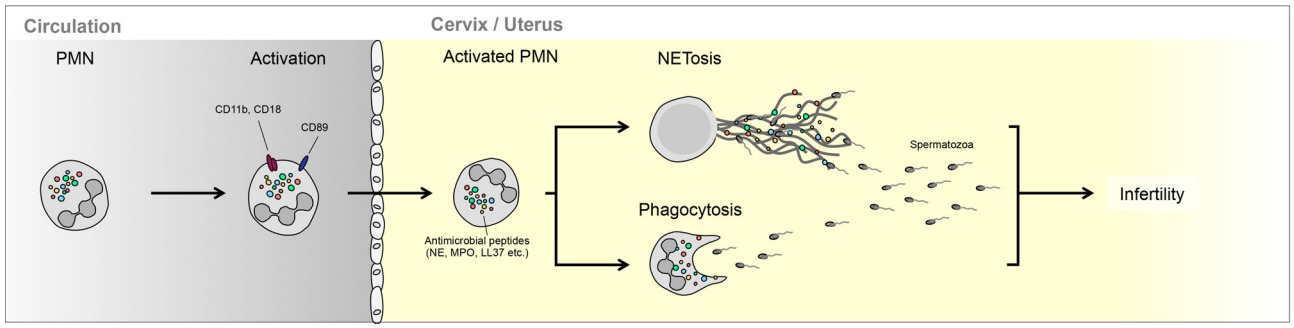

FIGURE 1 | Interaction between neutrophils and semen in the female reproductive tract. PMN can either phagocytize less motile spermatozoa or trap these in NETs. The ability of PMNs to interact with spermatozoa is regulated largely by seminal plasma, which can promote NETting (bovine) or enhance escape (equine).

horses, bovine spermatozoa cleared completely of SP showed low binding affinity to PMN, for a period as long as 3 h. This implies that less mobile bovine spermatozoa will be ensnared by NETs directly in the bovine vagina, due to the action of SP, while virile highly motile spermatozoa will enter the uterus cleared of SP and thereby evade interaction with any PMNs present in this part of the FRT (Alghamdi et al., 2009).

Apart from demonstrating remarkable species-specific differences, these results have major implications in how AI is carried out, and under which conditions SP protein fractions may have beneficial character or not (Alghamdi et al., 2009; Katila, 2012).

OCCURRENCE OF NETs IN PREECLAMPSIA

Although the vast majority of human pregnancies progress to term without any complications, many can be affected by disorders such as fetal growth restriction, preeclampsia, or preterm labor, which can result in maternal or fetal mortality/morbidity (Wilkinson, 2011). To a large extent these different pathologies may hold their origin in placental abnormalities (Brosens et al., 2011).

Human placentation is unique by the depth of invasion of the maternal endometrium by fetal tissues, but also by the extent in which maternal blood vessels are modified by fetal trophoblast (Huppertz, 2008; Pijnenborg et al., 2011). In this manner, toward the end of the first trimester, extravillous trophoblast cells enter the maternal endometrium and replace the endothelial cells of the maternal spiral arteries (Redman, 1997). This leads to a significant enlargement and relaxation of these vessels, thereby ensuring an optimal blood flow though the intervillous space.

Failure of such a modification is associated with either severe intra-uterine fetal growth restriction (IUGR) and/or early onset preeclampsia (Redman and Sargent, 2005; Huppertz, 2008; Brosens et al., 2011).

Preeclampsia is a disorder unique to human pregnancy, characterized by proteinuria and sudden elevation in blood pressure in previously normotensive pregnant women (Roberts and Redman, 1993). Currently the only effective therapy is delivery of the baby, which frequently results in extreme prematurity, as the onset of this disorder can occur as early as 20 weeks of gestation. If left untreated, preeclampsia can develop into eclampsia, a potentially lethal condition, characterized by epilepsy-like convulsions (Roberts and Redman, 1993).

Even though the placenta plays a central role in the underlying aetiology (Huppertz, 2008), preeclampsia is associated with systemic damage of the maternal endothelium and an anomalous maternal inflammatory response (Redman and Sargent, 2010). This inflammatory response is mediated in part by the release of placental micro-debris by the placental syncytiotrophoblast, the deportation of which is elevated in cases with preeclampsia (Gupta et al., 2005b; Hahn et al., 2005; Germain et al., 2007; Messerli et al., 2010).

With regard to PMNs, previous studies have shown that normal human pregnancy is associated with a pro-inflammatory phenotype of these cells, a feature which is significantly more pronounced in preeclampsia (Sacks et al., 1998). This was reflected by the elevated expression of cell surfaces markers such as CD11b, but also intracellular activation markers, such as iROS (Sacks et al., 1998). Additional evidence of peripheral PMN activation under these conditions is provided by the presence of elevated levels of NE in the plasma of patients with preeclampsia (Halim et al., 1996; Gupta et al., 2006b). This activation appears to be stimulated by the presence of placental micro-debris in the maternal circulation (Aly et al., 2004).

Prompted by our observations that preeclampsia was associated with significantly elevated fetal and maternal cell-free DNA in the maternal circulation (Zhong et al., 2001b, 2005a,b), we were very intrigued by the report that PMNs can release their nuclear DNA into the extracellular environment (Brinkmann et al., 2004). Consequently, we set out to determine whether a possible connection existed between these two phenomena.

For this purpose we used *in vitro* co-cultures using peripheral PMNs isolated from healthy controls and highly purified placental micro-debris (Gupta et al., 2005a). In our experiments we observed that placental micro-debris led to the activation of PMN as assessed by the elevated expression of CD11b (Gupta et al., 2005a). This activation by placental micro-debris was accompanied by the generation of NETs, in a time and dose dependent manner (Gupta et al., 2005a), with similar kinetics to what had been previously observed using bacterial agents (Brinkmann et al., 2004).

We also observed that NETs could be induced by other placentally derived factors, such as the cytokine IL-8. It is therefore possible that placentally derived micro-debris and inflammatory

cytokines (IL-8) may act in concert in the activation of PMNs and induction of NETs in pregnancy (Gupta et al., 2005a).

To assess whether these *in vitro* observations had any physiological relevance we examined placentae from normal healthy term deliveries or those affected by severe preeclampsia. PMN NETs could be detected in the intervillous space of normal placentae. This is to be expected as the normal placenta does deport micro-debris, which could lead to PMNs activation and ensuing NETosis, as part of the pro-inflammatory condition observed in normal pregnancy. The number of NETs in preeclamptic placentae was, however, dramatically elevated and appeared to fill the entire intervillous space in certain instances.

As preeclampsia is characterized by hypoxia-reperfusion damage (Burton and Jauniaux, 2004), the presence of large numbers of NETs directly in the intervillous space, the site of oxygen exchange between mother and fetus, may contribute to this.

Since NETs have recently been shown to promote thrombi in deep vein thrombosis (DVT) and in tumor model systems (Brill et al., 2012; Demers et al., 2012; Fuchs et al., 2012), it is possible that the occlusion of blood flow through the intervillous space by NETs could be exacerbated by such an additional event. The likelihood of such an event is high, as excessive fibrin deposition and infarction are frequently observed in preeclamptic placentae (Kitzmiller and Benirschke, 1973).

PMN NETs may also contribute to the widespread systemic damage to the maternal endothelium observed in preeclampsia (Powe et al., 2011), since endothelial cells are susceptible to cell death induced by NETting PMNs (Gupta et al., 2010).

Additional support suggesting that placentally derived factors contribute to PMN activation in preeclampsia is provided by the study of circulatory PMNs isolated from maternal blood isolated from the antecubital and uterine veins (Mellembakken et al., 2002). In cases with preeclampsia, it was observed that PMNs passing through the uteroplacental circulation appeared to have a more highly activated phenotype than those present in the peripheral circulation. No similar alteration was observed in normal pregnancies. These data therefore suggest that an inflammatory process occurs in the decidua and placental tissues during the development of preeclampsia (Mellembakken et al., 2002) (**Figure 2**).

It is currently unclear whether NETs represent an initiating lesion in preeclampsia, or are the result of another underlying placental deficiency. By using the data on maternal and fetal cell-free DNA levels in preeclampsia, it may be possible to tentatively infer regarding the time-point of NETs induction (Gupta et al., 2006a). In this context, elevations in cell-free fetal DNA have been suggested to be the result of a placental lesion, whilst elevations in maternal cell-free DNA could result from the generation of NETs (Gupta et al., 2006a). A number of studies have indicated that disturbances in cell-free fetal DNA levels occur early in gestation, prior to the onset of preeclampsia symptoms (Leung et al., 2001; Zhong et al., 2001a). In contrast, cell-free maternal DNA is only elevated once the symptoms become manifest (Zhong et al., 2001a). These data imply that two phases are present in preeclampsia; a pre-clinical phase involving a placental lesion, and a second clinical phase with symptoms involving a systemic inflammatory response by the mother (Roberts and Hubel, 2009). It would, hence, appear that NETs are associated with the manifest disorder, and not a preclinical initiating lesion (Gupta et al., 2006a).

NETs also do not appear to distinguish between early and late onset preeclampsia, as both seem to be associated with increased PMNs activity as assessed by increased levels of NE in maternal plasma in both forms, when compared to healthy pregnancies (Gupta et al., 2006b).

Currently it is still not clear whether PMN NETs are involved in other pregnancy-related disorders such as IUGR, recurrent fetal loss or preterm labor (Hahn et al., 2006). It is also evident that the underlying aetiology of multi-factorial syndromes, such as preeclampsia, will not involve a single lesion, but rather involve a number of different triggering events (Redman and Sargent, 2005; Brosens et al., 2011). These may include the imbalance of placentally produced angiogenic factors (Powe et al., 2011; Rana et al., 2012), or the dysregulation of inflammatory cytokine networks (Rusterholz et al., 2007; Granne et al., 2011).

A POSSIBLE ROLE OF NETs IN SPONTANEOUS FETAL LOSS

Although there is no direct evidence that NETs may be implicated in fetal loss, induced either via the presence of autoantibodies or infectious agents, there is accruing evidence that PMNs activation may play a crucial part in these events (Weiler, 2008; Lynch and Salmon, 2010; Girardi, 2011). Recurrent fetal loss is frequently associated with the presence of maternal antiphospholipid antibodies (aPL) (Weiler, 2008; Lynch and Salmon, 2010; Girardi, 2011). aPL via the interaction with beta-2-glycoprotein-I (β2GPI), binds to phosphotidylserine moieties on the trophoblast, thereby providing a key step for the activation of a series of coagulation factors, hence, the layman's term "sticky blood syndrome."

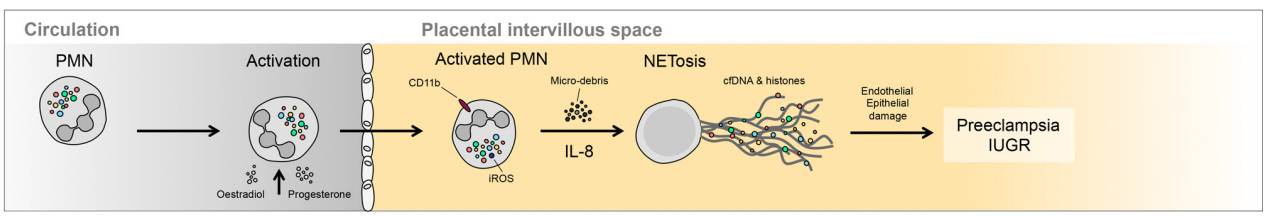

FIGURE 2 | Occurrence of NETs in preeclampsia. Large numbers of NETs have been detected directly in the intervillous space of preeclamptic placentae. These NETs appear to be triggered by the elevated release of highly inflammatory placental micro-debris.

The traditional view is that the presence of the aPL leads to activation of the clotting cascade, thereby leading to placental infarction and ensuing fetal demise (Weiler, 2008; Lynch and Salmon, 2010; Girardi, 2011). Support for this notion is provided by the clinical use of low molecular weight heparin, which has been shown to be effective in preventing fetal loss in most cases (Hahn et al., 2006).

Recent evidence indicating that aPL is associated with an inflammatory activation of PMNs via the complement system is leading to a change of the traditional dogma (Salmon and Girardi, 2008).

Evidence for such an interaction was largely provided by murine model systems. It was determined that when aPL were infused into pregnant mice, fetal demise was not associated with a deposition of fibrin or increased presence of thrombi, but rather that it involved the activation of the complement system, in particular components C3 and C5, and the repressive activity of Crry (Holers et al., 2002; Girardi et al., 2003). In addition, the innate arm of the immune system was implicated, as the decidua of treated mice exhibited considerable PMNs infiltration and elevated tissue factor (TF) expression (Redecha et al., 2007).

These findings were largely achieved via the dissection of the underlying molecular pathways using a series of knock-out murine models or pharmacologic inhibitors. In these examinations it was determined that elevated TF expression by PMNs appeared to be a crucial component in triggering fetal demise, which was the result of a complex chain of events (Redecha et al., 2007). The first of these was binding of aPL to the trophoblast, thereby catalyzing zymogenic cleavage of C5. This permits the active component C5a to bind to the C5aR on PMNs. This receptor-ligand interaction triggers increased cell surface expression of TF, itself a receptor for coagulation factors FVIIa and FXa. Such receptor TF/FVIIa-FXa complexes lead to activation of the G-protein coupled protease-activated receptor 2 (PAR-2), which in PMNs initiate ROS production and the release of pro-inflammatory cytokines (Redecha et al., 2007; Weiler, 2008; Girardi, 2011).

PMNs appear to be the final effector in this complement-triggered cascade, as their depletion via the use of appropriate antibodies effectively blocks aPL induced fetal loss (Pierangeli et al., 2005). For this reason it was suggested aPL induced fetal loss was the result of uncontrolled ROS production by complement activated PMNs in the feto-maternal interface, leading to oxidative damage of underlying placental tissue (Salmon and Girardi, 2008).

As the production of ROS is a vital component of the pathway triggering NETosis and the release of DNA into the extracellular environment (Fuchs et al., 2007), it is enticing to speculate that NETs may occur in aPL induced fetal loss. Furthermore, as the presence of NETs can be cytotoxic to closely adjacent cells (Gupta et al., 2010), it is possible that the occurrence of such entities can contribute to trophoblast injury apparent in this disorder.

An interesting observation is that PMNs from preterm infants or neonates do not appear capable of undergoing NETosis, or at least showed a very delayed response, when treated with potent stimuli such as platelet activating factor (PAF) and lipopolysaccharide (LPS), unlike normal adult PMNs (Marcos et al., 2009; Yost et al., 2009). This facet did not appear to involve a defect in signaling pathways, as neonatal PMNs expressed the required receptors (PAF-R and TLR4) and displayed normal responses, such as calcium mobilization, production of IL-8 in response to these stimuli. This deficit could also not be overcome by supplementation of intracellular ROS pools by treatment with glucose/glucose oxidase. It is not clear from this particular study how representative the relatively inert nature of neonatal PMN in cases with preterm delivery is, since previous reports have suggested that intrauterine activation of PMNs occurs under these conditions, leading to pulmonary haemorrhage in affected infants (Mehta and Petrova, 2006).

Although it may seem that the coagulation cascade is not directly involved in this particular model system, since fetal loss could not be hindered by anti-clotting agents such as hirudin, which prevent thrombus formation but not complement activation, it may nevertheless play an accessory role. This would especially be the case if NETs were implicated in aPL induced fetal loss, as these could provide the necessary stimulus and scaffold for clot formation (Fuchs et al., 2012). This supposition is based on recent data indicating that NETs stimulate the extrinsic and intrinsic coagulation pathways, by promoting platelet and RBC adhesion and by concentrating effector proteins and coagulation factors involved in hemostasis (Fuchs et al., 2010, 2012; Massberg et al., 2010; von Bruhl et al., 2012). In this context NETs have been found to be abundant in experimental DVT in baboons and mice, co-localizing with vWF, an important endothelial clotting factor (Brill et al., 2012). Apart from providing a DNA-based scaffold facilitating the binding of pro-coagulatory factors such as Factor XII, intrinsic PMN derived serine proteases such as NE and cathepsin G can stimulate clotting via the cleavage of coagulation mediators such as tissue factor pathway inhibitor (TFPI), or fibrin (Plow, 1980; Higuchi et al., 1992; Massberg et al., 2010; Semeraro et al., 2011).

Given that preeclampsia, IUGR and even fetal loss are broadly related to dysfunctions at the interface between innate immunity and haemostasis, it would be of cardinal importance to investigate the potential triggers of PMNs activation and NETosis in these pathologies. The trigger could be the interaction of neutrophils with activated cells, such as platelets or endothelium (Gupta et al., 2010; Saffarzadeh et al., 2012), or alternatively, involve hypoxia, inflammatory cytokines, or factors generated early in thrombotic events (Fuchs et al., 2007).

The scope of PMN activity associated with fetal loss may, however, be even broader. This is based on recent data suggesting that infections with *Brucella abortis* (Gupta and Bianchi, 1997) or *Listeria monocytogenes* (Knowles et al., 2011) leads to PMNs recruitment and activation, including release of IL-8 and ROS production. As *brucellosis* in cattle or *listeriosis* in humans can be directly associated with spontaneous abortion (Robbins and Bakardjiev, 2012), it is open to speculation whether NETs occur in infected placentae in these conditions, and thereby contribute to the process of fetal loss (**Figure 3**).

CONCLUSIONS

The activity of PMNs is modulated or altered during various phases of the reproductive cycle (Salamonsen and Lathbury, 2000;

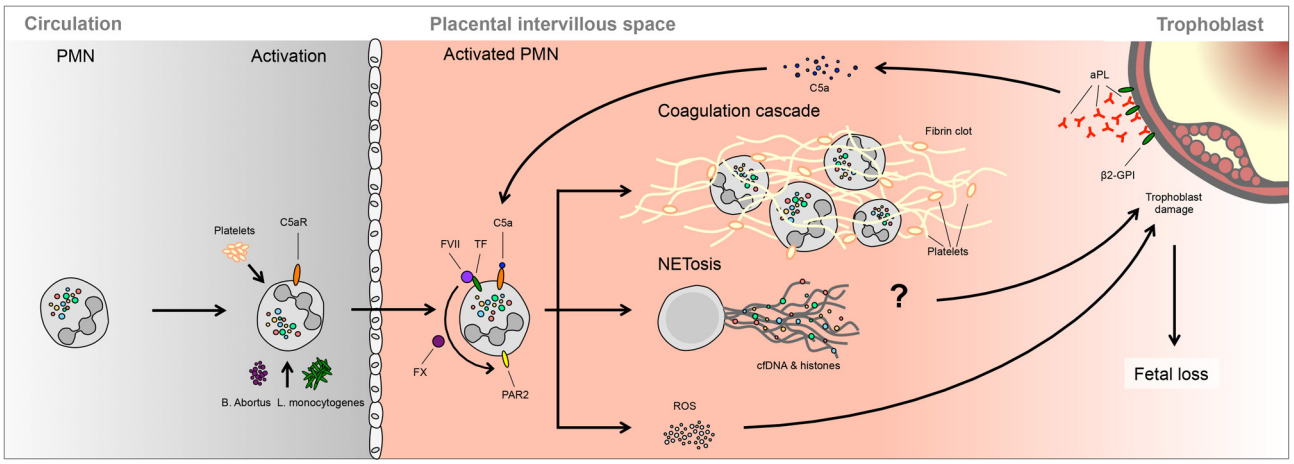

FIGURE 3 | A possible role of NETs in spontaneous fetal loss. In murine models, fetal loss triggered by aPL involves activation of the complement cascade, notably C3 and C5, which lead to PMNs activation via TF. Ensuing ROS production is proposed to lead to trophoblast damage, leading to fetal demise. It is not clear whether this process involves NETosis or PMNs activation of the clotting cascade.

Wood et al., 2007; Sasaki et al., 2009). This is most evident in the menstrual or oestrum cycle, in which the activity of circulatory PMNs is altered by the action of sex hormones, but also in the endometrial tissue, where PMNs play a role in tissue removal or modification. PMNs appear to play a significant role in post mating inflammatory response (Katila, 2012), as well as in the trapping and clearance of sperm by NETs (Alghamdi et al., 2004; Alghamdi and Foster, 2005). In these instances, the action of seminal fluid reveals interesting species specific differences, in that in horses it down-modulates PMN activity, thereby promoting sperm escape, whereas in cattle it promotes sperm entrapment by NETs (Alghamdi et al., 2004; Alghamdi and Foster, 2005).

The presence of large numbers of NETs directly in the intervillous space in preeclampsia (Gupta et al., 2005a), a crucial interface between mother and fetus, as well as the induction of NETosis by placentally-derived micro-vesicles or cytokines, suggest a role for these entities in this enigmatic disorder (Gupta et al., 2007).

Although still in the realm of speculation, new evidence implicating PMNs activation in fetal loss induced by auto-antibodies (Salmon and Girardi, 2008) or infectious agents (Robbins and Bakardjiev, 2012), may involve the occurrence of NETs.

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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.

Received: 11 October 2012; paper pending published: 29 October 2012; accepted: 12 November 2012; published online: 27 November 2012.

Citation: Hahn S, Giaglis S, Hoesli I and Hasler P (2012) Neutrophil NETs in reproduction: from infertility to preeclampsia and the possibility of fetal loss. Front. Immun. ***3****:362. doi: 10.3389/fimmu.2012.00362*

This article was submitted to Frontiers in Molecular Innate Immunity, a specialty of Frontiers in Immunology.

frontiers in
IMMUNOLOGY

MINI REVIEW ARTICLE
published: 21 January 2013
doi: 10.3389/fimmu.2012.00424

Reactive oxidants and myeloperoxidase and their involvement in neutrophil extracellular traps

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Neutrophils release extracellular traps (NETs) in response to a variety of inflammatory stimuli. These structures are composed of a network of chromatin strands associated with a variety of neutrophil-derived proteins including the enzyme myeloperoxidase (MPO). Studies into the mechanisms leading to the formation of NETs indicate a complex process that differs according to the stimulus. With some stimuli an active nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is required. However, assigning specific reactive oxygen species involved downstream of the oxidase is a difficult task and definitive proof for any single oxidant is still lacking. Pharmacological inhibition of MPO and the use of MPO-deficient neutrophils indicate active MPO is required with phorbol myristate acetate as a stimulus but not necessarily with bacteria. Reactive oxidants and MPO may also play a role in NET-mediated microbial killing. MPO is present on NETs and maintains activity at this site. Therefore, MPO has the potential to generate reactive oxidants in close proximity to trapped microorganisms and thus effect microbial killing. This brief review discusses current evidence for the involvement of reactive oxidants and MPO in NET formation and their potential contribution to NET antimicrobial activity.

Keywords: superoxide, hydrogen peroxide, hypochlorous acid

INTRODUCTION

Neutrophils release extracellular traps (NETs) in response to a diverse range of stimuli including a variety of microorganisms, microbial products, and chemokines (refer to the review by Guimaraes-Costa et al., 2012 for a more detailed list). NETs are composed of a scaffold of chromatin decorated with an assortment of neutrophil-derived proteins, including the enzyme myeloperoxidase (MPO; Urban et al., 2009). NETs are believed to contribute to host defense, supplementary to neutrophil phagocytosis, by trapping and potentially killing invading pathogens (Brinkmann et al., 2004). However, extended exposure of self-DNA and damaging neutrophil granule proteins may be detrimental to the host and NETs have been linked with autoimmunity (Kessenbrock et al., 2009; Lande et al., 2011) and other pathological conditions (Clark et al., 2007; Fuchs et al., 2010; Narasaraju et al., 2011; Caudrillier et al., 2012).

Activated neutrophils produce large amounts of superoxide ($O_2^{\bullet-}$) via their nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. $O_2^{\bullet-}$ dismutates to hydrogen peroxide (H_2O_2) leading to the formation of a variety of toxic oxygen derivatives, especially those formed by MPO-catalyzed reactions. Both the NADPH oxidase and MPO have been implicated in the regulation of NET formation. However, the specific reactive oxygen species (ROS) required remains to be clarified.

Myeloperoxidase catalyses the oxidation of chloride by H_2O_2 forming the strong oxidant hypochlorous acid (HOCl), the prime mediator of oxidative killing in the phagosome (Winterbourn and Kettle, 2012). MPO is present on NETs (Urban et al., 2009) and has the potential (given a supply of H_2O_2) to generate HOCl in close proximity to trapped bacteria, thus providing a prospective mechanism for oxidative NET-mediated killing. In this short review, we summarize experimental evidence for the involvement of ROS and MPO in the regulation of NET formation and discuss their potential contribution to NET antimicrobial activity.

ROS AND MPO IN NET FORMATION

Studies into the mechanisms of NET formation (NETosis) indicate a complex process that differs depending on the stimulus. Given the variability in NET inducers (Guimaraes-Costa et al., 2012) the existence of more than one pathway is perhaps not surprising. The term NETosis is sometimes used to describe only those forms of NET formation associated with cell death (Steinberg and Grinstein, 2007), but NETs can be released from living cells (Yipp et al., 2012), and here we use NETosis to describe any form of NET formation. NETs differ with respect to composition, timing, the involvement of cell death and dependency on reactive oxidants (Clark et al., 2007; Fuchs et al., 2007; Yousefi et al., 2009; Pilsczek et al., 2010). To date, the majority of inducers examined show dependency on an active NADPH oxidase and there is evidence that with some stimuli MPO is also involved.

NADPH OXIDASE DEPENDENCY

Evidence that an active NADPH oxidase is required for NET formation has come from studies using inhibitors of the oxidase, knockout mice, or neutrophils from patients with chronic granulomatous disease (CGD) whose NADPH oxidase is nonfunctional (Stasia and Li, 2008). Inhibition of the oxidase with diphenyleneiodonium chloride (DPI) prevents NETosis in response to several factors, including phorbol myristate acetate (PMA; Fuchs et al., 2007), an nitric oxide (NO) donor (Keshari et al., 2012), bacteria (Parker et al., 2012b), lipopolysaccharide (LPS; Yost et al., 2009), and complement factor 5a (C5a) after

priming with granulocyte/macrophage colony-stimulating factor (GM-CSF; Yousefi et al., 2009). Interestingly with *Staphylococcus aureus*, an early phase of NET release induced by secreted bacterial products is independent of the oxidase and of cell death, with dependency on these increasing over time (Pilsczek et al., 2010). The later release of NETs was possibly induced by bacterial phagocytosis, which would have been slow under the conditions employed in this study. Thus, two different forms of NET stimulation could have operated over the course of the experiments. From this study it might be assumed that activation of the oxidase leads to NET expulsion by cell death and that the oxidase is not required for release from viable cells. However, oxidase-dependent NET release from living cells has been reported (Yousefi et al., 2009).

Strong evidence for NADPH oxidase-dependent NETosis comes from the finding that CGD neutrophils do not form NETs when stimulated with PMA, bacteria (Fuchs et al., 2007), or GM-CSF + C5a (Yousefi et al., 2009). Exogenously added H_2O_2 restores the ability of CGD neutrophils to produce NETs (Fuchs et al., 2007), as does gene therapy to reconstitute NADPH oxidase function (Bianchi et al., 2009). Using a mouse model of CGD, Ermert et al. (2009) found that *gp91*$^{-/-}$ mice neutrophils do not make NETs when stimulated with PMA or *Candida albicans*. Furthermore, using genetically different inbred mouse strains these investigators observed that the level of NET formation correlated with the amount of ROS produced.

NET formation can also occur independently of oxidase activity. Not all stimulants activate the oxidase (Farley et al., 2012) and some that do may induce NETs independent of this. For example, the calcium ionophore ionomycin activates the NADPH oxidase yet induces NETs similarly in the presence or absence of DPI (Parker et al., 2012b). *S. aureus* leukocidins also induce NETs when oxidase activity is inhibited (Pilsczek et al., 2010). The oxidative burst was not measured in this study; however, similar concentrations of purified leukocidin combinations can induce ROS production (Colin and Monteil, 2003).

Although DPI is a general flavoenzyme inhibitor, the most likely explanation for its effect on NETosis is that it inhibits the NADPH oxidase, and this is supported by the CGD neutrophil and knockout mice studies. DPI does have other effects, including inhibition of mitochondrial complex I and inducible nitric oxide synthase (iNOS). However, even though an NO donor has been shown to induce NETs (Keshari et al., 2012), the low levels of iNOS in isolated human neutrophils make it unlikely that DPI prevents NETosis by inhibiting iNOS. Of note, a recent report describes DPI-sensitive NET induction by platelet activating factor, which does not activate the oxidase (Farley et al., 2012).

THE ROLE OF MPO

There is growing evidence that MPO is necessary for PMA-stimulated NETosis and the majority of studies indicate that an active enzyme is required. Inhibition of MPO decreases PMA-stimulated NETs (Akong-Moore et al., 2012; Palmer et al., 2012; Parker et al., 2012b) and neutrophils from MPO-deficient patients have reduced ability to produce NETs when stimulated with PMA. Metzler et al. (2011) found the level of NETs produced correlated with the degree of MPO deficiency and that neutrophils completely deficient in MPO could not make NETs. We observed just 3% of normal MPO activity was sufficient to allow PMA-induced NETosis (Parker et al., 2012b). Inhibition of this residual activity abrogated NET formation (**Figure 1A**).

Myeloperoxidase may not be required with all stimuli. We found inhibiting MPO in control donor neutrophils had no effect on *Pseudomonas aeruginosa*, *S. aureus*, or *Escherichia coli* NET induction (Parker et al., 2012b). MPO-deficient neutrophils also made NETs as efficiently as those from control donors when stimulated with *P. aeruginosa* and inhibition of residual MPO activity had no effect (**Figure 1B**; Parker et al., 2012b). In contrast to our observations, Akong-Moore et al. (2012) prevented *Pseudomonas*-induced NETosis with MPO inhibition. Our conditions favored phagocytosis (Parker et al., 2012b) and may account for the differences observed between the studies but this remains to be explored. Interestingly, MPO inhibition or knock out had no effect on NETosis in mouse neutrophils (Akong-Moore et al., 2012) indicating an apparent species-specific difference in NET formation. Of note, mouse neutrophils contain less MPO than human (Rausch and Moore, 1975).

Myeloperoxidase is reported to contribute toward NETosis, independent of its activity, by aiding chromatin decondensation (Papayannopoulos et al., 2010). Purified MPO increased nuclear decondensation in a cell-free system but the most dramatic increase occurred when MPO was added in conjunction with neutrophil elastase. In PMA-stimulated neutrophils, elastase translocated to the nucleus early in NETosis while MPO localized there later, when NET release was occurring (Papayannopoulos et al., 2010). Therefore, in neutrophils MPO may not play a direct role in chromatin decondensation.

To sum up, there is good evidence that MPO is important for PMA induction of NETs. From our studies, it would appear that this is not the case with bacteria. However, there are inconsistencies in the results from different laboratories that require explanation. Whether MPO is required with other physiological NET inducers is currently unknown. Nevertheless when MPO is needed, it appears that very little is actually required to facilitate NETosis.

ASSIGNING THE SPECIFIC ROS REQUIRED

Activation of the neutrophil NADPH oxidase leads to the production of a variety of ROS. Assigning which are required for NETosis is not simple. The site of oxidase activation and degree of degranulation, which vary depending on the stimulus, affect the relative amounts of the different ROS produced as well as access to different cell constituents. With soluble stimuli, such as PMA, and non-phagocytosed particulate stimuli, activation largely occurs at the plasma membrane although some occurs at intracellular sites (reviewed in Bylund et al., 2010; **Figure 1C**). As yet these are not well characterized. During phagocytosis, activation mainly occurs at the phagosomal membrane (Winterbourn and Kettle, 2012), but electron microscope evidence shows that some also occurs elsewhere in the cell (Robinson, 2008; **Figure 1D**).

The NADPH oxidase removes electrons from cellular NADPH and transfers them across a membrane to oxygen, forming $O_2^{\bullet-}$ in the extracellular environment, phagosome or a currently undefined intracellular compartment. $O_2^{\bullet-}$ is membrane impermeable but rapidly dismutates to membrane permeable H_2O_2. Some of

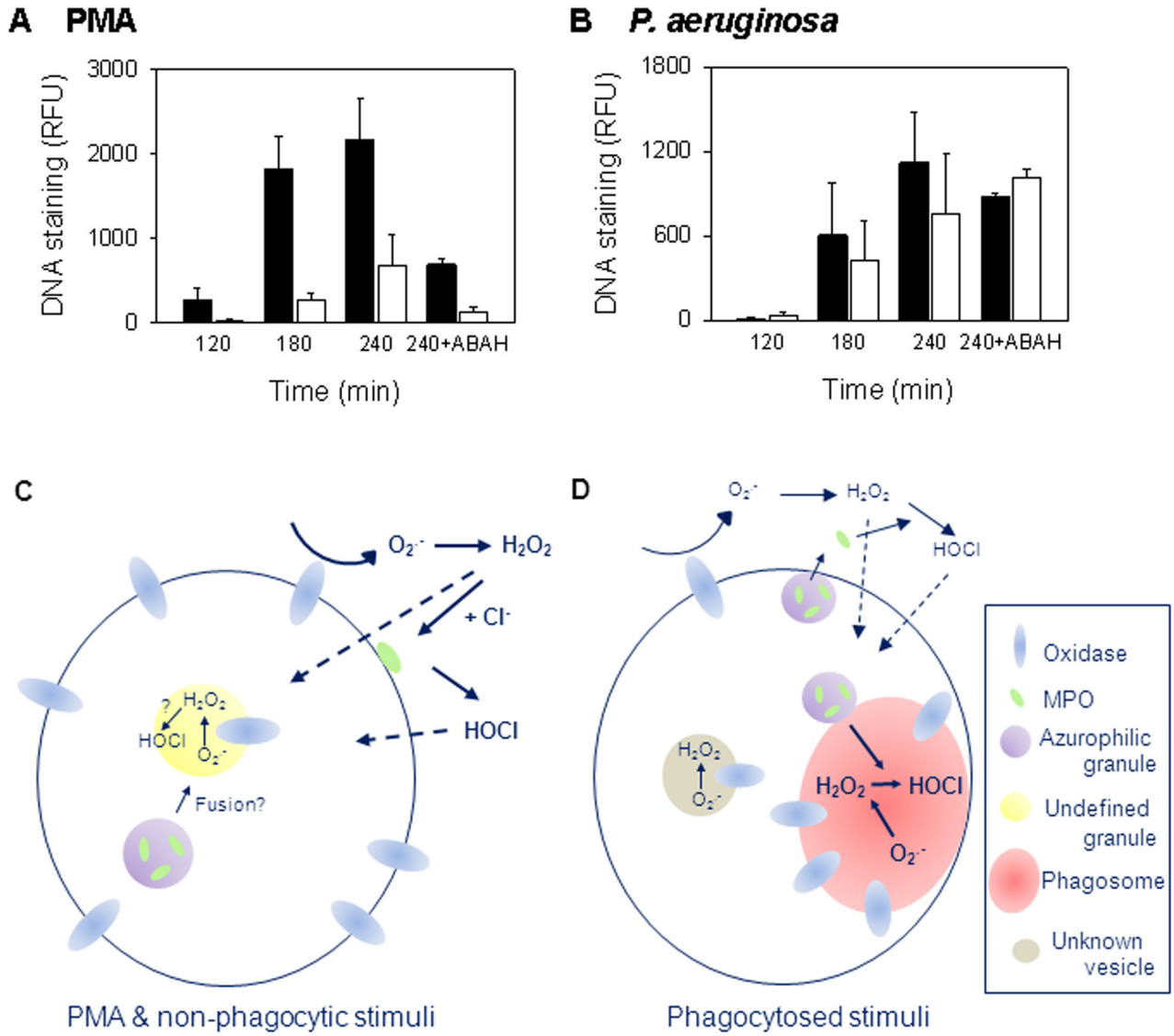

FIGURE 1 | Myeloperoxidase (MPO) is required for PMA but not bacterial induction of NETs. (A,B) The release of NETs from control (filled bars) and MPO-deficient (open bars) neutrophils measured over 4 h. MPO-deficient neutrophils formed NETs less efficiently with PMA, but not with *P. aeruginosa*, than neutrophils from control donors. To inhibit MPO, samples were incubated in the presence of 100 μM of the MPO inhibitor 4-aminobenzoic acid hydrazide (ABAH). Results are means ± SEM of two to three independent experiments. For PMA, $p = 0.02$ at 180 min; $p = 0.071$ at 240 min by *t*-test. Data obtained with permission from Parker et al. (2012b). **(C,D)** Schematic representations of the intra- and extracellular locations of oxidant production in response to **(C)** soluble and non-phagocytic stimuli, or **(D)** phagocytosis (reviewed in Bylund et al., 2010 and Robinson, 2008). Details are given in the text. With PMA, oxidant production is predominately extracellular while phagocytosis induces largely intracellular production.

the H_2O_2 produced extracellularly may diffuse into the cell while some may react with MPO outside the cell (**Figures 1C,D**). The production of HOCl in the extracellular environment requires MPO release, the timing or level of which varies with stimulus. In the phagosome, due to high MPO concentrations, essentially all of the H_2O_2 should react with MPO before it can diffuse out (Winterbourn and Kettle, 2012). H_2O_2 can also react to form hydroxyl radicals and singlet oxygen ($^1O_2$). However, the generation of these oxidants by neutrophils is considered to be very low (Winterbourn and Kettle, 2012). PMA gives a larger, more sustained oxidative burst than other stimulants that induce NETs. However, even with PMA, oxidase activity is over well before NETs are released. $O_2^{\bullet-}$ is produced within a minute of stimulation and continues for at least an hour but with the rate decreasing over this time (Decoursey and Ligeti, 2005). Similarly, oxidase activity continues for about 30 min following phagocytosis (Granfeldt and Dahlgren, 2001). Therefore, ROS produced must influence earlier rather than later events in NETosis.

By the nature of NADPH oxidase activation, it would seem it is likely that both the site of oxidant production and the nature of the oxidants produced are important in NET formation. Several groups have attempted to identify the specific ROS involved,

primarily by using enzyme inhibitors or oxidant scavengers. One of the difficulties with this approach is targeting these to the appropriate compartment. It is straightforward to scavenge oxidants that are generated extracellularly. However, where there is intracellular oxidant production, as with PMA (Bylund et al., 2010), this is much more difficult to intercept. Consequently, there are still many uncertainties about what specific ROS generated by the NADPH oxidase or MPO are required in NETosis. The following sections discuss the evidence available for individual species.

Hydrogen peroxide

Several studies have shown that exogenously added H_2O_2 is sufficient to induce NETs (Fuchs et al., 2007; Neeli et al., 2009; Lim et al., 2011). However, addition of an oxidant and observation of NETs does not necessarily mean that this oxidant is responsible with physiological stimuli. With PMA, addition of catalase to scavenge extracellular H_2O_2 has little or no effect on NETosis (Fuchs et al., 2007; Parker et al., 2012b). It is plausible sufficient H_2O_2 is generated intracellularly to induce NETs so that extracellular scavenging would have minimal effect. This was examined using polyethylene glycol-catalase (PEG-catalase) which is taken up by endocytosis (Beckman et al., 1988), though its intracellular compartment is unknown. PEG-catalase reduced but did not completely inhibit PMA-NETosis while bacterial induction of NETs was unaffected (Parker et al., 2012b). Most likely PEG-catalase did not gain access to the appropriate intracellular sites to exert a full effect. Use of catalase inhibitors, such as azide or amino-triazole, has given inconsistent results (Fuchs et al., 2007; Palmer et al., 2012; Parker et al., 2012b). However, these also inhibit MPO, which complicates interpretation of effects.

Superoxide

Addition of superoxide dismutase (SOD) to neutrophils has been shown to modestly increase PMA-induced NETs (Palmer et al., 2012; Parker et al., 2012b). This would accelerate removal of extracellular $O_2^{\bullet-}$ but have little effect on any generated intracellularly. Because most of the superoxide generated by neutrophils dismutates anyway, the presence of SOD would also make little difference to the amount of H_2O_2 produced (Winterbourn, 2008). At present we have no explanation for the SOD effect.

Hypochlorous acid and other MPO products

As the major strong oxidant produced by MPO, HOCl is a potential candidate for the oxidant responsible for MPO-dependent NET formation. Indeed, addition of HOCl to neutrophils has been reported to induce NETosis (Akong-Moore et al., 2012; Palmer et al., 2012). However, there are issues with interpreting these results. First, in our experience HOCl concentrations >50 μM are rapidly toxic to neutrophils (Carr and Winterbourn, 1997), whereas the concentrations used to induce NETs were several millimolar. Second, HOCl was added to RPMI which contains numerous scavengers, including >10 mM amino acids, which would consume the HOCl within seconds (Pattison and Davies, 2006). Although this would overcome toxicity, it would mean that very little HOCl would reach the neutrophils. Many products including amino acid chloramines would be formed, but it is unclear which would be responsible for NET formation. Third, addition of catalase to prevent extracellular HOCl formation, or removing HOCl with the potent scavenger methionine, did not inhibit PMA-stimulated NET formation (Parker et al., 2012b). Inhibition by >50 mM taurine was seen (Palmer et al., 2012), but interpretation of this observation depends on the specificity of these high concentrations. It is still possible that HOCl generated intracellularly could be involved, but more definitive evidence is needed before drawing this conclusion.

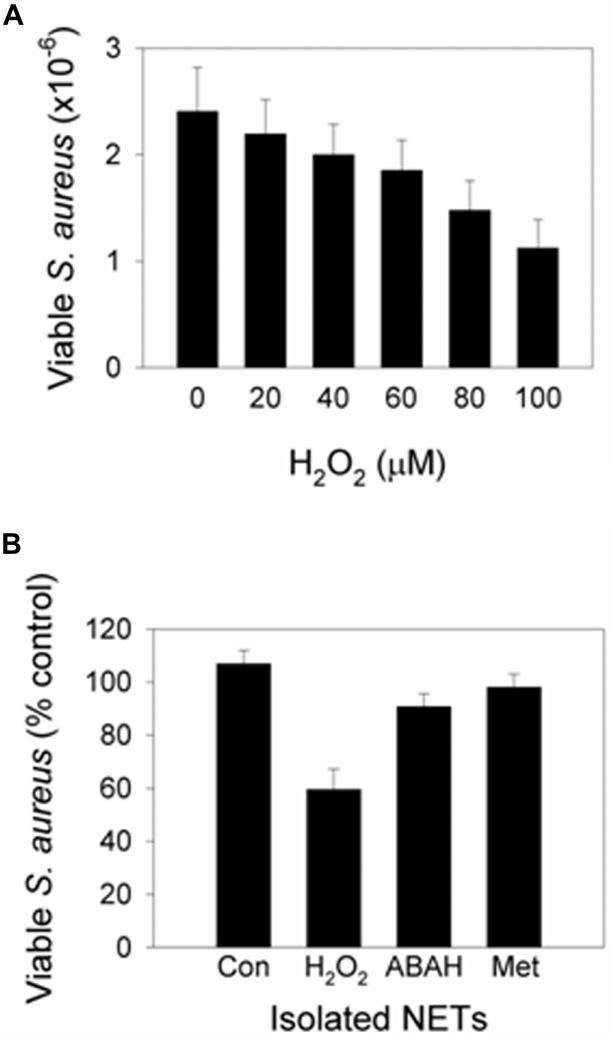

FIGURE 2 | Addition of H_2O_2 to NETs induces MPO-dependent killing. Neutrophils were stimulated with PMA to form NETs then incubated with *S. aureus* in the presence or absence of **(A)** varying concentrations of H_2O_2 or **(B)** 100 μM H_2O_2 (added in 20 μM aliquots every 5 min to facilitate MPO turnover). At the examined concentrations, H_2O_2 in the absence of NETs had no significant effect on *S. aureus* viability. **(A)** Bacterial numbers significantly decreased with ≥40 μM H_2O_2 ($p < 0.05$, *t*-test on normalized data, $n = 3$). **(B)** Bacterial viability decreased with H_2O_2 ($p < 0.001$), and inhibition of MPO with ABAH and scavenging of HOCl with methionine (Met) prevented killing ($p < 0.01$; one-way ANOVA with Holm–Sidak pairwise comparison, $n = 5$). Results are presented as percent of control cells (Con) incubated with NETs alone. Data obtained with permission from Parker et al. (2012a).

Alternative MPO products could be involved in NETosis. One example, singlet oxygen ($^1O_2$) has been implicated on the basis that NETs were observed after $^1O_2$ was generated using irradiated Photofrin (Nishinaka et al., 2011). However, while it is theoretically possible for neutrophils to generate $^1O_2$ from H_2O_2 and HOCl (Kiryu et al., 1999), it is a minor product (Hurst, 2012) and an unlikely candidate for NET regulation with other stimuli. MPO also catalyzes radical reactions, including lipid peroxidation. Interestingly, the radical scavenger Trolox inhibited PMA and LPS-induced NETosis in mouse neutrophils (Lim et al., 2011). This raises the possibility that a radical mechanism such as lipid peroxidation could be involved in the formation of NETs.

Summary of ROS required

In most cases, NADPH oxidase activity is needed for NET formation but the oxidants involved and their mechanisms of action are still unknown. The best, but not definitive, evidence is for H_2O_2 involvement, and with PMA a picture is emerging in which intracellularly generated MPO-derived ROS are important.

INVOLVEMENT OF ROS AND MPO IN NET-MEDIATED MICROBIAL KILLING

It has been postulated that the role of NETs *in vivo* is to trap and kill microorganisms and there are some excellent scanning electron micrographs of NETs entrapping both bacteria and fungi (Brinkmann et al., 2004; Beiter et al., 2006; Bruns et al., 2010). The evidence for direct killing by NETs is less convincing (Nauseef, 2012). Most studies have examined NET killing by incubating pre-formed NETs with bacteria then diluting and plating. In some instances, failure to release bacteria from NETs may have been interpreted as killing, a problem we encountered but overcame with DNase treatment to degrade NETs (Parker et al., 2012a). Using this method, several groups (Bruns et al., 2010; Menegazzi et al., 2012; Parker et al., 2012a) have observed that NETs on their own do not kill *S. aureus*, *Aspergillus fumigatus* conidia, or *C. albicans* blastospores.

EVIDENCE FOR MPO-MEDIATED NET KILLING

Myeloperoxidase is present on NETs (Brinkmann et al., 2004; Urban et al., 2009; Parker et al., 2012a) placing it in close proximity to ensnared bacteria. NET-bound MPO is active and able to generate HOCl (Parker et al., 2012a). In our study, incubation of *S. aureus* with isolated NETs had no effect on bacterial viability. However, killing was observed when H_2O_2 was added as a substrate for MPO (**Figure 2A**). MPO inhibition and a potent HOCl scavenger prevented killing (**Figure 2B**). Therefore, NET-MPO has the potential to generate HOCl and effect microbial killing. At a site of inflammation, neutrophils that have formed NETs will no longer be producing ROS. However, during inflammation there is continued infiltration and activation of neutrophils which should provide the H_2O_2 required. The close proximity of NET-MPO to trapped microorganisms would be expected to facilitate exposure of microbes to lethal concentrations of HOCl and avoid all the oxidant being scavenged by the surrounding media. *In vivo* imaging using HOCl sensitive probes and differential fluorescent detection of live/dead bacteria would confirm if this occurs in living organisms.

SUMMARY

There is good evidence that the enzymatic processes of the NADPH oxidase and MPO are important in NETosis but elucidation of the specific ROS and their reactions that regulate NET formation requires further investigation. While the use of scavengers and inhibitors is a useful aid to the study of ROS in NET formation, interpretation of results is confounded by limitations of specificity and getting sufficient concentrations to intracellular locales where the critical oxidant generation may occur. The intracellular pathways leading to chromatin decondensation and NET release are still being worked out. Once this information becomes available, the involvement of oxidants in individual steps can be investigated and a clearer picture should emerge.

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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.

Received: 10 October 2012; accepted: 23 December 2012; published online: 21 January 2013.

*Citation: Parker H and Winterbourn CC (2013) Reactive oxidants and myeloperoxidase and their involvement in neutrophil extracellular traps. Front. Immun. **3**:424. doi: 10.3389/fimmu.2012.00424*

This article was submitted to Frontiers in Molecular Innate Immunity, a specialty of Frontiers in Immunology.

frontiers in
IMMUNOLOGY

MINI REVIEW ARTICLE
published: 01 March 2013
doi: 10.3389/fimmu.2013.00045

NETosis and NADPH oxidase: at the intersection of host defense, inflammation, and injury

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Neutrophils are armed with both oxidant-dependent and -independent pathways for killing pathogens. Activation of the phagocyte nicotinamide adenine dinucleotide phosphate (NADPH) oxidase constitutes an emergency response to infectious threat and results in the generation of antimicrobial reactive oxidants. In addition, NADPH oxidase activation in neutrophils is linked to activation of granular proteases and generation of neutrophil extracellular traps (NETs). NETosis involves the release of nuclear and granular components that can target extracellular pathogens. NETosis is activated during microbial threat and in certain conditions mimicking sepsis, and can result in both augmented host defense and inflammatory injury. In contrast, apoptosis, the physiological form of neutrophil death, not only leads to non-inflammatory cell death but also contributes to alleviate inflammation. Although there are significant gaps in knowledge regarding the specific contribution of NETs to host defense, we speculate that the coordinated activation of NADPH oxidase and NETosis maximizes microbial killing. Work in engineered mice and limited patient experience point to varying susceptibility of bacterial and fungal pathogens to NADPH oxidase versus NET constituents. Since reactive oxidants and NET constituents can injure host tissue, it is important that these pathways be tightly regulated. Recent work supports a role for NETosis in both acute lung injury and in autoimmunity. Knowledge gained about mechanisms that modulate NETosis may lead to novel therapeutic approaches to limit inflammation-associated injury.

Keywords: NETs, NADPH oxidase, neutrophils, inflammation, injury

INTRODUCTION

Neutrophils are armed with a broad repertoire of tools for killing pathogens. Activation of the phagocyte nicotinamide adenine dinucleotide phosphate (NADPH) oxidase constitutes an emergency response to infectious threat and results in the generation of antimicrobial reactive oxidant intermediates (ROIs). In addition to oxidant-dependent host defense, neutrophils harbor proteases, antimicrobial peptides, lactoferrin, and other antimicrobial constituents that damage and kill microbes. Activation of NADPH oxidase in neutrophils is linked to activation of intracellular granular proteases and to generation of neutrophil extracellular traps (NETs). NETs are composed of an extracellular network of chromatin bound to granular and specific cytoplasmic proteins that can target extracellular pathogens.

NETosis can be induced *in vitro* by conditions that lead to robust NADPH oxidase activation [e.g., stimulation with phorbol myristate acetate (PMA)], and is triggered *in vivo* during states of emergency, such as infection and conditions mimicking sepsis, such as transfusion-associated acute lung injury (Caudrillier et al., 2012; Yipp et al., 2012). In this setting, NETosis in dead or dying neutrophils may amplify microbial killing. Whereas neutrophil apoptosis leads to non-inflammatory physiological cell death, NETosis results in the extracellular release of proteases and other injurious neutrophil constituents that can exacerbate inflammatory injury (Narasaraju et al., 2011; Caudrillier et al., 2012; Thomas et al., 2012).

We review the link between NETosis and NADPH oxidase activation and their effects on host defense and modulation of inflammation and injury. A greater understanding of these pathways may lead to novel therapeutic approaches to limit inflammation-associated injury.

HOW NEUTROPHILS DIE

One of the most important aspects of regulation of acute inflammation relates to its termination. Neutrophils recruited to sites of microbial invasion or tissue injury are activated by microbial products (e.g., endotoxin and formylated peptides), damage-associated molecular patterns (DAMPs; Zhang et al., 2010), and cytokines and chemokines within the inflammatory milieu. While this acute inflammatory response is critical for host defense, subsequent

termination of neutrophilic inflammation and transition to inflammatory responses that mediate tissue repair (e.g., M2 or alternative macrophage polarization; Sica and Mantovani, 2012) are necessary to limit tissue injury.

Neutrophil homeostasis involves production and death of an extraordinarily large population of cells. The lifespan of circulating neutrophils has been estimated to be <1 day; however, a recent *in vivo* labeling study showed that the average estimated circulating human neutrophil lifespan was considerably longer (5.4 days; Pillay et al., 2010). During infection and other major stressors, granulopoiesis and circulating neutrophil counts can increase dramatically; these conditions also modulate neutrophil survival and death as well as the mode of death.

Apoptosis is the default mode of neutrophil death. Apoptosis is stimulated by a number of factors [e.g., members of the tumor necrosis factor (TNF) cytokine family], and is regulated by specific caspases and members of the Bcl-2 family of proteins (Croker et al., 2011; Geering et al., 2011). Factors that can delay neutrophil apoptosis include lipopolysaccharide (LPS), granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and proinflammatory cytokines (Coxon et al., 1999; Klein et al., 2001; Garlichs et al., 2004; Akagi et al., 2008; Jun et al., 2011). Neutrophils are likely committed to apoptotic death by their constitutive co-expression of cell-surface Fas and Fas ligand, an autocrine mechanism that is suppressed by proinflammatory cytokines (Liles et al., 1996). In mice, phagocytosis of apoptotic neutrophils by macrophages reduces IL-23 production by these cells and downstream IL-17A production by T cells, thereby limiting granulopoiesis (Stark et al., 2005).

NETosis and necrosis of neutrophils are induced by different stimuli and have morphologically distinct features. Following toxin-induced necrosis, nuclear lobes in neutrophils lose their hypersegmented structure, but the nuclear envelope and granules remain intact (Fuchs et al., 2007). Nuclear decondensation and breakdown of the nuclear and granular membranes are unique features of NETosis (Fuchs et al., 2007). NETs are demonstrated by immunofluorescence showing mixing of nuclear (e.g., DNA and histones) and granular constituents [e.g., neutrophil elastase (NE)] on the extracellular surface of neutrophils (Brinkmann et al., 2010).

How neutrophils die likely affects their clearance and cross-signaling to monocytes/macrophages. At sites of inflammation, neutrophils undergo spontaneous apoptosis (Savill et al., 1989). In addition, neutrophil apoptosis can be induced by macrophages releasing death receptor ligands, such as TNF-α and Fas ligand (Brown and Savill, 1999; Yamashita et al., 1999; Renshaw et al., 2000). Macrophages recognize and ingest apoptotic neutrophils (Savill et al., 1989). Phosphatidylserine products are externalized by neutrophils early during apoptosis and stimulate phagocytosis of neutrophils by macrophages (efferocytosis), thus promoting resolution of inflammation (Frasch et al., 2011). In contrast, NETotic neutrophils display phosphatidylserine only after plasma membrane rupture (Fuchs et al., 2007). In addition, release of primary neutrophil granular proteins can recruit circulating monocytes to the site of inflammation, stimulate macrophages to produce cytokines, and enhance the ability of macrophages to phagocytose bacteria (Soehnlein et al., 2008a, 2009a,b).

GENERATION OF NETs

Neutrophil extracellular trap generation was first described by Brinkmann et al. (2004), who showed that neutrophils release granule proteins and chromatin that co-mingle in extracellular filaments that bind to and kill bacteria and degrade virulence factors. NETosis progresses through stages that are distinct from apoptosis and necrosis. The nuclei of neutrophils transition from hypersegmented lobes to a round, decondensed morphology. Peptidylarginine deiminase 4 (PAD4) converts histone tail arginine residues to citrulline, leading to loss of positive charge and chromatin decondensation (Neeli et al., 2009; Wang et al., 2009; Li et al., 2010; Farley et al., 2012). Later, the nuclear envelope and the granule membranes break down, allowing mixing of these components that are subsequently released as extracellular structures (Fuchs et al., 2007). Whereas NETosis has been considered to be an event following neutrophil death and breakdown of membranes, it is also possible for viable neutrophils to produce NETs (Yousefi et al., 2009; Yipp et al., 2012). Yipp et al. (2012) showed that during experimental infection, anuclear neutrophils with intact membranes formed NETs and were capable of migration and phagocytosis.

NETosis can be triggered by exposure of neutrophils to PMA, IL-8, LPS, interferon-gamma (IFN-γ), bacteria and fungi and their products, and complement-mediated opsonization (Brinkmann et al., 2004; Martinelli et al., 2004; Fuchs et al., 2007; Yamada et al., 2011; Saitoh et al., 2012; Yipp et al., 2012). Activated platelets can induce NETosis in neutrophils during bacterial sepsis, thereby capturing microbes and promoting their clearance (Clark et al., 2007; Phillipson and Kubes, 2011; McDonald et al., 2012). The proportion of activated neutrophils undergoing NETosis, the rapidity of NET generation, and the dependence on specific signaling pathways vary based on the stimulus (Hakkim et al., 2011). The Raf–MEK–ERK pathway is an upstream activator of NADPH oxidase in neutrophils and is involved in NETosis (Hakkim et al., 2011). Raf–MEK–ERK also upregulates expression of Mcl-1, an anti-apoptotic protein, suggesting that Raf–MEK–ERK may inhibit apoptosis to allow for NETosis (Hakkim et al., 2011). Several other signaling pathways can modulate NETosis. Inhibition of autophagy prevents chromatin decondensation in neutrophils and abrogates NETosis (Remijsen et al., 2011). Mammalian target of rapamycin (mTOR) mediates LPS-stimulated NET formation by post-transcriptional control of expression of hypoxia-inducible factor-1 alpha (HIF-1α; McInturff et al., 2012). IFN-γ produced by neutrophils can stimulate NETosis through an autocrine/paracrine process (Yamada et al., 2011).

In addition, specific neutrophil components that are NET constituents are also required for NET generation. NE is required for NET generation in pneumonia in mice (Papayannopoulos et al., 2010). In this model, NE traffics from primary granules to the nucleus where, together with myeloperoxidase (MPO), it degrades histones and promotes chromatin decondensation. MPO is also located in neutrophil primary granules and converts hydrogen peroxide to hypohalous acid, which has potent antimicrobial properties. MPO deficiency in humans leads to

failure to generate NETs following stimulation with PMA or *Candida* (Metzler et al., 2011).

Neutrophil extracellular traps are also regulated by inhibiting pathways. SerpinB1 is an inhibitor of the neutrophil serine proteases. SerpinB1-deficient mice develop increased inflammation, tissue injury, and mortality following bacterial and viral infection (Benarafa et al., 2007; Gong et al., 2011). SerpinB1 restricted NETosis through a pathway that involves translocation of SerpinB1 from the cytoplasm to the nucleus early during NETosis (Farley et al., 2012). Curiously, recombinant SerpinB1 also inhibited NETosis through mechanisms that are unclear (Farley et al., 2012). MUNC13-4, a member of the MUNC13 family of proteins, is involved in exocytosis of lytic granules in cytotoxic T lymphocytes, and its deficiency leads to familial hemophagocytic lymphohistiocytosis (Feldmann et al., 2003). In neutrophils, MUNC13-4 mediates ROI generation, exocytosis of primary granules, and phagolysosomal maturation, but also inhibits NETosis (Monfregola et al., 2012). Finally, serum endonuclease DNase1 promotes degradation of NETs (Hakkim et al., 2010). Pathways that inhibit NETosis may limit neutrophil-mediated injury.

RELATIONSHIP BETWEEN NADPH OXIDASE AND NETosis

The phagocyte NADPH oxidase is comprised of a membrane-bound cytochrome consisting of gp91^{phox} (phagocyte oxidase) and p22^{phox}. Upon activation, the cytoplasmic subunits, p47^{phox}, p67^{phox}, and p40^{phox} and rac translocate to the cytochrome. NADPH is oxidized to $NADP^+$, and electrons are transported down a reducing potential gradient that terminates when oxygen accepts an electron and is converted to superoxide anion. Neutrophil NADPH oxidase activation occurs in response to microbes and to stimuli that can mimic infectious threat, such as formylated peptides, opsonized particles, integrin-dependent adhesion (Mocsai et al., 2002; Graham et al., 2007), and to activation of specific pathogen recognition receptors, such as dectin-1, which recognizes fungal cell wall-associated beta-glucans (Gantner et al., 2003; Goodridge et al., 2011).

Chronic granulomatous disease (CGD), an inherited disorder of NADPH oxidase, is characterized by recurrent life-threatening bacterial and fungal infections. Patients with only residual NADPH oxidase activity in neutrophils have a better prognosis than those with completely absent oxidase function (Kuhns et al., 2010). CGD is also associated with severe inflammatory complications, such as Crohn's-like inflammatory bowel disease (Marciano et al., 2004; Segal et al., 2011).

Activation of NADPH oxidase in neutrophils is linked to the release of cationic proteins from an anionic proteoglycan matrix within primary granules (Reeves et al., 2002). In this model, the released neutrophil serine proteases become activated and can target phagocytized microbes. NADPH oxidase can also stimulate NETosis. PMA-stimulated NET generation requires NADPH oxidase, while MIP-2 induces NETosis independently of NADPH oxidase (Farley et al., 2012). In pneumococcal lung infection, NETosis of lung neutrophils was reduced, but not eliminated, in NADPH oxidase-deficient mice (Yamada et al., 2011). Neutrophils from CGD patients are defective in NETosis, and gene therapy results in restored NETosis in NADPH oxidase-competent neutrophils *in vitro* (Fuchs et al., 2007; Bianchi et al., 2009).

Neutrophil NADPH oxidase can also modulate apoptosis. NADPH oxidase stimulates phagocytosis-induced apoptosis (Coxon et al., 1996). TNF-α and Fas ligand can both induce apoptosis in neutrophils, but through distinct signaling pathways; NADPH oxidase was required for TNF-α-stimulated, but not Fas ligand-stimulated, apoptosis (Geering et al., 2011). Accelerating neutrophil death and clearance are likely to be important modes by which NADPH oxidase limits acute inflammation. Potentially, the intensity or kinetics of ROI generation may modulate the balance between apoptosis versus NETosis.

NADPH OXIDASE AND NETs IN HOST DEFENSE

NADPH oxidase can potentially target pathogens through a multistep process: direct antimicrobial effect of ROIs; intracellular activation of proteases that can target phagocytized pathogens; and generation of NETs that can attack extracellular pathogens. NETs can mediate host defense by trapping microbes thereby limiting their spread and by exposing them to high concentrations of several antimicrobial products. NET constituents can target different pathogens. For example, NE can degrade certain virulence factors of pathogens (Belaaouaj et al., 2000; Weinrauch et al., 2002). Calprotectin is a NET constituent that mediates nutritional immunity by sequestering divalent metal ions and targets *Candida* and *Aspergillus* species (Urban et al., 2009; Bianchi et al., 2011). However, the contribution of NET generation to host defense *in vivo* is difficult to determine because there are no genetic defects that selectively disable NETosis while leaving all other immune pathways intact. Therefore, it remains elusive whether the major role of NETs is to trap versus directly kill pathogens *in vivo*. In addition, the biological activity of individual cellular components released into NETs is undetermined. With these gaps in knowledge, the host defense contribution of NETosis versus that achieved by intracellular killing by intact neutrophils remains unsettled (Nauseef, 2012).

Specific pathogens may target NETs or exploit NET products to enhance bacterial invasion. For example, nuclease expression by *Staphylococcus aureus* degrades NETs and augments pathogen virulence *in vivo* (Berends et al., 2010). In addition, bacteria may exploit NET products to enhance microbial virulence. *Shigella flexneri* binds to cationic granular proteins expressed in NETs, which enhances bacterial adherence to and invasion of epithelial cells (Eilers et al., 2010). Paradoxically, excessive NETosis may impair host defense *in vivo*. SerpinB1-deficient mice had increased lung neutrophil NET generation but defective bacterial clearance compared to wild type mice in *Pseudomonas aeruginosa* pneumonia (Farley et al., 2012). While SerpinB1 may be required for host defense independently of its role in NETosis, these results raise the potential for injury caused by excessive NETosis abrogating antibacterial killing.

Neutrophil elastase and MPO are located in neutrophil primary granules, are constituents of NETs, and are required for NET generation in response to specific stimuli (described above). Therefore, deficiencies in these pathways can provide some insight into the role of NET constituents in host defense. The neutrophil serine proteases, NE, cathepsin-G (CG), and proteinase 3 (PR3),

are activated by lysosomal cysteine protease cathepsin C/dipeptidyl peptidase I (DPPI; Pham et al., 2004). Papillon–Lefèvre syndrome is a rare autosomal recessive disease resulting from loss-of-function mutations in the *DPPI* gene locus that is characterized by palmoplantar hyperkeratosis, periodontitis leading to loss of teeth, and severe bacterial infections, including liver abscesses (Almuneef et al., 2003; Pham et al., 2004). MPO-deficient mice have defective candidal killing *in vivo* (Brennan et al., 2001) and neutrophils from MPO-deficient patients are impaired in the ability to limit growth of extracellular *Candida albicans* (Metzler et al., 2011). However, MPO deficiency in humans is usually asymptomatic, although severe candidal infections have been observed in patients with co-existing diabetes (Cech et al., 1979a,b).

NADPH oxidase deficiency leads to a more severe phenotype than Papillon–Lefèvre or MPO deficiency. For example, patients with CGD are at high risk for invasive aspergillosis and specific bacterial pathogens (e.g., *Burkholderia cepacia*, *Serratia marcescens*, and *Nocardia* species; Winkelstein et al., 2000; van den Berg et al., 2009). Consistent with these clinical observations, CGD mice were highly susceptible to infection by *Aspergillus* and by *B. cepacia*, while $NE^{-/-} \times CG^{-/-}$ mice and DPPI-deficient mice were resistant (Vethanayagam et al., 2011).

Additional studies point to specific host defense functions of neutrophil proteases that are distinct from NADPH oxidase. Proteases in human neutrophils target *Streptococcus pneumoniae* (Standish and Weiser, 2009) and protease-deficient mice have increased susceptibility to pneumococcal pneumonia (Hahn et al., 2011). In contrast, neutrophil NADPH oxidase has variable effects on pneumococcal strains *in vitro*; CGD neutrophils have normal killing of pneumococci that produce peroxide, but defective killing of peroxide-deficient mutant strains (Pitt and Bernheimer, 1974; Shohet et al., 1974), suggesting that pathogen-derived reactive oxidants can complement the killing defect in CGD neutrophils.

Together, these observations in the clinic and in mouse models support NADPH oxidase, neutrophil serine proteases, and MPO having distinct functions in host defense. Interventions that deplete NETs *in vivo* (e.g., histone blocking antibody and DNase1; Caudrillier et al., 2012) may be useful to delineate specific host defense functions of NETs.

NADPH OXIDASE AND NETs HAVE DISTINCT EFFECTS ON INFLAMMATION AND INJURY

The generation of NETs can be a double-edged sword. On the one hand, they may promote pathogen killing. However, the same pathways that control microbial infection can also cause injury through a number of mechanisms. NET constituents can damage epithelial and endothelial cells, which can exacerbate inflammation-induced organ injury (Saffarzadeh et al., 2012). The interaction between NETs, platelets, and coagulation further illustrate the concept of the dual host defense and injurious potential of NETs. Platelet-derived defensins have intrinsic antimicrobial activities and can stimulate NETosis (Kraemer et al., 2011). NET constituents (NE, CG, and histones) can activate platelets and promote coagulation leading to intravascular thrombus growth that restrict tissue bacterial invasion (Fuchs et al., 2010, 2011; Massberg et al., 2010); however, these same mechanisms may promote an excessive coagulopathy and thrombosis resulting in endothelial cell injury and organ damage. Recent studies have shown that NETosis can drive transfusion-related acute lung injury and that depleting NETs was protective (Caudrillier et al., 2012; Thomas et al., 2012).

NETosis has also been implicated in the pathogenesis of autoimmune disorders, such as systemic lupus. Autoimmunity may be driven by a combination of factors related to aberrant NETosis, including direct damage to tissue, release of autoantigens that stimulate immune complexes, complement activation, and IFN-α release by plasmacytoid dendritic cells (Guiducci et al., 2010; Hakkim et al., 2010; Meyer-Hoffert and Wiedow, 2010; Garcia-Romo et al., 2011; Lande et al., 2011; Villanueva et al., 2011; Leffler et al., 2012). A subset of patients with systemic lupus have reduced ability to degrade NETs due to impaired serum DNase1 activity, and have a higher incidence of lupus nephritis (Hakkim et al., 2010).

While NETosis has, so far, been shown to enhance neutrophil-mediated injury, the role of NADPH oxidase is more complex and likely involves interplay of injurious and protective pathways. NET constituents such as neutrophil proteases generally lead to augmented inflammation and tissue injury (Adkison et al., 2002; Hu and Pham, 2005; Raptis et al., 2005; Akk et al., 2008; Soehnlein et al., 2008b). However, studies in NADPH oxidase-deficient mice point to a more complex interaction between NADPH oxidase and acute lung injury that is context-dependent. NADPH oxidase worsened the severity of acute lung injury following influenza virus challenge in mice (Imai et al., 2008). While wild type and NADPH oxidase-deficient mice had similar levels of lung injury following LPS challenge (Sato et al., 2002), NADPH oxidase-deficient mice had greater lung neutrophil sequestration, but less lung injury compared to wild type mice in *E. coli* sepsis (Gao et al., 2002). In contrast, NADPH oxidase was protective in acid aspiration-induced lung injury. NADPH oxidase-deficient mice had increased airway neutrophilic inflammation and acute lung injury (measured as albumin leak), but less injury per recovered neutrophil, compared to wild type mice (Segal et al., 2007; Davidson et al., 2013). In addition, NADPH oxidase was required for optimal activation of Nrf2, an ROI-inducible transcriptional factor that stimulates cytoprotective and anti-inflammatory responses (Davidson et al., 2013). Thus, NADPH oxidase likely has a dual effect on inflammation-induced lung injury. While the immediate effects of NADPH oxidase activation leads to ROI generation and possibly NETosis that are predicted to be injurious, NADPH oxidase can also protect against injury by limiting neutrophilic inflammation and by inducing cytoprotective pathways, such as Nrf2.

CONCLUSION

In response to infectious threat, neutrophils go to war. NADPH oxidase is rapidly activated by specific microbial stimuli, leading to ROI generation. NADPH oxidase can activate neutrophil granular proteases within the phagolysosome, thereby targeting phagocytized pathogens. In addition, NADPH oxidase stimulates NETosis, a process that targets extracellular pathogens. While important for host defense, NETs are

also implicated in the pathogenesis of a number of diseases, including acute organ injury and autoimmunity. Understanding mechanisms by which NADPH oxidase and its regulated pathways modulate inflammation and injury may identify novel therapeutic approaches.

There are several gaps in knowledge regarding NETosis. Although NETosis is induced by infection or conditions mimicking infectious threat, we do not have a good understanding of the molecular mechanisms that drive NETosis. NADPH oxidase can stimulate NETosis in certain settings and apoptosis in others; it is unclear how these dual effects are mediated. It is also unclear which pathways stimulate NETosis after neutrophil death and breakdown of membranes versus NETosis in living neutrophils. Additional questions relate to the relative contributions of NETosis versus intracellular killing of pathogens to host defense and whether the major function of NETs is to prevent spread of versus killing of pathogens.

Future translational areas of research involve the application of NETotic products as prognostic biomarkers for inflammatory disorders (e.g., sepsis, autoimmunity). For example, in patients with sepsis, the degree of NETosis may correlate with a better outcome due to enhanced bacterial clearance or a worse outcome reflecting inflammation-induced organ injury. Assays of NETotic markers (e.g., MPO–DNA complexes, NE) from cell-free fluid (e.g., plasma) may provide a quantitative measure of NETosis from archived human samples that could be correlated with clinical outcome in a variety of diseases. This knowledge may pave the way to therapeutic approaches to target NETs, such as use of histone neutralizing antibody or DNase1.

ACKNOWLEDGMENT

This work was supported by grants from the National Institutes of Health: AI079253 (to Brahm H. Segal).

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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.

Received: 18 December 2012; accepted: 07 February 2013; published online: 01 March 2013.

Citation: Almyroudis NG, Grimm MJ, Davidson BA, Röhm M, Urban CF and Segal BH (2013) NETosis and NADPH oxidase: at the intersection of host defense, inflammation, and injury. Front. Immunol. ***4****:45. doi: 10.3389/fimmu.2013.00045*

This article was submitted to Frontiers in Molecular Innate Immunity, a specialty of Frontiers in Immunology.

frontiers in
IMMUNOLOGY

ORIGINAL RESEARCH ARTICLE
published: 24 December 2012
doi: 10.3389/fimmu.2012.00391

Stable redox-cycling nitroxide Tempol inhibits NET formation

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To prevent the spread of pathogens neutrophils as the first line of defense are able to release Neutrophil Extracellular Traps (NETs), a recently discovered form of immune response. Reactive oxygen species (ROS) have been shown to be essential for many different induction routes of NET formation. Therefore, pharmacological inhibition of ROS generation has implications for research and medicine related to NETs. The application of diphenylene iodonium (DPI), an inhibitor of NADPH oxidase activity, is limited due to its toxicity to host cells as well as microbes. Therefore, we investigated the effect of 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (Tempol) a membrane-permeable radical scavenger on NET formation triggered by phorbol esters and *Candida albicans*. We quantified the amount of NETs with two complementary methods, using a microscopic analysis and an online fluorescence-based assay. In line with removal of ROS, Tempol reduced the amount of NET formation by neutrophils challenged with those stimuli significantly. Since Tempol efficiently blocks NET formation *in vitro*, it might be promising to test the effect of Tempol in experimental models of disorders in which NETs probably have hazardous effects.

Keywords: neutrophils, neutrophil extracellular traps, reactive oxygen species, nitroxide, Tempol, NET inhibition

INTRODUCTION

Polymorphonuclear neutrophils (PMNs) are circulating leukocytes and serve as a first line of defense against microbial infections, but are at the same time a main contributor to hazardous effects during inflammation (Nathan, 2006). In humans, approximately 2×10^{11} neutrophils are produced per day (Borregaard, 2010). These professional phagocytes contain a large reservoir of antimicrobial proteins, which they secrete or release into the phagosome containing engulfed microbes (Amulic et al., 2012). The cells express the phagocyte NADPH oxidase complex which enables them to produce superoxide (Segal et al., 2012). The superoxide anion is converted spontaneously or enzymatically driven to other Reactive Oxygen Species (ROS). The exposure to antimicrobial proteins and ROS concertedly kills the phagocytosed microbe (Nathan, 2006).

Neutrophil Extracellular Traps (NETs) have been described as an extracellular mechanism of neutrophils to trap and kill microbes (Brinkmann et al., 2004). NETs consist of nuclear chromatin decorated with antimicrobial proteins. ROS are required to form NETs, as neutrophils from chronic granulomatous disease (CGD) patients, unable to form ROS, do not release NETs (Fuchs et al., 2007). NETs can be induced by various stimuli, such as bacteria, fungi, parasites chemokine IL-8, and protein kinase C activation via Phorbol Myristate Acetate (PMA; Brinkmann et al., 2004; Guimaraes-Costa et al., 2009; Urban et al., 2009; Marcos et al., 2010; Parker et al., 2012). As a consequence of NET release upon these stimuli the neutrophil plasma membrane ruptures and thus, per definition, the NET-releasing cell dies (Fuchs et al., 2007). Other studies proposed alternative mechanisms of DNA trap release that is catapult-like and involves the release of mitochondrial instead of nuclear DNA from viable neutrophils (Yousefi et al., 2009). *Staphylococcus aureus* seems to be able to induce NET formation by triggering the neutrophils to expel their nuclei packaged in vesicles leaving the plasma membrane intact (Pilsczek et al., 2010; Yipp et al., 2012). These findings illustrate the complexity of the process of NETosis and therefore the urgent need for better tools to study the underlying mechanisms in more detail.

Apart from their positive role during infection by preventing dissemination of microbes (Yipp et al., 2012), NETs are assumed to have hazardous effects as well. Thus, NETs may also contribute to harm the host during uncontrolled inflammatory processes. For instance, NETs have been described to be involved in autoimmune and inflammatory disorders, such as Small-Vessel Vasculitis (SVV; Kessenbrock et al., 2009; Nakazawa et al., 2012), Systemic Lupus Erythematosus (SLE; Hakkim et al., 2010b; Villanueva et al., 2011; Leffler et al., 2012), amyloidoses (Azevedo et al., 2012), and in Transfusion-induced Acute Lung Injury (TRALI; Caudrillier et al., 2012; Thomas et al., 2012). Treatment with vitamin C reduced the load of NETs during experimental TRALI in mice (Caudrillier et al., 2012). This in particular indicates that potential NET inhibitors could prove valuable in medical applications for diseases where NET formation is involved.

Since a significant proportion of described NET release mechanisms depend on ROS (Hakkim et al., 2010a), any molecule that prevents the generation of ROS or drives their metabolism should

be able to inhibit the release of ROS-dependent NETs. Therefore, we reasoned that the ROS metabolizing compound Tempol (4-hydroxy-tetramethylpiperidin-1-oxyl) is able to interfere with NET formation. This low molecular weight compound is a stable, membrane-permeable redox-cycling nitroxide, mimetic to the super oxide dismutase (SOD) enzyme by scavenging superoxide radicals (Krishna et al., 1996). The great advantage of Tempol for any potential medical application is its very low toxicity in mammals (Wilcox, 2010). Other compounds that directly block ROS production, e.g., by inhibiting flavin enzymes of the NADPH oxidase complex or myeloperoxidase, such as diphenyleneiodonium (DPI) and sodium azide respectively, are either unspecific or toxic to cells (Riganti et al., 2004). Tempol can metabolize a variety of ROS and protect cells (Hahn et al., 1992) and animals (Goffman et al., 1992) from radiation damages.

To test the effect of Tempol on NET formation we used three different stimuli to induce NETosis: PMA, which has been shown to be ROS-dependent (Fuchs et al., 2007), the fungal pathogen *Candida albicans*, a physiological stimulus that also requires ROS (Ermert et al., 2009), and finally the chemokine IL-8, which has been suggested to induce NET formation ROS-independently (Marcos et al., 2010). For this purpose, the ROS production as well as the amount of netting neutrophils at different time points were determined microscopically and confirmed with an online fluorescence-based assay quantifying extracellular DNA or in more general terms cell death.

We show here that Tempol efficiently removes ROS produced by human neutrophils upon PMA and *C. albicans* stimulation, without negatively affecting phagocytic function of neutrophils. NET formation was inhibited by Tempol in a dose-dependent manner. PMA and *C. albicans*-induced NET formation efficiently as expected, whereas IL-8 did not trigger significant amounts of NETs. This study introduces Tempol as a valuable candidate compound to treat diseases in which NETs have hazardous effects to the host.

MATERIALS AND METHODS

ISOLATION OF NEUTROPHILS

Neutrophils were harvested from blood of healthy volunteers according to the recommendations of the local ethical committee (Regionala etikprövningsnämnden i Umeå) as approved in permit Dnr 09-210M. Fully informed consent was obtained, and all investigations were conducted according to the principles expressed in the Declaration of Helsinki. The isolation of neutrophils was performed as described previously (Fuchs et al., 2007). Briefly, neutrophils were separated from blood with two gradient centrifugation steps. The first one uses Histopaque 1119 (Sigma-Aldrich) to remove red blood cells while the second one uses discontinuous gradients of percoll (Amersham) that separate neutrophils from monocytes, lymphocytes, and residual red blood cells. Subsequently, the cells were washed with 1× PBS + 0.5% human serum albumin (HSA) and resuspended in RPMI 1640 medium without phenol red (Lonza).

CULTURE OF *C. ALBICANS*

An overnight culture of *C. albicans* (SC5314) was inoculated in YPD from a culture dish. After incubation the culture was diluted to a starting OD_{600} of 0.1 in fresh growth medium. To grow *C. albicans* in its yeast form the cells were incubated in YPD at 30°C for 4 h. To produce hyphae the same dilution was used in RPMI medium and the cell suspensions were incubated at 37°C for 4 h. The multiplicity of infection (MOI) for *C. albicans* hyphae was adjusted to the initial number of yeast cells that were used for inoculation, since under these conditions almost 100% filamentation can be expected. The factor 3×10^7 cells/ml at OD_{600} of 1 was used to convert the number of yeast cells in the logarithmic phase to the number of hyphae.

LUMINOL ASSAY

Neutrophil ROS production was determined by luminol bioluminescence as described previously (Ermert et al., 2009). Briefly, cells were seeded in a white 96 well plate at a concentration of 5×10^4 cells per well. Twenty microliters of Tempol in different concentrations or RPMI were added and the cells incubated for 15 min at 37°C. For the assay, luminol (Sigma-Aldrich) and horseradish peroxidase (Sigma-Aldrich) were added to a final concentration of 50 μM and 1.2 U/ml respectively. NET formation was induced just before starting the assay by the addition of PMA at final concentration of 100 nM, yeast or hyphae of *C. albicans* at an MOI of 3, or human recombinant IL-8 at a final concentration of 100 nM (Abcam). ROS were measured by luminescence catalysis in a Tecan Infinite 200 plate reader every 3 min for a period of 3 h.

CELL DEATH ASSAY

Neutrophil cell death or presence of extracellular DNA was measured by fluorescence with Sytox Green (Invitrogen) similar to previous descriptions (Fuchs et al., 2007; Ermert et al., 2009). Briefly, cells were seeded in a black 96 well plate with a concentration of 5×10^4 cells per well in a total volume of 160 μl. Subsequently, Tempol was added in different concentrations and plates were incubated for 15 min at 37°C. After this short incubation, Sytox Green, a membrane-impermeable DNA dye, was added to a final concentration of 2.5 μM, before cells were stimulated with PMA (100 nM final concentration), *C. albicans* yeast or hyphae (both MOI 3) or IL-8 (100 nM). The samples were monitored for fluorescence under cell culture conditions (37°C, 5% CO_2) in a plate-based fluorescence spectrophotometer (Fluostar Omega, BMG) in intervals of 10 min for a total of 10 h. Percent NET formation was calculated as percentage of lysis control. From this value, percent cell death of unstimulated neutrophils was subtracted at the respective time points. Data were presented as percent cell death.

PHAGOCYTOSIS ASSAY

Neutrophil phagocytosis was measured by using pHrodo *S. aureus* bioparticles according to the manufacturer's protocol (Invitrogen). This assay is based on increased fluorescence of the beads upon acidification in the phagosome.

Briefly, cells were seeded in a white 96 well plate at a concentration of 5×10^4 cells per well and pHrodo *S. aureus* BioParticles were added at a final concentration of 25 μg/well in a total volume of 150 μl. Samples containing Cytochalasin D (Sigma-Aldrich) at a final concentration of 10 μg/ml and incubated for 15 min at 37°C to block phagocytosis served as a negative control. As 100% control BioParticles in buffer at pH 4 were used. Tempol or RPMI

was added at different concentrations. Plates were measured in a fluorescence spectrophotometer (Fluostar Omega, BMG) every 30 min for 2 h.

CHEMOTAXIS ASSAY FOR HUMAN NEUTROPHILS USING TRANSWELL INLETS

To test bioactivity of human recombinant IL-8 (Abcam) neutrophil migration was measured in a transwell system. The neutrophils were labeled with a fluorescent cytoplasmic dye, BCECF-AM (Sigma-Aldrich) at a final concentration of 3.3 μM and 5×10^5 neutrophils were seeded on a fluorescence-impermeable transwell membrane (BD Falcon, HTS FluoroBlok Insert, 3.0 μM pore size). The transwell was placed into a 24-well tissue culture plate (BD Falcon) just before measurement. The lower compartment of the tissue culture plate contained 600 μl of RPMI with 0.05% HSA and 10 nM human recombinant IL-8 was added as chemoattractant. For base line migration the lower compartment contained medium only. As 100% control served 5×10^5 BCECF-AM labeled neutrophils without inserting the transwell system. The samples were monitored for fluorescence under cell culture conditions (37°C, 5% CO_2) in a fluorescence spectrophotometer (Fluostar Omega, BMG) in intervals of 1 min for a total of 10 min. Chemotaxis was plotted as percentage of total signal and base line signal was subtracted.

IMMUNOSTAINING OF NEUTROPHILS

Cells were seeded on cover slips coated with 0.01% poly-L-lysine (Sigma-Aldrich) in 24-well plates with a concentration of 1×10^5 cells per well in a total volume of 500 μl. Tempol was added to a final concentration of 30 mM and incubated for 15 min. Subsequently, cells were induced with PMA (100 nM final concentration) *C. albicans* yeast or hyphae (MOI 3) or IL-8 (100 nM) for 3 and 6 h at 37°C and 5% CO_2. After incubation the cells were fixed using paraformaldehyde (2% final concentration) for 30 min at RT. Specimens were stored at 4°C. For immune staining the cover slips were washed three times with PBS and permeabilized with 0.5% TritonX-100 (Merck) in PBS. Cells were blocked at 37°C for 1 h in blocking buffer containing 3% cold water fish gelatin (Sigma-Aldrich), 1% gelatin from bovine serum (Sigma-Aldrich), 5% donkey serum (Jackson Immuno research), and 0.25% Tween 20 (VWR). Antibodies directed against histone H1 (1.25 μg/ml; #BM465, Acris) and directed against neutrophil elastase (6 μg/ml; #481001, Calbiochem) were used and incubated for 1 h at 37°C. Secondary antibodies conjugated to Cyanine dyes (Cy 2 and Cy 3, Jackson Immuno research) were used. DNA was stained with DAPI and washed one time with double distilled water. Specimens were mounted in Mowiol 4-88 (Calbiochem) and images were captured using a 20× objective (Nikon 90i fluorescence microscope). The neutrophil elastase staining was confirmatory to validate the presence of NETs and is not presented here. Images were analyzed with NIS-Elements analysis software ver. 3.20.

MICROSCOPIC QUANTIFICATION OF NETS

We adapted previously described methods for NET quantification microscopically, such as Ermert et al. (2009), Papayannopoulos et al. (2010), Keshari et al. (2012), Parker et al. (2012). Microscopic image analysis was performed using histone immune-stained samples including five random images and counting at least 400 cells from each condition. ImageJ version 1.44p software was used to measure pixel areas by adjusting the threshold above background. Measured pixel areas were converted to μm^2 (With 200-fold magnification 472 pixels equal 100 μm^2). Particles comprising an area of less than 15 μm^2 were excluded from analysis. Human neutrophils have an average diameter of 10 μm, hence their average area in an unstimulated stage is approximately 80 μm^2 (assuming a circle and using $\pi \times r^2$). To quantify the number of cells that underwent NET formation we chose signal events that exceeded 100 μm^2 and thus were larger than the whole intact cell area. Events larger than 100 μm^2 were either considered as decondensed nuclei, an essential step prior to NETosis, or as released NETs. The total number of cells was counted and NET-forming cells were expressed as percentages of events larger than 100 μm^2 from the total number of events. Three independent experiments using neutrophils from three different donors were analyzed for each sample.

STATISTICAL ANALYSIS

Tukey one-way ANOVA was applied for statistical analysis of the data using the software GraphPad Prism v 5.00.

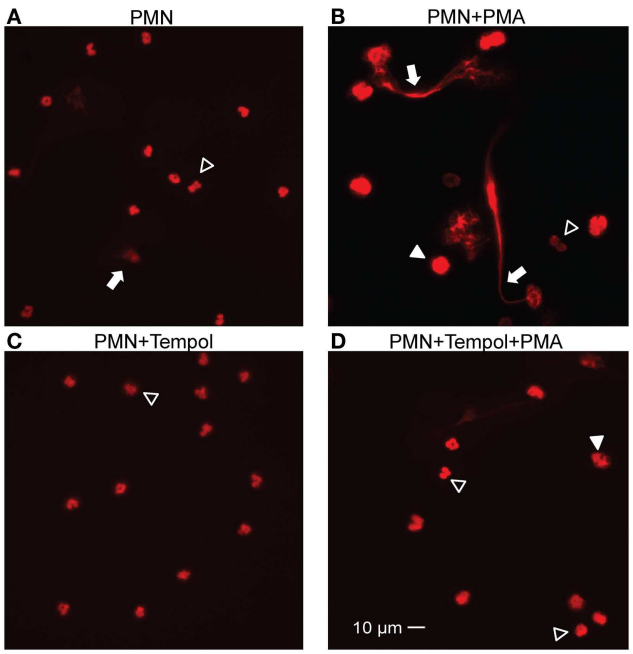

FIGURE 1 | Tempol reduces NET formation stimulated by PMA. Indirect immunofluorescence of PMA-stimulated PMNs (100 nM) for 6 h in the presence or absence of Tempol (30 mM). Unstimulated PMNs and PMNs incubated with Tempol only served as control. NET formation is visualized after fixation using staining of chromatin with a primary antibody directed against histone H1 and a Cy-3 conjugated secondary antibody. The representative microscopic images illustrate unstimulated PMNs **(A)**, PMNs stimulated with PMA **(B)**, PMNs treated with Tempol **(C)**, and PMNs treated with Tempol and stimulated with PMA **(D)**. NETs are indicated by arrows, decondensed nuclei with filled arrow heads. Open arrow heads show intact PMNs. Images were captured with a Nikon Eclipse 90i microscope and a Hamamatsu Orca-ER charge-coupled device camera using a 40× objective. Scale bar: 10 μm.

RESULTS

TEMPOL SCAVENGES ROS PRODUCED BY PMA-STIMULATED NEUTROPHILS

Protein kinase C (PKC) stimulation by the phorbol ester PMA is a well-known strong inducer of NET formation (Brinkmann et al., 2004). PMA-induced NET formation has additionally been shown to be ROS-dependent (Fuchs et al., 2007). Therefore, we tested the ROS scavenger Tempol for inhibition of NET formation. We stimulated neutrophils with PMA that have either been previously incubated with Tempol or with culture medium only. We analyzed the samples by microscopic immunofluorescence as described in Materials and Methods. After 6 h of incubation we observed NET formation in the mock-treated specimen. Patchy, web-like structures were present that stained positive for histone, a major component of NETs, and decondensation of nuclear chromatin could be observed (**Figure 1**). Decondensation of neutrophil nuclei is a hallmark of NET formation and occurs previous to the release of NETs (Fuchs et al., 2007). In contrast, the Tempol-treated neutrophils formed significantly less NETs and the majority of nuclei were lobulated indicating that they were still intact (**Figure 1**). Thus, we concluded that Tempol has NET-inhibitory activity.

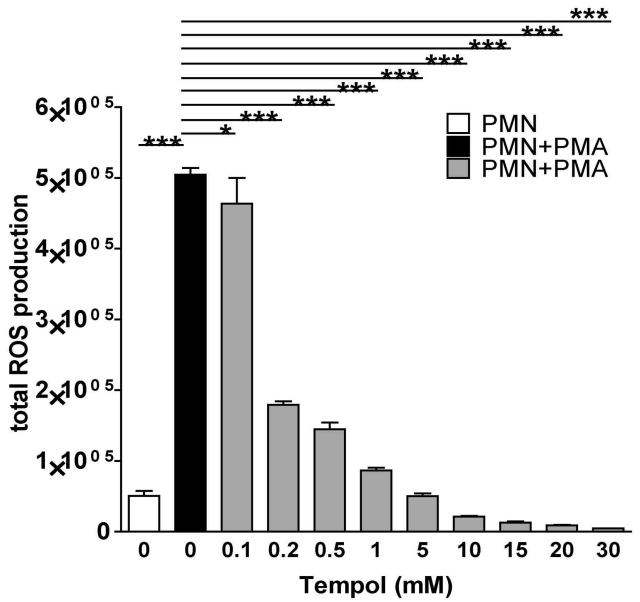

FIGURE 2 | Tempol scavenges neutrophil ROS triggered by PMA. The total amount of ROS generated by PMNs over the course of three 3 h is plotted against increasing concentrations of Tempol. The half maximal inhibitory concentration (IC50) is 0.2 mM Tempol. ROS amounts were calculated as area under the curve (AUC). White bar: unstimulated PMNs, black bar: PMNs stimulated with 100 nM PMA, grey bars: PMNs treated with different concentration of Tempol prior to stimulation with PMA. Significance was analyzed by Tukey one-way ANOVA (*$P \leq 0.05$ and ***$P \leq 0.001$). One representative experiment out of three independent experiments with three different donors is shown. Data are presented as means of three technical replicates ±SD.

First, we aimed to identify adequate concentrations of Tempol to remove ROS produced by neutrophils. For this purpose, we induced neutrophils with PMA in the presence of different concentrations of Tempol and subsequently measured the amount of ROS produced in a luminol-based plate assay. Tempol at a concentration of 0.2 mM removed approximately 50%, 1 mM 75% and 10 mM more than 90% of PMA-induced ROS (**Figure 2**). Thus, Tempol was able to remove PMA-induced neutrophil ROS efficiently and in a dose-dependent manner.

TEMPOL INHIBITS PMA-INDUCED ROS-DEPENDENT NET FORMATION

We next investigated the effect of Tempol on NET formation in a quantitative manner. To unambiguously determine NET formation we chose a microscopic approach. Neutrophils were stimulated with PMA in the presence of 0, 15, or 30 mM Tempol and then fixed after 3 and 6 h, respectively. The cells were labeled with an anti-histone antibody and analyzed by immunofluorescence microscopy. We adapted previously described methods to quantify NET formation microscopically, such as Ermert et al. (2009), Papayannopoulos et al. (2010), Keshari et al. (2012), Parker et al. (2012). Determining the area occupied by intact cells or NETs – as expressed in μm^2 – respectively, provides an appropriate measure to distinguish NETs from intact or non-NETotic dead neutrophils and thereby representing a means to assay NET

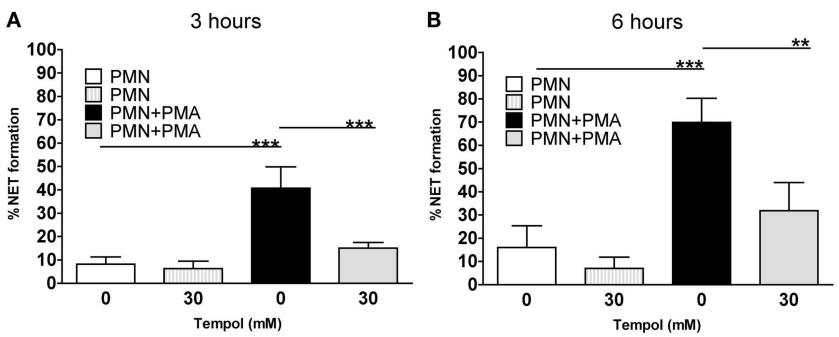

FIGURE 3 | Tempol prevents PMA-induced NET formation. Microscopic evaluation of NET formation by PMNs stimulated with 100 nM PMA reveals reduced formation of NETs in the presence of Tempol. Percentage of NET formation after 3 h **(A)** and after 6 h **(B)**. White bars: unstimulated PMNs, dashed gray bars: PMNs treated with Tempol only (30 mM), black bars: PMNs stimulated with PMA, gray bars: Tempol-treated PMNs stimulated with PMA. The percentage of cells undergoing NET formation was computed by using ImageJ as explained in materials and methods. Significance was analyzed by Tukey one-way ANOVA (**$P \leq 0.01$ and ***$P \leq 0.001$). Data are presented as means ± SD of three independent experiments with three different donors.

formation in a quantitative manner (see Materials and Methods). Using this microscopic approach we determined that 40% of PMA-stimulated neutrophils underwent NETosis after 3 h and 70% after 6 h (**Figure 3**). Notably, 30 mM Tempol reduced NET formation significantly to 15% after 3 h and to 30% after 6 h (**Figure 3**). Background levels of NETs from unstimulated neutrophils were usually around 20% after 6 h of incubation. In conclusion, Tempol treatment reduced PMA-induced NET formation to background levels, comparable to unstimulated neutrophils (**Figure 3B**).

To confirm these results with a microscopy-independent assay, we used a fluorescence-based cell death assay (CDA) taking advantage of the fact that the plasma membrane ruptures during NET release (Ermert et al., 2009). Neutrophils were treated with PMA in the presence of the membrane-impermeable DNA dye Sytox Green, only detecting extracellular DNA or DNA that is not surrounded by an intact membrane. The samples were monitored for fluorescence under cell culture conditions. NET formation was calculated as percentage of lysis control (equaling 100%) and additionally, background levels from unstimulated neutrophils were subtracted (see Materials and Methods).

In accordance to the microscopic analysis, Tempol reduced PMA-induced NET formation, as indicated by a decrease in fluorescence in the corresponding samples (**Figure 4A**). To be able to directly compare these results to the microscopic analysis, we extracted the values at 3 and 6 h from these measurements, respectively. The effect of Tempol was dose-dependent resulting in less than 10% cell death after 3 h (**Figure 4B**) and 20% cell death at 15 mM as well as 15% cell death at 30 mM Tempol after 6 h (**Figure 4C**). A specific mode of action for Tempol in reducing NET release is therefore highly probable. Notably, Tempol even decreased spontaneous cell death in the unstimulated control as verified by the same assay up to concentrations of 100 mM, clearly demonstrating that Tempol has very low toxicity at these concentrations (**Figure A1** in Appendix).

Taken together both the microscopic analysis and CDA confirmed that Tempol is able to block NET formation efficiently. Moreover, both assays resulted in highly similar levels of NET formation by PMA-stimulated neutrophils, suggesting that both assays are suitable to quantify NET formation.

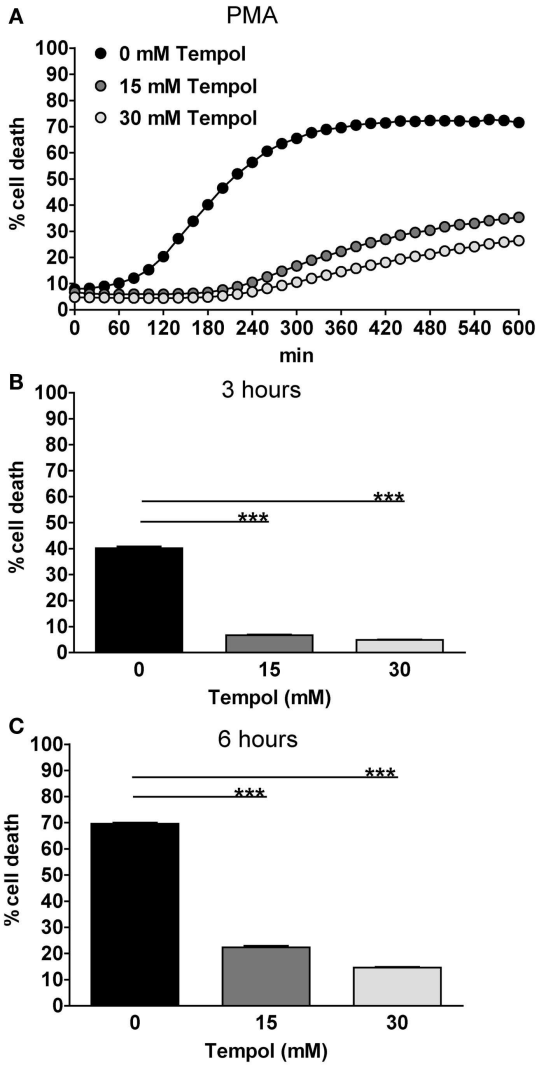

FIGURE 4 | Tempol prevents PMA-induced NET formation quantified by cell death assay. Tempol reduces PMN cell death in a dose-dependent manner. The rate of cell death of PMNs stimulated with the highly potent NET-inducer PMA in the presence or absence of Tempol was analyzed over a period of 10 h by measurement of DNA-staining by the cell-impermeable dye Sytox Green. **(A)** The *Y*-axis represents the relative amount of dead cells after normalization to the lysis control (100%). Background values (unstimulated cells) were subtracted from all values. To allow better comparison with microscopic analyses, the effect of Tempol on PMA-induced PMN cell death after stimulation for 3 h **(B)** and 6 h **(C)** is additionally depicted as bar panels. **(A)** Black curve: PMNs stimulated with 100 nM PMA without Tempol treatment, gray curves: PMNs treated with different concentrations of Tempol prior to stimulation with PMA. Significance was analyzed by Tukey one-way ANOVA $***P \leq 0.001$). One representative experiment out of three independent experiments with three different donors is shown. Data are presented as means of three technical replicates ±SD.

TEMPOL SCAVENGES ROS PRODUCED BY *C. ALBICANS*-STIMULATED NEUTROPHILS

Since PMA is a very potent, however non-physiological stimulus of NET formation, we used *C. albicans* to induce NETosis in neutrophils representing the most frequent fungal pathogen in humans. *C. albicans* is dimorphic (Sudbery et al., 2004) and both growth forms, yeast cells as well as filamentous hyphae are able to trigger NET formation by neutrophils (Urban et al., 2006). Therefore, we included both growth morphologies in this study.

To identify the Tempol concentrations which are sufficient to remove neutrophil ROS triggered by *C. albicans* and consequently potentially abolish NET formation, we measured ROS production in the presence of 0 mM up to 30 mM Tempol in the infection assays (**Figure 5**). Ten millimolar Tempol reduced yeast-triggered neutrophil ROS down to 2.5% of the amount produced in the absence of Tempol. ROS were further reduced in a dose-dependent manner to 0.5% at 30 mM Tempol. Similarly, hyphae-triggered ROS was reduced by Tempol, but with no further decrease at 30 mM Tempol. We concluded that 30 mM Tempol is sufficient to efficiently remove *C. albicans*-induced neutrophil ROS.

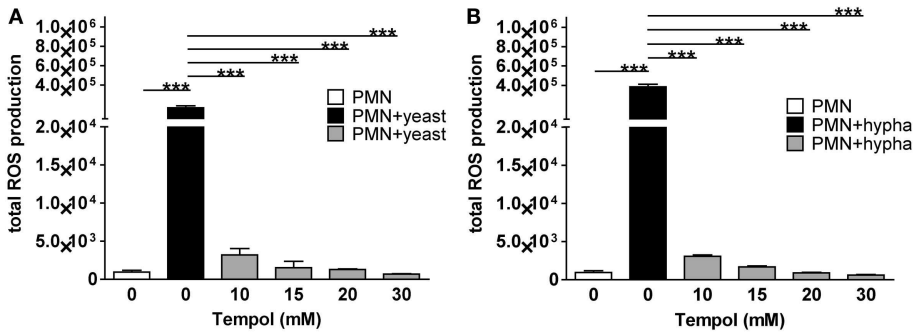

FIGURE 5 | Tempol scavenges neutrophil ROS triggered by different growth forms of *C. albicans*. The total amount of ROS generated by PMNs is plotted against increasing concentrations of Tempol present in the culture medium. PMNs were infected with *C. albicans* (MOI 3) in the presence of different concentrations of Tempol. The total amount of ROS produced over 3 h was calculated as area under the curve (AUC). PMNs infected with of *C. albicans* yeast **(A)** and hyphal cells **(B)**, respectively. White bars: unstimulated PMN, black bars: PMNs infected with *C. albicans*, gray bars: PMNs treated with different concentration of Tempol prior to stimulation with fungi. Significance was analyzed by Tukey one-way ANOVA (***$P \leq 0.001$). One representative experiment out of three independent experiments with three different donors is shown. Data are presented as means of three technical replicates ±SD.

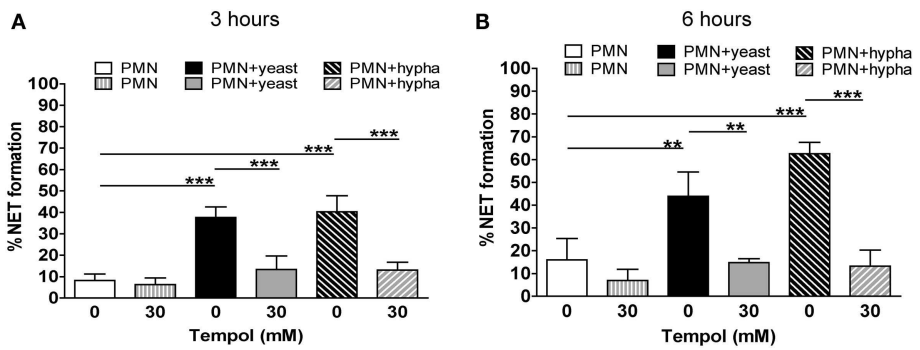

FIGURE 6 | Tempol prevents NET formation induced by different growth forms of *C. albicans*. Microscopic evaluation of *C. albicans* infected PMNs (MOI 3) demonstrates reduction of NET formation by Tempol. Percentage of NET formation after infection with *C. albicans* yeast or hyphal cells for 3 h **(A)** and 6 h **(B)**. White bars: unstimulated PMNs, dashed gray bars: PMNs treated with Tempol, black bars: PMNs stimulated with *C. albicans* yeast growth form, gray bars: PMNs treated with Tempol prior to infection with yeast, diagonal dashed black bars: PMNs stimulated with *C. albicans* hyphae, diagonal dashed gray bars: PMNs treated with Tempol prior to infection with hyphae. The percentage of cells undergoing NET formation was calculated as explained in materials and methods. Significance was analyzed by Tukey one-way ANOVA (**$P \leq 0.01$ and ***$P \leq 0.001$). Data are presented as means ±SD of three independent experiments with three different donors.

TEMPOL INHIBITS *C. ALBICANS*-INDUCED ROS-DEPENDENT NET FORMATION

As *C. albicans* induces NET formation in a strictly ROS-dependent manner (Ermert et al., 2009), we next investigated the effect of Tempol on NET formation induced by the fungal pathogen using the microscopic analysis described above. In these assays, yeasts induced 35% NETs after 3 h and 45% after 6 h of incubation (**Figures 6A,B**; solid bars). Thirty millimolar Tempol significantly reduced NET formation at 3 h to approximately 10% and at 6 h to 15% after stimulation with *C. albicans* yeasts. Similar results were obtained when hyphae were used to trigger NET formation (**Figures 6A,B**; dashed bars). Tempol reduced NET formation from 40% after 3 h and 65% after 6 h to 10 and 15%, respectively. Consequently, 30 mM Tempol was able to reduce NET formation induced by *C. albicans* yeast and hyphae to background levels.

To confirm the microscopic quantification we used the CDA (**Figure 7**) as described for PMA-stimulated neutrophils. Upon infection with yeast *C. albicans,* 40% neutrophil death was observed (**Figure 7A**) and 45% neutrophil death upon hyphae (**Figure 7B**) after 10 h of infection. To directly compare these results to the microscopic analysis, we extracted the values at 3 and 6 h, respectively (**Figures 7C–F**). Yeast induced 10 and 20% cell death above background of unstimulated neutrophils after 3 and 6 h, respectively (**Figures 7C,E**). Tempol at 15 and 30 mM reduced neutrophil cell death below 10% at all time points (**Figures 7C,E**). When hyphae were used for infection we observed 10% increase of neutrophil cell death above background after 6 h (**Figures 7D,F**). Again, Tempol reduced neutrophil cell death triggered by hyphae to 0%, indicating that Tempol inhibits ROS-dependent NET formation.

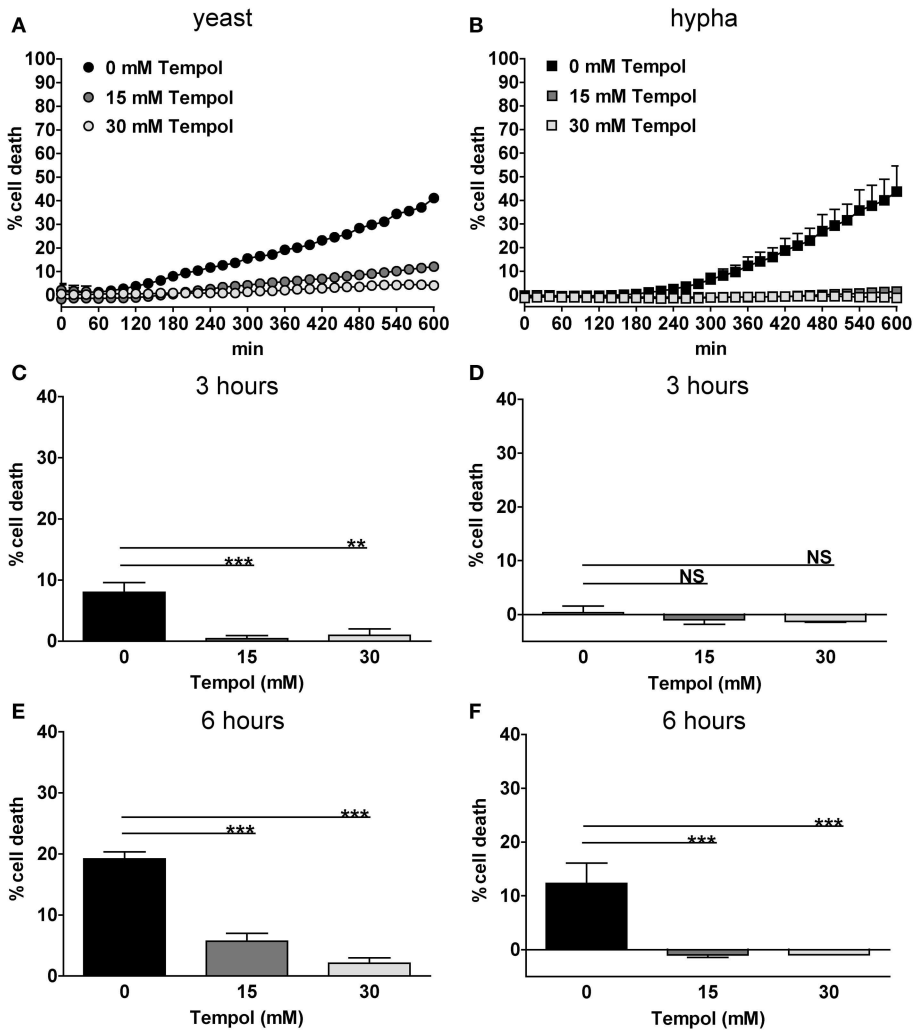

FIGURE 7 | Tempol prevents *C. albicans*-induced NET formation quantified by cell death assay. Tempol reduces cell death during infection with *C. albicans*. PMN cell death rates after infection with different growth morphologies of *C. albicans* in the presence or absence of Tempol were analyzed over a period of 10 h by measurement of DNA-staining by the cell-impermeable dye Sytox Green. The *Y*-axis represents the relative amount of dead cells after normalization to the lysis control (100%). Background values (unstimulated cells) were subtracted from all values. PMN cell death induced by the yeast **(A)** or the hyphal **(B)** growth form of *C. albicans* (MOI 3). Black curves: PMNs infected with *C. albicans*, gray curves: PMNs infected with *C. albicans* in the presence of different concentrations of Tempol. To allow for better comparison with microscopic analyses, the effect of Tempol on PMN cell death after infection with *C. albicans* at 3 h **(C,D)** and 6 h **(E,F)** time points was compared in respect of the different cell morphotypes used. Significance was analyzed by Tukey one-way ANOVA (NS: $P > 0.05$, $^{**}P \leq 0.01$, and $^{***}P \leq 0.001$). One representative experiment out of three independent experiments with three different donors is shown. Data are presented as means of three technical replicates ±SD.

Taken together, the results from the microscopic quantification and from the CDA are confirmatory and thus advantageous to singular assays. As the CDA is much less laborious as the microscopic analysis, this assay represents a valuable tool to quantify NET formation in a simple and robust manner.

IL-8 AND *C. ALBICANS* CO-STIMULATION DOES NOT INCREASE NET FORMATION BY HUMAN NEUTROPHILS

Since IL-8 was shown to prime neutrophils to produce more ROS upon stimulation with the staphylococcal product fMLP (Brechard et al., 2005), we aimed to elucidate whether treatment of neutrophils with IL-8 prior to infection with both morphotypes of *C. albicans* additionally enhances neutrophil NET formation. However, IL-8 and *C. albicans* did not synergize to result in increased NET formation as observed using the CDA (**Figures 8A,B**). The bioactivity of recombinant IL-8 was confirmed with a neutrophil chemotaxis assay showing that human neutrophils migrated significantly toward the chemoattractant (**Figure 8C**). We also investigated the contribution of IL-8 alone to ROS production and NET formation. We stimulated neutrophils with 100 nM IL-8 as described previously (Marcos et al., 2010). Compared to PMA or *C. albicans*, in our hands IL-8 did not induce significant amounts of ROS and neither a significant increase in NET formation as shown by microscopic analysis and the CDA (data not shown).

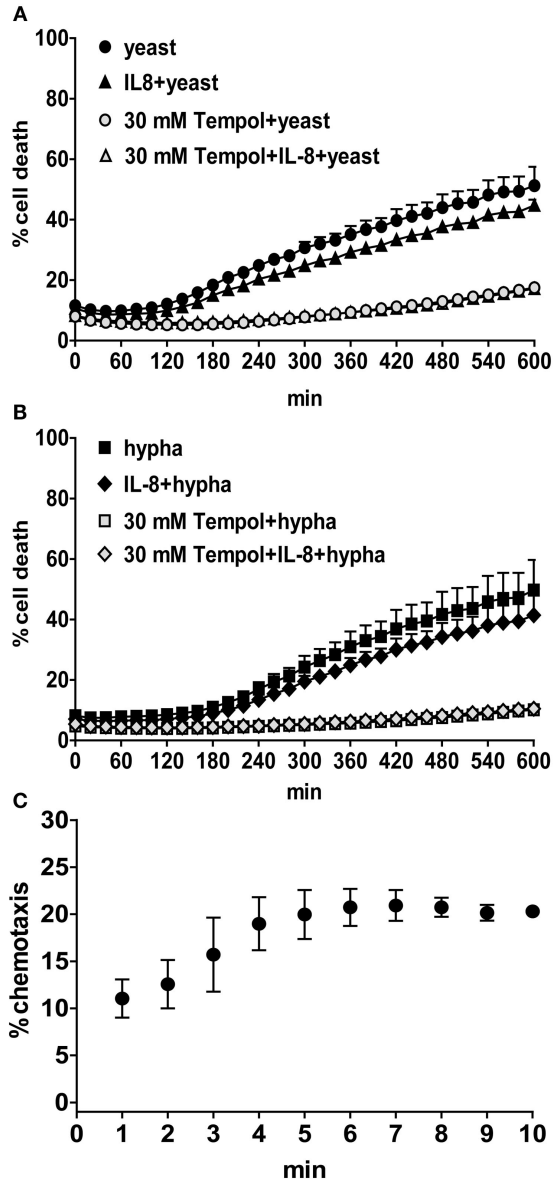

FIGURE 8 | During infection with *C. albicans* IL-8 does not enhance neutrophil NET formation. The rate of cell death of PMNs treated with 100 nM IL-8 for 30 min prior to infection with *C. albicans* (MOI 3) was analyzed over a period of 10 h by measurement of DNA-staining by the cell-impermeable dye Sytox Green. The *Y*-axis represents the relative amount of dead cells after normalization to the lysis control (100%). Background values (unstimulated cells) were subtracted from all values. Black symbols: PMNs infected with yeast [**(A)**, circles and triangles] or hyphal cells [**(B)**, squares and rhombuses] and additionally with IL-8 (triangles and rhombuses); gray symbols: PMNs additionally treated with 30 mM Tempol. One representative experiment out of three independent experiments with three different donors is shown. Data are presented as means of three technical replicates ±SD. Neutrophil chemotaxis was measured in a transwell assay **(C)**. The *Y*-axis represent the percentage of neutrophil migration toward 10 nM IL-8 normalized to 100% florescent labeled cells. The base line levels (migrated cells without chemoattractant) were subtracted from all values. One representative experiment out of three independent experiments with three different donors is shown. Data are presented as means of three technical replicates ±SD.

DISCUSSION

We showed that Tempol removed neutrophil-produced ROS effectively in a dose-dependent manner upon PMA and *C. albicans* stimulation. For both stimuli ROS are essential to result in NET formation. In line with this, Tempol reduced the amount of NET release upon PMA and *C. albicans* stimulation. We confirmed that Tempol effectively inhibits NETosis with two independent quantitative assays, using a microscopic approach as well as a fluorescence-based CDA. For PMA-stimulated NET formation both assays correlated extremely well leading to similar results. Using *C. albicans* as a physiological stimulus the microscopic and cell death analyses diverged to a larger extent. Percentage of NET release as determined microscopically was higher up to 6 h after infection with *C. albicans* as compared to neutrophil cell death, as determined by detection of DNA no longer protected by a membrane. Since neutrophil cell death increased up to 50% in later time points (up to 10 h), we assumed that there might be a delay of NET release after decondensation of nuclei. Decondensed nuclei are also classified as NETotic events in the microscopic analysis, whereas nuclei decondense already within an intact plasma membrane and, therefore, are not accessible to the CDA. Additionally, in contrast to the microscopic analysis, the background signal resulting from unstimulated neutrophils was subtracted at any given time point in the CDA. To illustrate the observed effect more clearly we randomly selected two more CDAs showing *C. albicans* infected neutrophils (**Figure A2** in Appendix). Overall, neutrophil cell death is lower upon *C. albicans* infection as compared to PMA stimulation, particularly when hyphae are used. The absolute values for neutrophil cell death vary the most for this microbial stimulus, probably due to donor variations and thus we did not combine independently performed CDAs, but rather representative experiments. Usually, neutrophil cell death upon infection with *C. albicans* yeast or hyphae ranges from 25 to 50% after 10 h. Regardless, in each experiment we detected NETs microscopically or by cell death and in both assays Tempol significantly reduced the signal. In contrast to previous findings, we did not observe a significant increase in NET formation upon IL-8 stimulation (Brinkmann et al., 2004; Marcos et al., 2010).

In more recent publications it has been described that alive neutrophils release NETs either from their mitochondria or from the nucleus both through an intact plasma membrane (Yousefi et al., 2009; Yipp et al., 2012). These observations notwithstanding, our findings are still valid for all different types of NET formation that may coexist, as we microscopically validate the presence of NET structures. Moreover, the CDA detects any extracellular DNA that is released irrespective of the fate of the netting neutrophil, since similar membrane-impermeable dyes were used in the mentioned studies (Yousefi et al., 2009; Yipp et al., 2012).

The formation of NETs has been the focus of a large amount of recent studies which addressed the role of these structures in health and disease (Remijsen et al., 2011; Brinkmann and Zychlinsky, 2012). Evidence accumulates that NETs, besides their beneficial role in infection, can also contribute to the pathogenesis of other diseases affecting the immune system in a negative way, e.g., promoting inflammation. The presence of NETs was reported for instance for SLE (Hakkim et al., 2010b; Villanueva et al., 2011;

Leffler et al., 2012), autoimmune SVV (Kessenbrock et al., 2009; Nakazawa et al., 2012), allergic asthmatic airways (Dworski et al., 2011), TRALI (Caudrillier et al., 2012; Thomas et al., 2012), amyloidoses (Azevedo et al., 2012), and cancer (Demers et al., 2012).

The emerging body of evidence for positive as well as negative NET-mediated effects on the health status of the host urge us to search for compounds that are able to interfere with the process of NET formation. A recent study attempted this by systematically screening for compounds that block NET formation. The authors identified NADPH oxidase activation and the Raf-MEK-ERK pathway to be crucial for NET formation (Hakkim et al., 2010a). Since ROS are crucial for many, although certainly not all, NET-inducing stimuli, we reasoned that any ROS interfering compound may used to block NETs. Tempol displays low toxicity in humans, as revealed by a clinical trial where Tempol was applied as topical treatment in a concentration as high as 400 mM (Metz et al., 2004). Systemic administration of 150 µl of 150 mM Tempol in mice resulted in 8.1 mM Tempol in the blood (Davis et al., 2010). Therefore, the amounts required for blockage of NET formation *in vitro* ranging between 10 and 30 mM seem ambitious to be reached *in vivo*, since our *in vitro* findings are 1.2- to 3.7-fold above this *in vivo* concentration. It is very likely that *in vivo* additional factors influence NET formation as compared to *in vitro* experiments. Therefore, future experiments in animals will have to give evidence on whether Tempol reduces NET formation *in vivo*. Application of Tempol seems plausible, since the neutrophils in our assays were not affected by Tempol concentrations as high as 100 mM (**Figure A1** in Appendix). Furthermore, Tempol is indeed less toxic than other compounds interfering directly with the activity of the phagocyte oxidase complex, such as DPI (Riganti et al., 2004). Therefore, it might be advantageous to remove ROS after production and not to prevent their production at all. Since Tempol is membrane-permeable, intracellular ROS, which are crucial signaling molecules (Cap et al., 2012), might be removed and this in turn could hamper signaling processes. Of note, we demonstrated that Tempol treatment did not negatively affect phagocytosis of neutrophils, but rather increase engulfment of staphylococcal particles (**Figure A3** in Appendix). However, it might not be recommendable to use Tempol in acute infections, since microbicidal mechanisms of phagocytes are undoubtedly affected by removal of ROS.

In conclusion, our study demonstrates that Tempol blocks PMA- and Candida-triggered NET formation *in vitro*. These findings suggest Tempol as a tool to study mechanisms of NET formation and may serve as a promising starting point to investigate the potential of Tempol for the reduction of *in vivo* NET formation.

ACKNOWLEDGMENTS

We would like to thank Marc Röhm for critically reading the manuscript and Patrik Rydén for advice in statistical analysis. Jenny Johansson Söderberg and Ulrich von Pawel-Rammingen we acknowledge for supplying reagents. This work was supported by grants from the Swedish Research Council VR-M (K2012-99X-21961-01-3), the Laboratory for Molecular Medicine Sweden (MIMS), the Medial Faculty Umeå (316-886-10), and the Cancer Research Foundation in Northern Sweden (AMP 11-684). Ava Hosseinzadeh acknowledges financial support from the J.C. Kempes Memorial Fund.

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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.

Received: 28 September 2012; accepted: 05 December 2012; published online: 24 December 2012.

Citation: Hosseinzadeh A, Messer PK and Urban CF (2012) Stable redox-cycling nitroxide Tempol inhibits NET formation. Front. Immun. ***3****:391. doi: 10.3389/fimmu.2012.00391*

This article was submitted to Frontiers in Molecular Innate Immunity, a specialty of Frontiers in Immunology.

APPENDIX

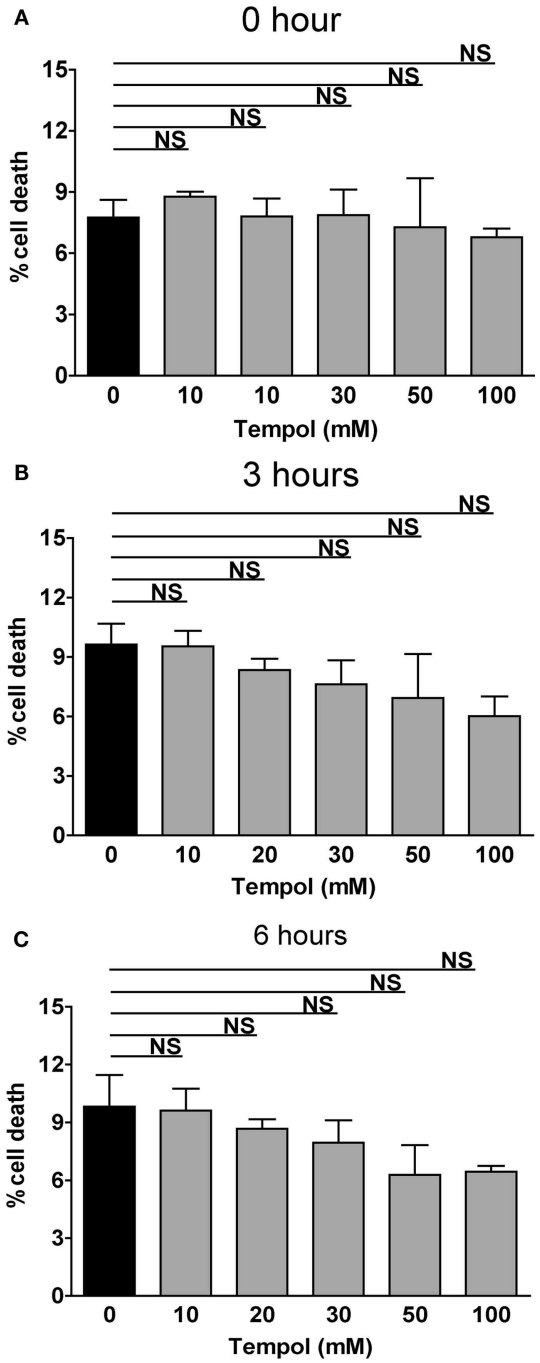

FIGURE A1 | Tempol alone is not toxic to human neutrophils. The rate of cell death of PMNs stimulated with Tempol was analyzed over a period of 6 h by measurement of DNA-staining by the cell-impermeable dye Sytox Green. The *Y*-axis represents the relative amount of dead cells after normalization to the lysis control (100%). PMNs treated with Tempol in a dose-dependent manner at time point 0 **(A)**, after 3 h **(B)**, and after 6 h **(C)**. Black bars: unstimulated PMNs, gray bars: PMNs treated with Tempol at different concentrations. Significance was analyzed by Tukey one-way ANOVA (NS: $P > 0.05$). One representative experiment out of three independent experiments with three different donors is shown. Data are presented as means of three technical replicates ±SD.

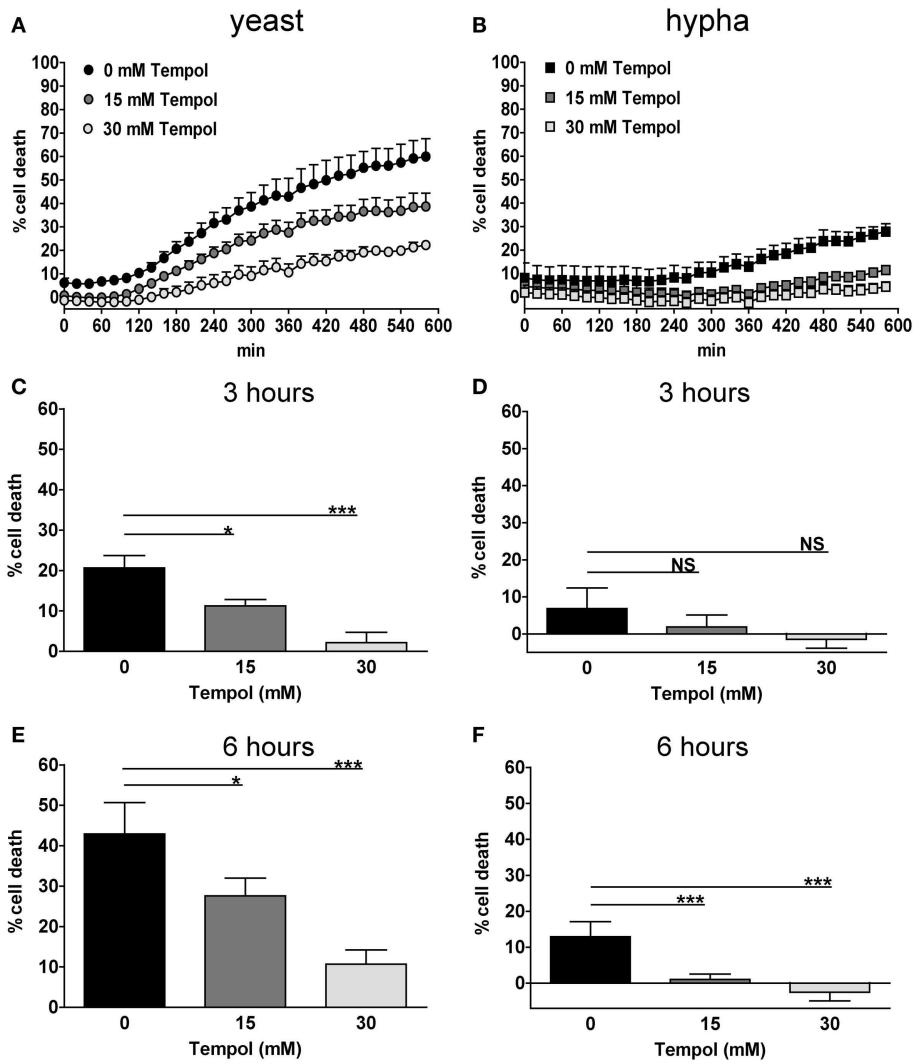

FIGURE A2 | Continued

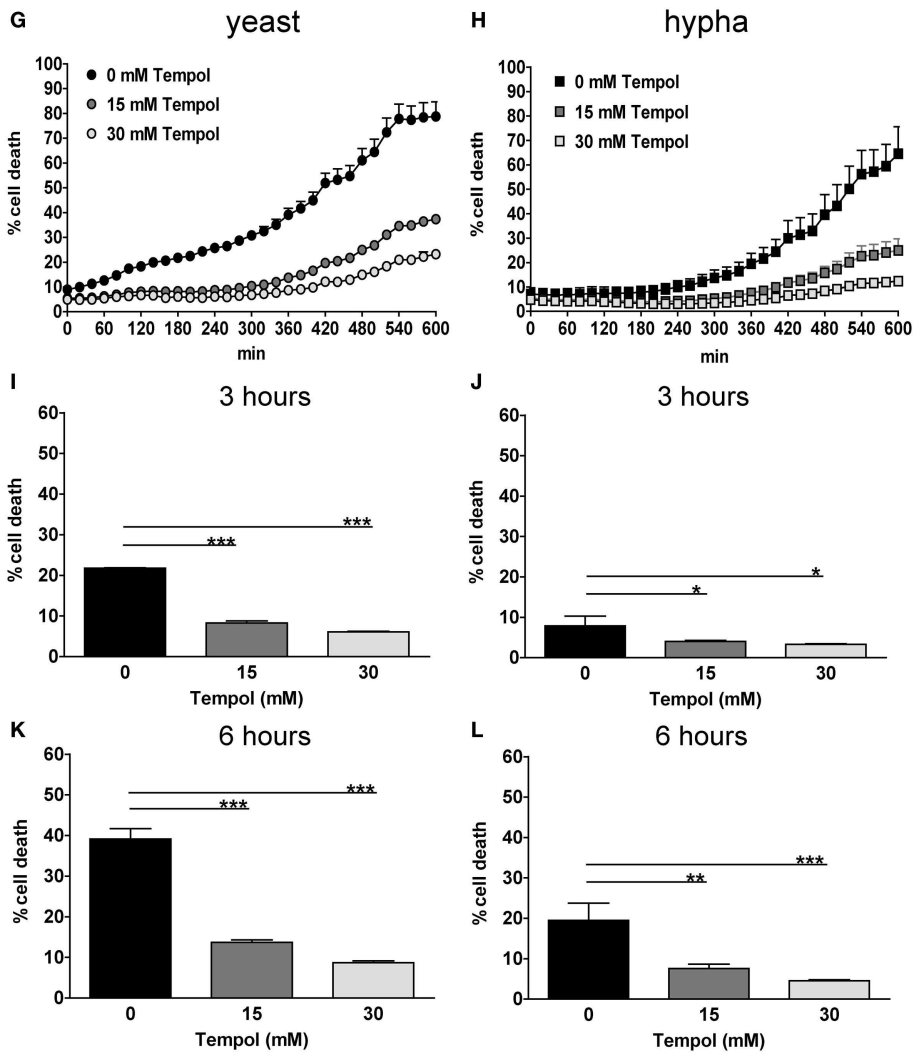

FIGURE A2 | Tempol prevents cell death induced by different growth forms of *C. albicans*, additional representative results. The addition of Tempol to the culture medium reduces cell death during infection with *C. albicans*. The rate of cell death of PMNs infected with different growth forms of *C. albicans* was analyzed over a period of 10 h by measurement of DNA-staining by the cell-impermeable dye Sytox Green. The *Y*-axis represents the relative amount of dead cells after normalization to the lysis control (100%). Background values (unstimulated cells) were subtracted from all values. Results are shown for two additional independent donors as rate of PMN cell death induced by yeast **(A,G)** or hyphal cells **(B,H)** of *C. albicans* (MOI 3). Black curves: PMNs infected with *C. albicans*, gray curves: PMNs treated with different concentrations of Tempol. Increasing concentrations of Tempol cause a reduction of PMN cell death upon infection with yeast **(C,I)** or hyphal cells **(D,J)** for 3 h. Percentage of dead cells after 6 h upon infection with yeast or **(E,K)** or hyphal cells of *C. albicans* **(F,L)**. Significance was analyzed by Tukey one-way ANOVA (NS: $P > 0.05$, $*P \leq 0.05$, $**P \leq 0.01$, and $***P \leq 0.001$). Data are presented as means of three technical replicates ±SD.

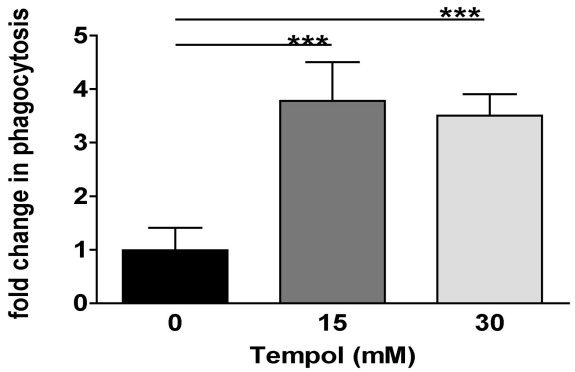

FIGURE A3 | Tempol enhances PMN phagocytosis of *Staphylococcus aureus* bioparticles. The effect of Tempol on PMN phagocytosis was analyzed using *Staphylococcus aureus* bioparticles. Cytochalasin D treatment was used as a means to block PMN phagocytosis as a negative control. Black bar: PMN phagocytosis without treatment was set as 1. Gray bars: Fold change of PMN phagocytosis upon treatment with 15 mM Tempol (dark gray) and 30 mM Tempol (light gray) as compared to untreated cells. Significance was analyzed by Tukey one-way ANOVA (***$P \leq 0.001$). Data are presented as means from two independent experiments with three technical replicates each ±SD.

frontiers in
IMMUNOLOGY

PERSPECTIVE ARTICLE
published: 05 December 2012
doi: 10.3389/fimmu.2012.00365

A NET outcome

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Neutrophils constitute a critical part of innate immunity and are well known for their ability to phagocytose and kill invading microorganisms. The microbicidal processes employed by neutrophils are highly effective at killing most ingested bacteria and fungi. However, an alternative non-phagocytic antimicrobial mechanism of neutrophils has been proposed whereby microorganisms are eliminated by neutrophil extracellular traps (NETs). NETs are comprised of DNA, histones, and antimicrobial proteins extruded by neutrophils during NETosis, a cell death pathway reported to be distinct from apoptosis, phagocytosis-induced cell death, and necrosis. Although multiple laboratories have reported NETs using various stimuli *in vitro*, the molecular mechanisms involved in this process have yet to be definitively elucidated, and many questions regarding the formation and putative role or function of NETs in innate host defense remain unanswered. It is with these questions in mind that we provide some reflection and perspective on NETs and NETosis.

Keywords: neutrophil, apoptosis, necrosis, phagocytosis, inflammation

NEUTROPHIL TURNOVER AND HOMEOSTASIS

Neutrophils are short-lived granulocytes that mature in bone marrow for several days (Bainton et al., 1971; Weissman et al., 2001). During maturation, these cells acquire key functional attributes, including the ability to phagocytose and kill microorganisms (Bainton et al., 1971; Glasser and Fiederlein, 1987; Weissman et al., 2001; Rosenbauer and Tenen, 2007; Pillay et al., 2010). After maturation, neutrophils are released into the bloodstream and circulate and/or marginate for 10–24 h before migrating into tissues, where they may function for an additional 1–2 days before they undergo apoptosis and are cleared by macrophages or dendritic cells (Cartwright et al., 1964; Fliedner et al., 1964; Bainton et al., 1971; Savill et al., 1989; Voll et al., 1997; Fadok et al., 1998; Huynh et al., 2002; Martin et al., 2003; Rigby and DeLeo, 2012). In addition, neutrophils in the total blood granulocyte pool (circulating and marginating) can be removed by the liver, spleen, and bone marrow, although the precise mechanism for this turnover process remains incompletely determined (reviewed by Summers et al., 2010). The neutrophil lifespan is highly regulated, as it is critical to remove spent/effete neutrophils as a means to prevent accidental release of cytotoxic molecules and associated host tissue damage (Edwards et al., 2003; Duffin et al., 2010; Bratton and Henson, 2011; Milot and Filep, 2011). Neutrophil turnover in an adult human is typically on the order of 10^{11} cells per day (Athens et al., 1961; Dancey et al., 1976; Rankin, 2010). While the hematopoietic system is able to regulate steady-state levels of circulating neutrophils, it can also be switched to an emergency granulopoiesis response to accommodate the increased demand for neutrophils during infection (Hirai et al., 2006; Panopoulos and Watowich, 2008).

The neutrophil lifespan is regulated by a balance of pro- and anti-apoptotic factors present in the environment. Cytokines and other factors such as interleukin (IL)-1β, IL-2, IL-4, IL-15, interferon-γ, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and lipopolysaccharide (LPS) can prolong and/or enhance neutrophil function and delay apoptosis for several days (Colotta et al., 1992; Duffin et al., 2010). Although enhancing neutrophil function and survival presumably favors elimination of invading microbes, the persistence of these cytotoxic host cells increases the potential for prolonged inflammation and host tissue damage. Therefore, it is not surprising that neutrophil turnover is a highly regulated process.

Molecular control of neutrophil turnover or apoptosis is mediated by several mechanisms, including extrinsic pathways induced by extracellular signals and intrinsic pathways induced by intracellular signals. These signals include those triggered by death receptors, which bind ligands that activate caspases to promote apoptosis, mitochondrial release of cytochrome *c*, and processes mediated by the BCL-2 protein family (Edwards et al., 2003; Duffin et al., 2010). Spontaneous or constitutive apoptosis in neutrophils is an example of intrinsic apoptosis. Apoptosis elicited by FAS, tumor necrosis factor (TNF)-α, or TNF-related apoptosis inducing ligand (TRAIL), caused by the binding of these extracellular ligands to the cognate receptor anchored on the cell surface, is an example of extrinsic pathway apoptosis (Kennedy and DeLeo, 2009; Duffin et al., 2010). Phagocytosis may also lead to neutrophil apoptosis (Watson et al., 1996; Kobayashi et al., 2002; Zhang et al., 2003; Kennedy and DeLeo, 2009). Neutrophil phagocytosis-induced apoptosis or phagocytosis-induced cell death (PICD) promotes the resolution of infection by disposing spent or effete neutrophils containing dead or partially digested microbes in a non-inflammatory manner (Kennedy and DeLeo, 2009). This process is described below in the context of the resolution of inflammation.

NEUTROPHILS AND THE INFLAMMATORY RESPONSE

The importance of neutrophils in the immune response is underscored by human diseases caused by defects in neutrophil function, which result in increased risk of infection from bacteria and fungi (Nauseef and Clark, 2010). For example, neutropenia, which can be medically induced by cytotoxic drugs or cancer therapy, is associated with significant morbidity (Bodey et al., 1966; Dale et al., 1979; Frøland, 1984; Tobias and Schleien, 1991). In addition, the inflammatory response, which from a cellular perspective is largely comprised of neutrophils, is critical for defense against invading microorganisms. On the other hand, timely resolution of the inflammatory response is an important process that returns the host immune system to pre-infection homeostasis. Historically, neutrophils were considered to have a passive part in inflammation resolution; however, this view has changed over time, and it is now known that neutrophils actively help to resolve inflammation by blocking and scavenging chemokines and cytokines (Ariel et al., 2006), and also produce pro-resolving lipid mediators (Ariel et al., 2006; Serhan et al., 2008). Thus, given that neutrophils contain and produce a vast array of cytotoxic molecules and contribute to the regulation of inflammation, it should not be unexpected that these cells are involved in – or are the primary cause of – a variety of inflammatory disorders. For instance, in chronic obstructive pulmonary disease, the aminopeptidase activity of leukotriene A4 hydrolase (LTA4H) is inhibited, causing accumulation of proline-glycine-proline, which in turn, promotes neutrophil recruitment and chronic lung inflammation (Weathington et al., 2006). In mouse models, recruitment of neutrophils has been shown to be involved in arthritis (Chou et al., 2010) and multiple sclerosis (Carlson et al., 2008; Liu et al., 2010). More notably, recent studies have demonstrated that neutrophils and neutrophil responses (rather than bacterial pathogens *per se*) are the cause of severe pneumonia and tissue destruction in animal models of bacterial respiratory tract infection (Bartlett et al., 2008; Diep et al., 2010). Thus, it is clear that unchecked neutrophil activation and neutrophil lysis are phenomena that can have a significant negative impact on health of the host.

RESPONSE TO INFECTION

Neutrophils are recruited rapidly to the site of infection in response to chemotactic stimuli released by the host and/or invading microorganism. Inasmuch as neutrophils are the most abundant leukocyte in humans, there can be a tremendous influx of neutrophils to the site of infection. At such sites, neutrophils bind and ingest microorganisms through a process known as phagocytosis (reviewed in Rigby and DeLeo, 2012). Ingested microbes are typically destroyed by the combined effects of NADPH oxidase-derived reactive oxygen species (ROS) and cytotoxic molecules delivered from cytoplasmic granules into the phagosome. Neutrophil granules contain numerous antimicrobial peptides (AMPs) and proteins, and matrix protein-degrading proteases, including alpha-defensins, cathelicidins, azurocidin, cathepsins, lactoferrin, lysozyme, proteinase-3, gelatinase, collagenase, and elastase (Faurschou and Borregaard, 2003; Nauseef and Clark, 2010; Rigby and DeLeo, 2012). Of note, these cytotoxic agents are normally targeted into the formed phagosome, thereby limiting inadvertent extracellular release and potential damage to host tissues (Nauseef and Clark, 2010).

It is well documented that neutrophil PICD occurs following ingestion of numerous microorganisms *in vitro* (Watson et al., 1996; Colamussi et al., 1999; Engelich et al., 2001; Kobayashi et al., 2003a,b, 2012; Kennedy and DeLeo, 2009), and *in vivo* this phenomenon likely promotes clearance of effete neutrophils containing dead or dying microbes (**Figure 1**; Kobayashi et al., 2012). Importantly, this process would prevent local host tissue damage that can occur if these spent host cells are not removed and undergo lysis, and thus ultimately promotes the resolution of inflammation (Whyte et al., 1993; Savill, 1997; Kobayashi et al., 2002, 2003a, 2012; Kim et al., 2004; Iyoda et al., 2005; Ariel et al., 2006; Kobayashi and DeLeo, 2009; Rigby and DeLeo, 2012). Such a process is considered normal for neutrophils during infection and healthy for the host. On the other hand, pathogenic microorganisms circumvent killing by neutrophils, and in doing so ultimately alter the normal process of neutrophil turnover during infection, by either delaying apoptosis or causing neutrophil lysis (Kobayashi et al., 2003a, 2010; DeLeo, 2004; Voyich et al., 2005). The resulting neutrophil lysis releases tissue-damaging molecules, not only allowing pathogen survival but also exacerbating the inflammatory response (**Figure 1**). This process can lead to disease and can be considered unhealthy for the host. As one example, some strains of *Staphylococcus aureus* are known to cause lysis of human neutrophils after phagocytosis (Rogers and Tompsett, 1952; Voyich et al., 2005, 2006; Kobayashi et al., 2010). Indeed, the possibility that *S. aureus* survive after phagocytosis and ultimately disseminate to cause disease (which can be explained at least in part by neutrophil lysis after trafficking) has been reviewed recently (Thwaites and Gant, 2011). These authors describe neutrophils as "Trojan horses" for the dissemination or metastasis of *S. aureus* (Thwaites and Gant, 2011). In accordance with the observations *in vitro*, *S. aureus* is an abundant cause of pyogenic infections in humans. Therefore, the ability of *S. aureus* to cause neutrophil lysis is likely a component of virulence.

NEUTROPHIL EXTRACELLULAR TRAPS AND NETOSIS

Until fairly recently, phagocyte biologists were content with a model of neutrophil function in which these phagocytes bind, ingest, and subsequently kill microorganisms. The idea that neutrophils would extrude DNA in a cytolytic process that captures microorganisms was unheard of – until Brinkmann et al. (2004) reported the formation of structures known as neutrophil extracellular traps (NETs). These unique structures, which are discussed in detail in this issue of *Frontiers in Immunology*, are composed of DNA, histones, and antimicrobial proteins, and can ensnare pathogens. Since the report by Brinkmann et al. (2004), extracellular traps have been shown to be produced *in vitro* by a number of different cell types, including neutrophils, mast cells, eosinophils, and endothelial cells (Palić et al., 2007; von Köckritz-Blickwede et al., 2008; Yousefi et al., 2008; Chuammitri et al., 2009; Katzenback and Belosevic, 2009; Aulik et al., 2010; Gupta et al., 2010; Wardini et al., 2010; Webster et al., 2010; Lin et al., 2011; Scapinello et al., 2011). Moreover, recent studies have investigated possible mechanisms for the induction of NETs. For example, it has been reported that formation of NETs requires activation of

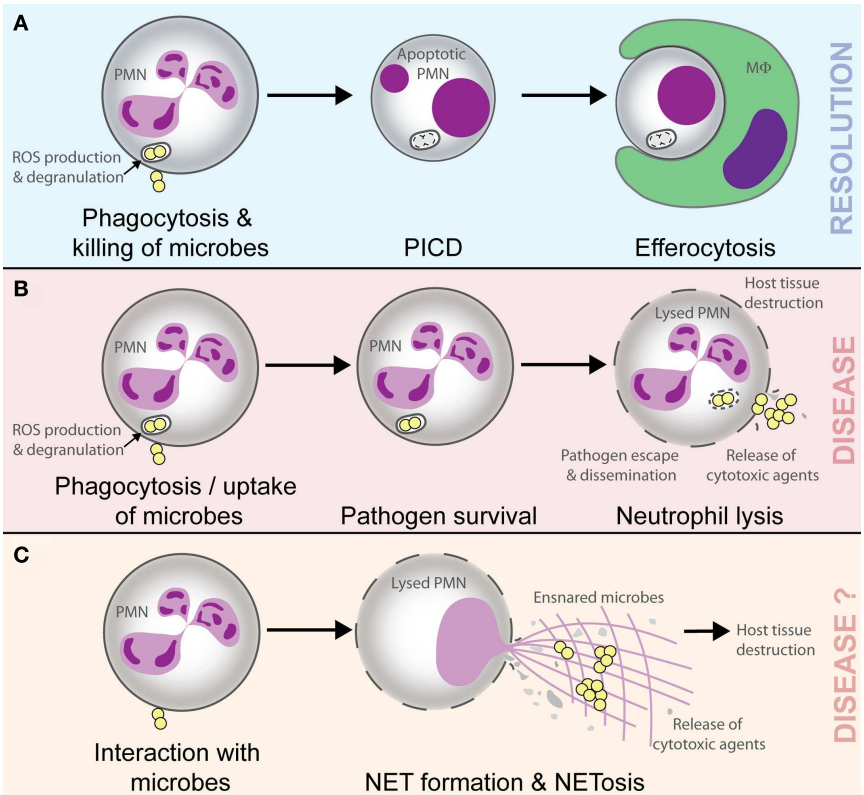

FIGURE 1 | Possible outcomes of the interaction of microbes with neutrophils. Phagocytosis and killing of microorganisms by neutrophils (polymorphonuclear leukocyte, PMN) triggers host cell apoptosis and ultimate removal by macrophages (MΦ) or dendritic cells. This process promotes resolution of the inflammatory response **(A)**. Pathogenic microbes such as *Staphylococcus aureus* can cause lysis of PMN after phagocytosis, thereby facilitating escape/dissemination of the invading pathogen and release of cytotoxic molecules that cause host tissue damage and disease **(B)**. NETs ensnare and may kill microbes, but there is accompanying lysis of neutrophils and release of cytotoxic molecules that are known to cause host tissue damage and promote inflammatory disease. In this regard, the outcome of NETosis and the formation of NETs should be similar to that in **(B**; i.e., disease; **C)**.

the Raf-MEK-ERK pathway through protein kinase C (Hakkim et al., 2011) and histone citrullination (Neeli et al., 2008; Li et al., 2010; Hemmers et al., 2011; Leshner et al., 2012). These findings suggest that NETosis and formation of NETs involves specific signal transduction events. Thus, it is tempting to advocate the importance of these structures in host defense due to the apparent simplicity and elegance of the phenomenon by which they occur. However, many questions remain about the role of NETs in host defense and the molecular mechanisms underlying their formation are incompletely characterized. In addition, the evidence for formation of NETs *in vivo* is not very compelling, and whether the formation of NETs is of benefit to the host remains an open question. Indeed, it was suggested early on that NETs form only under extreme circumstances and can injure host tissues (Clark et al., 2007).

While use of NETs appears as an alternative mechanism for pathogen control and elimination, production of NETs and NETosis (as a cytolytic process) seems at variance with the highly regulated control of neutrophil turnover (including PICD) and homeostasis, as discussed above. That is, utilization of NETs for host defense contrasts with the considerable effort made by the host to prevent inadvertent neutrophil lysis, release of cytotoxic agents, and post-lysis sequelae, such as inflammatory disorders. Notably, NETs have been implicated in a number of pathologic processes consistent with inflammatory disorders involving lysed neutrophils and cytotoxic molecules from neutrophils. For example, NETs can cause collateral damage in the form of endothelial and tissue damage (Clark et al., 2007; Ma and Kubes, 2008; Marin-Esteban et al., 2012) and may be partially responsible for sputum viscosity and tissue damage in cystic fibrosis patients (Papayannopoulos et al., 2011). NETs have been implicated in systemic lupus erythematosus and systemic vasculitis (Hakkim et al., 2010; Amulic and Hayes, 2011; Bosch, 2011; Garcia-Romo et al., 2011; Lande et al., 2011; Villanueva et al., 2011; Knight and Kaplan, 2012; Liu et al., 2012), gout, asthma, keratinocyte damage, and lupus nephritis (Mitroulis et al., 2011; Marin-Esteban et al., 2012), and may also be involved in the hyper reaction of the immune system by triggering physiological signals and causing pre-eclampsia (Gupta et al., 2005, 2006; Brinkmann and Zychlinsky, 2007). NETs are present in transfusion-related acute lung injury (Thomas et al., 2012), atherosclerotic carotid arteries (Döring, 2012), are toxic to vasculature (Clark et al., 2007; Gupta et al., 2010; Villanueva et al.,

Table 1 | Microbial susceptibility to NETs.

Species	Susceptibility	Reference
VIRUSES		
Feline leukemia virus	Modulates NET formation	Wardini et al. (2010)
Human immunodeficiency virus (HIV)-1	Infectivity reduced	Saitoh et al. (2012)
Influenza A H1N1	Modulates NET formation	Narasaraju et al. (2011)
BACTERIA		
Actinobacillus suis	Reduction in bacterial numbers	Scapinello et al. (2011)
Aeromonas hydrophila	Survives	Brogden et al. (2012)
Bacillus anthracis	Only unencapsulated strains killed	Papayannopoulos and Zychlinsky (2009); Szarowicz and Friedlander (2011)
Burkholderia pseudomallei	Reduction in bacterial numbers	Riyapa et al. (2012)
Escherichia coli	Reduction in bacterial numbers	Grinberg et al. (2008); Marin-Esteban et al. (2012)
Group A streptococcus	Survives	Buchanan et al. (2006); Lauth et al. (2009)
Group B streptococcus	Survives	Carlin et al. (2009)
Haemophilus influenzae	Survives	Juneau et al. (2011)
Listeria monocytogenes	Reduction in bacterial numbers	Ramos-Kichik et al. (2009)
Mannheimia haemolytica	Reduction in bacterial numbers	Aulik et al. (2010)
Mycobacterium canettii	Survives	Ramos-Kichik et al. (2009)
Mycobacterium tuberculosis	Survives	Ramos-Kichik et al. (2009)
Pasteurella multocida	Reduction in bacterial numbers	Scapinello et al. (2011)
Porphyromonas gingivalis	Survives	Delbosc et al. (2011); Palmer et al. (2011)
Pseudomonas aeruginosa	Survives	von Köckritz-Blickwede et al. (2008); Douda et al. (2011); Young et al. (2011); Khatua et al. (2012)
Salmonella typhimurium	Reduction in bacterial numbers	Brinkmann et al. (2004)
Shigella flexneri	Reduction in bacterial numbers	Brinkmann et al. (2004)
Staphylococcus aureus	Dependent on ratio	Döring et al. (2011)
Staphylococcus epidermidis	Survives	Cogen et al. (2010)
Streptococcus pneumonia	Survives	Beiter et al. (2006); Wartha et al. (2007); Midon et al. (2011)
Streptococcus pyogenes	Reduction in bacterial numbers	von Köckritz-Blickwede et al. (2008)
Streptococcus suis	Reduction in bacterial numbers	Scapinello et al. (2011)
Yersinia enterocolitica	Reduction in bacterial numbers	Casutt-Meyer et al. (2010)
Yersinia pestis	Survives	Casutt-Meyer et al. (2010)
PROTOZOA		
Eimeria bovis	Reduction in parasite numbers	Behrendt et al. (2010)
Leishmania amazonesis	Dependent on ratio	Guimarães-Costa et al. (2009)
Leishmania donovani	Survives	Gabriel et al. (2010)
Plasmodium falciparum	Trapped	Baker et al. (2008)
Toxoplasma gondii	Reduction in parasite numbers	Abi Abdallah et al. (2012)
FUNGI		
Aspergillus fumigatus	Growth inhibited	McCormick et al. (2010)
Aspergillus nidulans	Growth inhibited	Bianchi et al. (2011)
Candida albicans	Growth inhibited, blastospores survive	Urban et al. (2006); Menegazzi et al. (2012)
Candida glabrata	Growth inhibited	Springer et al. (2010)
Cryptococcus gatti	Survives	Springer et al. (2010)

2011; Saffarzadeh et al., 2012), and facilitate thrombosis where they could provide a scaffold for red blood cell adhesion (Fuchs et al., 2010, 2012; Van Den Berg and Reitsma, 2011; Brill et al., 2012). NETs may also contribute to cancer-associated thrombosis, since neutrophils from mice with experimentally induced cancers are more likely to form NETs than those from control mice (Demers et al., 2012). It is also of note that extracellular histones, a signature component of NETs, contribute to host death during sepsis (Xu et al., 2009). Whether the structures reported as NETs in these aforementioned inflammatory syndromes are distinct from the remains of necrotic neutrophils is unclear, but in any case the process or phenomenon is associated with a negative outcome for the host – similar to the prediction in the model described in **Figure 1**.

Given the association of NETs and NETosis with inflammatory disorders, and coupled with a highly regulated neutrophil turnover process, the frequency with which formation of NETs occurs should be fairly low. Indeed, even under optimal NET-inducing

conditions *in vitro*, only one-third of activated neutrophils, and perhaps as few as 10%, make NETs (Brinkmann and Zychlinsky, 2007; Fuchs et al., 2007; Munafo et al., 2009). The kinetics of NETosis vary depending on type and concentration of stimulus, isolation procedure of neutrophils, and the sensitivity of the detection method (Fuchs et al., 2012). Despite the fact that NET formation is stimulated by pathogens, it is still not clear whether NETosis that occurs during host-pathogen interactions is a programmed mechanism, a hijacking of host pathways by pathogen-produced factors, or simply an incidental component of neutrophil lysis. For instance, *S. aureus* is well known to cause lysis of neutrophils *in vitro* and *in vivo*, but the pathogen has also been reported to induce NETs (Brinkmann et al., 2004; Jann et al., 2009; Yipp et al., 2012). It is also not clear whether the release of NETs always leads to cell death (and the possibility of host tissue damage) or if it is an extrusion of DNA by intact cells (Yousefi et al., 2009; Remijsen et al., 2011; Guimarães-Costa et al., 2012; Yipp et al., 2012). Although it is difficult to understand how neutrophils can remain intact and viable after release of nuclear DNA, the question of whether NET formation always causes cytolysis or can occur with intact cells is important and must be resolved by the field.

One could hypothesize that the formation of NETs represents a directed host defense mechanism. If the process is host-directed, does this suggest there is an advantage to the use of NETs for removal of microbes versus traditional phagocytosis-based uptake and subsequent killing of microbial invaders? The antimicrobial activities of NETs have been ascribed to the histones, AMPs, and other cytoplasmic components associated with extracellular DNA. However, it is important to note that NETs provide a low concentration of AMPs compared to that present in the phagosome, and NETs lack the ability to produce microbicidal ROS. Published studies to date suggest that the formation of NETs does not lead to the universal killing of all microorganisms, although NETs can reduce the burden of selected of microorganisms *in vitro* (**Table 1**). This finding is perhaps not surprising, since solubilized azurophilic granule components isolated from disrupted neutrophils have varied capacity to kill different bacterial species (Bertram et al., 1986; Joiner et al., 1989; Levy et al., 1999; Palazzolo-Ballance et al., 2008; Nordenfelt et al., 2009). Moreover, several microorganisms are known to circumvent killing by NETs using a variety of strategies, including altering bacterial surface affinity to NETs (Wartha et al., 2007; Carlin et al., 2009; Juneau et al., 2011) and secreting NET-degrading DNases (Beiter et al., 2006; Buchanan et al., 2006; Midon et al., 2011; Palmer et al., 2011). As an alternative hypothesis, the formation of NETs (especially if it requires lysis of neutrophils) could be considered an incidental event rather than something intended by the host innate immune system. An incidental process seems more consistent with our understanding of the regulation of neutrophil turnover and homeostasis.

CONCLUDING PERSPECTIVE

Neutrophil extracellular traps have been suggested as an alternative or additional component of the innate host defense against microorganisms. Although progress has been made, many questions related to NET formation and function remain unanswered. Do NETs commonly occur *in vivo*? Compelling evidence is lacking. Are NETs formed by live neutrophils or does the process (i.e., NETosis) always result in cytolysis? If it is accompanied by neutrophil lysis, how does this phenomenon fit with what we know about the control of neutrophil turnover and the host efforts to prevent inflammatory syndromes? Importantly, is the pathway that leads to the formation of NETs a host-directed mechanism or simply an incidental phenomenon in neutrophils? These and other questions can only be answered by continued investigation into the biology and function of NETs.

ACKNOWLEDGMENTS

Thea Lu, Scott D. Kobayashi, and Frank R. DeLeo are supported by the Intramural Research Program of the National Institute of Allergy and Infectious Diseases, National Institutes of Health. Mark T. Quinn is supported by a grant (GM103500) from the National Institute of General Medical Sciences, National Institutes of Health.

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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.

Received: 10 October 2012; accepted: 16 November 2012; published online: 05 December 2012.

Citation: Lu T, Kobayashi SD, Quinn MT and DeLeo FR (2012) A NET outcome. Front. Immun. ***3****:365. doi: 10.3389/fimmu.2012.00365*

This article was submitted to Frontiers in Molecular Innate Immunity, a specialty of Frontiers in Immunology.

frontiers in IMMUNOLOGY

REVIEW ARTICLE
published: 24 January 2013
doi: 10.3389/fimmu.2013.00001

NET balancing: a problem in inflammatory lung diseases

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Neutrophil extracellular traps (NETs) are beneficial antimicrobial defense structures that can help fight against invading pathogens in the host. However, recent studies reveal that NETs exert adverse effects in a number of diseases including those of the lung. Many inflammatory lung diseases are characterized with a massive influx of neutrophils into the airways. Neutrophils contribute to the pathology of these diseases. To date, NETs have been identified in the lungs of cystic fibrosis (CF), acute lung injury (ALI), allergic asthma, and lungs infected with bacteria, virus, or fungi. These microbes and several host factors can stimulate NET formation, or NETosis. Different forms of NETosis have been identified and are dependent on varying types of stimuli. All of these pathways however appear to result in the formation of NETs that contain DNA, modified extracellular histones, proteases, and cytotoxic enzymes. Some of the NET components are immunogenic and damaging to host tissue. Innate immune collectins, such as pulmonary surfactant protein D (SP-D), bind NETs, and enhance the clearance of dying cells and DNA by alveolar macrophages. In many inflammatory lung diseases, bronchoalveolar SP-D levels are altered and its deficiency results in the accumulation of DNA in the lungs. Some of the other therapeutic molecules under consideration for treating NET-related diseases include DNases, antiproteases, myeloperoxidase (MPO) inhibitors, peptidylarginine deiminase-4 inhibitors, and anti-histone antibodies. NETs could provide important biological advantage for the host to fight against certain microbial infections. However, too much of a good thing can be a bad thing. Maintaining the right balance of NET formation and reducing the amount of NETs that accumulate in tissues are essential for harnessing the power of NETs with minimal damage to the hosts.

Keywords: neutrophil extracellular traps (NETs), lung inflammation, lung infection, surfactant protein D (SP-D), cystic fibrosis (CF), acute lung injury (ALI), neutrophil

INTRODUCTION

Although neutrophils are critical to our immune system in the event of microbial infections, an overabundance of neutrophils in circulation or in tissues has been implicated to be a problem in a number of lung diseases. Patients with inflammatory lung diseases such as cystic fibrosis (CF), severe asthma, chronic obstructive pulmonary disease (COPD), acute lung injury (ALI), acute respiratory distress syndrome (ARDS), and emphysema all exhibit various degrees of neutrophil influx; these neutrophils are a major contributor to these diseases (Downey et al., 2009; Grommes and Soehnlein, 2011). A massive influx of neutrophils is seen in acute pulmonary infections, pneumonia, and sepsis. Many of these lung conditions lead to ALI and tissue damage (Grommes and Soehnlein, 2011). Neutrophils and neutrophil extracellular traps (NETs) found in these inflammatory conditions cause tissue injury and severe inflammation in the lung (Villanueva et al., 2011; Saffarzadeh et al., 2012). NETs are extracellular DNA complexed with antimicrobial proteins, and help to fight infectious agents. However, an excess of NETs contributes to the pathology of a number of diseases. In the lungs, NETs have been identified in conditions of CF (Manzenreiter et al., 2012), ALI (Thomas et al., 2012), and infections with bacteria (Douda et al., 2011b), fungi (Bruns et al., 2010), and viruses (Narasaraju et al., 2011; Ng et al., 2012). In this review, neutrophil and NET functions during inflammation and infection will be discussed, followed by their contribution to tissue injury, autoimmunity, ALI, CF, and asthma. Lastly, we will discuss the targeting of NETs in therapy.

NEUTROPHIL FUNCTION AND RECRUITMENT DURING INFLAMMATION AND INFECTION

Neutrophils are an important component of our host defense against invading pathogens, often referred to as the immune system's first line of defense against infection. The neutrophil is the most abundant leukocyte comprising approximately 60% of all leukocytes found in circulating blood in humans. They are easily identified by their banded or multi-lobed nuclear structure, thus giving them their synonymous name of polymorphonuclear leukocytes (PMNs) (Nathan, 2006). Many of these neutrophils enter the lungs during infections and form NETs. Dysfunctions in NETosis and NET clearance can severely damage this vital organ.

ANTIMICROBIAL MECHANISMS OF NEUTROPHILS

The general dogma was that neutrophils fight against microorganisms by directly phagocytosing the targets or by releasing

toxic components via degranulation. Phagocytosis is one of the mechanisms identified in neutrophils that can directly engulf and digest potential pathogens as well as cell debris. Internalized pathogens are contained in phagosomes, where antimicrobial peptides from cellular granules and reactive oxygen species (ROS) produced by NADPH oxidase work together to create a toxic environment for most pathogens (Underhill and Ozinsky, 2002). Degranulation is the release of toxic ROS and antimicrobial granular proteins into the extracellular space. Neutrophil granules are categorized into three different types based on their contents: primary (azurophilic), secondary (specific), and tertiary (gelatinase). The presence of different types of granules in the neutrophils is dependent on the time of granule formation relative to the neutrophil maturation stage. This starts with the formation of primary granules, followed by secondary and tertiary granules (Gullberg et al., 1997). Primary granules contain MPO, elastase, cathepsin G, proteinase 3, defensins, and lysozyme; secondary granules contain collagenase, gelatinase, cystatin, lysozyme, and lactoferrin; tertiary granules contain gelatinase, lysozyme, and arginase. As such, a neutrophil will accumulate all three types of granules by the end of maturation. Collectively, these granules contain many antimicrobial proteins that function to fight infection in the lungs and other organs (Borregaard et al., 2007).

Neutrophils also indirectly defend the host against microbes by participating in elaborate cell signaling networks involving cytokines, chemokines, survival and growth factors that cause downstream pro-inflammatory effects. Neutrophils can secrete pro-inflammatory cytokines (e.g., TNF-α, IL-1β), CC and CXC chemokines (e.g., IL-8, IFN, IP-10, MIP-1α). The secretion itself is regulated by immunoregulatory cytokines (e.g., IFN-γ, IL-4, IL-13, IL-10) (Kasama et al., 2005). These factors can increase the production of various chemokines and cytokines to further regulate neutrophil functions (Kato and Kitagawa, 2006). Importantly, some of these factors can participate in recruiting more neutrophils or other leukocytes to the site of infection or sterile inflammation (Cassatella et al., 1997).

NEUTROPHIL MIGRATION INTO THE LUNGS

Typically, neutrophils are found in higher concentrations in the pulmonary capillaries compared to systemic blood even in the absence of inflammatory stimuli. This phenomenon allows neutrophils to readily migrate into the lungs in response to inflammatory stimuli. Neutrophils undergo cellular deformation in order to emigrate between endothelial cells of the pulmonary capillaries to reach the alveolar air space (Doerschuk et al., 1999). During inflammation, neutrophils become activated upon stimulation and may undergo processes of ROS production, degranulation, NETs formation, or other functions. Activation of neutrophils is required before migration into the lungs (Ley et al., 2007). Neutrophil activating factors may be derived from host [e.g., platelet activating factor (PAF), leukotriene B4, IL-8] or from pathogens [e.g., formylated peptide (fMLP) and lipopolysaccharide (LPS) (Krause et al., 1985; Martin et al., 1989; Anderson et al., 1991; Corteling et al., 2002; Mukaida, 2003)]. The chemokines that are most critical for neutrophil recruitment in the lungs include IL-8 (CXCL8) in humans, and MIP-2 (CXCL2) and KC (CXCL1) in rodents (Kobayashi, 2008). These chemokines are secreted by neutrophils themselves, epithelial cells, or macrophages (Cassatella et al., 1997; Matsukawa and Yoshinaga, 1999; Yamashiro et al., 2001; Kasama et al., 2005; Kato and Kitagawa, 2006).

NETs

Aside from the more traditional mechanisms of phagocytosis and degranulation, neutrophils can also generate NETs to directly combat microbes during inflammation and infection (Brinkmann et al., 2004). Takei et al. first described this novel form of neutrophil cell death to be distinct from apoptosis and necrosis in 1996 (Takei et al., 1996). This was later studied by Brinkmann et al. (2004), who coined the term NETosis for this cell death process. NETs are cast as decondensed chromatin fibers coated with antimicrobial histones and granular proteins (Brinkmann et al., 2004) (**Figure 1**). To date, NETs and NET-like structures have been identified by several labs as a host defense mechanism in many organisms including humans (Manzenreiter et al., 2012), mice (Ermert et al., 2009a), chickens (HETs) (Chuammitri et al., 2009), cats (Wardini et al., 2010), cattle (Aulik et al., 2010), fish (Palić et al., 2007b), insects (Altincicek et al., 2008), and even plants (Wen et al., 2009). Conservation of NET function across species suggests an evolutionary advantage of NETs in immune defense.

NET INDUCTION

The formation of NETs (NETosis) is stimulated by a variety of agents (**Table 1**). Microorganisms such as protozoa (Guimarães-Costa et al., 2009; Abi Abdallah et al., 2012), fungi (Urban et al., 2006, 2009; Ermert et al., 2009b), viruses (Narasaraju et al., 2011; Ng et al., 2012; Saitoh et al., 2012), bacteria (Brinkmann et al., 2004; Crotty Alexander et al., 2010), and bacterial component LPS (Douda et al., 2011b) can induce NETosis. Host-derived factors such as granulocyte/macrophage colony-stimulating factor (GM-CSF) with complement factor 5a (Yousefi et al., 2009), activated platelets (Clark et al., 2007; Caudrillier et al., 2012) and singlet oxygen (Nishinaka et al., 2011) also induce NETosis (**Table 1**). The pharmacological agent phorbol-12-myristate-13-acetate (PMA), a protein kinase C activator, is a known strong inducer of NETosis that is routinely used in studies of NETs. The potent neutrophil chemoattractant, IL-8, has also been shown to induce NETosis (Brinkmann et al., 2004; Gupta et al., 2005), but there has been some uncertainty regarding its ability to trigger NETosis in CF airways (Marcos et al., 2010, 2011).

NETosis MECHANISM

The process of NETosis requires mature neutrophils (Martinelli et al., 2004) and the presence of enzymes MPO, neutrophil elastase (NE), and peptidylarginine deiminase type IV (PAD4) (Neeli et al., 2008; Wang et al., 2009; Papayannopoulos et al., 2010; Metzler et al., 2011). Upon stimulation of the neutrophil, the nuclear envelope disintegrates to allow mixing of chromatin with granular proteins (Brinkmann et al., 2004; Fuchs et al., 2007). NE and MPO degrade histones and promote chromatin decondensation (Papayannopoulos et al., 2010). PAD4 mediates chromatin decondensation by hypercitrullinating positively charged

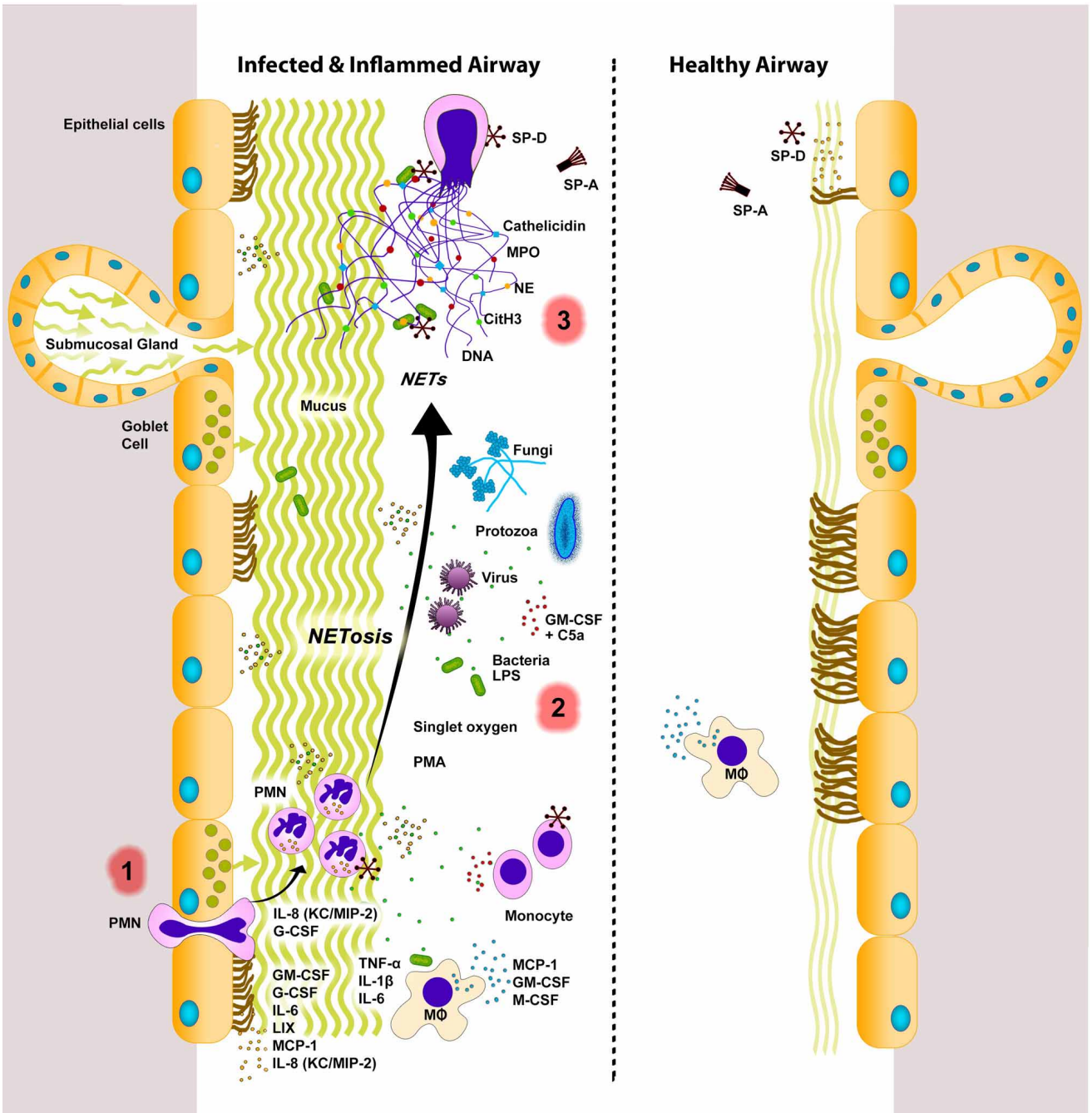

FIGURE 1 | NETs in infected and inflamed airways. Lungs respond to sterile injury or infection by secreting various signaling molecules. During infection and inflammation, various cells (e.g., endothelial, epithelial, immune cells) express inflammatory cytokines, chemokines, and growth factors to recruit monocytes (e.g., MCP-1) and neutrophils [e.g., IL8 (KC/MIP-2)] into the airway lumen. Neutrophils can be stimulated by a variety of agents (e.g., bacteria, viruses, fungi, protozoa, LPS, singlet oxygen, PMA, GM-CSF+C5a) to undergo NETosis. Release of cytotoxic DNA–protein complexes [e.g., citrullinated histone ($CitH_3$), neutrophil elastase (NE), myeloperoxidase (MPO), cathelicidin, other neutrophil proteases] not only increase mucus viscosity, but also contribute to lung damage that can perpetuate the vicious cycle of lung injury and inflammation. NETs are considered to be degraded by DNase enzymes. Macrophages can also internalize and remove DNA, as well as other cellular debris. A balance between NETosis and NET clearance is essential for effectively clearing infectious agents with minimal damage to the lungs. Dysregulation in these two processes can lead to lung injury and exacerbation of lung diseases. Innate immune collectins could help to maintain healthy lungs with minimal inflammation. SP-A, pulmonary surfactant protein A; SP-D, pulmonary surfactant protein D. PMN, neutrophils; MΦ, macrophages. Inflamed airways also have excess mucus. The putative sequence of NETotic events in the lungs are numbered as 1, 2, and 3. Note: Cytokines, chemokines, and growth factors are placed near their most probable source of secretion. However, the source and/or degree of cytokine secretion varies depending on the stimuli.

Table 1 | NETosis-inducing agents.

NETosis inducer	*In vitro*	*In vivo*	Reference(s)
BACTERIA			
Escherichia coli (P4 strain)	10 MOI; bovine	–	Clark et al., 2007; Grinberg et al. 2008; Yost et al., 2009
	0.01 MOI; human	–	
Pseudomonas aeruginosa (PA01)	0.1–10 MOI; human	1×10^6 CFU/mouse	Douda et al., 2011b; Young et al., 2011
Staphylococcus aureus	0.01–10 MOI; human	–	Brinkmann et al., 2004; Pilsczek et al., 2010
Shigella flexneri	0.01 MOI; human	$2.5–3.0 \times 10^{10}$/rabbit	Brinkmann et al., 2004
Salmonella enteric	0.01 MOI; human	–	Brinkmann et al., 2004
Group A *Streptococcus*	0.1 MOI; human	$5 \times 10^7–2 \times 10^8$ CFU/mouse	Buchanan et al., 2006; Crotty Alexander et al., 2010
Streptococcus pneumonia	0.01 MOI; human	1×10^7/mouse	Beiter et al., 2006
Mycobacterium tuberculosis	0.1–10 MOI; human	–	Ramos-Kichik et al., 2009
PROTOZOA			
Leishmania amazonensis	10 MOI; human	–	Guimarães-Costa et al., 2009
Leishmania donovani	10 MOI; human	–	Gabriel et al., 2010
Toxoplasma gondii	250 mU/ml; human, mouse	5×10^7/mouse	Abi Abdallah et al., 2012
Eimeria bovis	0.2 (sporozoites) MOI; bovine	–	Behrendt et al., 2010
FUNGI			
Aspergillus fumigates	5 (conidia) MOI; human	–	Bruns et al., 2010
Candida albicans	0.01 MOI; human	–	Urban et al., 2006
Aspergillus nidulans	0.5 (conidia) MOI; human	–	Bianchi et al., 2009
VIRUS			
Human immunodeficiency virus (p24 antigen)	1.0–2.4 ng/ml; human	–	Saitoh et al., 2012
Influenza A virus H1N1	20 MOI; human	100–500 PFU/mouse	Narasaraju et al., 2011
Influenza A virus H3N2	2 MOI; mouse	2×10^5 PFU/mouse	Ng et al., 2012
HOST FACTORS			
GM-CSF + C5a	25 ng/ml GM-CSF + 10^{-7} M C5a	–	Yousefi et al., 2009
IL-8 (CXCL8)	2.5–10 ng/ml; human	–	Gupta et al., 2005
MIP-2 (CXCL2)	100 nM; human	–	Marcos et al., 2010, 2011
Singlet oxygen	10 μg/ml Photofrin; human	–	Nishinaka et al., 2011
Platelet activating factor (PAF)	$10^{-10} – 10^{-7}$ M; human	–	Yost et al., 2009
Syncytiotrophoblast microparticles (STBM)	150 μg/ml; human	–	Gupta et al., 2005
OTHERS			
Glucose oxidase	200–1000 mU/ml; human	–	Yost et al., 2009
Calcium ionophore (ionomycin)	5 μg/ml; zebrafish 4 μM; human	–	Palić et al., 2007a; Neeli et al., 2008
Phorbol-12-myristate-13-acetate (PMA)	25–100 nM; human	–	Brinkmann et al., 2004; Remijsen et al., 2011
Bacterial component LPS, Panton-Valentine leukocidin	100 ng/ml; human	5–25 μg/mouse	Brinkmann et al., 2004; Clark et al., 2007; Pilsczek et al., 2010; Douda et al., 2011b

arginines of specific histones to relieve electrostatic coiling of the chromatin (Wang et al., 2009; Li et al., 2010; Leshner et al., 2012). These DNA–protein complexes are then released extracellularly as NETs.

As the discovery of NETs is relatively new, the mechanism of NETosis is not clearly understood. The majority of studies reveal that NETosis is dependent on the generation of ROS by NADPH oxidase; however, a few studies show that NETosis may also occur in a ROS-independent manner, for instance by stimulation with *Staphylococcus aureus* (Pilsczek et al., 2010). Patients with chronic granulomatous disease (CGD) have congenital defects in different subunits of NADPH oxidase (Nox2) that prevent their ability to generate ROS. Hence, the neutrophils of these patients are unable to perform phagocytic killing and NETosis, making them highly susceptible to life-threatening infections (Fuchs et al., 2007). The restoration of NADPH oxidase function and NET formation in these patients effectively protected them against microbial infections (Bianchi et al., 2009). Singlet oxygen is a member of the ROS family that has been shown to be essential for the formation of NETs. Singlet oxygen itself can trigger

NETosis independent of NADPH oxidase (Nishinaka et al., 2011). In addition to superoxide, autophagy has also been shown to be required for the generation of NETs (Remijsen et al., 2011). Recent evidence shows that the NETosis pathway requires cell signaling, of which p38 MAP kinase and Raf-MEK-ERK kinase pathways are involved (Hakkim et al., 2011; Keshari et al., 2012). Nonetheless, depending on the stimulus, the key components involved in the generation of NETs can vary (Parker et al., 2012) (**Table 2**).

ALTERNATIVE TYPES OF EXTRACELLULAR TRAPS

Extracellular DNA traps have been more recently documented to be not exclusive to only neutrophils. Extracellular DNA traps can also be generated from macrophages (METs) (Hellenbrand et al., 2013), eosinophils (EETs) (Yousefi et al., 2008; Dworski et al., 2011), and mast cells (MCETs) (von Köckritz-Blickwede et al., 2008; Lin et al., 2011). Nonetheless, all extracellular DNA traps are of an immune cell origin that contains a plethora of antimicrobial components. Most consider NETosis as a form of cell death distinct from classical apoptosis and necrosis since it requires histone hypercitrullination; however, the term "cell death" may not be entirely appropriate. In the late 1980's, Malawista et al. of Yale showed that enucleated neutrophils (i.e., cytoplasts) remain viable and are capable of killing microbes (Malawista et al., 1989). Recent studies also corroborate that cells do not necessarily die after the release of extracellular DNA traps (Yousefi et al., 2009; Pilsczek et al., 2010). Yipp et al. recently showed that neutrophils that undergo NETosis without lysis are viable and retain their ability to phagocytose bacteria (Yipp et al., 2012). In another study, neutrophils were viable after being primed with GM-CSF, then stimulated with LPS or C5a to release NETs of mitochondrial origin (Yousefi et al., 2009). A similar study by the same group showed that eosinophils were also viable after the release of EETs of mitochondrial origin (Yousefi et al., 2008). The reasoning for the viability of these cells after the release of extracellular DNA traps is thought to be caused by the type of DNA released; only mitochondrial DNA was extruded while nuclear DNA remained intact within the nucleus of the cell to allow neutrophils to continue its function. However, recent studies challenged this idea (Pilsczek et al., 2010; Yipp et al., 2012). In these studies, neutrophils remained viable after the release of NETs that are of nuclear origin. Once these neutrophils were stimulated with *S. aureus*, the neutrophils underwent a novel mechanism of rapid NETosis. NETs were released via a vesicular mechanism, in which vesicles budding from the neutrophil contained nuclear DNA (Pilsczek et al., 2010). However, the stimuli used for these studies were different. Depending on the stimulus, neutrophils can undergo a different form of NETosis (Parker et al., 2012).

Table 2 | Neutrophil components involved in NETosis.

Component	Function	Reference(s)
Neutrophil elastase (NE)	Chromatin decondensation	Papayannopoulos et al., 2010
Myeloperoxidase (MPO)	Chromatin decondensation;	Papayannopoulos et al., 2010; Metzler et al., 2011; Akong-Moore et al., 2012
HOCl	hypochlorite generation	
Peptidylarginine deiminase type IV (PAD4)	Chromatin decondensation; histone modification	Li et al., 2010; Leshner et al., 2012
Autophagy	NETosis pathway	Remijsen et al., 2011
NADPH oxidase	NETosis pathway	Fuchs et al., 2007
H_2O_2	Substrate for MPO	Akong-Moore et al., 2012
Singlet oxygen	Essential NETosis inducer	Nishinaka et al., 2011
Raf-MEK-ERK	NETosis pathway	Hakkim et al., 2011
ERK, p38 MAPK	NETosis pathway	Keshari et al., 2012

NET-MEDIATED TISSUE INJURY AND DISEASES

Despite the advantageous properties of NETs, their ineffective clearance and regulation can have pathological effects (**Figure 1**). The antimicrobial histones and peptides coating the NET-DNA are directly cytotoxic to tissue, and ineffective clearance of NETs causes deleterious inflammation of host tissue. NETs, and in particular extracellular histones, can directly cause epithelial and endothelial cell death (Xu et al., 2009; Saffarzadeh et al., 2012). Histone administration *in vivo* resulted in neutrophil margination, vacuolated endothelium, intra-alveolar hemorrhage, and macro- and microvascular thrombosis (Xu et al., 2009). Impaired degradation and clearance of NETs has also been shown to be linked to autoimmunity in patients with atherosclerosis (Döring et al., 2012), rheumatoid arthritis (Rohrbach et al., 2012), small-vessel vasculitis (SVV) (Kessenbrock et al., 2009), systemic lupus erythematosus (SLE) (Hakkim et al., 2010; Lande et al., 2011; Leffler et al., 2012; Liu et al., 2012), and Felty's syndrome (Dwivedi et al., 2012). PAD4 citrullinated histones in particular are highly immunogenic (Neeli et al., 2008). Autoantibodies against these modified histones are seen in patients with SLE (Liu et al., 2012), Felty's syndrome (Dwivedi et al., 2012) and a mouse model of rheumatoid arthritis (Rohrbach et al., 2012). The presence of autoantibodies in chronic inflammatory lung diseases has not been investigated, but the prolonged presence of NETs in the lungs may potentially elicit autoimmune responses.

In SLE patients, the self-DNA and antimicrobial peptides of NETs are immunogenic complexes that can activate plasmacytoid dendritic cells (pDCs) and serve as autoantigens to B cells in their production of anti-NET autoantibodies (Lande et al., 2011). Both anti-NET antibodies and DNase 1 inhibitors were found in the sera of SLE patients; these inhibitors prevented DNase 1 to access NETs for degradation (Hakkim et al., 2010). C1q deposited on NETs have also been shown to prevent NET degradation by directly inhibiting DNase 1 (Leffler et al., 2012). The deposition of C1q on NETs can activate complement to cause further neutrophil recruitment (Stokol et al., 2004; Leffler et al., 2012), which can further exacerbate the disease. Similarly in atherosclerosis, self-DNA and antimicrobial peptides of NET structures are autoantigenic and stimulate pDC-driven autoimmunity via TLR7/9 and production of type I IFN (Döring et al., 2012). As NETs derive autoantibodies, they can also form soluble immune complexes (ICs), which is hallmark of autoimmune diseases. Recently, Chen et al. showed that ICs can induce NETosis in mice *in vivo*

via FcγRIIA independent of NE, MPO, and NADPH oxidase (Chen et al., 2012). This study implicates that FcγR may play an important role in the NETosis pathway.

In SVV, anti-neutrophil cytoplasmic autoantibodies (ANCAs) are strongly associated with the disease (Kallenberg et al., 2006). Similar to ICs in SLE patients, ANCAs directed against proteinase-3 (PR3) and MPO can stimulate neutrophils in SVV to form NETs and promote autoimmunity (Sangaletti et al., 2012). These neutrophil proteins (PR3, MPO) are found attached to the chromatin scaffold of NETs and may be the host antigen source for the generation of ANCAs. The enhanced deposition of antimicrobial peptide LL37 (cathelicidin) onto NET-DNA was also observed in SVV (Kessenbrock et al., 2009). The binding of LL37 to NET-DNA can protect it from degradation (Lande et al., 2011) and has been shown to drive the autoimmune response and pathogenesis of SLE and psoriasis (Lande et al., 2007). As such, LL37 may have a role in the autoimmunity and pathogenesis of SVV as well as other NET-related diseases. All in all, these highly immunogenic NET structures result in the production of autoantibodies, further neutrophil recruitment and triggering of NETosis, which create a perpetuating cycle of autoimmune combat. Clearance of NETs from the lungs and other sites are essential for preventing NET-associated tissue and organ damage.

NETs AND SURFACTANT PROTEIN D (SP-D)

The lungs are lined with a pulmonary surfactant layer that contains surfactant proteins (SP-) A and D. These proteins help to prevent the lungs from infection and inflammation, especially because airways are constantly exposed to microorganisms and debris. SP-A and SP-D are innate immune collectins that can opsonize pathogens, and apoptotic and necrotic cells to signal their clearance by alveolar macrophages in the lungs and modulate pulmonary inflammation (Nayak et al., 2012). Specifically, SP-A and SP-D contain carbohydrate recognition domains and collagenous domains that can bind carbohydrate ligands of bacteria and DNA, respectively (Palaniyar et al., 2003a, 2004; Litvack and Palaniyar, 2010). The binding of these surfactant proteins to DNA and apoptotic cells enhances their clearance by alveolar macrophages (Schagat et al., 2001; Palaniyar et al., 2003a,b, 2005). SP-D in particular has a role in reducing apoptosis of alveolar macrophages and pro-inflammatory cytokines (Clark et al., 2002, 2003). As such, SP-A and SP-D have important roles in maintaining infection- and inflammation-free airways.

Recently, our lab showed that SP-D could simultaneously bind both NET-DNA and bacteria to help microagglutinate bacteria and promote bacterial trapping by NETs (Douda et al., 2011b). Currently, the factors that can suppress NETosis and promote the clearance of NETs are unknown. The binding of SP-D to DNA enhances the clearance of DNA by macrophages (Palaniyar et al., 2005); however, the role of SP-D on NET-DNA clearance is not clear. Preliminary studies from our lab suggest that SP-D can augment the clearance of NETs by alveolar macrophages (Douda et al., 2011a). There are a number of human inflammatory lung diseases that are characterized by decreased levels of bronchoalveolar SP-D. SP-D deficiency can lead to the accumulation of dying cells and increased production of anti-DNA auto-antibodies (Palaniyar et al., 2005). These studies suggest that SP-D is one of the important proteins for maintaining a balance of NETs in the lungs.

ACUTE LUNG INJURY (ALI) AND ACUTE RESPIRATORY DISTRESS SYNDROME (ARDS)

Infection-related conditions such as pneumonia, sepsis, and pulmonary infections with viruses, bacteria, or fungi can directly injure the lungs and cause ALI or ARDS. Non-infectious causes (sterile injury) such as high-tidal ventilation, hyperoxia, and pulmonary contusions also lead to ALI and ARDS (Matthay et al., 2012). ALI is described as a lung disease with acute onset and disruption of the alveolar-capillary interface that leads to increased microvascular permeability. As a result, protein-rich fluid from the capillaries leaks into the alveolar space causing pulmonary edema. ALI and ARDS have many different causes, but epithelial injury is the basis of ARDS, and it is a more severe form of ALI (Zhou et al., 2012). ALI/ARDS is characterized by a massive influx of neutrophils into the lungs causing neutrophilic inflammation. Excessive activation and migration of neutrophils into the lung is a hallmark of ALI. Neutrophils are important contributors to the progression of ALI/ARDS, and higher neutrophil concentration in the BAL fluid of patients with ARDS is often associated with greater severity of the disease (Grommes and Soehnlein, 2011). Excessive neutrophils and NETs contribute to the pathology of ALI, where NETs can directly induce lung epithelial cell death (Saffarzadeh et al., 2012).

NETs are also found in infection-related ALI models of influenza virus (Narasaraju et al., 2011; Ng et al., 2012), bacteria or bacterial component LPS (Li et al., 2010; Douda et al., 2011b; Barletta et al., 2012), and fungi (Urban et al., 2006, 2009; Hosogi et al., 2008; Bruns et al., 2010). Toll-like receptor 4 (TLR4) is a well-characterized pathogen recognition receptor that recognizes pathogen-associated molecular patterns found on pathogens such as viruses, fungi, and bacteria to initiate an immune response (Noreen et al., 2012). LPS is an important ligand of TLR4 that has been routinely shown to cause NETosis (Douda et al., 2011a; Barletta et al., 2012). In the presence of LPS, activated platelets containing TLR4, but not TLR4-deficient platelets migrate into the lungs (Andonegui et al., 2005). These activated platelets can bind to neutrophils to elicit neutrophil activation and induce NETosis (Clark et al., 2007; Caudrillier et al., 2012).

NETs can also be found in ALI models of sterile injury such as transfusion-related ALI (TRALI) (Caudrillier et al., 2012; Thomas et al., 2012). Plasma NETs are found in both ALI and TRALI patients. In addition to NETs, TRALI patients also have the antibody against human neutrophil alloantigen-3a (HNA-3a) in their blood. HNA-3a causes the most severe TRALI and has been shown to promote NETosis in human neutrophils *in vitro* (Thomas et al., 2012). Activated platelets have been shown to induce NETosis not only in TRALI, but also in severe sepsis and deep vein thrombosis (Clark et al., 2007; Brill et al., 2012; Caudrillier et al., 2012; Fuchs et al., 2012). NETs provide a platform for platelets to promote coagulation, thrombosis, and inflammation in vascular diseases such as atherosclerosis, sepsis, and thrombotic diseases (e.g., cancer-associated thrombosis) (Demers et al., 2012). As activated platelets can trigger

NETosis, the histone/DNA complexes of NETs too can activate platelets that further promote NETosis, thrombosis, and coagulation (Semeraro et al., 2011).

Neutrophils and platelets are both key players to the ALI pathology. In a TRALI mouse model, depletion of either neutrophils or platelets was protective (Looney et al., 2009). Comparably, the use of either aspirin or a glycoprotein IIb/IIIa inhibitor to target platelet activation effectively decreased NET formation and lung injury. To target NETs, a histone-blocking antibody and DNase 1 were used and shown to be protective against TRALI (Caudrillier et al., 2012). DNase 1 treatment alone during TRALI was able to improve blood oxygenation and prevent alveolar accumulation of NETs (Thomas et al., 2012). As such, targeting NETs may be a promising therapeutic approach in the treatment of ALI.

The extracellular DNA found accumulated in the airways of LPS-induced ALI and TRALI mice *in vivo* are attributed to NETs (Douda et al., 2011b; Caudrillier et al., 2012; Thomas et al., 2012). SP-D levels in BALF are reduced in patients with ARDS, children with respiratory syncytial virus (RSV) infection, and LPS-induced ALI mouse models (Hartl and Griese, 2006; Douda et al., 2011b). Decreased levels of SP-D may play a contributing factor to the impaired clearance of DNA from these lungs (Palaniyar et al., 2005; Douda et al., 2011b).

CYSTIC FIBROSIS (CF)

Another lung disease featuring chronic airway infections is CF. CF is caused by mutations in the CF transmembrane conductance regulator (CFTR) gene (Riordan et al., 1989), of which CF lung disease is the major cause of morbidity and mortality in these patients (Ratjen and Grasemann, 2012). CFTR is responsible for the modulation of bicarbonate and chloride secretion across airway epithelial cells, as well as for the regulation of sodium absorption via epithelial sodium channel (ENaC) (Stutts et al., 1997; Coakley et al., 2003; Berdiev et al., 2009). Patients with CF have impaired ion transport across the epithelium that ultimately leads to dehydration of the airway surface liquid (Matsui et al., 1998). Consequently, there is increased mucus viscosity and impaired mucociliary clearance (Henke and Ratjen, 2007). There are vast amounts of free DNA that accumulate in CF lungs that contribute to the increased mucus viscosity found in their airways (Henke and Ratjen, 2007). The DNA levels in their airways correlates with neutrophil count, and can be used as an index to inflammation and lung disease severity (Kirchner et al., 1996; Ratjen et al., 2005). Severe neutrophilic inflammation and dying of neutrophils is characteristic of CF lung disease. The origin of the DNA found in CF airways has been traditionally considered to be from necrotic neutrophils (Lethem et al., 1990). Studies conducted after the discovery of NETs have challenged this idea suggesting that the DNA is attributed to NETs as opposed to necrotic neutrophils (Marcos et al., 2010, 2011; Manzenreiter et al., 2012). Understanding the mechanisms that regulate neutrophil death in these airways will facilitate the identification of new therapeutic targets.

Although neutrophils and NETs play vital beneficial roles against infection, their success in host defense in CF patients is significantly compromised as patients often suffer chronic bacterial infections in their lungs. The microbiota present in CF airways is diverse, but eventual chronic pulmonary infections are dominated by opportunistic pathogens *Pseudomonas aeruginosa* and *Burkholderia cepacia* (Razvi et al., 2009; Fodor et al., 2012). In addition to the inability of neutrophils and NETs to eradicate bacteria, the DNA released from neutrophils can promote bacterial colonization and biofilm formation (Parks et al., 2009; Fuxman Bass et al., 2010). CF neutrophils can cast NETs against *P. aeruginosa*. However, evidence reveals that clinical strains of *P. aeruginosa* can acquire resistance to NET-mediated killing over the course of infection in CF airways (Young et al., 2011). The accumulation of bacteria, extracellular DNA and NET-associated enzymes such as MPO and elastases (neutrophil elastase, *Pseudomonas* elastase) worsen lung inflammation and tissue damage (Elizur et al., 2008; Voynow et al., 2008; Gupta et al., 2010; Xu et al., 2011; Dubois et al., 2012; Saffarzadeh et al., 2012). NE in the lungs can further exacerbate inflammation by inducing IL-8 expression for the recruitment of even more neutrophils (Nakamura et al., 1992). Neutrophils in CF airways exhibit a dysfunctional phenotype (Tirouvanziam et al., 2008). The gene expression profile and activation states of CF neutrophils and wild-type neutrophils are different (Adib-Conquy et al., 2008; Tirouvanziam et al., 2008; McKeon et al., 2010; Su et al., 2011), but the implications of these differences on neutrophil or NET function are not clearly understood.

Why more NETosis occurs in CF airways is unknown. Early stage CF lung disease is predominated by inflammation in the absence of any detectable infectious agents. At this stage, NETs are likely induced by host factors. As the lungs of CF patients are chronically infected with bacteria at later stages, it is likely that the source of NETosis stimulation may also be derived from bacterial components. The common pathogens (e.g., *S. aureus, P. aeruginosa, A. fumigatus, C. albicans*) that colonize the lungs of CF patients have been shown to be effective inducers of NETosis (Urban et al., 2006; Bruns et al., 2010; Pilsczek et al., 2010; Young et al., 2011). However, there is still debate on whether inflammation is secondary to chronic infection or vice versa (Becker et al., 2004; Verhaeghe et al., 2007). A number of studies reveal that inflammation and accumulation of neutrophils is seen early on in CF airways prior to the presence of any apparent infection (Tirouvanziam et al., 2000; Verhaeghe et al., 2007). Early CF airways have increased NF-κB activation and inflammatory cytokines such as IL-8, TNF, and GM-CSF (Khan et al., 1995; Rosenfeld et al., 2001; Verhaeghe et al., 2007). NF-κB is an inducible transcription factor that plays a key role in the regulation of cytokines and chemokines, cell adhesion molecules, acute phase proteins, and anti-microbial peptides during pulmonary inflammation (Batra et al., 2011). The contribution of these host-derived molecules on NETosis is unknown. IL-8 has been previously shown to induce NETosis in other studies (Brinkmann et al., 2004; Gupta et al., 2005, 2010), but its ability to induce NETosis in CF airways is uncertain (Marcos et al., 2010, 2011).

SP-A and SP-D levels are decreased in CF patients, where their concentration is inversely related to the degree of inflammation in early CF disease (Postle et al., 1999; Noah et al., 2003). Additionally, there is an inverse relationship between SP-D level

and neutrophil count in BALF (Griese et al., 2004). The lack of SP-D may have implications to the ineffective clearing of DNA in their lungs. The accumulation of NETs in the lung may lead to lung damage and exacerbate the disease by thickening the mucus layer. Aerosolized recombinant human DNase (rhDNase) is a therapeutic option used to treat patients with moderate to severe CF lung disease (Shak et al., 1990). It is used to break down polymerized DNA in the CF airways in order to reduce mucus viscosity. rhDNase treatment has shown to effectively reduce pulmonary exacerbations and improve lung function in some patients (Paul et al., 2004; Ratjen et al., 2005; Henke and Ratjen, 2007). However, DNase treatment does not help with the severe neutrophilic inflammation, chronic bacterial infection, and further deterioration of the lung. Neutrophils in CF lungs release uncontrolled extracellular proteases that destroy lung tissue, and exogenous protease inhibitors are ineffective in inhibiting these proteases (Griese et al., 2008; Voynow et al., 2008; Greene and McElvaney, 2009; Dubois et al., 2012). DNase can disrupt the ultrastructure of NETs, but DNase treatment can also dramatically increase the proteolytic activities of neutrophil enzymes (NE, cathepsin G, protease 3) bound to NETs (Dubois et al., 2012). Ultimately, NETs can harbor active proteases and protect these enzymes from exogenous protease inhibitors (Dubois et al., 2012). SP-D can be proteolytically degraded by active proteases HNE, *Pseudomonas* elastase, cathepsin G, and protease 3 *in vitro* (von Bredow et al., 2003). In CF lungs, SP-D is proteolytically damaged (Griese et al., 2003; von Bredow et al., 2003; Hirche et al., 2004), suggesting impaired host defense mechanisms of SP-D, which may contribute to the accumulation of NET-protein complexes and lung disease.

ASTHMA

Asthma is a chronic disorder characterized by heterogeneous inflammation of the airways involving eosinophilic and non-eosinophilic phenotypes. Patients with neutrophilic asthma (i.e., greater proportion of neutrophils than eosinophils in sputum) usually have greater disease severity with reduced response to corticosteroid therapy (Simpson et al., 2006; Haldar and Pavord, 2007). A recent study showed that the only biomarkers that could distinguish severe or moderate asthma from mild asthma are neutrophil count and IL-8, out of the eight potential biomarkers (IL-8, neutrophils, eosinophils, IL-1Rα, IL-1α, IL-5, IL-6, and RANTES) investigated in BALF (Sur et al., 2012). IL-8 is a known chemoattractant for neutrophils. The neutrophilic inflammation observed in severe asthmatics may be attributable to the increased expression of IL-8 in airway smooth muscle cells, and the increased number of IL-8 positive cells found in epithelia (Pepe et al., 2005; Shannon et al., 2008).

Recently, extracellular DNA traps have been identified in allergic asthmatic airways (Dworski et al., 2011). In the atopic asthmatic airways, eosinophils predominated and were the source of extracellular DNA traps (EETs) observed. Similar to an earlier study on EETs (Yousefi et al., 2008), the DNA were of mitochondrial origin, not nuclear (Dworski et al., 2011). Subjects with neutrophilic asthma had higher neutrophil counts and NETs than eosinophils and EETs. IL-8, neutrophil count, and NETs are all increased in neutrophilic asthma, and their contribution to disease severity is not clearly understood. The cause of NETosis in asthmatic airways is unknown. IL-8 is a potential trigger of NETosis in these airways as it has been previously shown to induce NETosis in other studies (Brinkmann et al., 2004; Gupta et al., 2005, 2010). Plasma levels of activated platelets also increase during seasonal allergic rhinitis and asthma (Kasperska-Zajac et al., 2008). Since activated platelets are known inducers of NETosis, their elevated levels in plasma may imply a role in their contribution to NETs.

TARGETING NETs IN THERAPY

The effective targeting of NET structures in therapy could benefit a multitude of diseases. The list of diseases associated with NETs has been constantly expanding since the discovery of NETs. This list includes SLE (Leffler et al., 2012), multiple sclerosis (Naegele et al., 2012), thrombotic diseases [cancer-associated thrombosis (Demers et al., 2012), deep vein thrombosis (Brill et al., 2012)], appendicitis (Brinkmann et al., 2004), sepsis (Clark et al., 2007), pre-eclampsia (Gupta et al., 2005), psoriasis (Lin et al., 2011), and HIV-1 (Saitoh et al., 2012). Current development of therapies to target NETs in inflammatory lung diseases include DNase (Shak et al., 1990; Hakkim et al., 2010), anti-histone antibodies (Xu et al., 2009, 2011; Semeraro et al., 2011), and antiproteases (Greene and McElvaney, 2009). DNase treatment is used for patients with ALI and CF to reduce pleural fluid viscosity by depolymerizing the DNA that accumulates in the lungs (Huggins et al., 2011). However, Dubois et al. showed that treating CF sputum with DNase could increase elastase activity (Dubois et al., 2012). Chronic inflammatory lung diseases already have elevated levels of proteases, which lead to lung damage and increased inflammation. Antiproteases are used in therapy to dampen the activity of these proteases (Greene and McElvaney, 2009). The use of exogenous protease inhibitors alone has been shown to be ineffective in CF sputum because NETs serve as a reservoir of these active proteases and protect them from inhibition (Dubois et al., 2012). As such, the combined use of DNase and antiproteases may be potentially helpful in controlling NET-mediated lung damage. The use of anti-histone antibodies has also been shown to be protective of NET-mediated lung damage in a TRALI mouse model (Caudrillier et al., 2012). This approach has yet to be investigated in humans; however, the use of anti-histone antibodies raises some concerns. Because extracellular histones are highly immunogenic and induce the production of autoantibodies (Liu et al., 2012), the use of anti-histone antibodies in therapy may promote autoimmunity. As an alternative, others have suggested using anionic polymers such as polysialic acid to neutralize histones (Saffarzadeh et al., 2012). MPO inhibitors have also been considered (Papayannopoulos et al., 2010). SP-D is another protein candidate that could regulate NETosis and NET clearance, and prevent autoantibody generations. Although an excess of NETs may lead to pathologies, moderate amounts are beneficial in protecting hosts against infections. Since NETosis involves components common to other essential pathways in the body (e.g., ERK pathway, p38 kinase pathway, autophagy pathway and intracellular microbial killing NADPH oxidases), careful consideration is required to design drugs to regulate NETosis.

CONCLUSION

Neutrophil function and NETs are critical components of our immune defense. Patients with CGD have impaired neutrophil function and cannot form NETs, making them highly susceptible to lethal infections. Although NETs are important, an excess due to the dysregulation of NETosis can lead to many pathologies. Exaggerated neutrophil recruitment, activation, and NET formation are characteristic of inflammatory lung diseases like CF and ALI. The prolonged presence of NETs is extremely deleterious to host tissue and can stimulate autoimmune responses due to its high immunogenicity. The effective clearance of these NET structures in the lungs may be important to the maintenance of healthy airways. Surfactant proteins A and D are innate immune proteins in the lungs that have shown to be important in the clearance of DNA and may also be important in the clearance of NETs. SP-D is also important in minimizing the production of anti-DNA autoantibodies, which may be protective against NET-mediated autoimmunity. While SP-D can bind to NETs, its role in NET clearance and in treating NET accumulated lungs is unknown. At present, DNase is the only clinically used treatment in targeting the NET structures of these NET-filled inflammatory lung diseases. The homeostasis between NET formation and clearance is essential in sustaining a healthy immune defense against potential pathogens that are constantly in contact with our lungs. The discovery and development of compounds that can help regulate NET formation and clearance would be highly beneficial in designing therapies for these diseases.

ACKNOWLEDGMENTS

This work was supported by the SickKids Foundation (Nades Palaniyar) and Canadian Institutes of Health Research Grant (MOP-134761; Nades Palaniyar). Olivia Z. Cheng was supported by RestraComp/Matching Funds Program Hospital for Sick Children Foundation Student Scholarship Program and a University of Toronto Fellowship Scholarship.

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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.

Received: 07 November 2012; accepted: 01 January 2013; published online: 24 January 2013.

Citation: Cheng OZ and Palaniyar N (2013) NET balancing: a problem in inflammatory lung diseases. Front. Immun. ***4****:1. doi: 10.3389/fimmu.2013.00001*

This article was submitted to Frontiers in Molecular Innate Immunity, a specialty of Frontiers in Immunology.

frontiers in IMMUNOLOGY

REVIEW ARTICLE
published: 13 December 2012
doi: 10.3389/fimmu.2012.00378

Host-protective effect of circulating pentraxin 3 (PTX3) and complex formation with neutrophil extracellular traps

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Pentraxin 3 (PTX3) is a soluble pattern recognition receptor which is classified as a long-pentraxin in the pentraxin family. It is known to play an important role in innate immunity, inflammatory regulation, and female fertility. PTX3 is synthesized by specific cells, primarily in response to inflammatory signals. Among these various cells, neutrophils have a unique PTX3 production system. Neutrophils store PTX3 in neutrophil-specific granules and then the stored PTX3 is released and localizes in neutrophil extracellular traps (NETs). Although certain NET components have been identified, such as histones and anti-microbial proteins, the detailed mechanisms by which NETs localize, as well as capture and kill microbes, have not been fully elucidated. PTX3 is a candidate diagnostic marker of infection and vascular damage. In severe infectious diseases such as sepsis, the circulating PTX3 concentration increases greatly (up to 100 ng/mL, i.e., up to 100-fold of the normal level). Even though it is clearly implied that PTX3 plays a protective role in sepsis and certain other disorders, the detailed mechanisms by which it does so remain unclear. A proteomic study of PTX3 ligands in septic patients revealed that PTX3 forms a complex with certain NET component proteins. This suggests a role for PTX3 in which it facilitates the efficiency of anti-microbial protein pathogen clearance by interacting with both pathogens and anti-microbial proteins. We discuss the possible relationships between PTX3 and NET component proteins in the host protection afforded by the innate immune response. The PTX3 complex has the potential to be a highly useful diagnostic marker of sepsis and other inflammatory diseases.

Keywords: PTX3, pentraxin, diagnosis, protein complex, anti-microbial protein, host-protection

INTRODUCTION

The release of neutrophil extracellular traps (NETs), first reported in 2004 (Brinkmann, 2004), is one of the anti-microbial actions of neutrophils. NETs are mesh-like structures that contain DNA as a backbone, with anti-microbial proteins attached (Amulic and Hayes, 2011). NETs trap microbes and form an anti-microbial-protein-rich microenvironment (Medina, 2009).

Pentraxin 3 (PTX3) was reported as one of the NET component proteins (Jaillon et al., 2007). PTX3 is a member of pentraxin family and mainly acts as a soluble pattern recognition receptor (PRR) in the innate immune response (Bottazzi et al., 2010). In NETs, PTX3 may participate in microbial recognition by facilitating the trapping of microbes. The circulating PTX3 level is known to be increased in certain diseases, and PTX3 may predominantly play a critical role in host protection. Interestingly, proteomic identification of the circulating PTX3 interacting proteins revealed that PTX3 formed a complex with NET component proteins (Daigo et al., 2012). This finding implies that the NET component proteins are active in pathogen recognition and clearance by tethering with each other in NETs and bloodstream. PTX3 appears to be a key tethering molecule to enhance the actions of NETs component proteins. In this review, we will discuss the host-protective roles of PTX3 in relation to NETs component proteins.

NETs

SOURCE, EXPRESSION, AND FUNCTION

Neutrophils are the major player in the innate immune system response against microbial pathogen invasion. One of the anti-microbial activities of neutrophils is the extrusion of NETs (Brinkmann, 2004). NETs are formed upon the activation of neutrophils by factors such as IL-8, lipopolysaccharide (LPS), phorbol 12-myristate 13-acetate (PMA), bacteria, fungi, and activated platelets (Brinkmann, 2004; Clark et al., 2007; Fuchs et al., 2007). Neutrophil death as a result of the extrusion of NETs is called "NETosis," which is a cell death pathway distinct from apoptosis or necrosis (Brinkmann and Zychlinsky, 2007; Steinberg and Grinstein, 2007). The release of NETs has also been reportedly observed without cell death (Yipp et al., 2012). Extracellular formations of this type are also observed in basophils and eosinophils (Schorn et al., 2012). NETs are mesh-like structures that consist of cellular DNA, along with bactericidal proteins, that reside in neutrophil granules and the nucleus. These proteins are connected to DNA fibers, and form a specialized microenvironment which facilitates the capture and killing of bacteria.

THE NET COMPONENT PROTEINS

Using a proteomic approach, Urban et al. identified 24 NET-associated proteins (Urban et al., 2009). These proteins are; nuclear components such as core histones; granular components such as neutrophil elastase (ELANE), lactotransferrin (LTF), cathepsin G (CTSG), myeloperoxidase (MPO), proteinase 3 (PRTN3), azurocidin 1 (AZU1), lysozyme C (LYZ), neutrophil defensins, and cytoplasmic proteins. In other proteins, histone H1, bactericidal permeability-increasing protein (BPI), pentraxin 3 (PTX3), and cathelicidin anti-microbial peptide (CAMP) are also defined as NET component proteins (Brinkmann, 2004; Jaillon et al., 2007; Lauth et al., 2009). Essentially all of these proteins possess anti-microbial activity.

PTX3

GENOME

Breviario et al. identified PTX3 as one of the IL-1β -induced genes in human umbilical vein endothelial cells (HUVECs) (Breviario et al., 1992). The human PTX3 gene is located on chromosome 3q band 25, consists of 1861 base pairs, and is translated into 381 amino acids (Breviario et al., 1992). PTX3 belongs to the pentraxin family, which included the acute phase proteins C-reactive protein (CRP) and serum amyloid P-component (SAP). As PTX3 has a longer N-terminal domain, it is classified as a member of the long-pentraxin subfamily. Unlike the more common short pentraxins CRP and SAP, the *PTX3* gene is highly conserved across species (Garlanda et al., 2005). The *PTX3* gene consists of three exons, among which the first and second exons encode the signal sequence peptide and the N-terminal domain, and the third exon encodes the C-terminal domain. In the promoter region of the *PTX3* gene, a number of potential enhancer binding sequences (Pu-1, AP1, NF-κB, SP1, and NF-IL6) are located (He et al., 2007).

STRUCTURE

After the processing of the signal sequence of the translated 1–17 amino acids, the mature PTX3 consists of two domains, i.e., the N-terminal domain (18–178 a.a.) and C-terminal domain (179–381 a.a.). The PTX3 C-terminal domain is a pentraxin-like domain, which is conserved among the pentraxin family with pentraxin signature (His-x-Cys-x-Ser/Thr-Trp-x-Ser). An N-linked glycosylation site (Asn220) is located in the C-terminal domain. In contrast to the C-terminal domain, the PTX3 N-terminal domain is a unique sequence unrelated to other proteins. The PTX3 protein forms an octamer via the inter-molecule disulfide bonds (Inforzato et al., 2008, 2010). Briefly, the N-terminal domain participates in the organization of a tetramer, and the C-terminal domain participates in the dimerization of the tetramer. Interestingly, the N-terminal tetramer formation has two states; a tetramer via the inter-disulfide bonds or non-covalent dimerization of the inter-disulfide-bonded dimer. This results in the asymmetric form of the full-length PTX3 (Inforzato et al., 2010).

EXPRESSION PATTERN

PTX3 mRNA expression is induced by primary inflammatory signals in certain cells, such as myeloid dendritic cells (Doni et al., 2003), peripheral blood leukocytes (Alles et al., 1994), mononuclear macrophages/phagocytes (Alles et al., 1994; Goodman et al., 2000), vascular endothelial cells (Breviario et al., 1992; Lee et al., 1993), smooth muscle cells (Klouche et al., 2004), fibroblasts (Lee et al., 1993; Goodman et al., 2000), adipocytes (Abderrahim-Ferkoune et al., 2003), glial cells (Polentarutti et al., 2000), cumulus oophorus cells (Salustri et al., 2004), mesangial cells (Nauta et al., 2005), and synovial cells (Luchetti et al., 2000). Transcriptional activation of PTX3 in response to the pro-inflammatory cytokines TNFα and IL-1β is regulated by NF-κB binding site in the PTX3 promoter (Altmeyer et al., 1995; Basile et al., 1997). Other pathways also regulate PTX3 expression in a cell- and signal-dependent manner. In detail, please refer to the excellent reviews cited (He et al., 2007; Ortega-Hernandez et al., 2009; Deban et al., 2011; Inforzato et al., 2011).

The characteristic PTX3 expression pattern is observed in neutrophils. In mature neutrophils, the PTX3 protein is abundantly present in granules, but PTX3 mRNA expression is not detected. In contrast, PTX3 mRNA expression is observed in progenitor neutrophils, such as promyelocytes and myelocytes/metamyelocytes (Jaillon et al., 2007). As PTX3 protein expression is observed in both neutrophil precursors and mature neutrophils, it is considered that the PTX3 protein is produced during the course of neutrophil maturation and mature neutrophils store it for use-on-demand. Immunostaining revealed that PTX3 is present in neutrophil granules and that it colocalizes with lactoferrin (Jaillon et al., 2007; Savchenko et al., 2011), suggesting that PTX3 localizes to specific granules. The stored PTX3 in neutrophils is released upon *E. coli*, *S. aureus* or zymosan stimulation, as well as PMA, ionomycin or TNFα treatment (Jaillon et al., 2007; Savchenko et al., 2011; Daigo et al., 2012). PTX3 release is not induced by IL-1β or latex bead stimulation (Jaillon et al., 2007). The released PTX3 localizes to NETs and plays a non-redundant role in pathogen resistance. Thus, PTX3 in neutrophils plays a distinctive role in the innate immune response due to its rapid secretion, as well as by its unique pattern of ready-to-use expression and storage.

CIRCULATING LEVELS

As the pentraxins CRP and SAP are well-known acute phase proteins, PTX3 may also be an acute phase biomarker. Under physiological conditions, the circulating PTX3 level is as low as approximately 2 ng/mL (Yamasaki et al., 2009). Recently, many studies on the circulating PTX3 level in clinical trials have been reported. These reports indicate that the PTX3 levels are significantly increased in certain infectious, cardiovascular, kidney, and female reproductive system diseases as well as other disorders (summarized in **Table 1**). In most cases, the PTX3 level correlates with both the severity and survivability of the disorder. In these diseases, the increases can reach up to 10∼100 times the control level in severe inflammatory and infectious diseases such as sepsis. In the case of sepsis, the plasma PTX3 dramatically increases to a level of up to 100 ng/mL (Muller et al., 2001) and the increase correlates with mortality (Mauri et al., 2010).

Although not included in **Table 1**, there are other infectious diseases, such as severe dengue virus infection (Mairuhu et al., 2005) and meningococcal disease (Sprong et al., 2009), in which the PTX3 levels are also increased. The PTX3 plasma concentration is increased in patients with acute myocardial

Table 1 | Circulating PTX3 levels measurements in clinical trials.

Disease category	Diseases	PTX3 concentration and significance	References
Physiological level		2.00 (1.95, 2.04)[a]	Yamasaki et al., 2009
Infectious diseases	Systemic inflammatory response syndrome (SIRS)	SIRS: 28.0 ± 5.6 Control: 1.04 ± 0.09[b] $p < 0.005$	Muller et al., 2001
	Pulmonary tuberculosis (TB)	TB: 3.21 Control: 0.98[c] $p < 0.0001$	Azzurri et al., 2005
	Sepsis	Sepsis: 26 (1, 202) Control: 6 (1, 12)[d] $p < 0.001$	Hill et al., 2009
	Febrile in the intensive/medium care unit (ICU/MC) or ward	In ICU/MC: 44.4 (13.6, 105.9) In ward: 14.2 (7.01, 25.1) Control: 2.30 (1.66, 3.67)[d] $p = 0.01$	De Kruif et al., 2010
	Bacteremia	Non-survivor: 44.8 (10.7, 69.4) Survivor: 6.4 (3.4, 13.5)[d] $p < 0.001$	Huttunen et al., 2011
Cardiovascular diseases	Unstable angina pectoris (UAP)	UAP: 6.09 (4.34–7.85) Control: 2.30 (2.03–2.55)[e] $p = 0.00003$	Inoue et al., 2007
	Chronic heart failure (CHF)	CHF: 3.06 (2.38, 4.23) Control: 1.91 (1.35, 2.60)[d] $p = 0.001$	Kotooka et al., 2008
		Cardiac event: 6.0 (4.3, 9.3) Event-free: 3.2 (2.0, 5.5)[d] $p < 0.001$	Ishino et al., 2008
	Heart failure (HF)	Cardiac event: 6.22 (5.59) Event-free: 2.99 (2.95)[d] $p < 0.001$	Suzuki et al., 2008
		HF: 3.28 (1.51, 2.90) Non-HF: 2. 18 (1.51, 2.90)[d] $p < 0.001$	Matsubara et al., 2011
	Coronary artery disease (CAD)	CAD with inflammatory rheumatic disease (IRD): 1.96 ± 0.98 Control: 1.21 ± 0.59[b] $p < 0.001$	Hollan et al., 2010
	Aortic valve stenosis (AS)	AS: 3.5 ± 1.9 Control: 2.1 ± 0.8[b] $p < 0.05$	Naito et al., 2010
	Acute coronary syndrome (ACS)	ACS: 1.73 ± 0.82 Control: 0.50 ± 0.39[b] $p < 0.001$	Ustundag et al., 2011
		ACS: 0.36 (0.225, 1.39) Control: 0.015 (0, 0.06)[f] $p < 0.0001$	Kume et al., 2011
	Hypertension	Anti-hypertensive mediation Pre-treatment: 35.25 ± 5.45 Post-treatment: 0.14 ± 0.19[b] $p < 0.0001$	Parlak et al., 2012
	Acute ischemic strokes	Non-survivor: 18.0 (8.2, 26.1) Survivor: 6.4 (3.4, 11.8)[d] $p < 0.001$	Ryu et al., 2012
	Giant cell arteritis (GCA)	GCA: 23.31 ± 4.06 Control: 3.97 ± 0.28[g] $p < 0.003$	Baldini et al., 2012
Kidney diseases	Hemodialysis (HD)	HD: 3.03 ± 1.81 Uremic patients: 2.34 ± 1.19 Control 1.03 ±0.4[b] $p < 0.001$	Malaponte et al., 2007
		HD: 1.87 (1.34, 2.50) Control: 1.11 (0.86, 1.51)[d] $p < 0.001$	Xu et al., 2011
		Renal transplant patients: 5.78 (1.09–20.36) HD group: 1.65 (0.24–7.89)[h] $p = 0.0001$	Argani et al., 2012

(Continued)

Table 1 | Continued

Disease category	Diseases	PTX3 concentration and significance	References
	Chronic kidney disease (CKD)	Stage 5 CKD: 5.7 (0.9, 64.3) Stage 3 to 4 CKD: 2.2 (0.4, 16.0) Control: 1.8 (0.1, 9.1)[d] $p < 0.001$	Tong et al., 2007
		Stage 5 CKD: 5.3 (1.0, 58.0) Control: 1.8 (0.1, 9.2)[d] $p < 0.001$	Suliman et al., 2008
		CKD: 7.7 (1.8, 32.9) Control: 1.3 (0.1, 2.7)[f] $p < 0.001$	Yilmaz et al., 2010
		CKD: 3.80 ± 2.35 Control: 2.15 ± 0.93[b] $p < 0.0001$	Nishi et al., 2011
		CKD with periodontitis: 6.3380 ± 2.74875 CKD: 5.4100 ± 2.65296 Healthy: 1.8350 ± 0.75977[b] $p = 0.000$	Pradeep et al., 2012
Female reproductive system diseases	Preeclampsia (PE)	PE: 13.8 (3.9, 32.3) Control: 2.2 (1.2, 3.8)[d] $p < 0.001$	Cetin et al., 2006
		PE: 22.64 (18.56, 26.34) Control: 13.17 (8.55, 16.54)[d] $p < 0.001$	Hamad et al., 2012
	Pelvic inflammatory disease (PID)	PID: 9.3 ± 1.01 Control: 2.27 ± 0.12[b] $p < 0.001$	Chang et al., 2011
	Polycystic ovary syndrome (PCOS)	PCOS: 1.0 ± 3.6 Control: 0.8 ± 0.8[b] $p = 0.021$	Aydogdu et al., 2012
Others	Severe Psoriasis (sP)	sP: 2.84 ± 0.94 Control: 1.22 ± 0.47[b] $p < 0.0001$	Bevelacqua et al., 2006
	Ulcerative colitis (UC) and crohn's disease (CD)	Active UC: 8.22 ± 5.48 Active CD: 5.80 ± 3.59 Control: 1.76 ± 1.02[b] $p < 0.05$	Kato et al., 2008
	Obesity	Obesity: 0.99 ± 0.09 Control: 0.63 ± 0.05[g] $p < 0.01$	Miyaki et al., 2010
	Central obesity in abdominal obesity patients	Central obesity: 3.00 ± 2.61 Control: 1.33 ± 0.81[b] $p < 0.01$	Shim et al., 2010
	Severe traumatic brain injury (TBI)	non-survivors 9.95 (6.42) Survivors 5.46 (4.87)[b] μg/mL $p < 0.001$	Gullo Jda et al., 2011
	Obstructive sleep apnea (OSA)	Moderate-to severe OSA: 2.36 (1.79, 2.98) Control: 1.53 (1.14, 2.04)[f] $p < 0.01$	Kasai et al., 2011
	Schizophrenia (SZ)	SZ with the metabolic syndrome: 388.2 (504.1) SZ: 430.4 (523.0) Control: 213.6 (524.0)[d] pg/mL $p < 0.001$	Beumer et al., 2012

PTX3 concentrations are shown in ng/mL, unless indicated.

[a] *Geometrical mean (confidence interval).*

[b] *Mean ± SD.*

[c] *Geometrical mean.*

[d] *Median (interquartile range).*

[e] *Mean (95% confidence interval).*

[f] *Median (25th percentile, 75th percentile).*

[g] *Mean ± SEM.*

[h] *Median (Minimum-Maximum).*

Table 2 | Responses to certain disorders in PTX3-knockout and PTX3-transgenic mice.

Category	Experiment summary	Result summary	References
Lung injury	Murine hepatitis virus strain 1 (MHV-1) infection	Causing greater severity of acute lung injury (ALI)[a]	Han et al., 2012
	Ventilator-induced lung injury (VILI)	Faster development of VILI[b]	Real et al., 2012
	LPS instillation	Causing greater severity of ALI[a]	Han et al., 2011
Vascular damage	Coronary artery ligation and reperfusion	Worsen heart damage[a]	Salio et al., 2008
	Atherogenic diet feed	Increased atherosclerotic lesion area in PTX3 and ApoE-double KO mice	Norata et al., 2009
	Ischemia and reperfusion of the superior mesenteric artery	Prevent tissue injury and mortality[a]	Souza et al., 2009
		Increased tissue injury and mortality[b]	Souza et al., 2002
Infection	LPS-induced endotoxemia	Increased survival ratio[b]	Dias et al., 2001
	CLP-induced sepsis	Increased survival ratio[b]	Dias et al., 2001
	Pulmonary infection by *Aspergillus fumigatus*	Decreased survival ratio[a]	Garlanda et al., 2002
	Pulmonary infection by *Klebsiella pneumoniae*	Faster lethality by a high inoculum administration[b]	Soares et al., 2006
		Delayed lethality by a mid-to-low inoculum administration[b]	Soares et al., 2006
	Murine cytomegalovirus (MCMV) infection	More susceptible to MCMV infection[a]	Bozza et al., 2006
	Influenza virus infection	More susceptible to influenza virus infection[a]	Reading et al., 2008
Others	Fas-deficient (lpr) C57BL/6 (B6) mice with mild lupus-like autoimmunity	Aggravate autoimmune lung disease in PTX3-KO $B6^{Lpr}$ mice	Lech et al., 2011
	Kidney ischemia reperfusion injury	Less kidney injury and inflammation[a]	Chen et al., 2012
	Subcutaneous injection of Matrigel containing FGF2 and/or TSG-6	Abolishing of vascularization inhibition in PTX-KO mice	Leali et al., 2012
	Rolling interaction of PMNs in the mesenteric venules	Increased rolling interaction frequency[a]	Deban et al., 2010
	Sexual system	Subfertile[a]	Varani et al., 2002
	Kainate-induced seizures	More widespread seizure-related neuronal damage in the forebrain of PTX3-KO mice	Ravizza et al., 2001

[a] *PTX3-knockout mouse study.*

[b] *PTX3-transgenic mouse study.*

infarction (Peri et al., 2000). During pregnancy, the serum PTX3 level slightly increases as the pregnancy progresses (Larsson et al., 2011). A higher PTX3 level is observed in preeclampsia (Cetin et al., 2006; Rovere-Querini et al., 2006). Finally, the serum PTX3 level is reported to be a biomarker for lung carcinoma (Diamandis et al., 2011). Thus, the circulating PTX3 level increases non-specifically in various infections and inflammatory disorders. For the purpose of diagnostic measurement, the dynamics of the PTX3 complex, such as the NET component proteins should be monitored (more details are discussed below).

FUNCTION

PTX3 has been postulated to play a variety of roles in innate immunity, inflammatory regulation, and female fertility (Bottazzi et al., 2006; Garlanda et al., 2009; Inforzato et al., 2011; Cieslik and Hrycek, 2012). PTX3-knockout and transgenic mice studies have indicated that the predominant role of PTX3 occurs in host protection in the case of lung injury, infection, vascular damage, as well as certain other disorders (summarized in **Table 2**). Briefly, the resistance against pathogens such as *Aspergillus fumigatus*, *Paracoccidioides brasiliensis*, and *Klebsiella pneumoniae* has been reported (Garlanda et al., 2002; Diniz et al., 2004; Soares et al., 2006). In addition to its anti-pathogenic activity, PTX3 also has been shown to play a role in protecting against severe inflammatory reactions in animal models of sepsis (Dias et al., 2001), seizure-induced neurodegeneration (Ravizza et al., 2001) and acute myocardial infarction (Salio et al., 2008). In addition, PTX3 participates in extracellular matrix deposition. PTX3 is involved

in the organization of hyaluronan in the viscoelastic matrix of cumulus oophorus (Scarchilli et al., 2007). It is considered that these functions of PTX3 are exhibited synergistically along with the binding of specific ligands (the details are provided in section "Ligands").

Of note, among the studies in PTX3-knockout and transgenic mice, there are some reports of an opposite effect of PTX3 on host-protection. In an intestinal ischemia and reperfusion model, Souza et al. reported an increased injury and lethality in the PTX3-transgenic mice that seemed to be associated with elevation of the TNFα concentration and aggravation of the inflammatory response (Souza et al., 2002). They also reported the suppression of tissue injury and lethality after ischemic and reperfusion in PTX3-knockout mice. PTX3 administration to these PTX3-knockout mice reversed this suppression (Souza et al., 2009). Other groups have also reported an adverse effect of PTX3 in acute ischemic lung injury (Chen et al., 2012) and ventilator-induced lung injury (Real et al., 2012). In the case of *Klebsiella pneumoniae* infection, faster lethality was observed when a higher inoculum was administered to PTX3-transgenic mice, but the lethality was conversely delayed when a middle or low inoculum was administered (Soares et al., 2006). Taking these bi-phasic functions of PTX3 in host-defense into account, more detailed accounts of the disease-specific mechanisms of PTX3 need to be elucidated to achieve useful clinical applications.

LIGANDS

The multiple host-protective functions of PTX3 arise from the capacity for the recognition and binding to ligands. The reported PTX3 ligands are classified as follows: (1) complement components; (2) Fungi, bacteria, microbial components, and viruses; (3) selectin P; (4) extracellular matrix proteins and (5) growth factors (Presta et al., 2007; Mantovani et al., 2008; Deban et al., 2009; Moalli et al., 2011). Some of these ligands bind to PTX3 in a PTX3-domain specific manner, while others require full-length PTX3 for binding (Deban et al., 2009; Bottazzi et al., 2010).

Table 3 | List of the NET component proteins and proteins belonging to the PTX3 complex.

NET component proteins			Proteomic identification of PTX3 complex in sepsis
Cellular localization	**Protein name**	**Gene name**	
Granules	Neutrophil elastase	ELANE	
	Lactotransferrin	LTF	
	Azurocidin	AZU1	Yes
	Cathepsin G	CTSG	
	Myeloperoxidase	MPO	Yes
	Proteinase 3	PRTN3	
	Lysozyme C	LYZ	
	Neutrophil defensin 1 and 3	DEFA1 and 3	Yes (DEFA1)
	Pentraxin 3	PTX3	Target protein
	Bactericidal permeability-increasing protein	BPI	
	Cathelicidin anti-microbial peptide	CAMP	
Nucleus	Histone H1	H1F0	
	Histone H2A	H2A	Yes
	Histone H2B	H2B	
	Histone H2B-like	H2B	
	Histone H3	H3	Yes
	Histone H4	H4	Yes
	Myeloid cell nuclear differentiation antigen	MNDA	
Cytoplasm	S100 calcium-binding protein A8	S100A8	
	S100 calcium binding protein A9	S100A9	
	S100 calcium-binding protein A12	S100A12	
Cytoskeleton	Actin (beta and/or gamma 1)	ACTB, ACTG1	Yes
	Myosin-9	MYH9	Yes
	Alpha-actinin (1 and/or -4)	ACTN1, ACTN4	
	Plastin-2	LCP1	
	Cytokeratin-10	KRT10	Yes
Peroxisomal	Catalase	CAT	
Glycolytic enzymes	Alpha-enolase	ENO1	Yes
	Transketolase	TKT	Yes

1. PTX3 binds to certain select complement components, such as C1q (Inforzato et al., 2006), C4b-binding proteins (Braunschweig and Józsi, 2011), ficolins (Ma et al., 2009; Gout et al., 2011), mannose-binding lectin 2 (MBL) (Ma et al., 2011), factor H (Deban et al., 2008; Kopp et al., 2012), factor H-like protein 1 (Kopp et al., 2012) and factor H-related protein 1 (Kopp et al., 2012) for the regulation of the complement pathways in the innate immune response. The interaction of PTX3 and C1q elicits a dual consequence in the classical complement pathway. When C1q binds to immobilized PTX3, the classical complement pathway is activated; however, the binding of C1q to PTX3 in the fluid phase inhibits complement activation (Nauta et al., 2003). PTX3 can also activate the lectin pathway by binding to the ficolins and MBL. PTX3 enhances complement deposition by ficolin-2 on the *Aspergillus fumigatus* surface (Ma et al., 2009), and PTX3-MBL binding enhanced C4 and C3 deposition as well as the phagocytosis of *Candida albicans* (Ma et al., 2011). PTX3 is not only involved in complement activation, but also acts as a complement inhibitor to regulate excessive complement activation by binding to C4b-binding proteins and factor H. Please refer to the review by Doni et al. for more detail (Doni et al., 2012).
2. In the protection afforded against infection, PTX3 recognizes certain fungi, bacteria, microbial moieties, and viruses. PTX3 binds to microbial pathogens such as *Pseudomonas aeruginosa* (Garlanda et al., 2002), *Salmonella typhimurium* (Garlanda et al., 2002), *Aspergillus fumigatus* (Garlanda et al., 2002), and *Paracoccidioides brasiliensis* (Diniz et al., 2004). PTX3-knockout mice are susceptible to invasive pulmonary aspergillosis due to inappropriate Th1 and Th2-helper-cell-mediated resistance (Garlanda et al., 2002). Macrophages from PTX3-transgenic mice exhibit improved phagocytosis of *Paracoccidioides brasiliensis* as well as an enhancement of the production of nitric oxide (NO) (Diniz et al., 2004). PTX3 also binds to outer membrane protein A from *Klebsiella pneumoniae* (KpOmpA) in order to modulate the inflammatory response triggered by KpOmpA (Jeannin et al., 2005). PTX3 binds to cytomegalovirus and influenza virus type A for the inhibition of infection (Bozza et al., 2006; Reading et al., 2008). Upon binding to influenza virus, PTX3 exerts anti-viral activity by the inhibition of hemagglutination, the neutralization of virus infectivity and the inhibition of viral neuraminidase (Reading et al., 2008).
3. As an inflammatory modulator, PTX3 binds to selectin P. The N-linked glycosidic moiety of PTX3 contributes to the binding of selectin P, and this binding dampens neutrophil recruitment at the sites of inflammation (Deban et al., 2010). Importantly, in a model of acid-induced acute lung injury, both exogenous PTX3 and endogenously released PTX3 administration suppress neutrophil recruitment (Deban et al., 2010). This suggests a negative feedback role of PTX3 that dampens the excessive neutrophil recruitment via selectin P.
4. PTX3 takes part in extracellular matrix formation by binding to TNFα-induced protein 6 (TNFAIP6 or TSG-6) and inter-α-trypsin inhibitor (IαI) (Salustri et al., 2004; Scarchilli et al., 2007; Ievoli et al., 2011). PTX3-knockout mice exhibit a defect in female fertility because of the defects in ovulation (Varani et al., 2002) and the organization of the cumulus oophorus extracellular matrix (Salustri et al., 2004). The PTX3-TSG-6 and PTX3-IαI binding events are considered to be essential for the organization of hyaluronan in the viscoelastic matrix of cumulus oophorus (Inforzato et al., 2011; Moalli et al., 2011).
5. PTX3 binding to fibroblast growth factor 2 (FGF-2) regulates endothelial cell proliferation and angiogenesis, smooth muscle cell (SMC) activation, and intima thickening after arterial injury (Rusnati et al., 2004; Camozzi et al., 2005). PTX3-FGF2 binding can inhibit the proliferation and chemotactic activity of FGF2 in SMCs by interfering with the interaction of the FGF2 and FGF receptors (Camozzi et al., 2005).

Taking these results, it is clear that the protective effects of PTX3 are realized in coordination with specific PTX3 ligands. Therefore, we carried out a proteome-wide identification of PTX3 ligands and complexes in septic patient serum and plasma.

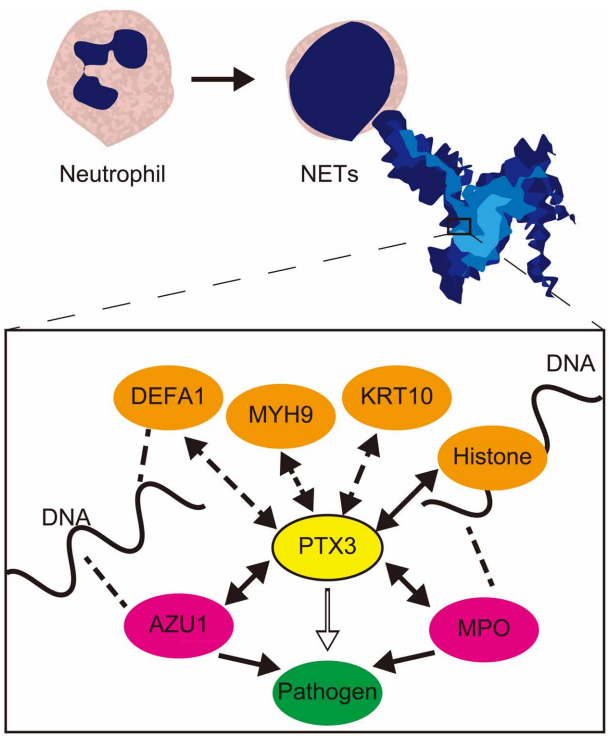

FIGURE 1 | Schematic relationship and role of PTX3 and NET component proteins in pathogen recognition and clearance. NET component proteins which were identified as PTX3 complex (Daigo et al., 2012) are associated PTX3 in NETs. Among these, the confirmed direct interaction of AZU1 and MPO to PTX3, and formerly reported histone-PTX3 interaction (Garlanda et al., 2005) are designated by two-way arrows. These bindings facilitate pathogen clearance efficiency of AZU1 and MPO. The pathogen recognition and anti-pathogenic action are designated by open arrow and closed arrow in box, respectively. Two-way arrows with dashed lines designate other potential interactions to PTX3. The indirect association to DNA via histone or basic proteins such as DEFA1, AZU1, and MPO, which DNA associations are designated by dashed lines, maintains PTX3 localization in NETs. PTX3, pentraxin 3; DEFA1, neutrophil defensin 1; MYH9, Myosn-9; KRT10, Cytokeratin-10; AZU1, azurocidin 1; MPO: myeloperoxidase.

PTX3 and its complex component proteins were immunoprecipitated by anti-PTX3 antibody-crosslinked magnetic beads, and the isolated fractions were subjected to shotgun proteomics analysis for label-free relative quantitation via spectral counting (Daigo et al., 2012). The identified proteins included the known PTX3 ligands such as C1q, ficolins, TSG-6, and IαI, as mentioned above. Additionally, the ficolin-binding proteins of mannan-binding lectin serine protease 1 and 2 (MASP1 and MASP2) (Ma et al., 2009), and the TSG-6 binding proteins of the versican core protein (VCAN) and thrombospondin-1 (THBS1) (Salustri et al., 2004) were included in the proteins that were identified. As these proteins were identified in pooled normal human plasma with artificially spiked recombinant PTX3, these appear to be stable circulating PTX3 complexes. Nevertheless, the disease-specific dynamics of these binding levels need to be investigated further, as do the specific functions of these PTX3 complexes in sepsis.

NET COMPONENT PROTEINS AS PTX3 LIGANDS: A NEWLY RECOGNIZED PROTECTIVE ROLE

In the effort to identify the PTX3 ligand in septic patient fluids, a novel finding is that the NET component proteins were included (Daigo et al., 2012) (**Table 3**). A detailed investigation revealed that azurocidin 1 (AZU1) and myeloperoxidase (MPO) directly bind to PTX3. AZU1 and MPO belong to the NET component proteins (Urban et al., 2009) and exert bactericidal activity (Watorek, 2003; Klebanoff, 2005). AZU1 preferably binds to the PTX3 N-terminal domain, with a pattern of calcium ion dependency. In contrast to AZU1, MPO binds to both the PTX3 N-terminal and C-terminal domains, and does not require calcium ions. Further investigation of the PTX3-AZU1 interaction revealed that the AZU1 binding affinity to PTX3 was 22 ± 7.6 nM, and that AZU1 and PTX3 are partially co-localized in NETs (Daigo et al., 2012).

From these results, it is suggested that PTX3 may enhance the bactericidal efficiency of AZU1 and MPO in terms of both pathogen recognition and AZU1 and MPO binding (**Figure 1**). The mechanism by which PTX3 localizes in NETs has not yet been determined, but it is possible that PTX3 localization arises from an interaction with histones or the basic proteins AZU1, MPO, and defensin, along with a simultaneous association between these basic proteins and DNA (**Figure 1**). It is not clear at present whether the PTX3-AZU1 and PTX3-MPO binding events in the bloodstream take place within or outside of NETs. Either or even both of these are possible, and these complexes may be active in pathogen recognition and also involved in clearance. In septic patients, the plasma levels of AZU1 are increased, but do not significantly correlate with mortality (Berkestedt et al., 2010). As useful biomarkers of sepsis not yet available (Pierrakos and Vincent, 2010), the binding levels of PTX3-AZU1 and PTX3-MPO in septic plasma have the important potential to fulfill this purpose.

CONCLUSION

Recent proteomic investigation of the circulating PTX3 complex components has revealed new and pivotal roles of PTX3 in the innate immune response, along with a pattern of binding to the NET component proteins. In NETs, PTX3 brings the NET component proteins into close proximity with the pathogens that PTX3 capture in order to enhance pathogen clearance. Also, in the bloodstream, PTX3 forms a complex with bactericidal proteins for the recognition and clearance of pathogens. These activities of PTX3 in concert contribute to the host-protective effect. In addition, the dynamic changes that occur in PTX3 and its complex proteins may become specific biomarker for severe inflammatory diseases.

ACKNOWLEDGMENTS

This work was supported by Japan Grants-in-Aid for Scientific Research 20221010 from the Ministry of Education, Culture, Sports, Science and Technology, and collaborative research of the University of Tokyo and JSR Corporation. We thank Dr. Kevin Boru of Pacific Edit for review of 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.

Received: 28 September 2012; paper pending published: 16 October 2012; accepted: 26 November 2012; published online: 13 December 2012.

Citation: Daigo K and Hamakubo T (2012) Host-protective effect of circulating pentraxin 3 (PTX3) and complex formation with neutrophil extracellular traps. Front. Immun. ***3****:378. doi: 10.3389/fimmu.2012.00378*

This article was submitted to Frontiers in Molecular Innate Immunity, a specialty of Frontiers in Immunology.

frontiers in IMMUNOLOGY

ORIGINAL RESEARCH ARTICLE
published: 05 March 2013
doi: 10.3389/fimmu.2013.00056

In vivo and *in vitro* studies on the roles of neutrophil extracellular traps during secondary pneumococcal pneumonia after primary pulmonary influenza infection

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Seasonal influenza virus infections may lead to debilitating disease, and account for significant fatalities annually worldwide. Most of these deaths are attributed to the complications of secondary bacterial pneumonia. Evidence is accumulating to support the notion that neutrophil extracellular traps (NETs) harbor several antibacterial proteins, and trap and kill bacteria. We have previously demonstrated the induction of NETs that contribute to lung tissue injury in severe influenza pneumonia. However, the role of these NETs in secondary bacterial pneumonia is unclear. In this study, we explored whether NETs induced during pulmonary influenza infection have functional significance against infections with *Streptococcus pneumoniae* and other bacterial and fungal species. Our findings revealed that NETs do not participate in killing of *Streptococcus pneumoniae in vivo* and *in vitro*. Dual viral and bacterial infection elevated the bacterial load compared to animals infected with bacteria alone. Concurrently, enhanced lung pathogenesis was observed in dual-infected mice compared to those challenged with influenza virus or bacteria alone. The intensified NETs in dual-infected mice often appeared as clusters that were frequently filled with partially degraded DNA, as evidenced by punctate histone protein staining. The severe pulmonary pathology and excessive NETs generation in dual infection correlated with exaggerated inflammation and damage to the alveolar-capillary barrier. NETs stimulation *in vitro* did not significantly alter the gene expression of several antimicrobial proteins, and these NETs did not exhibit any bactericidal activity. Fungicidal activity against *Candida albicans* was observed at similar levels both in presence or absence of NETs. These results substantiate that the NETs released by primary influenza infection do not protect against secondary bacterial infection, but may compromise lung function.

Keywords: NETs scoring, neutrophils, primary influenza, secondary pneumonia, *Streptococcus pneumoniae*, mouse models

INTRODUCTION

Secondary bacterial super-infections are the most frequent complications among fatal cases of seasonal influenza and of pandemic influenza. Primary influenza virus infection predisposing to secondary bacterial pneumonia was highlighted by autopsy analyses during the 1918 influenza H1N1pandemic, as over 95% of deaths were attributed to secondary bacterial infections (Simonsen, 1999; Johnson and Mueller, 2002; Morens et al., 2008; Estenssoro et al., 2010). Bacterial pathogens including *Streptococcus pneumoniae* (*S. pneumoniae*), *Staphylococcus aureus (S. aureus), and Haemophilus influenzae (H. influenzae*) are frequently associated with secondary infections (Kyaw et al., 2006). The incidence of bacterial pneumonia was 25–56% in patients infected with 2009 pandemic swine influenza H1N1 virus, especially severely ill patients (Dominguez-Cherit et al., 2009; Kumar et al., 2009; Palacios et al., 2009; Estenssoro et al., 2010). Similarly, most fatalities due to seasonal influenza are linked to bacterial super-infections (McCullers, 2006; Lei et al., 2010). The extent of influenza-induced cytopathic damage and the magnitude of immune response in the lungs influence the morbidity and mortality in bacterial super-infections. Histopathologically, secondary bacterial infections following influenza exhibit marked inflammation, bronchopneumonia, bacteremia, and diffuse damage in multiple lobes of the lungs (McCullers and Rehg, 2002; McCullers, 2004; Kash et al., 2011). Evidence is accumulating to support that severe outcomes in secondary bacterial complications are due to exaggerated inflammatory responses and neutrophil-predominant infiltrations (Morens et al., 2008; Karlström et al., 2011). Induction of lung inflammatory cytokines (e.g., TNF-α, IL-1β) and chemokines (e.g., mouse KC, MIP-1α)

with accompanying intense neutrophil influx within the damaged areas after challenge with *S. pneumoniae* following primary influenza implicates the role of neutrophils in lung pathogenesis (Smith et al., 2007). Although bacterial infections are most common, secondary fungal infections with *Aspergillus* or *Zygomycota* species are also occasionally encountered in severe influenza infections. Invasive zygomycosis was reported in patients who succumbed to pandemic H1N1-2009 virus infection (Guarner et al., 2006; Bal et al., 2012).

Our previous findings demonstrate the participation of neutrophil extracellular traps (NETs) in lung pathogenesis during influenza (Narasaraju et al., 2011). NETs were initially identified for their role in bactericidal activity, and were implicated in innate immunity (Brinkmann et al., 2004; Fuchs et al., 2007). DNA fibers in NETs form web-like structures and harbor several antibacterial proteins that aid in trapping and killing bacteria or other microbial pathogens. However, the prolonged presence of NETs is linked with host tissue damage and risk for development of auto-reactivity against various components in NETs. Furthermore, NETs have been observed in inflammatory, autoimmune, and vascular diseases (Baker et al., 2008; Kessenbrock et al., 2009; Garcia-Romo et al., 2011). The close attachment of DNA strands in NETs carrying cytotoxic proteases leads to thin endothelial damage documented in sepsis and small vessel vasculitis (Garcia-Romo et al., 2011). We previously reported NETs embroiled with thin alveolar-capillary surfaces of the lungs during severe influenza (Narasaraju et al., 2011). NETs-associated proteins including histone and myeloperoxidase (MPO) are directly involved in inducing cytotoxic effects in alveolar epithelial and endothelial cells (Saffarzadeh et al., 2012).

Although NETs generation is evident in bacterial infections such as *S. pneumoniae,* there are hitherto no *in vivo* reports on the characterization of NETs induction during secondary bacterial pneumonia following primary influenza. It is not known whether NETs produced during influenza exacerbates secondary bacterial pneumonia or if they play beneficial roles by trapping and killing bacteria. This study characterized the induction of NETs during secondary bacterial pneumonia following primary influenza. Our findings revealed increased release of NETs during secondary bacterial infection. The presence of significant NETs release during influenza did not reduce bacterial replication in the lungs. Moreover, *in vitro* NETs stimulation did not significantly alter the gene expression of several antibacterial proteins, and these NETs did not exert any bactericidal activity. These results indicate that NETs released during primary influenza do not confer a protective role.

MATERIALS AND METHODS

MICROORGANISMS, ANIMALS, AND ETHICS APPROVAL

Influenza virus A/Puerto Rico/8/34 H1N1 (PR8) obtained from the American Type Culture Collection was propagated in embryonated eggs, and viral titers were determined as described previously (Narasaraju et al., 2011). *S. pneumoniae* serotype 19F was cultured in brain-heart infusion broth supplemented with 5% fetal calf serum under anaerobic conditions. *Klebsiella pneumoniae* K15 (*K. pneumoniae*) was cultured in Luria–Bertani broth under aerobic conditions. For experiments, both bacterial species were harvested at their mid-logarithmic phases at 37°C. Bacterial optical density (OD) was measured at 600 nm, and cell numbers were calculated by growth curves based on OD and standard colony counts of serial dilutions. *S. aureus* and *Pseudomonas aeruginosa* (*P. aeruginosa*) were also employed for *in vitro* experiments to study NETs generation and degradation. All bacterial strains were clinical isolates from Singapore. *Candida albicans* (*C. albicans*, a clinical isolate from Singapore) was cultured on Sabouraud dextrose agar under aerobic conditions at 28°C. Cell numbers were counted with a hemocytometer. Female BALB/c and C57BL/6 mice (7–10 weeks old) were used, and housed in micro-isolator cages in an animal BSL-2 laboratory. All animal protocols (050/11 and 117/10) were approved by the Institutional Animal Care and Use Committee, National University of Singapore.

VIRAL AND BACTERIAL INFECTIONS OF MICE

For all infections, animals were anesthetized with a mixture of 75 mg/kg ketamine and 1 mg/kg medetomidine, and revived with Antisedan (atipamezole hydrochloride) solution given by intraperitoneal injection. C57BL/6 mice were infected intratracheally with lethal doses of influenza PR8 virus, i.e., 250 plaque-forming units (PFU) or ∼1 LD_{50}. Seven days after influenza infection, mice were challenged with sub-lethal doses of 10^5 colony-forming units (CFU) of *S. pneumoniae* 19F in 50 µl of phosphate-buffered saline (PBS) via the intratracheal route. Animals infected with bacteria alone also received the same bacterial challenge dose.

HISTOPATHOLOGIC ANALYSES

Lungs from each animal in all groups were fixed in 4% formaldehyde, dehydrated, and embedded in paraffin. Lung sections (4 µm thick) were stained with hematoxylin and eosin. A semi-quantitative histopathologic scoring system was employed in a blinded manner by experienced histopathologists. To generate each lung injury score, multiple fields were examined per slide at 400 × magnification. Within each field, points were assigned according to predetermined criteria as described previously (Matute-Bello et al., 2001). The points for each category were added and weighted according to their relative importance, and the injury score was calculated according to the following modified formula: (alveolar hemorrhage) + 2 × (alveolar infiltrate) + 3 × (fibrin) + (alveolar septal congestion).

DETECTION AND QUANTIFICATION OF NETS IN LUNG SECTIONS BY IMMUNOSTAINING

Immunohistochemical analyses of formalin-fixed lung sections were performed. Briefly, lung sections were deparaffinized in Histo-Clear, rehydrated through an ethanol series, permeabilized with 0.025% Triton X-100 in Tris-buffered saline (TBS) for 10 min, and blocked with 3% bovine serum albumin in TBS for 1 h. The sections were then incubated overnight at 4°C with 1:1000 dilutions of mouse monoclonal anti-histone H2B antibody and rabbit polyclonal anti-MPO antibody (Abcam). After washing thrice with TBS, the sections were incubated with 1:250 dilutions of anti-mouse Alexa Fluor 488 and anti-rabbit

Alexa Fluor 555 secondary antibodies (Molecular Probes), as well as DAPI (Invitrogen) at room temperature for 1 h. The sections were then washed with TBS, mounted with anti-fade mounting medium (Invitrogen), and examined under an IX81 Olympus confocal microscope. All images were processed using the FV10-ASW 3.0 viewer.

For quantification of NETs, the slides were scanned using a high resolution MIRAX MIDI system (Carl Zeiss), and 20 fields per slide were captured randomly at 40 × magnification using the Pannoramic viewer. We designed a scoring system to quantify NETs in lung sections microscopically based on the distribution of structures of NETs (**Table 1**). To identify NETs, we selected two major morphologic patterns of NETs formation (individual single strands or clusters of DNA) co-stained for MPO, histone H2B, and DAPI, and assigned scores based on their appearance in each field. The areas of the clusters were measured using ImageJ software, and were used as differential criteria to assign scores. The total score was calculated as the sum of scores of 20 fields. For comparison of NETs counts, the mean scores were derived for three groups ($N = 6$ each), i.e., primary influenza, primary bacterial, and secondary bacterial infections.

MEASUREMENT OF BACTERIAL LOAD AND VIRAL mRNA IN MURINE LUNGS

Mice were euthanized at 30% weight loss, and the left lobes of lungs were harvested under sterile conditions in liquid nitrogen. The lungs were homogenized in PBS using a gentleMACS dissociator (Miltenyi Biotec). Pneumococcal colony counts of lung homogenates were determined by plating 10-fold serial dilutions on tryptic soy agar plates supplemented with 5% sheep erythrocytes. For quantification of influenza viral nucleoprotein mRNA, real-time RT-PCR was performed using primers 5′-GGGTGAGAATGGACGAAAAA-3′ and 5′-TCCATCATTGCTTTTTGTGC-3′ as described previously (Yamada et al., 2012).

ISOLATION OF BONE MARROW-DERIVED NEUTROPHILS FROM MICE

Neutrophils were isolated using Percoll-gradient according to a previous protocol (Ermert et al., 2009). Briefly, bone marrow from 7–10-week-old female BALB/c mice was flushed out of the tibia and femur in Dulbecco's PBS without Ca^{2+} and Mg^{2+} (Biowest), homogenized with a 22-gauge needle, and passed through a 70-μm cell strainer to obtain single cell suspensions. Cells were washed once, and layered on discontinuous Percoll gradient, i.e., 78, 69, and 52% Percoll in PBS, and centrifuged for 30 min at 1500 × g. Mature neutrophils were recovered from the interphase between 69 and 78% Percoll. The purity of mature neutrophils was greater than 90% as assessed by modified Giemsa staining.

Table 1 | Scoring system for the quantitative evaluation of NETs in the lungs of infected animals.

Category	Number	Area (μm^2)	Score
None	N.A.	N.A.	0
Single strand	<5	N.A.	1
Single strand	≥5	N.A.	2
Cluster, small	1	<500	2
Cluster, small + single strands/clusters	>1	<500	4
Cluster, medium	1	500–5000	6
Cluster, medium + small clusters/strands	>1	500–5000	8
Cluster, large ± small clusters/strands	N.A.	>5000	10

Total score, sum of scores in 20 fields under 400× magnification. Areas of demarcated NETs clusters were computed using ImageJ software.
N.A., Not applicable.

COLLECTION OF BRONCHOALVEOLAR LAVAGE FLUID (BALF)

BALF was collected from female BALB/c mice infected intratracheally with 500 PFU of PR8 virus (lethal dose) or control PBS (mock infection) at 5 days post-infection (dpi) as described previously (Narasaraju et al., 2011). The BALF was centrifuged, and the aliquots of cell-free supernatants were stored at −80°C until further use.

QUANTIFICATION OF NETS *in vitro* AND EFFECT OF INHIBITORS OF REDOX ENZYMES ON NETS PRODUCTION

We previously demonstrated that NETs generation is induced by redox enzymes such as MPO and superoxide dismutase (SOD) when neutrophils were co-cultured with influenza-infected alveolar epithelial cells (Narasaraju et al., 2011). To substantiate whether redox enzymes released into the alveolar space during infection contribute to NETs formation, we incubated neutrophils isolated from bone marrow of uninfected mice with BALF collected from influenza-infected or mock-infected animals. Neutrophils (0.5×10^6) were incubated with infected BALF for 15 min, 30 min, 1 h, 1.5 h, and 2 h in 8-well chamber slides. At the end of each time-point, the supernatant was removed, the NETs were fixed with formaldehyde, stained, mounted, and observed under fluorescence microscopy. At least 10 fields were captured per well under 400× magnification. NETs were quantified as the percentage of positive events (neutrophils undergoing NETosis) out of total neutrophils in an average of 10 fields (Berends et al., 2010). To determine whether the induced redox enzymes are involved in NETs release, neutrophils were pre-treated for 15 min with 10 μM diphenyleneiodonium chloride (DPI, an inhibitor of NADPH oxidase), 100 μM diethylthiocarbamate (DETC, an inhibitor of SOD), or 100 μM of 4-aminobenzoic hydrazide (ABAH, an inhibitor of MPO) followed by incubation with uninfected or infected BALF for 2 h. NETs were quantified as described above.

GENE EXPRESSION OF ANTIMICROBIAL PROTEINS DURING *in vitro* NETS GENERATION BY QUANTITATIVE REAL-TIME RT-PCR

We previously demonstrated that MPO activation induces NETs, and mice challenged with lethal influenza show induced MPO activity in BALF. Incubation of neutrophils with BALF collected from influenza-infected mice significantly induces NETs generation (Narasaraju et al., 2011; Ng et al., 2012). In this study, we evaluated the gene expression of specific proteins during active NETs generation. We selected six proteins associated with bactericidal activity, i.e., cathelicidin, lactotransferrin, pentraxin-3, matrix metalloproteinase-9 (MMP9), S100A8, and S100A9.

For NETs induction, neutrophils (1.5×10^6 per well) were incubated with 150 μl of BALF isolated from influenza-infected or mock-infected mice for 15 or 30 min, 1, 1.5, or 2 h. RNA was extracted using the RNeasy RNA purification kit (Qiagen), and reverse-transcribed with MMLV reverse transcriptase (Promega). The resultant cDNAs were subjected to real-time PCR analysis using LightCycler SYBR Green PCR mix (Roche). The following gene primers were employed, i.e., mouse cathelicidin-related antimicrobial peptide (5′-GGCTGTGGCGGTCACTATC-3′ and 5′-GTCTAGGGACTGCTGGTTGAA-3′); pentraxin-3 (5′-CGCAGGTTGTGAAACAGCAAT-3′ and 5′-ATGCACGCT TCCAAAAATCTTC-3′); MMP9 (5′-GCAGAGGCATACTT GTACCG-3′ and 5′-TGATGTTATGATGGTCCCACTTG-3′); lactotransferrin (5′-CCGCTCAGTTGTGTCAAGAAA-3′ and 5′-CATGGCATCAGCTCTGTTTGT-3′); S100A8 (5′-AAATCAC CATGCCCTCTACAAG-3′ and 5′-CCCACTTTTATCACCATC GCAA-3′); S100A9 (5′-GCACAGTTGGCAACCTTTATG-3′ and 5′-TGATTGTCCTGGTTTGTGTCC-3′); and GAPDH as the housekeeping gene control (5′-CTTCATTGACCTCAACTACA-3′ and 5′-ATATTTCTCGTGGTTCACAC-3′).

ANTIBACTERIAL AND ANTIFUNGAL ACTIVITIES, AND ANALYSIS OF NETS DEGRADATION AND BACTERIAL ENTRAPMENT

Bactericidal activity of neutrophils was ascertained by co-incubating neutrophils with *S. pneumoniae* or *K. pneumoniae*. Neutrophils (0.5×10^6) were incubated with 150 μl of infected or uninfected cell-free BALF for 1 or 2.5 h to generate NETs. *S. pneumoniae* 19F or *K. pneumonia*e K15 bacteria were added at multiplicity of infection (MOI) of 0.2, 0.1 or 0.01 for 120, 30 or 90 min, respectively. Cytochalasin B (10 μg/ml) was added to each test well 15 min prior to addition of bacteria to inhibit phagocytosis. After incubation, the contents of each well were scraped thoroughly, and serial dilutions were plated onto 5% sheep blood agar (for *S. pneumoniae*) and Luria–Bertani agar (for *K. pneumoniae*). After overnight incubation at 37°C, colony counts were carried out to determine the number of CFU.

Fungicidal activity was ascertained in a similar manner by incubating 0.5×10^6 neutrophils for 2.5 h with infected or control BALF in 0.1% gelatin-coated wells, to which *C. albicans* was added at MOI of 0.1. Cytochalasin B (10 μg/ml) was added to each test well 15 min prior to addition of *C. albicans* to inhibit phagocytosis. After incubation, the contents of each well were scraped thoroughly, and serial dilutions were plated onto Sabouraud dextrose agar. After overnight incubation at 37°C, colony counts were performed to determine the number of CFU.

To ascertain whether bacteria can cause degradation of NETs DNA, we incubated 0.5×10^6 neutrophils with *S. pneumoniae*, *K. pneumoniae*, *S. aureus*, and *P. aeruginosa* at MOI of 1. After incubation for 2 h, NETs were stained with DAPI and observed under fluorescence microscopy. In addition, NETs were induced with infected BALF, and *S. pneumoniae* was added at MOI of 0.01, 0.1, 1, 10, 100, and incubated for 2 h. NETs were quantified as described previously. To visualize bacterial entrapment in NETs, bacteria were stained with a rabbit polyclonal antibody against *S. pneumoniae* 19F for 45 min at room temperature and counterstained with Alexa Fluor 555 for 1 h at room temperature. Bacterial entrapment in NETs was evaluated as the percentage of total NETs showing bacterial entrapment in an average of 10 fields (Berends et al., 2010).

STATISTICAL ANALYSES

Statistical analyses were performed using Student's *t*-test for pairwise comparison, and ANOVA with Tukey *post-hoc* correction for comparison of more than 2 groups. Non-parametric data were analyzed using Kruskal–Wallis test with *post-hoc* Mann–Whitney pairwise comparison and Bonferroni correction. A value of $P < 0.05$ was considered statistically significant. The data were expressed as mean ± SE.

RESULTS

SYNERGISTIC EFFECTS OF PRIMARY INFLUENZA AND SECONDARY BACTERIAL INFECTION ON MICROBIAL REPLICATION AND LUNG PATHOGENESIS

To determine the synergistic effects of primary influenza and secondary bacterial pneumonia, mice were challenged with lethal doses of influenza, followed by sub-lethal *S. pneumoniae* infection. The effects of co-infection on microbial replication in the lung micro-environment were determined. Interestingly, co-challenge with *S. pneumoniae* generally did not alter virus replication, and viral RNA levels declined similar to animals infected with virus alone. However, significant reduction of virus titers in dual-infected animals was found at 5 dpi (**Figure 1A**). In contrast, dual infection enhanced bacterial multiplication significantly higher than the animals infected with bacteria alone, indicating the additive effect of influenza on bacterial load (**Figure 1B**). Influenza infection alone exhibited inflammation predominantly with neutrophils and macrophages, while sub-lethal infection with *S. pneumoniae* alone produced relatively mild inflammatory responses. Infection with bacteria alone for two days did not cause severe pathology: alveolar septal congestion was similar to influenza-infected animals; focal neutrophil-specific cellular infiltrations were observed in the alveolar air spaces, but excessive neutrophils were found within the capillaries and small blood vessels. Co-infection with influenza and *S. pneumoniae* culminated in multi-lobular pulmonary damage compared to infection with virus or bacteria alone. Alveolar air spaces were filled with proteinaceous material and fibrin deposition in dual-infected lungs. The increased inflammatory cell recruitment in dual-infected mice is likely to be driven by elevated levels of lung cytokines and chemokines due to influenza infection prior to bacterial challenge. Influenza infection alone intensified alveolar septal congestion, with mild-to-moderate inflammation mainly with macrophages and neutrophils (**Figure 1C** and **Table 2**). No significant difference in overall histopathology score was found between influenza only and dual infection groups, although there was heightened inflammation and tissue consolidation in dual-infected animals. However, the histopathology score was the lowest in mice sub-lethally infected with *S. pneumoniae* (**Figure 1D**).

QUANTIFICATION OF INCREASED NETS RELEASE IN MICE CHALLENGED WITH *S. pneumoniae* FOLLOWING PRIMARY INFLUENZA

We previously demonstrated that during influenza infection, NETs are induced predominantly in areas of tissue damage.

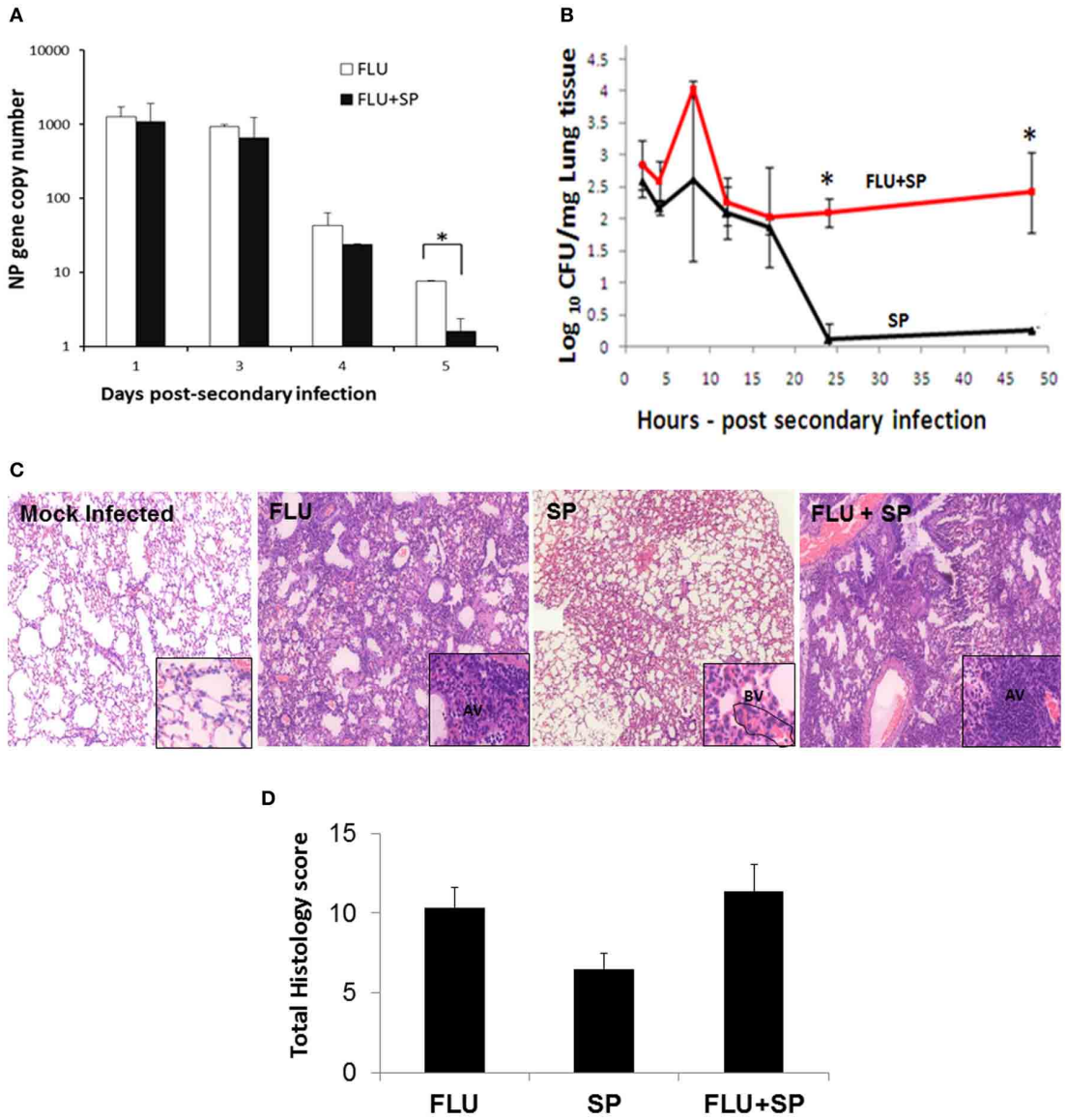

FIGURE 1 | Effects of influenza and/or *S. pneumoniae* infections on their replication and on lung pathology. (A,B) C57BL/6 mice were infected with sub-lethal doses (30 PFU) of influenza virus (FLU) or mock-infected with PBS (control). Seven days after influenza infection, mice were challenged with sub-lethal doses of *S. pneumoniae* 19F for viral and bacterial co-infection experiments (FLU + SP). For bacterial infection alone (SP), mice were infected with sub-lethal doses of *S. pneumoniae* 19F. $N = 3$ per animal group. *Denotes $P < 0.05$. **(A)** Expression of the viral nucleoprotein gene was measured by real-time RT-PCR analysis until 5 days post-secondary infection. **(B)** Growth of *S. pneumoniae* in the lung homogenates of mice with dual infection and bacterial infection only was determined by the number of CFU until 48 h post-secondary infection. **(C,D)** C57BL/6 mice were infected with lethal doses of influenza virus (250 PFU) or mock-infected with PBS (control). Seven days after influenza infection, mice were challenged with sub-lethal doses of *S. pneumoniae* (FLU + SP). Influenza infection alone (FLU). *S. pneumoniae* challenge only (SP). **(C)** Histopathologic analyses reveal alveolar septal congestion and inflammation in the lungs of influenza-infected mice, while infection with bacteria alone for 48 h did not cause severe pathology. Dual infection culminated in extensive pulmonary damage and severe pneumonia. Mock-infected animals showed normal alveolar architecture. Mice infected with influenza only had severe inflammation in the alveoli, whereas the inflammation was mainly found in blood vessels (BV) in animals infected with bacteria alone. Dual-infected mice demonstrated severe inflammation in the alveoli (AV). Magnification of 10× (main panels) and 40× (inserts). **(D)** Histopathology scores of lungs showing the mean values ± SE of 6 animals per group. The *P*-values were not significant between the groups.

Enhanced NETs release and DNA fibers of the NETs entangled with the alveolar epithelium and small blood vessels are observed in lethal influenza infection (Narasaraju et al., 2011). To determine whether secondary bacterial challenge influences NETs induction, we evaluated the NETs release during primary infections with influenza or *S. pneumoniae* alone, and during dual infection. NETs were identified by triple labeling with histone H2B, MPO, and DAPI (**Figure 2A**). We performed quantification

Table 2 | Histopathology and NETs scoring of lungs of infected animals.

Infection group	Animal number	Alveolar septal consolidation	Alveolar hemorrhage	Intra-alveolar fibrin	Cellular infiltrates	Total histo-pathology score	Total NETs score
Influenza only	23	2	2	1	2	7	35
	24	2	1	3	4	10	28
	25	2	1	3	2	8	17
	31	3	1	6	6	16	31
	32	2	1	3	4	10	21
	33	3	1	3	4	11	35
Streptococcus pneumoniae only	29	2	0	0	0	2	33
	30	2	1	0	0	3	16
	7.9	2	0	0	0	2	23
	37	2	1	0	0	3	33
	38	2	0	0	2	4	36
	7.10	2	1	0	0	3	35
Influenza + *Streptococcus pneumoniae*	26	2	2	6	6	16	61
	27	2	1	0	2	5	22
	28	2	1	3	3	9	54
	34	2	1	3	4	10	36
	35	2	1	6	6	15	39
	36	2	1	6	4	13	71

of NETs in the lung tissues based on the appearance of NETs as individual, small, medium, or large clusters as depicted in **Figure 2B**. The system for scoring of the unique morphologic structures is indicated in **Table 1**, while the cluster area calculation is exemplified in **Figure 2C**. At least 20 fields were counted for each mouse lung under 40× magnification. Dual-infected animals displayed prominently increased and large clusters of NETs in their alveoli and small airways. In contrast, the lungs of mice infected with bacteria or influenza virus alone displayed mainly individual NETs or small clusters of NETs. Prominent staining of MPO and histone in the clusters indicated that these structures were formed mainly by neutrophils. The average total NETs scores were calculated, and revealed that NETs induction was significantly intensified in the dual infection group (**Figure 2D**) compared to the primary influenza group.

DEGRADATION OF NETS IN THE PRESENCE OF *S. pneumoniae* BOTH *in vivo* AND *in vitro*

The large clusters of NETs found in dual-infected murine lungs, exhibited typical bundles of NETs with elongated DNA fibers. However, we also noted degrading NETs, as shown by scattered staining of histone protein within these clusters, which also closely stained for MPO (**Figure 3A**). In contrast, influenza infection alone displayed predominantly continuous staining for histone or MPO. These results indicate partial degradation of NETs in the presence of *S. pneumoniae*, which is known to produce endonucleases that aid the bacteria to escape from being trapped inside the NETs (Beiter et al., 2006; Buchanan et al., 2006). To ascertain whether neutrophils recruited during pulmonary influenza infection produce NETs when they encounter invading bacteria, we incubated neutrophils isolated from influenza-infected mice with different bacteria. Incubation of neutrophils with *S. pneumoniae* resulted in extensive NETs (**Figure 3B**), compared to incubation with *S. aureus, P. aeruginosa*, or *K. pneumoniae* (data not shown). Bacteria entrapped within the NETs were also observed. Furthermore, partial degradation of NETs with disconnected and punctate staining for DAPI was observed in neutrophils incubated with *S. pneumoniae* (**Figure 3B**, arrowheads). To assess the interaction of NETs with pneumococci, we incubated NETs (induced by BALF from influenza-infected mice) with bacteria at different MOI. Augmented bacterial entrapment was observed with increasing MOI (**Figures 3C,D**). To investigate bacterial DNase activity, salmon sperm DNA was incubated with bacterial pellets and supernatants, which revealed that *S. pneumoniae* possessed potent DNase activity, whereas *K. pneumoniae* did not (**Figure A1**). Incubation of neutrophils with influenza-infected BALF and *S. pneumoniae* at different MOI also resulted in some reduction of total NETs, indicating partial degradation of NETs by

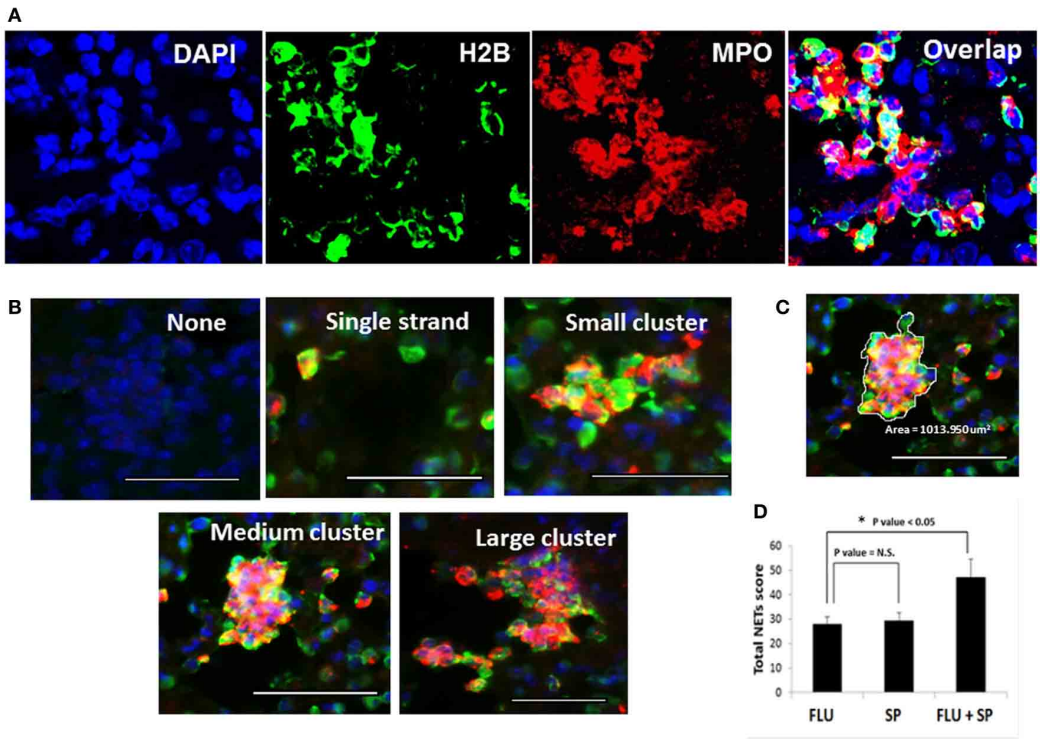

FIGURE 2 | Detection and quantification of NETs in the lungs of infected mice. Detection of NETs induced in lung tissues of mice infected with lethal doses of influenza virus followed by *S. pneumoniae* challenge. **(A)** For identification of NETs, lung sections were stained with histone H2B (green), MPO (red), and DAPI (blue). **(B)** NETs formation was analyzed microscopically based on triple immunostaining, and by their morphologic characteristics, i.e., appearing as individual NETS, small, medium, or large clusters of NETs. All scale bars represent 50 μm. **(C)** A representative cluster with area demarcated for calculation by ImageJ software. **(D)** Quantification of the total NETs score was obtained from at least 20 fields of each lung section. Values represent the means ± SE of 6 animals per group. *Indicates $P < 0.05$; N.S. = Not significant difference.

S. pneumoniae (**Figure A2**). Taken together, these results suggest the degradation of NETs by pneumococci as an evasion strategy.

REDOX ENZYMES INDUCED DURING INFLUENZA INFECTION CONTRIBUTE TO NETS FORMATION

We observed that NETs were generated to a greater degree when neutrophils were incubated with influenza-infected BALF (**Figure 4A**). Upregulation of several cytokines, chemokines, and redox enzymes is observed during influenza infection. We previously identified that redox enzymes produced by infected epithelial cells stimulate NETs release (Narasaraju et al., 2011). To confirm whether the redox enzymes present in BALF potentially contribute to NETs formation, we incubated neutrophils in the presence of inhibitors of MPO, NADPH oxidase, and SOD. We found significant inhibition of NETs release by MPO and NADPH oxidase inhibitors, but not with SOD inhibitor (**Figure 4B**). These findings confirmed our previous findings that redox enzymes play a significant role in NETs generation during influenza pneumonia.

EXPRESSION OF ANTIMICROBIAL GENES IN NEUTROPHILS DURING NETS FORMATION

We previously showed that NETs induction during influenza is regulated by MPO activity, with increased MPO activity found in BALF from animals with lethal influenza. Incubation of neutrophils with BALF collected from influenza-infected mice significantly induced NETs generation. We performed kinetics of NETs release during incubation with BALF, which revealed intensified NETs release with incubation time (**Figure 4A**). Incubation of neutrophils with BALF from uninfected mice or from mice challenged with sub-lethal influenza generated less NETs. These studies underscore the notion that NETs are induced in the lung micro-environment in the presence of enzymes such as MPO (Narasaraju et al., 2011). We evaluated the gene expression of 6 selected antimicrobial proteins that are associated with NETs, which were identified by DNA microarray analysis of lungs of mice challenged with lethal influenza. Incubation of influenza-infected BALF with neutrophils did not significantly alter the gene expression of cathelicidin, S100A8, S100A9, lactotransferrin, pentraxin-3, and MMP9 (data not shown).

NETS DO NOT EXERT BACTERICIDAL ACTIVITIES BUT STILL POSSESS ANTIFUNGAL PROPERTIES

To determine whether NETs induced during influenza can kill bacteria, we incubated neutrophils (stimulated with influenza-infected BALF) with *S. pneumoniae* or *K. pneumoniae* in the presence of cytochalasin B (an inhibitor of phagocytic activity). The generation of NETs was more prominent when neutrophils

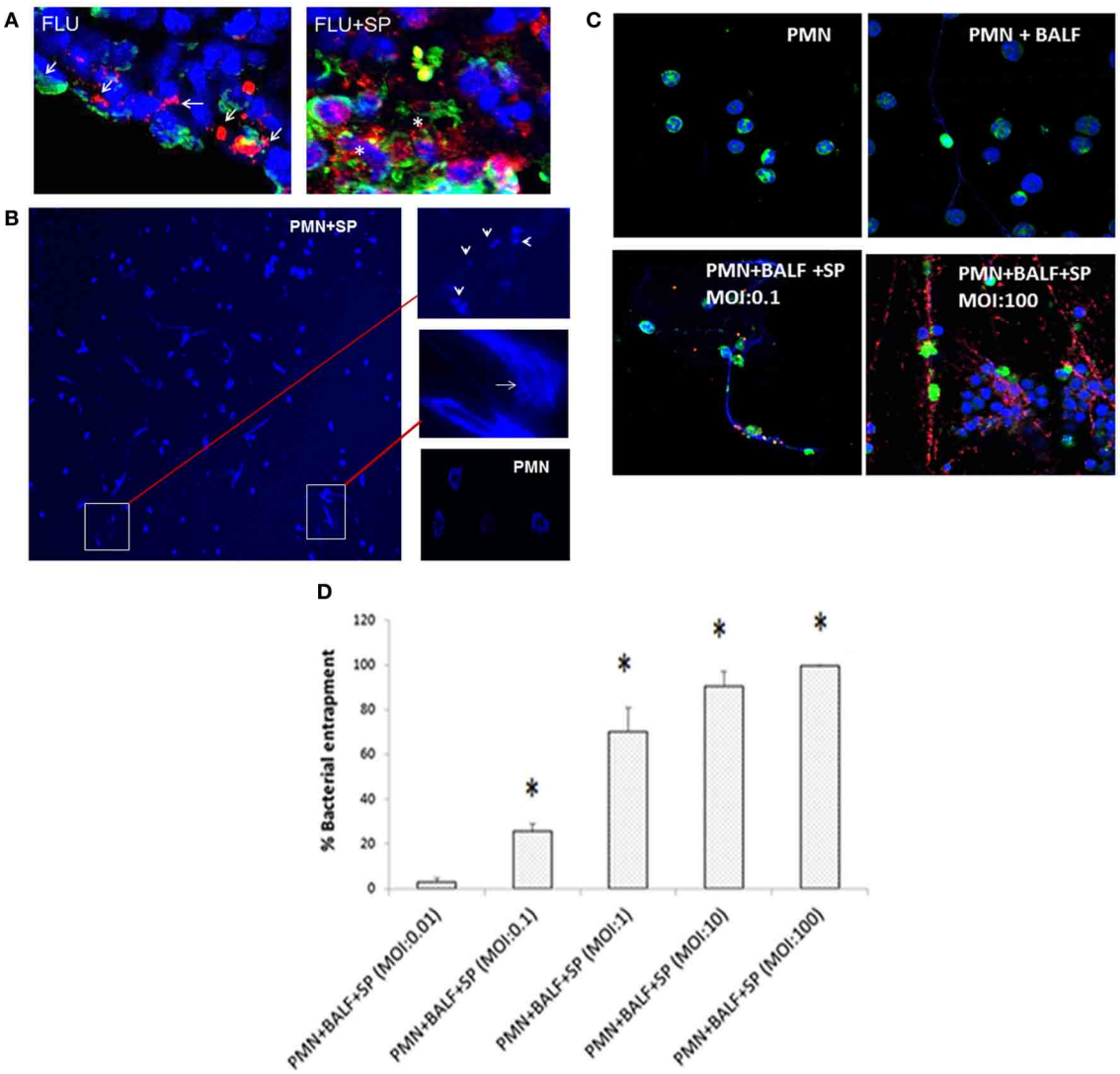

FIGURE 3 | Partial degradation of NETs and bacterial entrapment within NETs associated with *S. pneumoniae* 19F. (A) Lungs of mice infected with influenza alone (FLU) exhibit continuous staining (arrows) of MPO (red) and histone H2B (green). However, lungs of mice with viral and bacterial co-infection (FLU + SP) revealed large clusters of NETs often filled with degraded DNA, appearing as punctate staining (asterisks) of MPO and histone H2B. **(B)** To ascertain whether *S. pneumoniae* and other bacterial species cause NETs degradation, neutrophils were isolated from the lungs of influenza-infected mice (Narasaraju et al., 2011), incubated with *S. pneumoniae, K. pneumoniae, P. aeruginosa*, or *S. aureus* at a neutrophil:bacterial ratio of 1:10, and stained with DAPI. Extensive NETs were notable when neutrophils were incubated with *S. pneumoniae* (PMN + SP), compared with incubation with the other 3 bacterial species (data not shown). NETs were negligible for the untreated neutrophils (PMN) serving as negative control. Partially degraded NETs were observed when incubated with *S. pneumoniae*, suggesting DNA degradation in NETs by endonucleases produced by *S. pneumoniae*. Arrow indicates bacteria trapped inside the NETs, while arrowheads depict partially degraded NETs. **(C)** Experiments were performed to study NETs interactions with bacteria. Normal mouse bone marrow-derived neutrophils (PMN) served as negative control. These neutrophils were also incubated with BALF from influenza-infected mice (PMN + BALF). Neutrophils with infected BALF were also incubated with *S. pneumoniae* at various MOI (PMN + BALF + SP). Immunostaining for NETs and bacteria was performed for tests and controls. *S. pneumoniae* bacteria (red) were found entrapped within the DNA fibers (blue) and histone H2B (green) depending on MOI. Higher MOI of 100 displayed greater accumulation and entrapment of bacteria in the vicinity of NETs compared to lower MOI of 0.1. **(D)** Bacterial entrapment on NETs was significantly enhanced with increasing MOI. Values represent the means ± SE of at least 3 independent experiments. *Denotes $P < 0.05$ vs. MOI of 0.01.

were incubated with *S. pneumoniae* compared to *K. pneumoniae*. However, these NETs did not possess any bactericidal activity (**Figures 5A,B**). Although invasive fungal infections are not commonly associated with influenza infections, the expression of proteins such as S100A8, S100A9 may also exert antifungal effects. Hence, we investigated whether the NETs induced during influenza possess antifungal activity against *C. albicans*. As shown in **Figure 5C**, we found significant difference in antifungal activity in the presence of NETs compared with the mock control without neutrophils. However, neutrophils incubated with

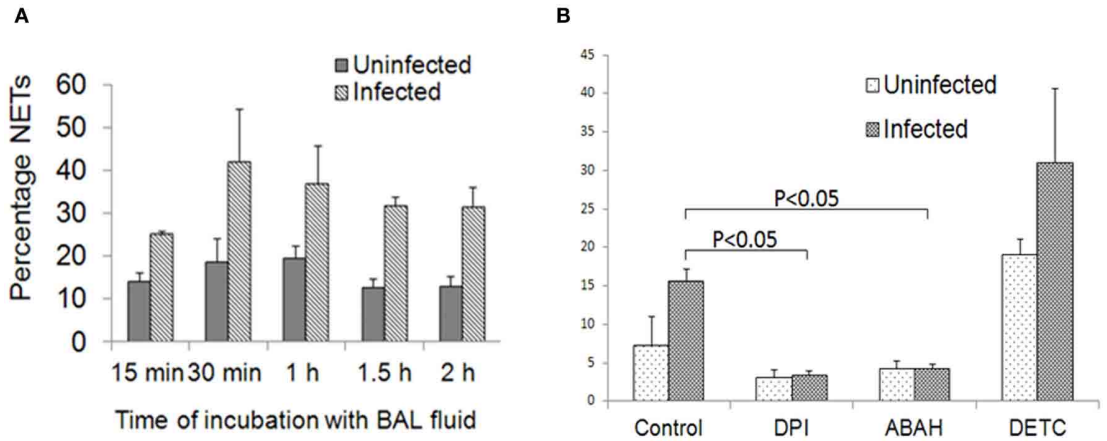

FIGURE 4 | Redox enzymes mediate NETs induction during influenza infection. (A) Neutrophils isolated from bone marrow of uninfected mice produced NETs when stimulated with BALF collected from influenza-infected animals. NETs were identified as early as 30 min after incubation. The *P*-values for the various time-points were not significant. **(B)** Incubation of neutrophils with inhibitors of redox enzymes revealed significant reduction in NETs formation in the presence of DPI and ABAH only. Values represent the means ± SE of at least 3 independent experiments. $P < 0.05$ vs. control incubated with infected BALF.

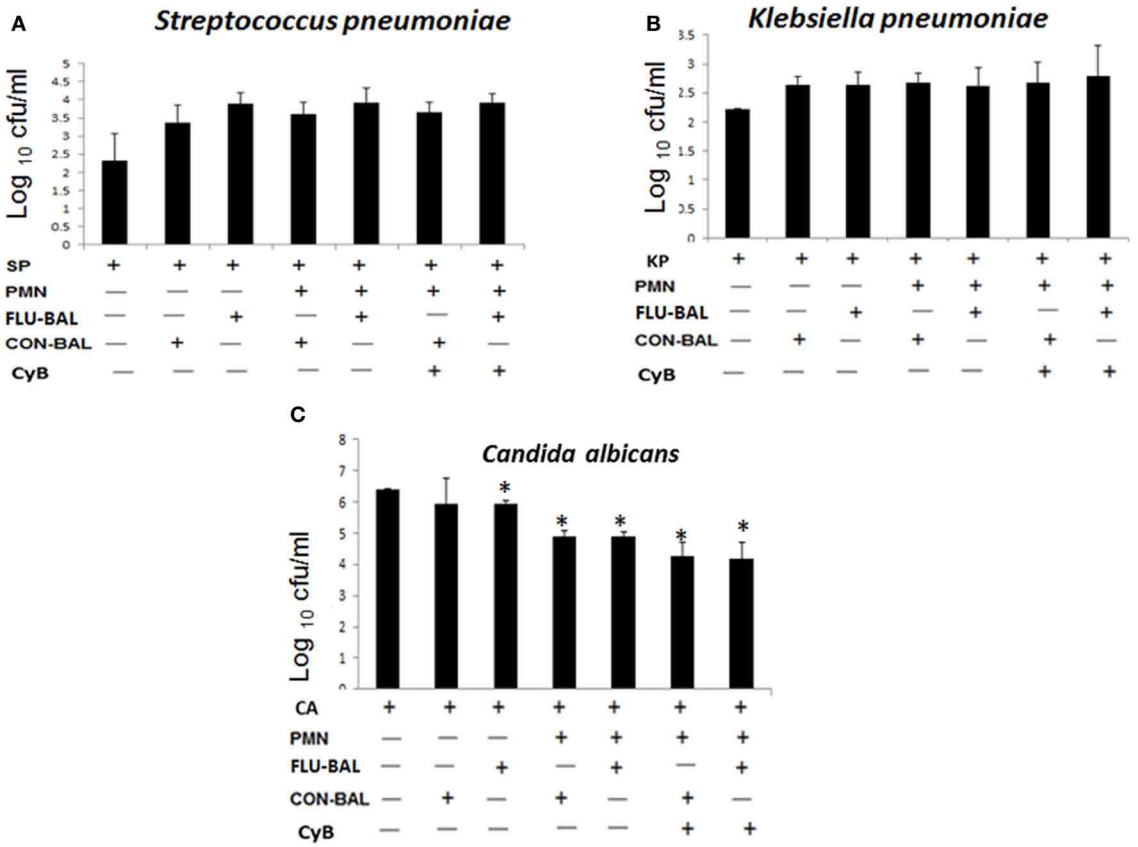

FIGURE 5 | Bactericidal and fungicidal activities of neutrophils and NETs *in vitro*. To determine the antimicrobial activity of NETs, neutrophils (PMN) were incubated with virus-infected BALF (FLU-BALF) or uninfected control BALF (CON-BALF) in the presence of **(A)** *S. pneumoniae* 19F or SP, **(B)** *K. pneumoniae* K15 or KP, and **(C)** *C. albicans* or CA with or without cytochalasin B (CyB). After incubation for 2.5 h, the remaining bacteria were plated on sheep blood agar or Luria–Bertani agar, and colonies were counted 24 h later. For colony counts of *C. albicans*, overnight incubation was carried out, followed by plating on Sabouraud dextrose agar. Values represent the means ± SE of at least 3 independent experiments. *Denotes $P < 0.05$ vs. untreated *C. albicans* only control.

influenza-infected BALF revealed antifungal effects similar to those neutrophils incubated with uninfected BALF.

DISCUSSION

Secondary bacterial infection with *S. pneumoniae* was the most predominant critical complication in the 2009 swine influenza H1N1 pandemic (Palacios et al., 2009; Isais et al., 2010; Rice et al., 2012). Although bacterial super-infections have been recognized since the 1918 influenza pandemic, the synergism between influenza and bacterial co-pathogens, and the detailed pathogenic contributions of dual infections to increased morbidity and mortality are not completely understood. Our previous studies linked excessive neutrophil recruitment and release of NETs with lung damage in mice challenged with lethal influenza (Narasaraju et al., 2011). NETs have been characterized for their role in bacterial killing (Brinkmann et al., 2004; Fuchs et al., 2007). In this study, we investigated whether NETs induced during primary influenza influence secondary bacterial replication and pathologic events in the lungs. Our findings revealed that prior challenge with influenza augmented bacterial growth compared to bacterial infection alone. Extensive NETs were prominent during dual infection, which revealed severe pulmonary pathology and tissue consolidation. Furthermore, we observed partially degraded NETs in the lungs of dual-infected mice, as well as when neutrophils were incubated with *S. pneumoniae in vitro*. There were no significant changes in gene expression of several proteins associated with NETs upon stimulation of neutrophils with BALF acquired from influenza-infected mice, and these NETs failed to exhibit any bactericidal activity. Taken together, our studies revealed that NETs induced during influenza infection do not participate in bacterial killing, but may exacerbate lung pathology during secondary bacterial pneumonia.

The higher mortality rates in bacterial super-infections following influenza are associated with increased respiratory failure, inflammation, and bacteremia. The lethal synergism of dual infections varies with the challenge dose of virus or bacteria, and the duration of infections (McCullers and Rehg, 2002; Smith et al., 2007). In our current model, we analyzed lethal influenza, followed by sub-lethal *S. pneumoniae* challenge, because we found extensive NETs generation only during lethal influenza challenge. Although bactericidal effects of NETs are well-explored, most studies have been confined to *in vitro* experiments (Brinkmann et al., 2004; Young et al., 2011; Marin-Esteban et al., 2012). We evaluated the *in vivo* scenario of the pre-existing NETs within the lung micro-environment at the time of bacterial challenge. Despite the presence of excessive NETs in dual-infected mice, there was augmented bacterial replication, suggesting that the NETs released in the lungs are not involved in clearance of *S. pneumoniae.* This study warrants future investigations to explore the effects of NETs on other bacterial pathogens, since *S. pneumoniae* is known to generate endonucleases that can digest NETs. We designed a scoring system for the quantification of NETs based on their appearance in the lungs. Clusters formed due to excessive neutrophils, and NETs released within the damaged areas were more frequently noted in dual-infected animal lungs, while individually formed NETs were predominant in mice infected with influenza alone. Although MPO and histone proteins strongly stained within the clusters, we could not exclude the presence of other cellular debris. Interestingly, DNA fibers in large clusters of NETs in the lungs of dual-infected mice appeared partially degraded, as evident from the punctate appearance of histone and MPO. Degradation of NETs may be mediated by endonucleases that are secreted by *S. pneumoniae*. This phenomenon was further supported by our *in vitro* experiments demonstrating that incubation of neutrophils isolated from influenza-infected mice with *S. pneumoniae* led to partial NETs degradation. Degradation of NETs releases their associated proteins consisting mainly of histones, elastase, and other proteins. Extracellular histones released by either NETs or dying cells can potentially contribute to inflammation and tissue injury during sepsis and other inflammatory responses (Warr and Jakab, 1979; Xu et al., 2009; Saffarzadeh et al., 2012). Histones released by degraded NETs can inflict cytopathic effects in alveolar epithelial and endothelial cells mediated through toll-like receptor-mediated signaling, e.g., TLR2, TLR4 (Kessenbrock et al., 2009; Small et al., 2010; Xu et al., 2011). The results suggest the potential role of NETs and their degrading components in enhanced immunopathology observed during secondary bacterial pneumonia following influenza, and warrants further investigations into the role of NETs in lung injury.

Dual-infected mice suffered greater lung damage compared to influenza infection alone. Sub-lethal *S. pneumoniae* infection alone displayed relatively mild pathology compared with the influenza-infected group. The accumulation of neutrophils and NETs was more obvious in blood vessels rather than air spaces in bacterial infection alone. Although heightened bacterial colonization was observed in dual infection, the influenza virus titers did not change up to 3 days post-bacterial infection, but decreased thereafter possibly due to viral clearance. These results are congruent with previous findings that co-infection of bacteria as early as 2 days after influenza infection does not enhance viral replication, but increases bacterial colonization (Kash et al., 2011). There are possible explanations for the enhanced bacterial loads in dual-infected animals. Firstly, prior damage by influenza exposes the cellular basement membrane to allow the bacteria to disseminate deeper into the lungs. Furthermore, platelet-activating factor receptor (PAFr) is enhanced during influenza infection, which facilitates bacterial attachment to the cell membrane. Blocking PAFr significantly abrogates bacterial multiplication (McCullers and Rehg, 2002). Our data indicated that the presence of NETs did not influence bacterial replication, but may indirectly provide an optimal environment for bacterial growth by increasing alveolar-capillary damage. To substantiate the *in vivo* evidence supporting the lack of bactericidal activity of NETs, we stimulated NETs *in vitro* in the presence of BALF acquired from mice with lethal influenza. We found extensive NETs induction, but there were no significant alterations in gene expression of several antimicrobial proteins during NETs formation. These NETs failed to suppress bacterial growth, and exhibited no significant difference in fungal replication when compared to neutrophils stimulated with uninfected BALF. The lack of antibacterial activity of NETs may be due to the relatively low amount of antibacterial proteins

in NETs, and whether these proteins are still active after NETs release. Nevertheless, these results concur with a recent report that NETs can trap bacteria and other pathogens, but may not kill these microorganisms (Menegazzi et al., 2012).

In conclusion, these studies demonstrate that secondary *S. pneumoniae* infection following primary influenza intensified NETs generation and lung pathogenesis. NETs did not participate in bacterial killing as evidenced by both *in vivo* and *in vitro* experiments. Infection with *S. pneumoniae* may cause partial degradation of NETs, which could in turn release NETs-associated proteins, thus contributing to the pulmonary pathology in dual infection.

ACKNOWLEDGMENTS

We are grateful to M. C. Phoon, S. H. Lau, K. T. Thong, Y. Yamada, N. Li, K. S. Tan, J. P. Hsu, and the staff members of the Confocal Microscopy Unit at the Clinical Research Center, National University of Singapore for their assistance. This study was supported by the National University of Singapore and the Singapore-MIT Alliance for Research and Technology.

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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.

Received: 29 October 2012; accepted: 12 February 2013; published online: 05 March 2013.

*Citation: Narayana Moorthy A, Narasaraju T, Rai P, Perumalsamy R, Tan KB, Wang S, Engelward B and Chow VTK (2013) In vivo and in vitro studies on the roles of neutrophil extracellular traps during secondary pneumococcal pneumonia after primary pulmonary influenza infection. Front. Immunol. **4**:56. doi: 10.3389/fimmu.2013.00056*

This article was submitted to Frontiers in Molecular Innate Immunity, a specialty of Frontiers in Immunology.

APPENDIX

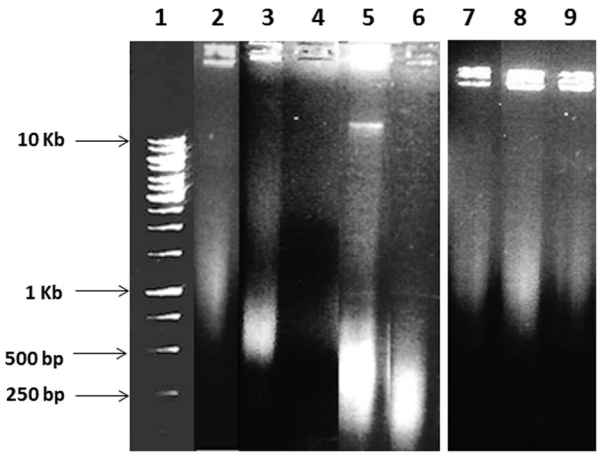

FIGURE A1 | Analysis of degradation of salmon sperm DNA by *S. pneumoniae* and *K. pneumoniae*. Salmon sperm DNA (2 μg) was treated with either bacterial pellet (10^7 cells) or double-filtered supernatant (3 μl) of *S. pneumoniae* 19F or *K. pneumoniae* K15 at 37°C for 1 h in DNase buffer (10 mM Tris, 3 mM $MgCl_2$, 5 mM $CaCl_2$, pH 7.4), and the reaction was stopped with 0.5 M EDTA. The samples were then subjected to 1% agarose gel electrophoresis. Lanes: (1) DNA ladder. Salmon sperm DNA was incubated with (2) DNase buffer; (3) DNA alone; (4) BHI broth; (5) *S. pneumoniae* pellet; (6) *S. pneumoniae* supernatant; (7) LB broth; (8) *K. pneumoniae* pellet; and (9) *K. pneumoniae* supernatant. The DNA smearing below 500 bp in lanes 5 and 6 revealed that salmon sperm DNA was digested by both pellet and supernatant of *S. pneumoniae*, indicating secreted DNase activity. However, *K. pneumoniae* did not digest the DNA as shown by relatively intact salmon sperm DNAs.

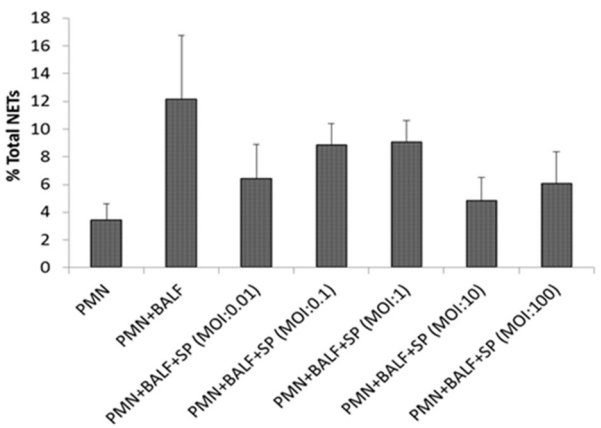

FIGURE A2 | Quantification of NETs degradation by *S. pneumoniae in vitro*. Mouse bone marrow-derived neutrophils (PMN) served as negative control. Neutrophils were stimulated for 2 h with BALF from influenza-infected mice (PMN + BALF), which were then incubated with *S. pneumoniae* 19F (PMN + BALF + SP) at MOI of 0.01, 0.1, 1, 10, and 100 for 2 h. After incubation, the remaining NETs were quantified as previously described. Compared with the PMN + BALF group, infection with different MOI of bacteria resulted in modest reduction of total NETs, implying only partial degradation of NETs by *S. pneumoniae* 19F. Values represent the means ± SE of at least 3 independent experiments. The *P*-values were not significant between the PMN + BALF + SP groups versus the PMN + BALF group.

frontiers in
IMMUNOLOGY

MINI REVIEW ARTICLE
published: 14 December 2012
doi: 10.3389/fimmu.2012.00382

Neutrophils cast extracellular traps in response to protozoan parasites

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Release of extracellular traps by neutrophils is a now well-established phenomenon that contributes to the innate response to extracellular bacterial and fungal pathogens. The importance of NETs during protozoan infection has been less explored, but recent findings suggest an emerging role for release of neutrophil-derived extracellular DNA in response to this class of microbial pathogens. The present review summarizes findings to date regarding elicitation of NETs by *Toxoplasma gondii*, *Plasmodium falciparum*, *Eimeria bovis*, and *Leishmania* spp.

Keywords: protozoan parasite, neutrophils, extracellular traps, anti-microbial defense, *toxoplasma*

INTRODUCTION

Neutrophil granulocytes, or polymorphonuclear leukocytes (PMN), are the most numerous of innate immune cells. They are regarded as one of the most important of the innate defender cells due to the fact that they are the first to arrive at a site of infection or inflammation, and they come pre-armed with an arsenal of anti-microbial effector molecules. Neutrophils are produced in the bone marrow and are released into the blood after they have matured and acquired their characteristic granules. The latter particles serve as the storage depot for enzymes involved in host defense and also sometimes host tissue damage (Borregaard, 2010).

Polymorphonuclear leukocytes arriving at a site of infection or inflammation do so in response to a chemotactic gradient of IL-8 in humans or MCP-2 in mice. Circulating neutrophils undergo a step-by-step migration process involving rolling adhesion, extravasation and accumulation at sites of infection (Faurschou and Borregaard, 2003; Ley et al., 2007). Here, neutrophils eliminate pathogens through both phagocytosis-dependent and -independent mechanisms. During phagocytosis-dependent killing, the resulting microbe-carrying phagosome fuses with lysosomes, as well as primary and secondary granules resulting in pathogen destruction. This method of killing relies on both oxidative and non-oxidative mechanisms. The oxidative mechanism involves production of reactive oxygen species through the activity of the NADPH oxidase enzyme complex, while non-oxidative mechanisms rely on delivery and activation of antimicrobial peptides and proteases (Faurschou et al., 2002; Faurschou and Borregaard, 2003). Granule contents may also be released into the extracellular milieu, enabling phagocytosis-independent killing at the cost of collateral damage to host tissue.

NEUTROPHIL EXTRACELLULAR TRAPS

A landmark study by Brinkmann et al. (2004) identified a previously unrecognized neutrophil anti-microbial mechanism that is an important component of extracellular killing. This involves a novel process in which nuclear chromatin decondenses and DNA is ejected into the extracellular environment, ensnaring and inactivating tissue pathogens. Neutrophil extracellular traps (NETs) are made up of a DNA backbone studded with histones and laced with a number of anti-microbial peptides that together form an extracellular mesh that traps and kills microbial pathogens (Wartha et al., 2007; Kaplan and Radic, 2012). The protein components of NETs include bacterial permeability-increasing protein (BPI), myeloperoxidase, cathepsin G, lactoferrin, gelatinase, peptidoglycan recognition proteins (PGRPs), calprotectin, and elastase (Weinrauch et al., 2002; Brinkmann et al., 2004; Cho et al., 2005; Urban et al., 2006, 2009; Fuchs et al., 2007). Release of extracellular traps has now been described in neutrophils isolated from several species including humans, mice, cows, horses, cats, chickens, and even fish (Brinkmann et al., 2004; Alghamdi and Foster, 2005; Palic et al., 2007; Chuammitri et al., 2009; Ermert et al., 2009; Aulik et al., 2010; Wardini et al., 2010). NET formation is mostly associated with extracellular bacteria, but in this review we summarize new findings that protozoan parasites also evoke this response. We dwell only briefly on mechanisms of NET formation, which has been expertly reviewed recently by pioneers in the field (Remijsen et al., 2011; Brinkmann and Zychlinsky, 2012).

NETosis: A NEW FORM OF PROGRAMED CELL DEATH

The process by which NET formation occurs has been termed NETosis and is now understood to be a form of programed cell death that is independent of both apoptosis and necrosis. As such, NETosis endows the neutrophil with the extraordinary ability to exert anti-microbial effects well beyond death. Although long viewed as a form of cell death, a recent study showed that neutrophils release NETs *in vivo* without undergoing lysis while maintaining crawling and phagocytic activity (Yipp et al., 2012). Several nuclear and cytoplasmic events must take place to complete NETosis (summarized in **Figure 1**). These events involve

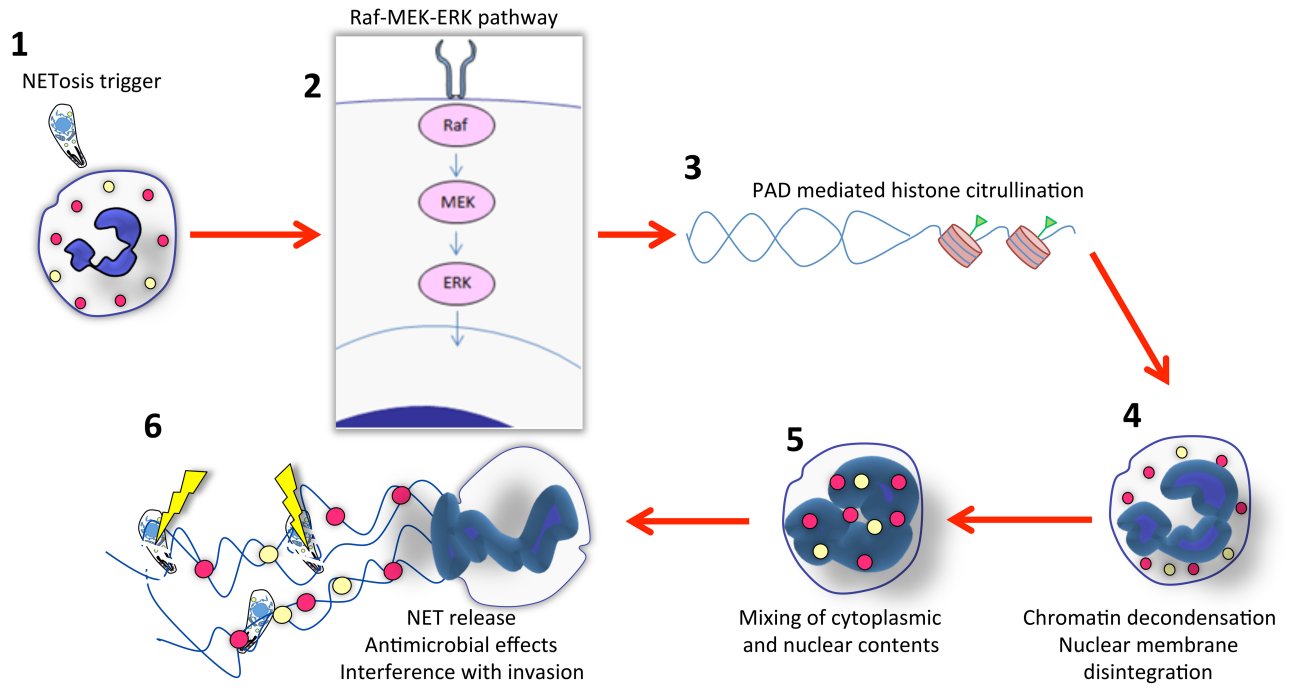

FIGURE 1 | Outline of NET formation. (1) Initiation of NETosis generally occurs through engagement of cell surface receptors. For parasites such as *Toxoplasma,* the receptor ligand interaction is not known. While invasion itself is not required, it is possible that adhesion enables high local concentrations of parasite ligands that trigger the NET cascade. **(2)** Evidence indicates involvement of the Raf–MEK–ERK pathway during NET formation elicited by several stimuli, including *Toxoplasma.* In most cases, NADPH oxidase is also involved (not shown here). **(3)** Signaling to the nucleus results in chromatin modification. Histone citrullination mediated by peptidylarginine deiminase (PAD) appears to be a prerequisite for NET release. **(4)** Concurrent with chromatin decondensation, the nuclear membrane disintegrates. **(5)** This results in mixing of cytoplasmic, granule (yellow and red circles) and nuclear contents. **(6)** Finally, DNA with associated histones and granule molecules are released into the extracellular environment entrapping microbes in the vicinity. For protozoa such as *Toxoplasma* this results in some parasite killing, but the major effects of NETs may be to interfere with invasion. There is evidence that other protozoa such as *Leishmania donovani* possess mechanisms to avoid NET-mediated killing.

peptidylarginine deiminase (PAD)-mediated histone citrullination, followed by chromatin decondensation, nuclear membrane disintegration, and the eventual mixing of both nuclear and cytoplasmic effector proteins before the final step, which is the expulsion of a protein-loaded NET into the extracellular milieu (Brinkmann and Zychlinsky, 2007; Fuchs et al., 2007; Papayannopoulos and Zychlinsky, 2009; Wang et al., 2009). In addition, most studies indicate that NET formation is dependent on a functional NADPH-oxidase complex, and that myeloperoxidase and neutrophil elastase also regulate NET release (Fuchs et al., 2007; Papayannopoulos et al., 2010; Metzler et al., 2011). Recently, Hakkim et al. (2011) identified a signaling pathway involved in extracellular trap formation that involves a Raf–MEK–ERK pathway and that inhibition of this pathway leads to inhibition of NET formation (**Figure 1**).

Neutrophils have now been shown to extrude NETs in response to many molecular triggers as well as to intact pathogens. Some of the most important molecular triggers are: LPS, PMA, GM-CSF/LPS, IL-8, glucose oxidase, Ca^{2+} ionophore, thapsigargin, TNF, and LPS-activated platelets (Brinkmann et al., 2004; Gupta et al., 2005, 2010; Clark et al., 2007; Jaillon et al., 2007; Neeli et al., 2008; Marcos et al., 2009; Wang et al., 2009; Yost et al., 2009; Yousefi et al., 2009). Bacterial and fungal pathogens that induce NET formation include *Staphylococcus aureus*, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Shigella flexneri*, *Salmonella typhimurium*, *Escherichia coli*, *Mycobacterium tuberculosis*, *Listeria monocytogenes*, *Aspergillus fumigatus*, *Aspergillus nidulans*, and *Candida albicans* among others (Brinkmann et al., 2004; Beiter et al., 2006; Buchanan et al., 2006; Urban et al., 2006; Grinberg et al., 2008; Bianchi et al., 2009; Ramos-Kichik et al., 2009; Bruns et al., 2010). More recently, Saitoh et al. (2012) published a report showing the importance of NET formation in mediating defense against human immunodeficiency virus-1, adding to the repertoire of pathogens involved in NET formation.

NETosis AND PROTOZOA

While most studies have focused on the effect of NETs on bacterial and fungal pathogens, little attention has been paid in the past to the role of NET formation in the response to protozoan infection. This is beginning to change. It is now clear that these important pathogens also possess the requisite signals to trigger NET release, although how this impacts the course of infection is not entirely clear. To date, NET formation has been described during responses to Apicomplexan species (*Toxoplasma gondii*, *Plasmodium falciparum*, and *Eimeria bovis*) and to Trypanosomatids (*Leishmania amazonensis*, *Leishmania chagasi*, *Leishmania donovani*, and *Leishmania major*; **Table 1**; Baker et al., 2008; Guimaraes-Costa et al., 2009; Behrendt et al., 2010; Gabriel et al., 2010; Abi Abdallah et al.,

Table 1 | NET induction by protozoa.

Protozoan	Protozoan stage	PMN origin	Infectivity compromised?	*In vitro*/*in vivo* evidence	Reference
T. gondii	Tachyzoite	Human/mouse	Yes	Both	Abi Abdallah et al. (2012)
P. falciparum	Trophozoites	Human	ND	*In vivo*	Baker et al. (2008)
E. bovis	Sporozoite	Bovine	Yes	*In vitro*	Behrendt et al. (2010)
L. donovani	Promastigote	Human	No	*In vitro*	Gabriel et al. (2010)
L. major	Promastigote	Human	No	*In vitro*	Guimaraes-Costa et al. (2009), Gabriel et al. (2010)
L. amazonensis	Promastigote	Human	Yes	*In vitro*	Guimaraes-Costa et al. (2009)
L. amazonensis	Amastigote	Human	ND	*In vitro*	Guimaraes-Costa et al. (2009)
L. chagasi	Promastigote	Human	ND	*In vitro*	Guimaraes-Costa et al. (2009)
L. braziliensis	ND	Human	ND	*In vivo*	Guimaraes-Costa et al. (2009)

ND, not determined.

2011, 2012). Notably, these are all intracellular parasites, raising the question of how extracellular traps could significantly impact infection. However, these pathogens must eventually emerge from their intracellular niche to invade other cells, and clearly at this point they are vulnerable to extracellular immune mediators such as NETs. Further impacting infection, release of extracellular traps could also contribute to immunopathology associated with some protozoa.

Toxoplasma gondii

Toxoplasma is a ubiquitous obligate intracellular protozoan parasite with the ability to infect most warm-blooded animals. It normally causes asymptomatic infection in immunocompetent adults and children but can cause severe disease in immunocompromised hosts and poses significant risks for pregnant women (Montoya and Liesenfeld, 2004; Dubey, 2007). There is evidence that neutrophils play an important role during *Toxoplasma* infection, inasmuch as they are rapidly recruited to the site of infection and they produce a variety of chemokines and cytokines in response to the parasite (Bliss et al., 1999, 2000; Del Rio et al., 2001, 2004).

In addition to cytokine and chemokine production during *Toxoplasma* infection, we recently demonstrated that PMN encounter with parasites elicits NET formation (Abi Abdallah et al., 2012). We employed neutrophils elicited in the peritoneal cavity after a thioglycollate injection and determined that mouse neutrophils produce NETs in response to co-incubation with *T. gondii* as determined by immunofluorescence staining for histone H3 and direct DNA staining with DAPI. In addition, NETs were digested using micrococcal nuclease and DNA concentration was measured using a commercially available DNA measuring kit. We also confirmed that DNA release by mouse PMN is a controlled process and not the result of random cell lysis by showing that cells retained lysozyme intracellularly after NET formation. The release of NETs occurred in a parasite strain-independent fashion given that all three major clonal lineages of *Toxoplasma* induced the response in a comparable manner. Using cytochalasin D to block parasite invasion of cells, we determined that *Toxoplasma* induces NETs in an invasion-independent manner. We assessed the viability of parasites entrapped within NETs and determined that approximately 25% of parasites in close association with NETs were no longer viable compared to 99% viability of the same parasite population cultured in the absence of PMN. Importantly, addition of DNase to our cultures reduced parasite killing to levels seen in the absence of neutrophils, directly implicating NET formation in toxoplasmacidal activity.

To obtain *in vivo* evidence for NET release during *Toxoplasma* infection we developed a pulmonary model of infection, in which parasites were introduced into mice intranasally. This method of infection induced a large influx of neutrophils into the lung, and we observed colocalization of parasites and PMN. In these mice, bronchoalveolar lavage fluid (BALF) contained a high concentration of dsDNA. This was most likely due to NET release insofar as BALF from neutrophil-depleted animals did not accumulate significant amounts of dsDNA. Importantly, neutrophil depletion prior to infection resulted in a higher number of viable parasites recoverable from the lung compared to non-depleted controls. While we documented modest killing of *Toxoplasma* within NETs, we speculate that their more significant function may be to physically entrap parasites thereby interfering with invasion (**Figure 1**).

We observed a similar NET response in the neutrophil-like human promyelocytic leukemia cell line HL-60, and freshly isolated human peripheral blood neutrophils displayed particularly vigorous NET release in response to *Toxoplasma* (**Figure 2**). Using a chemical inhibitor of ERK1/2, we identified a role for this mitogen-activated protein kinase in the signaling pathway leading to *Toxoplasma*-initiated NET release in human PMN.

Plasmodium falciparum

Malarial disease is caused by an obligate intracellular protozoan parasite of the genus *Plasmodium*. Annual cases globally are estimated to be in the range of 215–659 million with the World Health Organization estimating that upward of 780,000 fatal cases occur each year (Breman and Brandling-Bennett, 2011). The malaria sporozoite is transmitted through the bite of an infected mosquito.

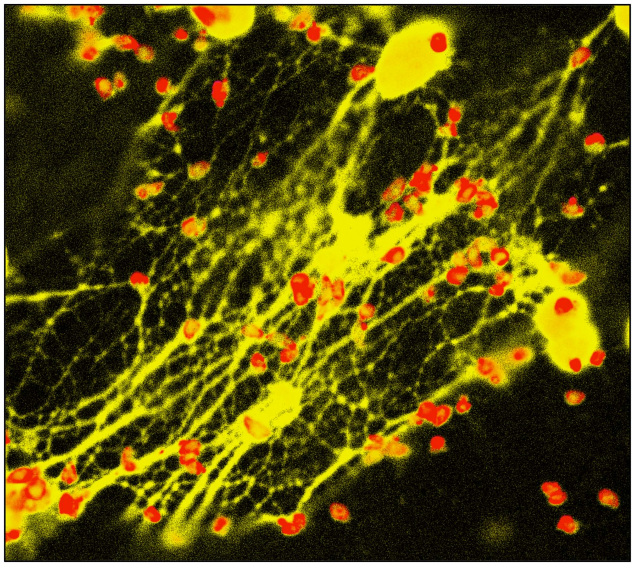

FIGURE 2 | NET formation triggered by *Toxoplasma*. Human peripheral blood neutrophils were co-incubated with *Toxoplasma* in the presence of cytochalasin D to prevent invasion. 4 hrs later, cells were fixed and stained with antibody to the tachyzoite surface molecule SAG-1 (pseudo-colored red). PMN nuclei and NETs were visualized using DAPI (pseudo-colored yellow).

After a relatively silent period in the liver, merozoites emerge and invade circulating red blood cells where they undergo explosive cycles of growth followed by re-invasion. Infection and subsequent remodeling of the erythrocyte cell membrane results in the many clinical manifestations of the disease, including cerebral malaria (Bei and Duraisingh, 2012).

In a field study conducted in Nigeria, patients with active malaria infections were tested for the presence of NETs (Baker et al., 2008). Blood samples were collected from children under the age of 6 diagnosed with clinically uncomplicated *P. falciparum* infections. The researchers found that all children tested exhibited evidence of NET-like structures circulating in the blood and that those structures contained entrapped parasitized erythrocytes and trophozoites. They further found that infected children possessed elevated levels of antinuclear antibodies (ANA) and that in the majority of those children the levels of ANA that are reactive with dsDNA were above the predictive level for autoimmunity. These results provide a preliminary indication that NET formation could contribute to pathogenesis of malaria in children. It was speculated that NET-triggered induction of anti-DNA antibodies could also negatively impact efforts to develop CpG-based vaccines, not just against malaria but against other infections that elicit significant NET release.

Eimeria bovis

A number of *Eimeria* spp. induce enteritis in livestock, making them pathogens of great veterinary and economic relevance. *E. bovis* and *E. zuernii* protozoa are very well known to induce intestinal lesions and are especially pathogenic to calves and young cattle. Sporozoites liberated from an oocyst invade various cells types, in which they form a parasitophorous vacuole where they continue to mature through different life-cycle stages, eventually rupturing the host cell and invading neighboring cells (Daugschies and Najdrowski, 2005).

Given the early role neutrophils play in the context of infections and their documented importance during *E. bovis* infections, Behrendt et al. (2010) sought to characterize the role NETs play during infection. Using bovine neutrophils, they found that *E. bovis* sporozoites induce vigorous NET formation. In fact, PMN exposed to sporozoites responded faster and stronger in terms of NET formation when compared to PMA. The strongest NETosis response occurred in response to viable sporozoites relative to inactivated *Eimeria* or parasite lysates. We made similar observations in the NET response to *Toxoplasma*. As previously described by others, this study showed that *E. bovis* NETosis induction is NADPH-oxidase dependent. The authors found exposure of parasites to PMN led to decreased infectivity and they speculated that this was a result of the NET-mediated parasite immobilization rather than direct killing.

Leishmania spp.

Leishmania parasites infect millions of people around the world and are the causative agent of leishmaniasis. The parasite is transmitted by the bite of an infected female sandfly and, depending on the parasite species, infection can cause disease with a variety of clinical manifestations. These can range from disfiguring and scaring lesions in cutaneous and mucosal leishmaniasis to potentially lethal visceral leishmaniasis (also known as kala-azar). There are two major parasite stages that have been defined for *Leishmania*: amastigotes and promastigotes. Amastigotes normally reside within macrophages while promastigotes reside inside the sandfly vector and are the form of the parasite that is inoculated after a blood meal (Turco and Descoteaux, 1992; Duthie et al., 2012).

Two recent studies examined the role NET play in the early stages of a *Leishmania* infection. Guimaraes-Costa et al. (2009) looked at the induction of NETs by *Leishmania* species. In their study it was found that *L. amazonensis* promastigotes induce NET formation, and they also found evidence for decreased viability of parasites. Both promastigotes (*L. amazonensis*, *L. major*, and *L. chagasi*) and amastigotes (*L. amazonensis*) were found to elicit NET formation. Interestingly, this group concluded that lipophosphoglycan (LPG) was responsible for NET induction based upon add back experiments using purified LPG. It was further concluded that histones within NETs mediated parasite inactivation based upon the observation that anti-histone antibodies abrogated killing. Furthermore, the authors observed a killing effect on promastigotes upon incubation with purified H2A histone. Interestingly in a recent study, Wang et al. (2011) also found that histones H2A and H2B could efficiently kill *Leishmania* promastigotes.

Using human neutrophils, Gabriel et al. (2010) showed that *L. donovani* promastigotes induce reactive oxygen species-independent NET production. They also observed that NET induction is *L. donovani* strain independent, and in addition they found that *L. major* promastigotes displayed the same activity. Using genetically engineered parasites, these investigators found

that NET induction by *L. donovani* promastigotes is independent of both parasite surface LPG and GP63 (a promastigote surface metalloprotease), both of which have been implicated in establishment of infection in mammalian hosts. Interestingly, while LPG does not elicit NET formation, it appears to mediate resistance to killing by these structures. This is because while wild-type parasites retain viability in the presence of NETs, mutant parasites lacking LPG display decreased viability under the same conditions. Although these results suggest that NETs have limited antimicrobial effect against normal *Leishmania* promastigotes, these structures may play an entrapment role in interfering with the ability of the parasites to enter host cells. Thus, while these two studies clearly document NET formation in response to *Leishmania* parasites, they differ in some key respects. The Saraiva group (Guimaraes-Costa et al., 2009) found that LPG induces NET release, and that these structures possess leishmanicidal activity. In contrast, the Descoteaux group found that NETs are induced independently of LPG, and indeed that LPG expression renders parasites resistant to NET-mediated killing (Gabriel et al., 2010). These differences might be attributable to variation in LPG structure, differences in strains of parasites used, or possibly differences in how the experiments were conducted. Regardless, it is interesting that an immunohistochemical analysis of cutaneous *Leishmania* lesions from biopsies of patients in Brazil revealed extracellular regions of DNA and histone suggesting NET activity during in vivo infection (Guimaraes-Costa et al., 2009).

CONCLUDING REMARKS

Release of extracellular traps is now regarded as an important neutrophil function that, remarkably, went unrecognized until only recently. Compared to studies on other microbial pathogens, the role of NET formation in response to protozoan parasites is relatively limited. Nevertheless, it is clear from the handful of studies reviewed here that protozoan pathogens elicit NET release (**Table 1**). In some cases, entrapment appears to interfere with invasion of host cells. There is evidence indicating that NETs directly kill entrapped parasites, and it also appears that at least some protozoans possess mechanisms to evade killing by NETs. Finally, there is evidence that by triggering release of neutrophil DNA, protozoan infection may lead to autoantibody formation, in turn contributing to disease pathogenesis. Determining how protozoans trigger NET release, how NETs impact infection, and how protozoans deal with the threat of NET entrapment are important areas of future investigation.

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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.

Received: 27 September 2012; accepted: 29 November 2012; published online: 14 December 2012.

Citation: Abi Abdallah DS and Denkers EY (2012) Neutrophils cast extracellular traps in response to protozoan parasites. Front. Immun. ***3****:382. doi: 10.3389/fimmu.2012.00382*

This article was submitted to Frontiers in Molecular Innate Immunity, a specialty of Frontiers in Immunology.

frontiers in
IMMUNOLOGY

ORIGINAL RESEARCH ARTICLE
published: 06 March 2013
doi: 10.3389/fimmu.2013.00048

A proposed role for neutrophil extracellular traps in cancer immunoediting

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Upon activation, neutrophils release fibers composed of chromatin and neutrophil proteins termed neutrophil extracellular traps (NETs). NETs trap and kill microbes, activate dendritic cells and T cells, and are implicated in autoimmune and vascular diseases. Given the growing interest in the role of neutrophils in cancer immunoediting and the diverse function of NETs, we searched for NETs release by tumor-associated neutrophils (TANs). Using pediatric Ewing sarcoma (ES) as a model, we retrospectively examined histopathological material from diagnostic biopsies of eight patients (mean ± SD age of 11.5 ± 4.7 years). TANs were found in six patients and in two of those we identified NETs. These two patients presented with metastatic disease and despite entering complete remission after intensive chemotherapy had an early relapse. NETs were not identified in the diagnostic biopsies of two patients with localized disease and two with metastatic disease. This study is the first to show that TANs in ES are activated to make NETs, pointing to a possible role of NETs in cancer.

Keywords: cancer, cancer immunoediting, Ewing sarcoma, neutrophils, neutrophil extracellular traps

INTRODUCTION

Upon activation, neutrophils release neutrophil extracellular traps (NETs), which are fibers composed of chromatin and neutrophil proteins. NETs are released via a novel form of cell death called NETosis (Brinkmann and Zychlinsky, 2012). NETosis requires the production of reactive oxygen species (ROS; Fuchs et al., 2007), myeloperoxidase (MPO; Metzler et al., 2011) and on the translocation of neutrophil elastase (NE) from azurophilic granules to the nucleus (Papayannopoulos et al., 2010). Eventually, neutrophils release NETs that trap and kill microbes, activate dendritic cells (DCs) and T cells, and are implicated in autoimmune and vascular diseases (Brinkmann and Zychlinsky, 2012).

We postulated that NETs also have a role in cancer. The immune system can identify and destroy nascent tumor cells in a process termed cancer immunosurveillance. However, the immune system can also promote tumor progression. Together, these host-protective and tumor-promoting actions of immunity are referred to as cancer immunoediting (Kim et al., 2007). To our knowledge, there are no data on the possible role of NETs in this process.

Ewing sarcoma (ES) is the second most common primary bone cancer that afflicts adolescents and young adults. Although advances in diagnosis, surgery, chemotherapy, and radiation have substantially improved the survival rate of patients with localized ES to nearly 70%, the long-term outcome for those with metastatic or recurrent disease remains poor (Potratz et al., 2012). The importance of the immune system in anti-tumorigenic reactions against ES cells was previously shown. Therapy-naive ES patients manifest an inflammatory microenvironment with a high expression of type 1-associated chemokines and infiltration of $CD8^+$ T lymphocytes, expressing corresponding chemokine receptors. Patients with higher numbers of tumor-infiltrating $CD8^+$ T lymphocytes demonstrated a significant overall survival benefit (Berghuis et al., 2011). Potential targets for immune recognition specific to ES are protein products of the gene fusion EWS-ETS (Meyer-Wentrup et al., 2005) and other tumor-associated antigens like the ganglioside antigen GD2 (Kailayangiri et al., 2012). ES cells were also shown to be sensitive to natural killer (NK) cells (Cho et al., 2010) and to tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), a member of the TNF superfamily with strong anti-tumor activity and minimal toxicity to most normal cells and tissues (Mitsiades et al., 2001). By contrast, several mechanisms of immune escape are evident. Bone marrow T cells with a regulatory phenotype T(reg) were found at significantly higher numbers in patients with primary metastatic ES compared with localized ES (Brinkrolf et al., 2009). In addition, complete or partial absence of human leukocyte antigen (HLA) class I expression was observed in 79% of ES tumors. Lung metastases consistently lacked HLA class I with sequential tumors, demonstrating a tendency toward decreased expression with disease progression (Berghuis et al., 2009).

Given the absence of data in the literature on the role of NETs in cancer immunoediting, we used histopathological material from diagnostic biopsies of pediatric ES patients to identify NETs in the tumor bed.

MATERIALS AND METHODS

PATIENTS

We aimed to examine histopathological material from diagnostic biopsies of all ES patients ($n = 14$), who were treated in our Pediatric Hemato-Oncology Department, Dana Children's Hospital, Tel Aviv Sourasky Medical Center, Tel Aviv, Israel, between July 2009 and July 2011. The histopathological material was taken from diagnostic biopsies, before initiation of chemotherapy or radiotherapy. All patients were previously healthy with no background systemic diseases or immunodeficiency and were free of infection during removal of the biopsy samples. Three patients were diagnosed elsewhere and therefore their histopathological material was not available and in another three the remaining material was insufficient for NET analysis. Of the remaining eight patients, six were males and two were females with a mean ± SD age of 11.5 ± 4.7 years. Further data are summarized in **Table 1**. The study was approved by the Institutional Review Board of Tel Aviv Sourasky Medical Center, Tel Aviv, Israel.

TREATMENT PROTOCOL

Newly diagnosed non-metastatic ES patients received the Children's Oncology Group (COG) AEWS0031 regimen B (i.e., chemotherapy intensification with alternate courses given every 2 weeks; Womer et al., 2012). ES patients with lung metastasis at diagnosis were treated according to the European EURO-E.W.I.N.G. 99 protocol R2 arm [i.e., consolidation with high-dose chemotherapy (busulfan–melphalan) and peripheral blood stem cell rescue; Ladenstein et al., 2010].

NETs ANALYSIS

Hematoxylin and eosin (HE) stained sections of the ES biopsies were analyzed by a board-certified pathologist (Jonathan Ben-Ezra) for the presence of tumor-associated neutrophils (TANs). When TANs were identified, additional six 5-μm-thick sections were cut from the same block and stained for the presence of NETs as previously described (Brinkmann et al., 2004). In short, paraffin sections were hydrated before antigen retrieval by heating at 37°C for 50 min with target retrieval solution, pH 9 (Dako, Hamburg, Germany). The sections were blocked with 1% bovine serum albumin (BSA), 3% donkey serum (Millipore GmbH, Schwalbach/Ts., Germany), 3% cold fish gelatin (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), 0.05% Tween, and 0.025% Triton for 30 min at room temperature, and incubated with primary antibodies against CD99 (AbD Serotec, Düsseldorf, Germany) and MPO (Dako, Hamburg, Germany) overnight at 4°C in a humid chamber. After washing, donkey anti-mouse Cy3 and donkey anti-rabbit Alexa 488 (Dianova, Hamburg, Germany) were used as secondary antibodies. The sections were also stained with the bisbenzimid DNA dye "Hoechst" H33342 (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and Draq5 DNA dye (New England Biolabs GmbH, Frankfurt/M., Germany). Confocal images were captured using Leica software (TCS-SP, Leica, Mannheim, Germany).

RESULTS

Clinical and histopathological data on the eight ES patients that were included in the study are provided in **Table 1**. NETs were identified in the diagnostic biopsies of 2/8 patients: a 17-year-old-male with a large ES mass of the left iliac bone, L5 vertebrae involvement, and multiple lung metastases (patient 1) and a 15-year-old-male with ES of the right proximal femur and suspected lung metastases (patient 2). Both were treated with the EURO-E.W.I.N.G. 99 protocol and after entering complete remission underwent consolidation therapy with autologous stem cell transplantation. Both had an early relapse, 12 and 18 months after stem cell transplantation (22 and 27 months after diagnosis, respectively). In comparison, NETs were not found in the biopsies of four patients: a 3-year-old female and a 12-year-old male with

Table 1 | Clinical and histopathological data.

Pat. No.	M/F	At diagnosis			Diagnostic biopsy		Chemotherapy-induced necrosis*** (%)	Months from diagnosis	
		Age (years)*	Primary disease site	Lung/bone metastasis	Neutrophils	NETs		Follow-up	Relapse
1	M	17	Lt. Iliac bone	yes	yes	yes	100****	30	22
2	M	15	Rt. proximal femur	yes	yes	yes	99	33	27
3	F	3	Lt. scapula	no	yes	no	97–98	33	no
4	M	12	Lt. iliac bone	no	yes	no	95****	31	no
5	M	9	Lt. iliac bone	yes	yes	no	99****	23	no
6	M	8	L4 vertebrae	yes	yes	no	100	13	no
7	F	16	Rt. distal fibula	no	no	NA**	100	43	no
8	M	13	Rt. metatarsal bones	no	no	NA**	100	41	no

M/F, male/female; NA, not applicable.
**Mean age ± SD, 11.5 ± 4.7 years.*
***Given that neutrophils were not identified in the tumor bed, further investigation for NETs was not performed.*
****The percentage of chemotherapy-induced necrosis, which is used as a prognostic factor for patient outcome, is measured during definitive surgery (i.e., after six courses of neo-adjuvant chemotherapy).*
*****Also received radiotherapy according to protocol.*

localized disease and 9-year-old and 8-year-old males with lung metastases at diagnosis. These four patients are free of disease 33, 31, 23, and 13 months after diagnosis, respectively. In another two patients with localized disease, TANs were not identified, thus further investigation for NETs was not performed.

In the HE sections of both patients who demonstrated NETs (**Figures 1A,B,E,F**), we observed small round blue cells characteristic of ES. In patient 1, neutrophils invaded the ES cell mass; whereas in patient 2, neutrophils were adjacent to the ES cells inside, clearly defined necrotic tissue. In both patients, we observed material that is suggestive of NETs (thick black arrows). Adjacent sections with immunofluorescence staining demonstrated the presence of NETs (**Figures 1C,D,G,H**). In both patients, neutrophils were observed where MPO (green) is localized to the granules, indicating non-activated cells (thick white arrows). However, in many areas, MPO was localized to delobulated nuclei (thin white arrows) and to extracellular DNA stained in red (white arrowheads), attesting to the presence of NETs. In patient 1, NETs were formed in close contact with ES cells, which are labeled with CD99 antibodies (blue). Interestingly, CD99 appears degraded in areas where neutrophils are present. In patient 2, NETs were formed in the interface between ES cells and necrotic tissue. In the necrotic areas of both patients, we observed extracellular DNA that is not associated with MPO, indicating that these are not NETs.

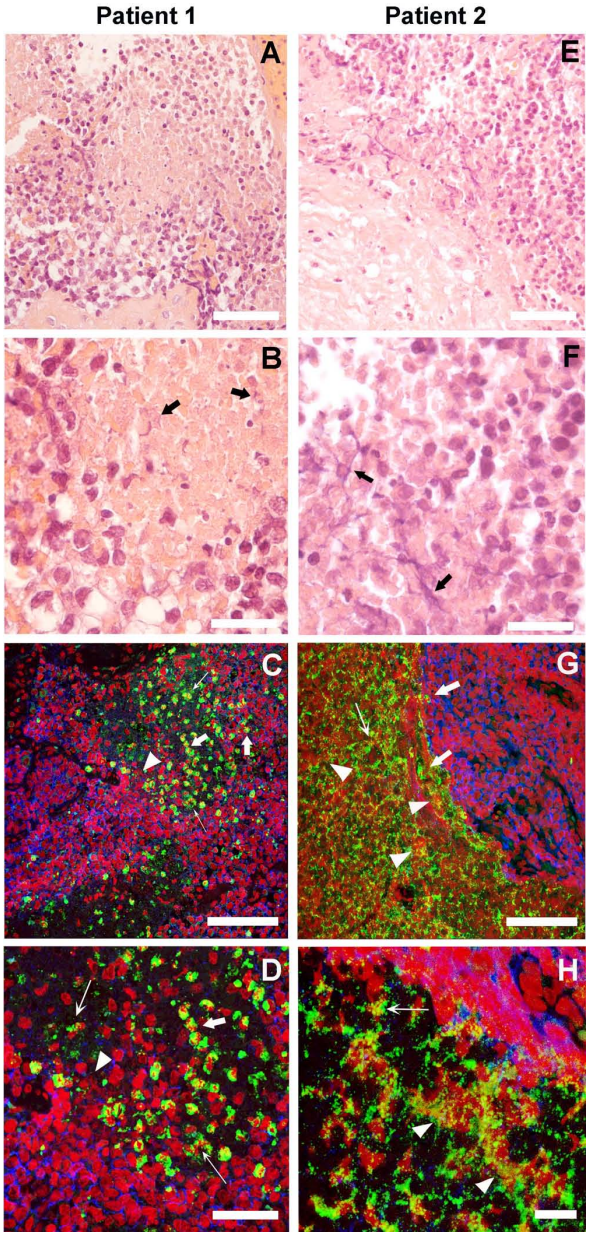

FIGURE 1 | Neutrophil extrcellular traps (NETs) in Ewing sarcoma. The histological HE stained sections of two patients in low **(A,E)** and high **(B,F)** magnification suggest the presence of NETs (thick black arrows). Immunofluorescence staining for myeloperoxidase (MPO; green), CD99 (blue), and DNA (red) of sections adjacent to those shown in HE at low **(C,G)** and high **(D,H)** magnification shows projections of a z-stack of confocal sections 5-μm thick. Naïve Neutrophils are located where MPO is in granules (thick white arrows). Neutrophils in the process of making NETs are found where MPO is localized to delobulated nuclei (thin white arrows) and NETs are located where MPO is colocalized with extracellular DNA (white arrowheads; scale bars, 25 μm).

DISCUSSION

This study is the first to show that TANs in ES are activated to make NETs and thus points to a possible role for NETs in cancer. In the past, most of the research in the field of cancer immunoediting was confined to the role of T lymphocytes, NK cells, macrophages, and DCs (Kim et al., 2007); less attention was given to the role of neutrophils that are an essential effector cell of the innate immune system, serving as the first line of defense against infectious microorganisms. Neutrophils have either pro- or anti-tumor activity, depending on factors such as type of cancer and cytokine profile of the tumor microenvironment (Souto et al., 2011). For example, transforming growth factor (TGF)-β within the tumor induces a population of TANs with a pro-tumorigenic phenotype. However, in the absence of TGF-β, TANs are cytotoxic to tumor cells and express abundant pro-inflammatory cytokines (Fridlender et al., 2009). Neutrophils can promote tumor growth by secretion of matrix metalloproteinase (MMP)-9 that prevents tumor cell apoptosis in the lungs (Acuff et al., 2006) and can promote tumor angiogenesis and neovascularization (Masson et al., 2005). Nevertheless, neutrophils can also be cytotoxic to tumor cells by producing several types of ROS (Lichtenstein, 1987; Dallegri et al., 1991). Notably, in a mouse model of breast cancer, neutrophils were shown to inhibit metastatic seeding by generating hydrogen peroxide (Granot et al., 2011). Neutrophils also produce defensins, which can lyse cancer cells, recruit other immune cells, such as DCs, and have anti-angiogenetic properties (Al-Benna et al., 2011).

We speculate that NETs could have anti-tumorigenic effects, for example by actual killing of tumor cells or activating the immune system. Alternatively, NETs could have a pro-tumorigenic activity by facilitating metastases. Indeed, the three-dimensional structure of NETs may serve to physically capture tumor cells and prevent their dissemination to adjacent tissues. Several components of NETs have been shown to be cytotoxic to tumor cells. MPO was shown to kill B-16 melanoma cells and inhibit their growth in mice after implantation (Odajima et al., 1996). It is of note that

MPO deficient patients may have an exceptionally high incidence of cancer (7/14 patients, 50%; Lanza et al., 1988). NETs can kill activated endothelial cell (Gupta et al., 2010), probably through histones (Saffarzadeh et al., 2012), damaging tumor-feeding blood vessels. NE secreted by TANs can cleave cyclin E (CCNE) to its low molecular weight isoforms and thus promote their presentation to cytotoxic T lymphocytes (Mittendorf et al., 2012). NETs modulate the link between innate and adaptive immune responses by activating plasmacytoid DCs through toll-like receptor 9 (TLR9), an intracellular receptor that recognizes DNA. This NET-mediated activation is important in autoimmune diseases like psoriasis (Skrzeczynska-Moncznik et al., 2012) and systemic lupus erythematosus (Lande et al., 2011). We postulate that tumor antigens caught on NET components could be displayed to DCs and cause their activation. NETs also prime T cells by TCR signaling that requires direct contact (Tillack et al., 2012). Alternatively, NETs, which harbor potent proteases, could be pro-tumorigenic by degradation of the extracellular matrix and promotion of metastases. NETs may also form a barrier between cancer cells and the immune system, thus assisting cancer cells to escape immune recognition. The fact that both our patients with metastatic disease and NET formation relapsed may point to the pro-tumorigenic mechanism of NETs.

Chemotherapy-induced necrosis is one of the most important predictors of outcome in non-metastatic ES patients. Less than 90% necrosis after neo-adjuvant chemotherapy predicts a higher relapse rate (Lin et al., 2007). Recently, van Maldegem et al. (2012) suggested the need for additional biomarkers to predict outcome and allow patient stratification into different risk groups. Our patients had a good histological response to chemotherapy including the two patients who relapsed (**Table 1**); therefore, the presence of NETs could possibly become an additional prognostic marker in ES and other cancers.

Our study is limited by its retrospective design. We examined biopsies that underwent standard histopathological processing and were not necessarily handled with the same care required for NETs preservation. Thus, we cannot rule out that NETs were not found in other samples because they were damaged during material handling or because only a small part of the tumor was sampled. The study is also limited by its small sample size, which precludes statistical analysis to firmly prove the clinical relevance of NET formation in ES. A larger prospective cohort of patients with diverse types of malignancies is advocated. However, we present these preliminary data due to the novelty of our finding, supporting the notion that NETs have a role in cancer immunoediting.

To conclude, this study provides the first data on the release of NETs by TANs in ES. Given that both patients with metastatic disease and NET formation relapsed may point to a pro-tumorigenic mechanism of NETs and may constitute an important prognostic marker in ES and in other cancers. This intriguing topic may have a significant contribution to our understanding of innate immune responses against cancer and potentially lead to the development of new therapeutic strategies in the battle against cancer.

ACKNOWLEDGMENTS

We would like to thank R. L. Berger, Kfar Saba, Israel, for writing and editing assistance.

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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.

Received: 29 November 2012; accepted: 08 February 2013; published online: 06 March 2013.

Citation: Berger-Achituv S, Brinkmann V, Abed UA, Kühn LI, Ben-Ezra J, Elhasid R and Zychlinsky A (2013) A proposed role for neutrophil extracellular traps in cancer immunoediting. Front. Immunol. ***4****:48. doi: 10.3389/fimmu.2013.00048*

This article was submitted to Frontiers in Molecular Innate Immunity, a specialty of Frontiers in Immunology.

frontiers in IMMUNOLOGY

HYPOTHESIS AND THEORY ARTICLE
published: 18 March 2013
doi: 10.3389/fimmu.2013.00067

Identification of macrophage extracellular trap-like structures in mammary gland adipose tissue: a preliminary study

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PAD4-mediated hypercitrullination of histone H4 arginine 3 (H4R3) has been previously found to promote the formation of Neutrophil Extracellular Traps in inflamed tissues and the resulting histone H4 citrulline 3 (H4Cit3) modification is thought to play a key role in extracellular trap (ET) formation by promoting chromatin decondensation. In addition to neutrophils, macrophages have also recently been found to generate functional extracellular traps (METs). However, a role for PADs in ET formation in macrophages has not been previously described. Transcripts for PAD2 and PAD4 are found in mature macrophages and these cells can be induced to citrullinate proteins, thus raising the possibility that PADs may play a direct role in ET formation in macrophages via histone hypercitrullination. In breast and visceral white adipose tissue from obese patients, infiltrating macrophages are often seen to surround dead adipocytes forming characteristic "crown-like structures" (CLS) and the presence of these lesions is associated with increased levels of inflammatory mediators. In light of these observations, we have initiated studies to test whether PADs are expressed in CLS macrophages and whether these macrophages might form METs. Our preliminary findings show that PAD2 (and to a lesser extent, PAD4) is expressed in both in the macrophage cell line (RAW 264.7) and in CLS lesions. Additionally, we provide evidence that macrophage-derived extracellular histones are seen around presumptive macrophages within CLS lesions and that these histones contain the H4Cit3 modification. These initial findings support our hypothesis that obesity-induced adipose tissue inflammation promotes the formation of METs within CLS lesions via PAD-mediated histone hypercitrullination. Subsequent studies are underway to further validate these findings and to investigate the role in PAD-mediated MET formation in CLS function in the mammary gland.

Keywords: peptidylarginine deiminases, PAD2, ETosis, macrophage extracellular traps, adipose tissue inflammation, histone citrullination, deimination, crown-like structures

INTRODUCTION

ETosis is a recently described cell death-associated phenomenon that results in the release of a complex lattice of chromatin that contains DNA, histones, and other associated proteins (Amulic and Hayes, 2011; Liu et al., 2012; Saffarzadeh et al., 2012). These extracellular chromatin webs can be used to entrap and kill microbial organisms. Initially, this phenomenon was described in neutrophils, termed NETosis (Neutrophil Extracellular Traps), but subsequent studies have found that this mechanism also exists in other cell types such as macrophages, eosinophils, and mast cells. Given the ever-expanding array of cell types that can form extracellular traps (ET), this activity is now more generally referred to as ETosis. Various infectious agents such as bacteria, fungi, and protozoa, have been found to induce ETosis, as have cytokines, such as IL-8, and chemicals, such as phorbol myristate acetate (PMA) (Brinkmann et al., 2004).

At the molecular level, ETs were first shown to be comprised of decondensed DNA scaffolds and various neutrophil granular proteins (Brinkmann et al., 2004). Later proteomic studies further concluded that ETs contain histones (the most abundant fraction), elastase, glycolytic enzymes, and several other cytosolic proteins (Urban et al., 2009). Immunofluorescence (IF) studies have also shown additional components in ETs such as NADPH oxidase subunits, pentraxin-3, and cathelicidin. Interestingly, the localization of these proteins to NETs was not supported by subsequent biochemical studies, suggesting that some of these components may be loosely associated with ETs (Guimaraes-Costa et al., 2012). While the precise mechanisms underlying ET induction and formation are not completely understood, several morphological features of ETosis have been observed across various studies. These features include loss of distinct segregation of euchromatin and heterochromatin, disappearance of the lobular nuclear architecture in neutrophil nuclei, granular membrane disruption, and nuclear membrane swelling. ET induction is thought to occur when proteolytic enzymes are released from cytosolic granules where these enzymes then cause a collapse of the nuclear envelope,

leading to disruption of plasma membrane integrity and, eventually, release of ETs into the extracellular space (Fuchs et al., 2007; Remijsen et al., 2011).

While many studies have now documented a role for NETs in an array of activities, much less is known about ETosis in other cell lines such as macrophages. Two recent studies found that the bacterial organisms *Histophilus somni* and *Mannheimia haemolytica* can induce ETs in bovine macrophages (METs) (Aulik et al., 2012; Hellenbrand et al., 2013). Additionally, another study showed that human THP-1-derived macrophages and the RAW 264.7 macrophage cell line formed METs in response to *E. coli* toxins. Interestingly, while MET formation has been documented in tissue macrophages, it has yet to be reported in peripheral blood monocytes (Aulik et al., 2012).

PAD enzymes catalyze the conversion of positively charged arginine residues to neutrally charged citrulline in a hydrolytic reaction termed citrullination or deamination. The resulting loss of charge at this site can dramatically alter the target protein's tertiary structure as well as its ability to interact with other proteins (Wang et al., 2009; Mohanan et al., 2012). The N-terminal tails of histones such as H3 and H4 are arginine-rich and appear to represent major target for PAD enzymes. For example, numerous reports have shown that PAD4 and, more recently, PAD2, regulate gene expression via citrullination of histone H4R3 and H3R26, respectively (Wang et al., 2009; Cherrington et al., 2012). While the mechanisms by which histone citrullination regulates gene transcription are not fully understood, we recently demonstrated that PAD2-catalyzed histone citrullination promoted localized chromatin decondensation at target gene promoters, thus likely facilitating binding of the basal transcriptional machinery (Zhang et al., 2012). On a more global level, we have also recently shown that activation of PAD4 in neutrophils promotes histone hypercitrullination, global chromatin decondensation, and NET formation (Wang et al., 2009). In this previous study, we showed by transmission electron microscopy that activation of PAD4 in HL60 granulocytes promoted the conversion of multi-lobular heterochromatic nuclei into a more round euchromatic nuclear pattern (Wang et al., 2009). Additionally, we demonstrated that TNF-α treatment of blood neutrophils resulted in the release of extracellular chromatin that was extensively citrullinated at histone H4R3. The link between the H4Cit3 modification and NET formation is very strong and this modification is now routinely utilized to document the presence of ETs in cells and tissues (Neeli et al., 2008; Wang et al., 2009). In addition to TNF-α, LPS and H_2O_2 have also been shown to induce PAD-mediated histone deimination (Neeli et al., 2008). Importantly, the requirement of citrullination in NET formation *in vivo* was recently documented by investigators who showed that PAD4$^{-/-}$ mice have reduced ability to form NETs in response to various stimuli. Additionally, the investigators found that these mice are more susceptible to bacterial infections (Li et al., 2010). More generally, PAD activity is also closely associated with non-microbial induced immune-mediated inflammatory activity such as that seen in autoimmune arthritis, colitis, and chronic obstructive pulmonary disease. Along these lines, excessive PAD-mediated ETosis has also recently been found to play a role in deep vein thrombosis and cystic fibrosis (Marcos et al., 2010).

Obesity is a major public health concern and, among other things, is a risk factor for hormone receptor-positive breast cancer in postmenopausal women (Cleary and Grossmann, 2009; van Kruijsdijk et al., 2009). Numerous studies in obese humans and animals have shown that macrophages infiltrate visceral adipose tissue and surround dead adipocytes forming a characteristic "crown-like structure" (CLS) morphology. These macrophages are believed to primarily function at these sites by "cleaning up" the remnants of dead/dying adipocytes via phagocytosis of lipids, cytoplasmic debris, and karyorrhectic remnants (Cinti et al., 2005). Additionally, these immune cells produce proinflammatory mediators that are found in the circulation of obese women and have been linked to breast cancer progression.

Recent studies have shown that CLS lesions occur in white adipose tissue of the breast in obese women and in the mammary gland of experimental models of obesity (Morris et al., 2011; Subbaramaiah et al., 2011). Importantly, in mouse models of obesity, CLS occur in association with activation of NF-κB and elevated levels of inflammatory mediators including TNF-α, IL-1β, and COX-2 (Subbaramaiah et al., 2012). Increased levels of TNF-α at these sites is thought to enhance inflammatory activity due to a paracrine signaling loop between TNF-α, saturated fatty acids, and adipocytes (Suganami and Ogawa, 2010). Given that TNF-α can induce ET formation in neutrophils, that PADs are intimately associated with inflammation, and that macrophages can form ETs, we predicted that macrophages may undergo ETosis in CLS lesions. As outlined below, we performed a preliminary study to test this hypothesis. Our findings suggest that METs do form in CLS structures, thus laying the groundwork for future studies to investigate the functional role for METs in the resolution of inflamed white adipose tissue in the mammary gland.

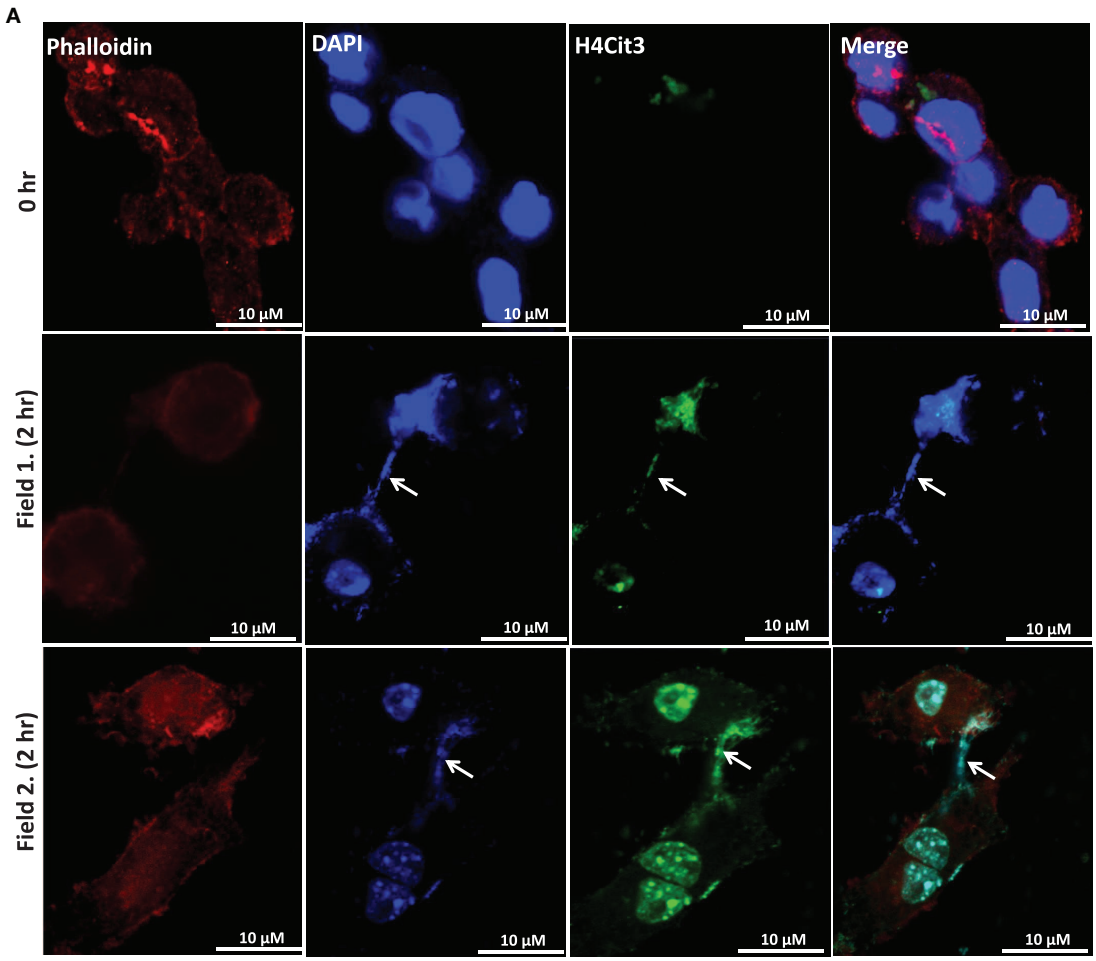
MATERIALS AND METHODS

RAW 264.7 CELL CULTURE AND MET INDUCTION

RAW 264.7 mouse macrophage cells were grown in RPMI 1640 (Cellgro, Manassas, VA, USA) supplemented with 10% fetal bovine serum containing penicillin-streptomycin. The cells were grown in a sterile, humidified incubator, and maintained in these conditions at 37°C and 5% CO_2. To perform IF staining, macrophages were grown on 12 mm glass coverslips for 2 days at 37°C (Fisher Scientific, Hanover Park, IL, USA). The RAW 264.7 macrophages were treated with recombinant TNF-α (20 ng/ml, R&D systems, Minneapolis, MN, USA) for 2 h, then washed in 1× phosphate buffered saline (PBS), fixed in 4% cold paraformaldehyde for 20 min, washed in 1× PBS, and IF staining was performed as described below.

MAMMARY GLAND ADIPOSE TISSUE FROM OBESE MICE

Mammary gland adipose tissue sections were prepared from a dietary model of obesity as described previously (Hong et al., 2009; Subbaramaiah et al., 2011). Ovariectomized C57BL/6J mice (Jackson Laboratories) at 5 weeks of age were given high fat diet (60 kcal% fat, D12492i, Research Diets) to generate obese mice. Mice were fed *ad libitum* for 10 weeks and sacrificed to collect mammary gland tissue. Tissue samples were formalin fixed for histological and immunohistochemical analyses.



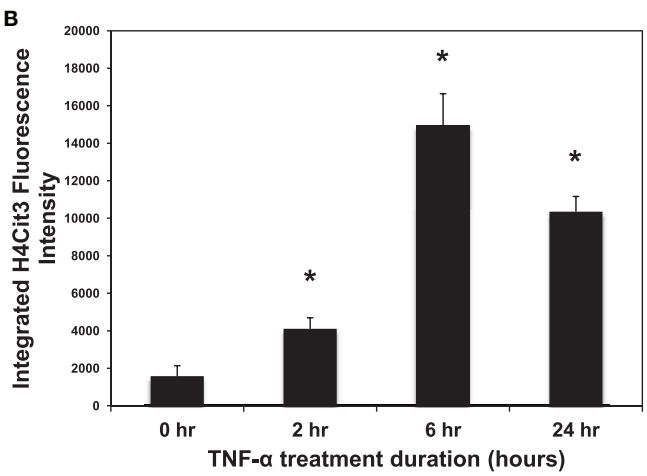


FIGURE 1 | (A) H4Cit3 immunofluorescence staining in RAW 264.7 macrophages following 0 and 2 h TNF-α treatment. Cultured RAW 264.7 macrophage cells appear to form METs following TNF-α stimulation and these METs contain citrullinated histones. DAPI staining (blue) shows that DNA extends beyond the Phalloidin-stained cell borders. Anti-Histone H4Cit3 staining shows presence of citrullinated histones within METs (Arrows). Two separate fields from the 2 h treatment group (Fields 1 and 2) are included for reference. **(B)** Integrated anti-H4Cit3 fluorescence intensity quantitation at 0, 2, 6, and 24 h of TNF-α treatment. The RAW 264.7 macrophages were treated with 20 ng/ml of TNF-α, fixed, stained with the anti-H4Cit3 antibody, and imaged by indirect immunofluorescence at the indicated time points. Five unique fields were imaged by confocal microscopy at each time point and the mean integrated fluorescence intensity was calculated using ImageJ software analysis. Results were analyzed by ANOVA and the graphs represent mean ± standard deviation (*p-value < 0.01).

The animal protocol was approved by the Institutional Animal Care and Use Committee at Weill Cornell Medical College.

mRNA ISOLATION AND RT-PCR

RNA was isolated from the RAW 264.7 macrophage cells using the Qiagen RNeasy mini kit, including on-column DNAse treatment to remove genomic DNA (Qiagen # 74104). The purified RNA was reverse-transcribed using the Applied Biosystems High Capacity RNA-to-cDNA kit according to manufacturer's protocol (Applied Biosystems # 4387406, Foster City, CA, USA). The cDNA (100 ng) was mixed with Go-Taq DNA polymerase (Promega # M3005, Madison, WI, USA) and primer set. The following primer pairs were used (size of the amplicons are in parentheses): PAD2 primer set #1 (400 bp): Fwd – 5′-AGAAGGGAGGCTCTGAGGTC-3′ and Rev – 5′-CTGGCCAGAGAATTGAGGAC-3′; PAD2 primer set #2 (188 bp): Fwd – 5′-CAAGATCCTGTCCAATGAGAG-3′ and Rev – 5′-ATCATGTTCACCATGTTAGGGA-3′; PAD4 primer set #1 (406 bp): Fwd – 5′-TCTCCCTGCTGGACAAGTCT-3′ and Rev – 5′-AGCCCAGTGAGCTCTGACAT-3′; PAD4 primer set #2 (193 bp): Fwd – 5′-CTACTCTGACCAAGAAAGCC-3′ and Rev – 5′-ATTTGGACCCATAACTCGCT-3′; GAPDH primer set #1 (324 bp): Fwd – 5′-CCCACTAACATCAAATGGGG-3′ and Rev – 5′-ATCCACAGTCTTCTGGGTGG-3′; and GAPDH primer set #2 (209 bp): Fwd – 5′-GGGCATCTTGGGCTACAC-3′ and Rev – 5′-GGTCCAGGGTTTCTTACTCC-3′. PCR was performed using the following set up: 5 min at 95°C, 30 cycles of (30 s, 94°C; 30 s, 58°C; 30 s, 72°C), 5 min at 72°C. The PCR products were analyzed on a 1% agarose gel using Geldoc (BioRad) software.

IMMUNOHISTOCHEMISTRY AND IMMUNOFLUORESCENCE

Immunohistochemistry (IHC) and IF experiments followed our previously described protocol (Cherrington et al., 2010). Briefly, paraffin embedded tissue sections were deparaffinized and rehydrated in 3 × 5 min washes in xylene followed by single sequential 5 min washes in 100, 95, and 75% EtOH. Slides were then incubated for 10 min in 0.5% hydrogen peroxide in methanol to quench endogenous peroxidases. Antigen retrieval was performed by boiling slides 2× for 10 min in 0.01 M sodium citrate pH 6.8 and, after cooling, slides were washed in 1× PBS. Tissue slides and fixed coverslips containing RAW264.7 cells were then blocked in 10% normal goat serum and 2× casein (Vector Labs, Burlingame, CA, USA) for 20 min at room temperature in a humidified microprobe chamber. Slides were blotted to remove excess blocking solution and then primary antibody diluted in 1× PBS was applied to the slides for 2 h at room temperature. Slides or cover slips were then stained with either anti-PAD2 (ProteinTech #122100-1-AP, Chicago, IL, USA) at a 1:100 dilution, anti-PAD4 (Sigma-Aldrich # P4749) at a 1:100 dilution, or with anti-histone H4 Citrulline 3 (Millipore-Upstate # 07-596) at a 1:100 dilution. Following 3× washing with 1× PBS, slides were incubated with a 1:200 dilution of biotinylated secondary antibody (in 1× PBS) for 1 h at room temperature and then washed 3× in 1× PBS. For IHC, slides were incubated in DAB chromagen (Vector Labs) solutions according to the manufacturer's protocol, washed and then counterstained with Gill's hematoxylin, and coverslipped. For each experiment, duplicate slides were treated with control rabbit IgG antibody at the appropriate concentration as a negative control. For immunofluorescence, slides were incubated in streptavidin conjugated-488 or 555 (Invitrogen), washed, and then mounted using Vectashield containing DAPI (Vector Labs). As a negative control, duplicate slides were treated with control rabbit IgG at the appropriate concentrations.

In order to quantitate changes in levels of citrullinated histones following TNF-α treatment, a time course experiment was performed evaluating H4Cit3 immunofluorescence intensity at 0, 2, 6, and 24 h. RAW 264.7 macrophages were treated with 20 ng/ml of TNF-α, fixed, and H4Cit3 immunofluorescence staining was performed using the method described above. Five different fields from each time point were imaged using confocal microscopy and the mean integrated fluorescence intensity was calculated using ImageJ software analysis as previously described by Gavet and Pines (2010). The results were then analyzed by one-way ANOVA to detect statistical differences and a p-value < 0.01 was considered significant. All values are presented as mean ± SD.

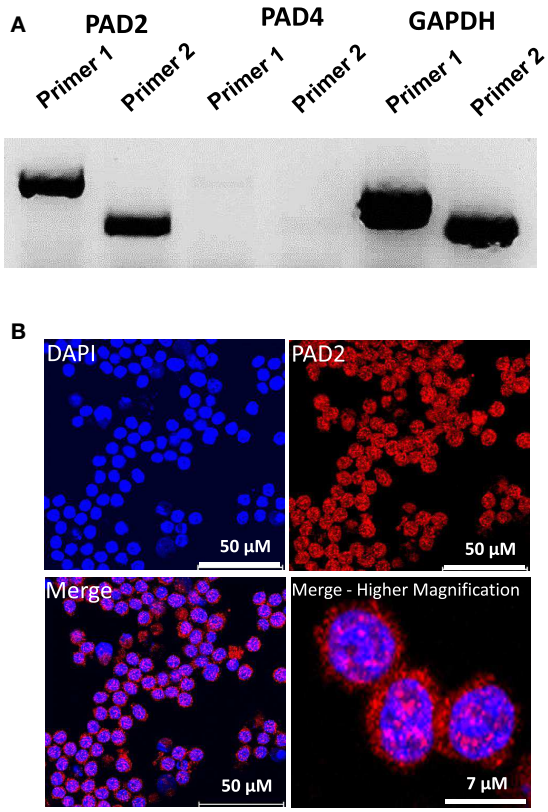


FIGURE 2 | PAD2 expression in cultured RAW 264.7 macrophage cells. **(A)** RT-PCR for PAD2 and PAD4 mRNA expression in cultured RAW 264.7 macrophages. Two unique primer sequence sets were used to test for PAD2 and PAD4 mRNA expression levels. RAW 264.7 macrophages were found to strongly express PAD2 mRNA. GAPDH mRNA expression levels were used as the internal control for RT-PCR. **(B)** Anti-PAD2 immunofluorescence labeling shows positive staining in the nucleus and cytoplasm of RAW 264.7 macrophages. Nuclei are stained with DAPI.

RESULTS AND DISCUSSION

TNF-α APPEARS TO INDUCE MET FORMATION IN RAW 264.7 MACROPHAGES

Given that TNF-α induces ET formation in neutrophils, we first tested whether TNF-α could promote ET formation in the RAW 264.7 macrophage cell line. As shown in **Figure 1A**, we found that TNF-α treatment led to DAPI-stained chromatin in characteristic strands outside of ∼5–10% of the phalloidin-bound RAW264.7 cells. Two different microscopic fields from 2 h treatment groups are shown in **Figure 1** as representative images. By contrast, these characteristic strands were not seen in untreated control cells. This observation supports the hypothesis that TNF-α induced the release of chromatin from the cell nucleus into the extracellular space. As a further test of this hypothesis, we then stained the TNF-α-treated RAW264.7 cells with the anti-H4Cit3 antibody. Results show that the extracellular chromatin appeared to be extensively citrullinated at H4R3, thus suggesting that PAD-mediated histone hypercitrullination promotes ETosis in macrophages. In an effort to quantitate the extent to which TNF-α promoted the formation of extracellular chromatin in RAW264.7 cells, we quantitated the staining intensity for the anti-H4Cit3 antibody at different time points following treatment. Results show that the mean integrated fluorescence intensity at 2, 6, and 24 h was significantly increased (as determined by ANOVA) when compared to the 0 h time point ($p < 0.01$). We also note that the H4Cit3 intensity gradually increased until the 6 h time point, while levels were lower at the 24 h time point. This result supports the hypothesis that, as with neutrophils, TNF-α stimulates histone hypercitrullination and ETosis in macrophages.

PAD2 IS A LIKELY CANDIDATE FOR CATALYZING HISTONE HYPERCITRULLINATION DURING ETosis IN MACROPHAGES

While PAD4 is the only PAD family member to have been shown to be required for ETosis, both PAD2 and PAD4 have been previously shown to be expressed in macrophages. Therefore, as an initial investigation into which PAD family member may catalyze ET production in RAW264.7 cells, we first investigated mRNA expression levels of PAD2 and PAD4 in this cell line. Surprisingly, RT-PCR analysis found that PAD4 expression was very low in RAW264.7 cells (**Figure 2A**), suggesting that this family member may not play a critical role in macrophage function. We note, however, that we have yet to test for PAD4 levels in *in vivo*-derived macrophages. Given the high level of PAD2 mRNA observed in these cells, we then carried out indirect immunofluorescence to test whether RAW264.7 macrophages also expressed PAD2 protein. Results showed PAD2 staining was strong in these cells (**Figure 2B**) and appeared to localize, in part, to DAPI-poor euchromatic regions of the RAW 264.7 cell nucleus (**Figure 2B**, bottom right panel). While not conclusive, this finding supports the hypothesis that PAD2 is a primary driver of histone hypercitrullination, chromatin decondensation, and ET formation in macrophages. We note that the localization of PAD2 to the cell nucleus fits well with this hypothesis. Additionally, this hypothesis is further supported by our recent finding that both global and promoter-specific chromatin decondensation is catalyzed by PAD2 in breast cancer cell lines (Zhang et al., 2012).

EVIDENCE SUPPORTING THE HYPOTHESIS THAT METs EXIST IN MOUSE MAMMARY GLAND CLS LESIONS

Our findings in the RAW264.7 cell line suggested that PAD2-catalyzed histone hypercitrullination may promote ETosis in CLS-localized macrophages. In order to begin testing this hypothesis, we next investigated whether PAD2 was expressed in the nucleus of macrophages in CLS lesions. Results show that PAD2 expression appeared to be robust in these cells (**Figure 3**). Importantly, similar to the RAW264.7 cells, PAD2 was found to concentrate in the DAPI-poor regions of the presumptive

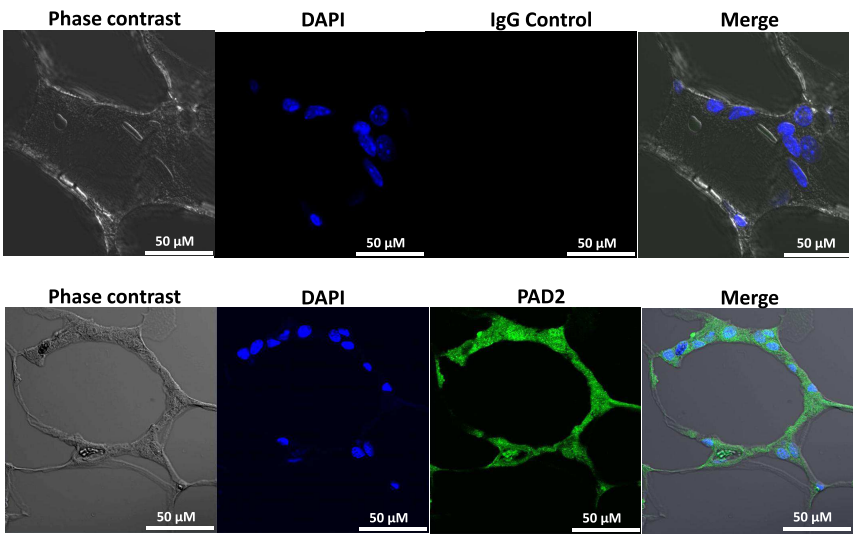


FIGURE 3 | PAD2 expression in cells within CLS lesions of the murine mammary gland. Immunofluorescence staining of mammary gland adipose tissue sections with anti-PAD2 antibodies shows that cells within the CLS lesions appear to express PAD2. Nuclei are stained with DAPI. Non-specific rabbit IgG was used as a negative control.

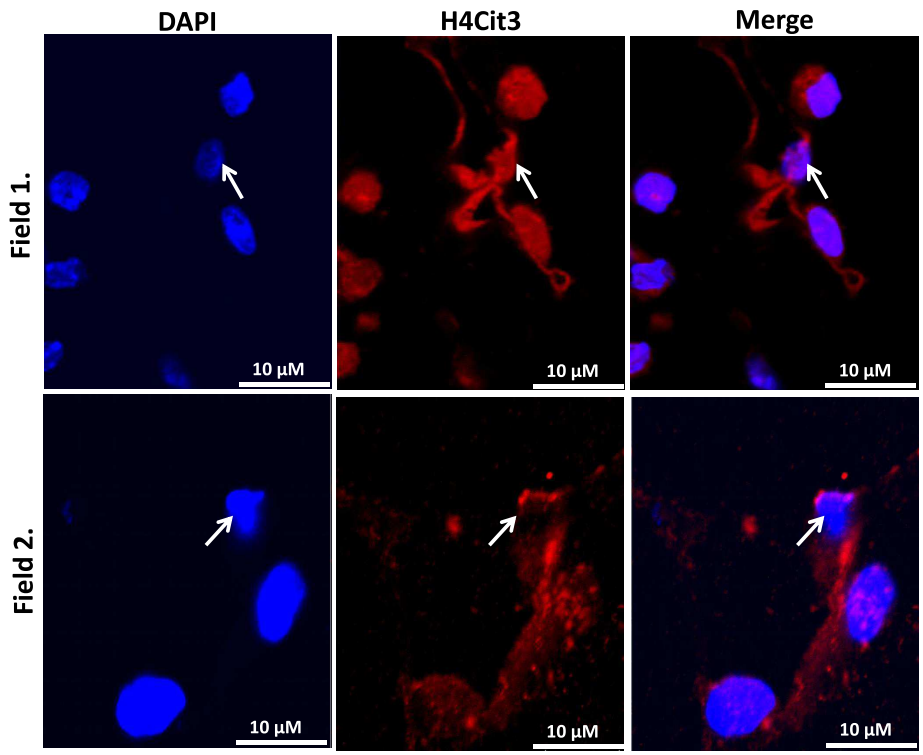


FIGURE 4 | Extracellular trap-like structures within CLS lesions of the murine mammary gland stain positive for the histone H4Cit3 modification. Immunostaining of mouse mammary gland adipose tissue with the anti-H4Cit3 antibody shows that extranuclear citrullinated histones appear to extend into the extracellular space between cells within CLS lesions. Arrow highlights nuclei from which the extracellular histones appear to originate.

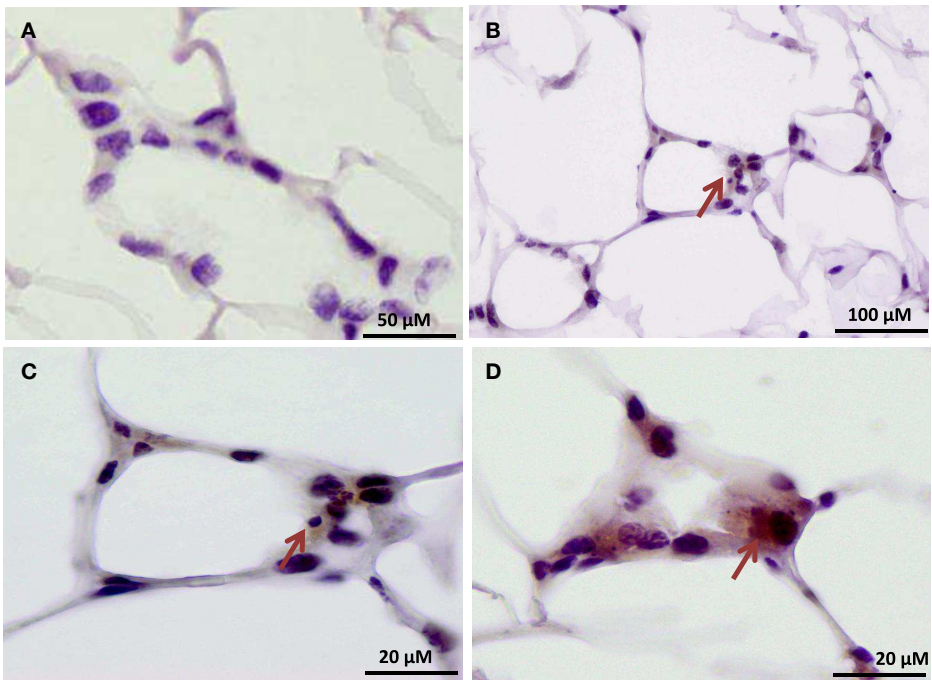


FIGURE 5 | Immunohistochemical localization of the histone H4Cit3 modification within CLS lesions of the mammary gland. (A) Negative control showing adipose tissue sections stained with non-specific rabbit IgG and hematoxylin counterstain. **(B–D)** Arrows indicate CLS lesions with positive immunostaining for the H4Cit3 modification. A higher magnification of the CLS lesion from **(B)** is given in **(C)**.

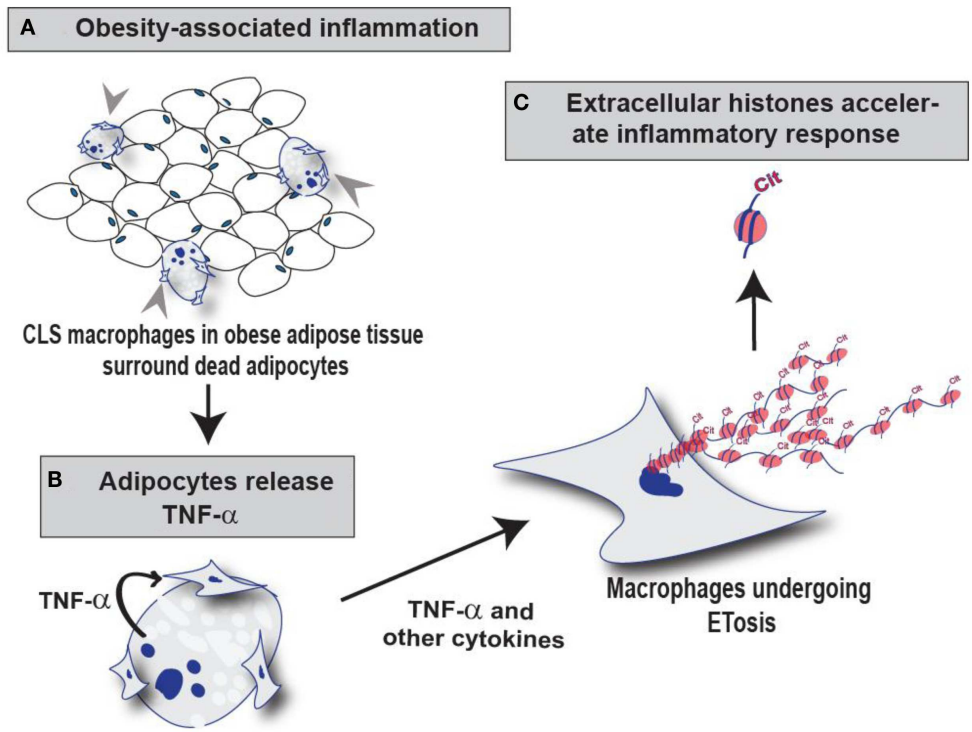


FIGURE 6 | Schematic illustration of the role of PAD2-mediated histone citrullination during MET formation in CLS lesions. (A) Macrophages infiltrate obese adipose tissue and surround dead adipocytes forming the CLS lesions. **(B)** TNF-α and other cytokines released from adipocytes may stimulate ETosis in these surrounding CLS macrophages thus promoting PAD2-mediated histone citrullination, chromatin decondensation, and extracellular chromatin scaffold formation. **(C)** We hypothesize that the citrullinated histones released during MET formation may regulate the inflammatory microenvironment of mammary adipose tissue by influencing the macrophage infiltration and/or macrophage activation.

macrophage cell nucleus, again suggesting that PAD2 localizes to euchromatic regions of the nucleus. We then stained these samples with the anti-H4Cit3 antibody and found positive staining for the histone H4Cit3 modification extending from numerous CLS cell nuclei (**Figure 4**). This staining is highlighted by arrows in the two representative images (**Figure 4**, Field 1 and Field 2) and appears to extend into the extracellular space between CLS cells. In order to more precisely refine the localization of H4Cit3-stained chromatin, we next carried out immunohistochemical staining of the CLS-containing sections using the anti-histone H4Cit3 antibody. Results showed that CLS cell nuclei were frequently stained with this antibody (**Figure 5**). Importantly, the anti-H4Cit3 staining is particularly intense in fragmented nuclear particles and, in such cases, the staining appeared to extend well into the cell cytoplasm and extracellular space.

Outcomes from this study suggest that macrophages undergo ETosis following TNF-α stimulation *in vitro* and within mammary gland CLS lesions in obese mice. Our IF and IHC experiments using the anti-H4Cit3 antibody (a well validated marker for ET chromatin) finds that histone hypercitrullination is seen in extracellular chromatin in both RAW264.7 cells and in CLS macrophages, thus supporting the hypothesis that the observed ETosis is likely PAD-mediated. Additionally, we show that PAD2, but not PAD4, expression is robust within the nucleus of RAW264.7 cells and CLS-localized macrophages, thus identifying PAD2 as a strong candidate for catalyzing ETosis formation in macrophages. These preliminary findings provide support for the hypothesis that METs occur in CLS lesions of mammary gland adipose tissue from obese mice and lay the groundwork for future studies aimed at identifying a role for METs in CLS function. Given the close ties between PAD activity and inflammation, we favor the hypothesis that the release of hypercitrullinated histones from the macrophage nucleus into the extracellular space during MET formation plays a critical role in promoting inflammatory signaling pathways within microenvironment of mammary gland adipose tissue (**Figure 6**). Experiments are currently underway to test this hypothesis.

ACKNOWLEDGMENTS

The authors would like to thank Dr. Ling Qi for generously providing the RAW 264.7 macrophage cell line. The grant support for Scott A. Coonrod was a DOD Era of Hope Scholar Award (W81XWH-07-1-0372). Andrew J. Dannenberg was supported with funding from NCI 1R01CA154481, the Breast Cancer Research Foundation, and the Botwinick–Wolfensohn Foundation (in memory of Mr. and Mrs. Benjamin Botwinick).

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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.

Received: 07 December 2012; accepted: 01 March 2013; published online: 18 March 2013.

Citation: Mohanan S, Horibata S, McElwee JL, Dannenberg AJ and Coonrod SA (2013) Identification of macrophage extracellular trap-like structures in mammary gland adipose tissue: a preliminary study. Front. Immunol. ***4****:67. doi: 10.3389/fimmu.2013.00067*

This article was submitted to Frontiers in Molecular Innate Immunity, a specialty of Frontiers in Immunology.

frontiers in IMMUNOLOGY

CLINICAL CASE STUDY
published: 12 November 2012
doi: 10.3389/fimmu.2012.00333

Abundant neutrophil extracellular traps in thrombus of patient with microscopic polyangiitis

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This is a case study of a patient diagnosed with microscopic polyangiitis (MPA) and complicated with deep vein thrombosis (DVT), who died of respiratory failure despite treatment. Autopsy revealed severe crescentic glomerulonephritis and massive alveolar hemorrhage. The thrombus contained abundant neutrophils. Although it is reported that patients with ANCA-associated vasculitis (AAV) have an increased risk of DVT, it remains elusive why they are prone to thrombosis. A recent study has demonstrated the presence of neutrophil extracellular traps (NETs), a newly recognized mode of neutrophil cell-death, in glomerular crescents of MPA patients. Interestingly, NETs were identified in the thrombus as well as in the glomerular crescents in the present case. When compared to other thrombi unrelated to MPA, the amount of NETs was significantly greater in the MPA patient. On the other hand, NETs are critically involved in thrombogenesis because histones within NETs can bind platelets and blood coagulants. Although this is important in regard to containment of microbes within NETs, excessive NETs could cause thrombosis. The collective findings suggest the possibility that thrombosis could be critically associated with MPA via NETs, and that NETs could be a therapeutic target in MPA patients.

Keywords: MPO-ANCA, microscopic polyangiitis, neutrophil extracellular traps, deep vein thrombosis, histone-citrullination

CASE PRESENTATION

A 56-years-old woman was admitted to the section of Internal Medicine because of fever and tender swelling of the left leg that began 2 weeks ago. Urinalysis revealed microhematuria (30–49/high power field) and proteinuria (100–300 mg/dl). Hematological examinations showed leukocytosis with white blood cell counts of 16,410/μl, anemia with hemoglobin of 6.9 g/dl, and normal platelet counts of 24.0×10^4/μl. Blood chemistry demonstrated elevated levels of blood urea nitrogen (22.9 mg/dl) and creatinine (2.85 mg/dl). The serum level of C-reactive protein was also elevated (7.39 mg/dl). Myeloperoxidase-anti-neutrophil cytoplasmic antibody (MPO-ANCA) was positive (836 units/ml); while, other autoantibodies, including proteinase 3-ANCA, anti-glomerular basement membrane antibody, and anti-phospholipid antibody, were negative. In coagulation tests, fibrin degradation products and D-dimers were markedly elevated (28.1 μg/ml and 21.7 μg/ml, respectively). Contrast-enhanced computed tomography showed bilateral infiltrative shadows in the lower lobules of the lungs with pleural effusion and thrombosis in the left common iliac vein. Based on these findings, the patient was diagnosed with microscopic polyangiitis (MPA) complicated with pneumonia and deep vein thrombosis (DVT). Immediately, a filter was inserted into the inferior vena cava in order to prevent fatal pulmonary embolism. Although alveolar hemorrhage was considered to be a differential diagnosis for pneumonia, antibiotic treatment was initiated because bacterial infection could not be ruled out at this time. However, she developed dyspnea and hemoptysis 5 days later. Bronchoscopy and bronchoalveolar lavage were not conducted because of the respiratory distress; however, alveolar hemorrhage due to MPA was still considered. Therefore, combination therapy of corticosteroid (intravenous administration of 0.5 g methylprednisolone for 3 consecutive days followed by oral administration of 40 mg/day prednisolone) and cyclophosphamide (intravenous administration of 500 mg/day cyclophosphamide) was started 5 days after the admission. However, she died of respiratory failure 4 days later.

Autopsy revealed diffuse crescentic necrotizing glomerulonephritis without immunoglobulin deposition and massive alveolar hemorrhage with neutrophil infiltration (**Figures 1A,B**). The glomerular findings were consistent with pauci-immune crescentic glomerulonephritis of MPA. Alveolar hemorrhage was also considered as a sign of MPA, though typical capillaritis could not be identified in the lungs. The thrombus was relatively, fresh and contained abundant neutrophils (**Figures 1C,D**). In the thrombus, no microbe was detected by special staining techniques, including Gram stain, Giemsa stain, and Periodic acid-Schiff reaction.

BACKGROUND

MPA is an ANCA-associated vasculitis (AAV), in which pauci-immune crescentic glomerulonephritis develops with

generation of MPO-ANCA. Alveolar hemorrhage due to capillaritis in the lungs is a frequent complication and is sometimes fatal. It is reported that AAV patients have an increased risk of developing DVT, especially during the active stage of the disease (Stassen et al., 2008). Vasculitis possibly triggers thrombosis through the action of inflammatory cytokines and other substances related to the injury of vascular endothelial cells. However, the formation of thrombus does not always occur in the affected vessels. Thus, it remains elusive why AAV patients are prone to thrombosis.

A recent study has demonstrated the presence of neutrophil extracellular traps (NETs), a newly recognized mode of neutrophil cell-death, in glomerular crescents of MPA patients (Kessenbrock et al., 2009). Kessenbrock et al. suggested that MPO-ANCA could bind with activated neutrophils and accelerate NETs formation. Intrinsically, NETs play roles in the innate immune response to microbes, in which the meshwork is composed of DNA fibers that comprise histones and antimicrobial proteins including MPO (Brinkmann et al., 2004). Under physiological condition, NETs are induced following phagocytosis in order to trap and kill surviving microbes, and are adequately digested subsequently. However, aberrant formation and disordered regulation of NETs could be implicated in the production of MPO-ANCA and subsequent development of MPA (Nakazawa et al., 2012; Ray, 2012). In addition, the extracellular DNA in NETs could accelerate MPO-ANCA production via activation of plasmacytoid dendritic cells and B cells in a toll-like receptor 9-dependent manner (Hurtado et al., 2008).

On the other hand, NETs are critically associated with thrombosis because histones within NETs can bind platelets and blood coagulants (Xu et al., 2009; Fuchs et al., 2010). NETs induce the formation of a firm thrombus with red blood cells and fibrin. Although the synergy of antimicrobial and pro-thrombotic functions of NETs is considered to be valuable in the inclusion of microbes in the NETs, excessive NETs formation conversely causes thrombosis. Thus, we focused on NETs in order to understand the association of thrombosis with MPA.

RESULTS AND DISCUSSION

In the present case, the immediate initiation of immunosuppressive therapy was precluded because the possibility of bacterial pneumonia could not be totally ruled out. Unfortunately, the inevitable delay in the initiation of treatment could be attributed to the patient demise. Therefore, development of alternative therapeutic strategies other than immunosuppressive therapy is desirable for treatment of patients with MPA.

Using the autopsy materials, we investigated the presence of NETs in the glomeruli and thrombus. As previously shown (Kessenbrock et al., 2009), NETs were identified in the glomerular crescents (**Figures 1E–G**). Interestingly, NETs were also identified in the thrombus (**Figures 1H–J**). Citrullination of histones is essential for the induction of NETs (Li et al., 2010). It is considered that histone-citrullination correlates with chromatin decondensation during NETs formation. Thus, we next investigated the

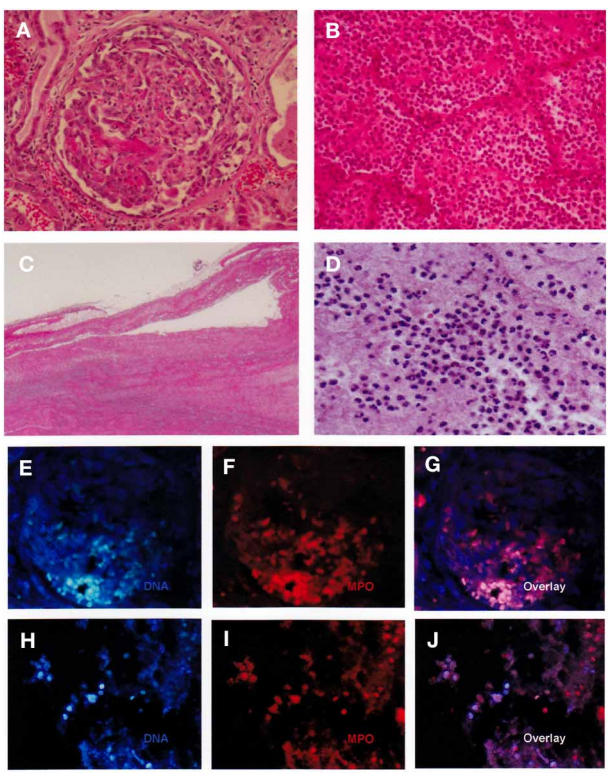

FIGURE 1 | Autopsy findings. (A) Crescentic necrotizing glomerulonephritis. **(B)** Alveolar hemorrhage with neutrophil infiltration. **(C, D)** DVT: neutrophils were abundant in the thrombus. **(E–G)** NETs in the glomerulus. Blue: DNA stained by DAPI. Red: MPO. NETs were present in the crescent. **(H–J)** NETs in the thrombus. The detection of NETs was performed similar to the renal specimens. Original magnification: ×40 **(C)**, ×200 **(B)**, × 400 **(A, D, E–J)**.

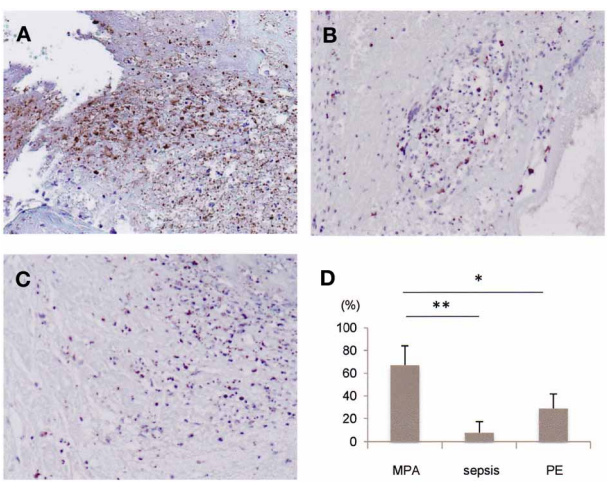

FIGURE 2 | Comparison of NETs in thrombi derived from patients with diverse diseases. In order to detect citrullinated histones in thrombi from patients with MPA **(A)** and other diseases [**B**: bacterial sepsis, **C**: post-operative pulmonary embolism (PE)], immunohistochemistry was performed using anti-citrullinated H3 antibody. Original magnification: ×200 **(A–C)**. **(D)** Comparison on the amount of NETs among thrombi derived from patients with MPA (present case), bacterial sepsis, and post-operative PE. $*p < 0.05$, $**p < 0.01$.

degree of histone-citrullination in the thrombus. Results showed that extensive histone-citrullination was observed in the thrombus of the MPA patient (**Figure 2A**). When compared to other thrombi not associated with MPA, namely, the thrombi from a patient who died of bacterial sepsis (**Figure 2B**) and from one who died of post-operative pulmonary embolism (**Figure 2C**), the area of histone-citrullination was larger in the MPA patient. In order to quantify the degree, five photographs under high power view (×400) were taken at random. The area of citrullinated H3 was quantified by Image J software and then standardized by the numbers of neutrophils counted in the serial sections with hematoxylin and eosin staining. Mann-Whitney *U*-test was employed for statistical analysis. As shown in **Figure 2D**, the amount of NETs was significantly greater in the MPA patient in comparison with other thrombi unrelated to MPA. These findings suggest that the thrombus in the MPA patient contains abundant NETs, and thrombosis is certainly associated with MPA via NETs.

DVT can lead to fatal pulmonary embolism. However, the administration of anti-coagulants to patients with MPA should be discreet because they are prone to pulmonary hemorrhage. Based on the understanding of the role of NETs in the pathogenesis of thrombosis in MPA, active regulation of NETs could be a novel therapeutic strategy. It is known that peptidylarginine deiminase 4 (PAD4), which citrullinates histones, and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which generates reactive oxygen species, are essential for NETs formation (Li et al., 2010; Remijsen et al., 2011). Therefore, inhibitors of these enzymes are regarded as possible candidates for active regulation of NETs. Actually, an NADPH oxidase inhibitor ameliorated the influenza A virus-induced lung inflammation in which excessive NETs were involved (Vlahos et al., 2011). Additionally, inhibitors of PAD4 and NADPH oxidase may be effective against MPA itself because NETs are involved not only in the pathogenesis of thrombosis, but also in the production of MPO-ANCA (Nakazawa et al., 2012; Ray, 2012). The inhibition of NETosis is promising for the patients with MPO-AAV.

CONCLUDING REMARKS

The quick initiation of treatment is important against MPA especially with a fulminant clinical course. The present case suggests the possibility that thrombosis and glomerulonephritis could be associated with NETosis, and that NETs could be a therapeutic target in MPA patients.

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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.

Received: 04 September 2012; accepted: 20 October 2012; published online: 12 November 2012.

Citation: Nakazawa D, Tomaru U, Yamamoto C, Jodo S and Ishizu A (2012) Abundant neutrophil extracellular traps in thrombus of patient with microscopic polyangiitis. Front. Immun. ***3****:333. doi: 10.3389/fimmu.2012.00333*

This article was submitted to Frontiers in Molecular Innate Immunity, a specialty of Frontiers in Immunology.