Published in final edited form as:

Cell Microbiol. 2016 October ; 18(10): 1349–1357. doi:10.1111/cmi.12652.

Point-Counterpoint: Pondering Neutrophil Extracellular Traps (NETs) with healthy skepticism

William M. Nauseef¹ and Paul Kubes²

¹Inflammation Program and Department of Internal Medicine, Roy J. and Lucille A. Carver College of Medicine, University of Iowa, and Veterans Administration Medical Center, Iowa City, IA 52240

²Department of Physiology and Pharmacology, Immunology Research Group, Snyder Institute for Chronic Diseases, University of Calgary, Calgary, AB T2N 4N1, Canada

Abstract

The authors engage in a dialogue that evaluates critically the state of the study of Neutrophil Extracellular Traps (NETs), a phenomenon currently the object of considerable interest, with the goal of identifying those aspects that merit clarification in order to assign the process its proper place in our current understanding of cell biology. Since the seminal observations in the Zychlinsky laboratory that described the extrusion of filaments of nuclear DNA associated with histones and granule proteins from neutrophils stimulated *in vitro*, many investigators have examined the phenomenon of NET formation in numerous and diverse settings. However, an overview of work in this rapidly growing field prompts several fundamental questions about NETs, both those created *in vitro* and those found *in vivo*, including their precise composition, the mechanisms by which they arise, their clinical relevance, and the interrelationship of those observed *in vitro* and *in vivo*. In this discussion, the authors challenge interpretation of data from some experimental settings and provide recommendations for specific studies that would address the concerns raised, improve understanding of the biological relevance of NETs, and strengthen the field.

Preamble

Our intention in the conversation to follow is to evaluate critically the state of the study of Neutrophil Extracellular Traps (NETs), a phenomenon currently the object of considerable interest in order to identify aspects that merit clarification and thereby to assign the process its proper place in our current understanding of cell biology. To set the starting point for this dialogue, we need to introduce both the subject and the discussants.

NETs are lattice-like structures composed of nuclear DNA, histones, and at least 24 associated proteins (Urban *et al.*), including neutrophil granule proteins [typically

Corresponding author information: Dr. William M. Nauseef, Inflammation Program and Department of Medicine, Roy J. and Lucille A. Carver College of Medicine, University of Iowa, D160 MTF, 2501 Crosspark Road, Coralville, IA 52241 (USA), Tel. +1 319 335 4278, Fax +1 319 335 4194, william-nauseef@uiowa.edu; Dr. Paul Kubes, Department of Physiology and Pharmacology, Immunology Research Group, Snyder Institute for Chronic Diseases, University of Calgary, Calgary, AB T2N 4N1, Canada, pkubes@ucalgary.ca.

myeloperoxidase (MPO) and neutrophil elastase (NE) but also lactoferrin and cathepsin G] that form when neutrophils undergo cytolysis after exposure *in vitro* to a variety of agonists (Brinkmann *et al.*). In most experimental settings, NET formation requires a functional NADPH oxidase to produce oxidants, which promote release of NE. Chromatin decondensation during NET formation requires histone citrullination mediated by peptidylarginine deaminase 4 (PAD4) as a prerequisite for DNA release (Neeli *et al.*, Wang *et al.*). Although lysis of neutrophils occurs during NET formation under most circumstances, neutrophils *in vivo* can release nuclear DNA and maintain structural integrity in a process referred to as “vital NETosis” (Yipp *et al.*, Yipp *et al.*). In addition, neutrophils primed with granulocyte-macrophage colony stimulating factor (GM-CSF) and subsequently stimulated with Toll-like receptor 4 (TLR4) agonists or C5a release NETs composed of mitochondrial, not nuclear, DNA and do so without undergoing lysis (Yousefi *et al.*).

With respect to the discussants and in the interest of full disclosure, we share our individual perspectives. As someone involved in the study of human neutrophils for nearly four decades, **WMN** interprets the presence of DNA aggregated with cationic granule proteins as the fortuitous fate of cellular contents released when neutrophils undergo necrosis, either as an accidental or programmed event. Published studies do not convince **WMN** that the generation of these aggregates after neutrophil lysis represents a form of programmed cell death, as currently defined (Galluzzi *et al.*), nor is the chance trapping of microbes in DNA-neutrophil granule meshwork a host defense strategy. Circulating DNA in predisposed individuals contributes to autoimmunity, but the link to circulating NETs seems tenuous.

On the other hand **PK** who has been studying neutrophils *in vivo* for close to 30 years is convinced that the neutrophil does not chase after bacteria, many of which move 10 times the speed of a crawling neutrophil, but rather lay traps to help ensnare pathogens. Nowhere is this more important than in the vasculature where neutrophils lack the ability to catch bacteria out of the mainstream of blood and as such release NETs to catch these invaders. **PK** does have some reservations about the use of extracellular DNA as the exclusive marker of NETs, particularly in the context of sterile injury where a large necrotic component exists and in infections where the bacteria have potent lytic molecules. In addition, discovery of the mechanisms that lead to NET release when using phorbol myristate acetate (PMA), should also be validated using more biologically relevant stimuli.

However, we agree on two critical points. First, we accept without question the observed phenomena reported in the literature by our respected colleagues but rather challenge, in some settings, the interpretation of the data. Second, we share the perspective that healthy skepticism inspires critical and rigorous examination of the data on which theories are based, refines appreciation of nuances, and often strengthens the field. This scientific rigor has always led to better understanding of biologic processes; helping us to accept the existence of DNA and evolution, HIV, dendritic cells and antigen presentation, nitric oxide as well as suppressor T cells (now known as T regulatory cells). All of these discoveries were initially challenged, some severely, all are now accepted as real entities, and all play important roles in biology. However all had to go through proper rigorous testing and not blind faith to be fully accepted by the scientific community. Through this polarized review we hope to bring to the fore questions about NETs that require further research.

1. What constitutes a NET?

WMN

The Zychlinsky lab identified filamentous aggregates of nuclear chromatin and granule proteins after neutrophils were treated with phorbol myristate acetate (PMA) and named the novel structures NETs (Brinkmann *et al.*). Since then, the presence of DNA, histones, and cationic granule proteins [typically myeloperoxidase (MPO) and neutrophil elastase (NE)] has served as the operational definition of NETs. However, in some settings, exceptions commonly occur, when the NADPH oxidase, granule proteins, citrullination, or histones are not involved (Yipp *et al.*, Masuda *et al.*, Sorensen *et al.*). For example, peptidylarginine deiminase 4 (PAD4), the enzyme that mediates histone citrullination, is essential for NET formation in murine models of Group A streptococcal infection (Li *et al.*) but dispensable in influenza infections (Hemmers *et al.*). DNA, the only component apparently always present in NETs, need not arise from NETs. For example, in a recent report on responses of macrophages to sterile hepatic injury, Wang and Kubes describe the deposition of DNA released from damaged cells whose nuclei have been nibbled by recruited peritoneal macrophages (Wang *et al.*). The authors underscore that these structures lack the neutrophil proteases associated with NETs, but what is the relationship of these and similar structures to what others describe as granule protein-free NETs?

The remarkable variability and diversity of NET formation in different tissue settings suggests either a biological process with an extraordinary complexity of modulation, for example as typifies receptor-dependent signaling, or a nonspecific and somewhat stochastic event accompanying neutrophil death in different contexts. For example, NETs from neutrophils that originate in the oral cavity form independent of the NADPH oxidase, neutral serine proteases and $\beta 2$ integrins and are resistant to DNase (Mohanty *et al.*). Are these NETs or something else?

For uniformity in investigation of phenomena, firm definitions need to be established. Morphology alone is subjective and non-specific and alone cannot be used. For example, the appearance of fibrin clots in scanning electron microscopy mimics that of NETs and both can be disrupted by DNase-I treatment (Krautgartner *et al.*). Fixation methods using ethanol or acetone can produce morphologic changes that would be defined as NETs (Masuda *et al.*), and entities defined by morphologic criteria cannot be quantified.

These challenges are not unique to the study of NETs and arise at the dawn of examination of any phenomenon. For example, ambiguities in identifying different types of cell death compelled the Nomenclature Committee for Cell Death to establish biochemical criteria by which to classify specific forms of cell death (Galluzzi *et al.*). Investigators studying NETs would benefit by establishing similar criteria for their field.

PK

It is clear that there are at least two if not three mechanisms of NET production and they do not all lead to the demise of the neutrophil, causing tremendous confusion in the field. Moreover, not all neutrophils are created equal and while all neutrophils have the capacity to lyse and release their DNA labelled with proteases and other components in response to

PMA, only about 25% of neutrophils release their DNA content in response to for example *S.aureus*. Whether this reflects the age of the NET producing neutrophil or an entirely separate lineage of neutrophils also remains unknown. In line with this, it was shown that senescent neutrophils have a higher potential to release NETs (Zhang *et al.*). Most studies that show examples of NETs show images of DNA with nearby neutrophils. Whether there are lysed neutrophils not visible in these images or whether the adjacent neutrophils are the source of the NETs remains unclear as almost always still images rather than videos of the process are provided. Fuchs and colleagues were one of the few to show a lysing neutrophil producing a NET in response to PMA (Fuchs *et al.*).

The PMA-induced NET production does require oxidants and elastase release (Fuchs *et al.*), while the non-lytic NET production is not as dependent on oxidants but also makes use of elastase (Kolaczowska *et al.*). The latter may require a vesicular component (Pilschek *et al.*) However because of the inability to transfect primary neutrophils, the cell biology of this vital netosis mechanism is woefully lacking. Moreover only one-quarter of neutrophils make NETs and there are no good markers to identify which neutrophil should be tracked in a field of view. Clearly, the cell biology delineating exact mechanisms of formation of these DNA-containing vesicles is clearly warranted and as such requires skepticism until this issue can be resolved. There are commonalities to the formation of NETs via lysing and non-lysing mechanisms including the importance of PAD4 for hypercitrullination of histones, the need for elastase in almost all experimental systems, and the presence of histones distinguishing NETs from fibrin or bacterial DNA. However, there are exceptions even to these rules.

It is worth noting that the one exception to the presence of histones in NETs is when DNA is released from mitochondria. Indeed, one group has shown that neutrophils can release DNA from mitochondria in response to GM-CSF and subsequent short-term TLR4 or C5a receptor stimulation, which does not lead to death of the neutrophils. These NETs lack histones and as such are more difficult to identify as true NETs, particularly in infections where bacteria release DNA, but PCR can be used to delineate the source (Yousefi *et al.*). In addition, eosinophils, mast cells, macrophage, basophils and even endothelium have been postulated to make extracellular traps (reviewed elsewhere), however it is PK's contention that the absence of several proteins critical for linking the nucleoskeleton to the cytoskeleton (Olins *et al.*) that renders neutrophils nearly uniquely susceptible to NET production and that much caution is needed when ascribing nuclear extracellular trap production to cells that lack these structural defects. As mentioned above, macrophage will nibble at dead nuclei giving the appearance of NETs, when in fact the source is simply dismantling of necrotic cells and can occur in the absence of neutrophils (Wang *et al.*). If indeed there are 3 ways in which NETs can be produced, it would help the field to have a clear understanding of which pathway is being used.

2. What distinguishes a NET from the debris remaining after a neutrophil has undergone cytolysis due to primary or secondary necrosis?

WMN

This is a corollary of point #1. Frequently, reports identify DNA and MPO in tissues as evidence for the presence of NETs in *in vivo* settings. However, how can the presence of DNA and MPO serve as unambiguous surrogate markers for NETs when both circulate in plasma, especially in pathologic settings such as trauma, infection, and clinical exacerbations of autoimmune diseases? MPO is present in plasma of healthy individuals; in a survey of ~25,000 subjects in the United Kingdom, plasma concentrations of MPO ranged from 454 to 951 pM (Meuwese *et al.*). Levels are increased in patients with atherosclerotic heart disease (Nicholls *et al.*) and in other inflammatory disorders, acute and chronic (Davies *et al.*). Soluble MPO normally circulates in plasma in an inactive complex with ceruloplasmin and increases when neutrophils are activated (Sokolov *et al.*, Chapman *et al.*).

Plasma of healthy humans contains circulating cell-free DNA, which predominantly originates from leukocytes (Chan *et al.*, Sun *et al.*). Levels increase during pregnancy, in the presence of tumors, and after trauma (Chan *et al.*, Sun *et al.*, Snyder *et al.*), with the increases coming from non-leukocyte tissues. Acute inflammatory pathologic states, such as myocardial and cerebral infarction, and active autoimmune diseases can increase DNA levels to > 500 ng/ml, well above the level in normal plasma (~36 ng/ml) (Snyder *et al.*). Mapping the nucleosome occupancy by transcription factor footprinting can identify the tissue source of the cell-free DNA, as is currently being done to establish the origin of tumors in selected patients and to monitor early signs of organ transplant rejection (Masuda *et al.*, Snyder *et al.*).

Taken together, these data undermine the supposition that the coincident presence of DNA and neutrophil granule proteins is sufficient and unambiguous evidence of NET formation. In fact, some studies suggest that circulating DNA present during sepsis reflects tissue damage and not NETs. In a study using the cecal ligation and puncture model of infection, the authors identified circulating citrullinated cell-free DNA associated with histone H3 in septic but not control animals (Hamaguchi *et al.*). However, neutrophils isolated from septic mice did not have increased citrullination of DNA, and depletion of neutrophils did not significantly alter the amount of circulating DNA, prompting the authors to conclude that the cell-free DNA circulating in septic mice was not of neutrophil origin and thus not evidence for NETs in that experimental setting.

Identification of the tissue from which circulating cell-free DNA originates in inflammatory settings may clarify the ambiguity, although the majority of free DNA in normal plasma arises from leukocytes and only evidence that the source was other than from neutrophils would be informative.

PK

There is no question there are conditions in which cell free DNA can be found entirely independent of NETs in plasma. Moreover, there is no question that NETs remain a difficult

structure to track. As such the Kubes lab and others have always measured the colocalization of 3 separate molecules in an attempt to identify NETs. First, one should label DNA as the backbone to ensure that the other molecules measured are indeed associated with DNA. Sytox green intercalates into DNA and so serves this function very well and does not enter live cells thereby not staining intracellular DNA. However, this observation alone means very little. Indeed Kolaczowska *et al.* (Kolaczowska *et al.*) have nicely demonstrated large areas of DNA that turned out to be dead or dying cells that were sufficiently permeabilized to allow for penetrance of Sytox green. Histones are a second component that needs to be measured because many bacteria will release their own DNA, thought to help with biofilm production and this free floating DNA especially in situations like cecal ligation and puncture, almost certainly drowns out the lesser signal that might be expected from NETs. In addition, DNA from dead and dying cells could further complicate these results. Finally, neutrophil elastase attached to chromatin does suggest the source of the DNA is neutrophils and emphasizes that this is not just free DNA. However, the elastase and DNA need to be shown to be co-localized and not simply detected in plasma. Intriguingly, elastase is rapidly inactivated by plasma anti-proteases whereas DNA associated elastase appears to retain its proteolytic activity (Belorgey *et al.*). As such, elastase can be used as a third marker of NETs and its proteolytic activity can be detected *in vivo* in close proximity to NETs (Kolaczowska *et al.*). However, without being able to co-localize these molecules, the jury is out whether you are or are not studying NETs.

Another important point to keep in mind is that DNA has a fibronectin binding site. In tissues, NETs have been observed to be immobilized to extracellular matrix (Yipp *et al.*). NETs could also be attached to the vessel wall. In a study from 1997, Ward and colleagues reported that histones avidly bound von Willebrand factor in a receptor ligand pair but the authors concluded that “While the vWF-histone interaction has no conceivable physiological role....”. Perhaps now there is a reason for this interaction. In fact, imaging NETs *in vivo* in the lab of **PK** has revealed immobilized NETs on the surface of endothelium, an interaction that could be disrupted by blocking or shedding vWF (Kolaczowska *et al.*). As such it is quite likely that NETs can be formed without much free-floating DNA. Therefore, the measure of free DNA does not prove NET production but just as importantly, it also does not disprove the production of NETs. **PK** therefore agrees that much more rigorous means are necessary to absolutely confirm the production of NETs *in vivo*, particularly in the setting of sterile necrotic injury as well as in the setting of infections where the pathogen is capable of producing lytic compounds that lead to necrosis and cell free DNA.

PK would agree that the approaches to measure NETs *in vivo* are not optimal. Measuring free DNA, depleting neutrophils and then arguing the existence of NETs or the lack of NETs is not accurate. Ideally a bi-specific antibody that would detect DNA bound to a protein like elastase simultaneously would be a very useful tool.

3. Do NETs represent a specific form of regulated cell death?

WMN

Cell death is currently defined as a state of irreversible permeabilization of the plasma membrane and complete fragmentation. Cell death can be accidental, as occurs after

physical, chemical, or mechanical assault, or regulated (aka programmed) (Galluzzi *et al.*). In the former, death is ~immediate, does not involve specialized cellular components or signaling pathways, and thus resists inhibition by pharmacologic or genetic intervention. Tissue damage and cell death from trauma or burns serve as clinical examples of accidental cell death. Regulated or programmed cell death, on the other hand, takes time to develop after exposure to initiating stimuli, with time needed to initiate biochemical signals that will subsequently culminate in engagement of irreversible pathways that execute cell death. Pathways driving programmed cell death can be inhibited pharmacologically or genetically.

Although exposure of neutrophils to PMA or *S.aureus*-derived toxins promotes NET formation *in vitro*, identical structures are generated by electroporation (800V at 25 μ F) of freshly isolated neutrophils (Malachowa *et al.*, Malachowa *et al.*), suggesting that creation of NETs *in vitro* more likely represents accidental rather than programmed cell death.

One form of programmed cell death, necroptosis, can culminate in cytolysis (Linkermann *et al.*, Wallach *et al.*). Necroptosis depends on activities of receptor interacting protein kinases (RIPK) 1 and 3 and the executioner protein mixed lineage kinase like protein (MLKL). The genetic absence of critical components in the pathway or pharmacologic inhibition of RIPK1 with necrostatin-1 or of MLKL with necrosulfonamide blocks necroptosis. Two studies have examined the role of the RIPK-MLKL pathway in PMA-triggered NET formation and observed opposite results. One implicates RIPK1 and MLKL; necrostatin-1 inhibits NET formation and neutrophils from RIPK3^{-/-} mice fail to form NETs (Desai *et al.*). The opposite results, namely no role for necroptosis, were observed in the other study (Amini *et al.*). As noted in the editorial accompanying these jointly published reports (Naccache *et al.*), two differences in the experimental design may provide clues to the mechanistic basis for the conflicting results. The negative report studied neutrophils stimulated in suspension and treated with PMA for only 15 minutes (Amini *et al.*), whereas the positive study used neutrophils adhered to glass and treated with PMA for 120 minutes (Desai *et al.*). Although the verdict on the relationship between NET formation and RIPK-MLKL pathway remains uncertain at this time, it is noteworthy that the positive study reported that urate crystals, the provocative agent in gout, also trigger neutrophil cytolysis via necroptosis, a finding confirmed independently by another group (Mulay *et al.*).

PK

What most cells that live long lives can do is upregulate cell death pathways through protein synthesis. By contrast, platelets and red blood cells die by pathways that do not require protein synthesis. In fact these cells have most molecules presynthesized and stored. The neutrophil, which lives the shortest half-life of all three cell types, also pre-synthesizes many of its proteins. As such it would not be inconceivable that neutrophils could also pre-plan their death. Indeed, phosphatidylserine (PS) can be rapidly mobilized to the outer leaflet of the plasma membrane of neutrophils and lead to their demise via phagocytosis or efferocytosis, a fate not dissimilar to that of platelets and red blood cells. However, as is typically the case for most events in neutrophils, PS expression by stimulated neutrophils is more complicated. PS can be expressed on the surface of neutrophils by agonists such as fMLF but then rapidly internalized (Frasch *et al.*). WMN has seen the same after feeding

neutrophils *S.aureus* (unpublished observation). Thus, the transient PS expression by neutrophils bears no relation to apoptosis whatsoever. More recent work has begun to explore the signaling pathways that could block rapid NET production. Indeed, Hakkim *et al.* described a role for Raf-MEK-ERK pathway of NET production (Hakkim *et al.*) and while this suggests a role for specific kinases, this pathway was upstream of oxidant production and as such did not block the signaling molecules directly upstream of NET release. More recently, Van Avondt and colleagues (Van Avondt *et al.*) demonstrated that the inhibition of Signal Inhibitory Receptor on Leukocytes-1 (SIRL-1) could prevent NET production without affecting oxidant production, for the first time highlighting a NET-specific signaling pathway.

A major issue that is plaguing the field is the signaling pathways upstream of NETosis leading to cell death, and the pathways upstream of vital NETosis which does not necessarily lead to neutrophil cell death. The two pathways may partly reflect differences between *in vitro* and *in vivo* observations. This is discussed below.

Although there is no question that severe manipulation of cells through, for example, physical shearing or extraordinary electroporation can lead to the release of nuclear material extracellularly, as was shown by Malachowa *et al.* (Malachowa *et al.*), these perturbations would release all material large enough to leak through pores. It would have been interesting in this study to look systematically whether electroporation leading to DNA release through fragmented plasma membrane also had elastase, hypercitrullinated histones and MPO bound to the DNA backbone. It is possible that all these molecules did attach to DNA *in vitro* following electroporation. However one could make the same argument that necroptosis and electroporation have similar outcomes. Testing to see whether similar molecular mechanisms underlie NETs induced by *S.aureus* versus electroporation would further strengthen the case that NETs induced by *S.aureus* is accidental. However the above mentioned histone hypercitrullination, a need for elastase, a need for certain signalling molecules are unlikely to be required when one blows holes in membranes using electroporation. Concluding that electroporation is similar to how *S.aureus* induces NET release requires more detailed examination.

4. What, if any, relationship exists between NETs created *in vitro* and observed *in vivo*?

WMN

Nonphysiologic agonists such as PMA or artefactual manipulations can cause neutrophil cytolysis and create entities with the surrogate markers for NETs, as illustrated above in the example of electroporation. However, neutrophils can release DNA without cell lysis or death, a process referred to as “vital NETosis” (Yipp *et al.*). Live neutrophil NET formation must involve distinct molecular mechanisms that preserve the integrity of the plasma membrane and the functional capacity of neutrophils. Furthermore, many of the features of *in vivo* NET formation differ strikingly from those associated with *in vitro* neutrophil cytolysis, strongly suggesting that the mechanisms underlying each differ.

The requirements for *in vivo* NET formation and live cell DNA extrusion need identification in order to establish an informed consensus on the biologically relevant agonists to serve as standards for *in vitro* NET formation. At that point, careful scrutiny of the proposed requirements, be it activity of the NADPH oxidase, MPO, NE, or other cationic granule proteins, can be examined rigorously in the *in vitro* setting, where a more reductionist experimental approach can be pursued, and the relationship between structures created *in vitro* and *in vivo* be established.

PK

There is no question that with PMA *in vitro* one sees many neutrophils lyse. *In vivo* to date we have primarily seen NET production independent of cell death. Indeed, Yipp *et al.* (Yipp *et al.*), demonstrated vital NETosis reporting that after releasing their nuclear material through vesicular transport, these anuclear neutrophils could continue to crawl and phagocytose bacteria. How could a neutrophil survive without a nucleus? This observation is reminiscent of earlier work (Korchak *et al.*, Roos *et al.*, Malawista *et al.*, Malawista *et al.*) in which centrifugation of intact neutrophils through Ficoll-Hypaque gradients containing cytochalasin B created anuclear cells called cytoplasts. Cytoplasts from resting neutrophils are anuclear and contained fewer granules (from 10,200 granules/100 resting neutrophils to 21) and mitochondria (from 524 mitochondria/100 resting neutrophils to 6) than do normal intact neutrophils (Korchak *et al.*) but retained the capacity to perform many of their critical functions including phagocytosis, NADPH oxidase activation, crawling, chemotaxis, and killing of ingested microbes (Malawista *et al.*). Clearly, many functions can be performed by neutrophils independent of their nucleus and protein synthesis, and recently it has been shown that NETosis was a form of death independent of protein synthesis (Sollberger *et al.*). In addition, Malawista and colleagues showed that the anuclear neutrophils could be frozen and then revived 30 days later from a deep freeze to resume normal function.

What remains unknown is what happens to the cytoplasts. Indeed, how neutrophils die after they make NETs is not entirely clear in vital NETosis but presumably they get taken up by macrophage due to increased expression of PS as one of a number of eat me signals. Actually draining pus from human abscesses reveals ghost cells that have no nucleus yet still appear to have an intact membrane raising the question, what are these cells (Yipp *et al.*). Of course these could be any type of cells not necessarily neutrophils, but it is strange that such anuclear cells that have clearly failed to fragment their membranes and not been phagocytosed should be present in this type of infectious nidus. One caution worth mentioning is that while *in vitro* one can see a cell explode, this may not be as easy to capture *in vivo* and because the remnants may not be retained in the field of view, it could be incorrectly concluded that neutrophils do not die during NET release.

5. Is the presence of platelets with or without thrombi a necessary feature for *in vivo* NET formation?

WMN

In vivo formation of NETs depends heavily on the presence of platelets as a critical component (Yipp *et al.*). Platelets interact with neutrophils and other innate immune cells in

a wide range of clinical settings, including infection, inflammation, and autoimmune diseases (Kral *et al.*, Maugeri *et al.*). In addition, circulating DNA released from cells associates in a receptor-dependent fashion with the plasma membranes of leukocytes (Bennett *et al.*) and platelets (Clejan *et al.*), in the latter case triggering platelet aggregation and activation (Fiedel *et al.*). To me, this process accurately describes events currently considered *in vivo* NET formation. Consistent with that interpretation, TLR4-activated platelets, as would occur during endotoxemia or some bacterial infections, bind neutrophils and trigger transfusion lung injury (TRALI) and the creation of structures called NETs (Caudrillier *et al.*). Furthermore, fragments of neutrophils containing *both* nuclei and free granules have been identified in pulmonary capillaries during the generalized Shwartzman reaction (Horn *et al.*).

Are these structures and processes manifestations of the complex and synergistic interactions between platelets and circulating immune cells and not exclusively linked to the release of neutrophil DNA? Is it possible that the structures observed *in vivo* and now called NETs represent the same or related structures to those reported previously in the setting of leukemia, when malignant myeloid cells aggregate together and with platelets to produce thrombi in pulmonary and CNS vasculature, sometimes with devastating consequences (McKee *et al.*). Identification of an essential role for platelets in the *in vivo* setting would be an important and clinically relevant advance in understanding and would underscore the synergistic interactions among circulating cells during acute inflammation.

PK

Since the *in vitro* observation that platelets bound neutrophils in a flow chamber following TLR4 stimulation leading to NET formation (Clark *et al.*), a series of studies from a number of groups have suggested a role for platelets in NET production *in vivo* (Caudrillier *et al.*). Platelet-neutrophil interactions have been documented to lead to NET production in sepsis, TRALI, deep vein thrombosis and other conditions. NETs can also contribute to thrombosis as NET-attached serine proteases, including neutrophil elastase, enhance coagulation in a process involving local proteolysis of the coagulation suppressor tissue factor pathway inhibitor (Massberg *et al.*). A relationship between platelets, neutrophils, NETs and cancer has also been suggested (Guglietta *et al.*). In fact, it is tempting to ask whether NETs can at all be formed in the absence of platelets *in vivo*. A major challenge is to deplete all platelets in model organisms, inasmuch as 5–10% of residual platelet numbers may be sufficient to induce NETs. These types of platelet-depletion experiments have been sufficient to reveal a need for platelets in LPS-dependent and *E.coli* dependent NET formation (McDonald *et al.*). We have shown that in response to LPS, TLR4 on platelets and neutrophils is necessary to induce NETs. Clearly, platelets may be an important checkpoint activator for NET production in response to Gram negative bacteria. But the jury is out on whether platelets are necessary for NET production with *S.aureus*. We have not been able to reduce NET production in platelet-depleted mice exposed to *S.aureus* using platelet depleting techniques. As mentioned this could be due to less than 100% efficiency in platelet depletion (unpublished results). Indeed, the few residual platelets could be mediating NETs. However, *S.aureus* could be capable of stimulating NET production independent of platelets. *In vitro* isolation of neutrophils without any contaminating platelets is also not trivial and so *in vitro*

data need to be interpreted cautiously in terms of the importance of platelets. Nevertheless, Pilszczek and colleagues did report that human neutrophils produced NETs *in vitro* in response to *S.aureus* (Pilszczek *et al.*), presumably in the absence of platelets. Nevertheless, we did observe better NET production with *S.aureus* if platelets are present (unpublished observations).

6. What, if any, is the biological relevance of NETs?

WMN

Since the original observations of NET formation *in vitro*, NET formation has been linked with an extraordinarily broad range of biological events – host defense, response to iron chelators, tissue damage, autoimmunity, and even metabolic diseases such as diabetes. Recent reviews list as many as 22 different pathological conditions, including a variety of infections, inflammatory bowel disease, cystic fibrosis, chronic otitis media, pancreatitis, and atherosclerosis, all linked to NET formation (Remijsen *et al.*). In some cases, the extrapolations seem, from my perspective of neutrophil biology, to be far-fetched. For example, the suggestion that patients with CGD improve clinically after transplantation because their newly acquired neutrophils now can form NETs ignores a much more profound improvement after transplantation, namely acquisition of neutrophils that can generate oxidants in phagosomes. Focusing selectively on the antimicrobial activities within neutrophil phagosomes, the site of most, if not all, neutrophil-mediated antibacterial action, reconstitution of H₂O₂-generating activity corrects the defect in killing ingested *S.aureus* seen in CGD neutrophils (Gerber *et al.*).

Naïve neutrophils kill *S.aureus* primarily within phagosomes and only a fraction of neutrophils in a population (10 to ~22%) undergo NET formation (Fuchs *et al.*). In light of these data, one would not necessarily anticipate a dramatic increase in the frequency or severity of infection in the absence of NETs. That expectation is met by recently published observations of humans, not knock-out mice, whose neutrophils are unable to generate NETs. Patients with Papillon-Lefèvre Syndrome lack all neutrophil serine proteases and their neutrophils fail to form NETs when stimulated with PMA (Sorensen *et al.*, Roberts *et al.*). Although affected patients suffer from severe periodontal disease, none experiences more frequent or more severe systemic infection. At first blush, the absence of serious systemic infection in this patient population would seem strong evidence that NET formation, as assessed *in vitro*, contributes little to host defense. I think that such a judgement would ignore the possibility that redundant systems intervene and thereby minimize the impact of the inability to form NETs. However, the data are consistent with the observation that a relatively small fraction of neutrophils form NETs *in vitro* (Fuchs *et al.*). One would anticipate that large scale NET formation and the associated release of cytoplasmic contents of neutrophils that can act as danger associated molecular patterns would promote excessive inflammation rather than augment host defense. Furthermore, employment of wholesale cytolysis as a host defense strategy seems antithetical to behavior typical for neutrophils. In a thoughtful commentary (Malachowa *et al.*), Malachowa *et al.* review the many mechanisms that neutrophils utilize to keep their potent and toxic arsenal in

check and to avoid unintended activation. In that light, they reason, cytolytic NET formation appears inconsistent with the overall behavior of neutrophils.

Taken together, these data and considerations undermine the oft-stated claim that NET formation, as judged by *in vitro* provocation with PMA, is a major component of neutrophil-mediated host defense.

PK

Like anything in science, a new discovery often has a large wave of followers and a large group of nay-sayers. Through rigorous scientific interrogation the significance of a particular biologic process eventually becomes clear. In the original study by Zychlinsky and colleagues the proposal was that NETs were formed to trap bacteria. This has now been shown *in vitro* and *in vivo* for many different pathogens. However, mice that do not make NETs have no phenotype unless challenged. But it is important to remember that mice are harbored in specific pathogen-free environments often in more pristine conditions than their investigators. Indeed, mice whose neutrophils lack oxidants, proteases, ligands for recruitment molecules like P-selectin or E-selectin (PSGL-1), or individual TLRs all fail to show any spontaneous signs of infection. Similarly, mice lacking molecules that do indeed cause NETs including MPO, elastase, NADPH oxidase and PAD4 do not manifest spontaneous infections especially in mice kept in a clean environment. However, following challenge in the laboratory with significant bacterial inoculums leads to phenotypes in a number of the aforementioned mouse mutants for some but not all pathogens. Therefore, a lack of spontaneous disease in a single knockout does not necessarily negate the importance of a particular pathway and may simply highlight the importance of the biologic process due to multiple redundant pathways. Clearly, there are multiple killing mechanisms exercised by immune cells and inhibition of a single pathway may simply not be sufficient *in vivo*.

Of course the skeptics are correct in questioning a detrimental role for NETs even in normal physiologic processes. For example, in normal wound healing, which has been optimized by millions of years of evolution, there is a need for platelets, neutrophils, monocytes and macrophages, and in their absence healing is delayed. It behooves us to find rationales to produce NETs in these normal biologic processes. In fact as far as infections are concerned, we recently proposed that NET formation may be important only in bacteremias that have become systemic reaching sufficiently high levels of inflammatory mediators to induce platelet stimulation and NET production (Yipp *et al.*). But neutrophils detect LPS at much lower concentrations than do platelets and as such the latter seems to function as a barometer ensuring that NETs are not produced anytime the neutrophil is activated. It may be that NETs are particularly important in certain types of infections. For example, if phagocytosis is not an option during large hyphae infection, NET release may be an alternative backup plan to help eradicate infection. Indeed, neutrophils will release more NETs as the size of a pathogen increases (Branzk *et al.*). Simultaneously, under these conditions, releasing DAMPs including NETs may be critical to allow for neutrophil swarming and eradication of a multicellular organism.

However, as a last thought, and to give credibility to the field, surely there are situations where NETs are not formed and more publications highlighting these observations are

necessary. In minor localized trauma for example, NET production is very difficult to see despite the presence of platelets and neutrophils (Kolaczowska *et al.*). Clearly the right stimuli are missing and it is these stimuli that will be so very important to identify.

Suggestions going forward

“Be not the first by whom the New are try’d, Nor yet the last to lay the Old aside”

[Alexander Pope’s *Essay on Criticism* from 1711]

- Use of PMA as the only agonist in a study should be stopped. Rather, use of PMA should be reserved to serve only as a positive control to ensure DNA reporters are working.
- Identification and definition of components of NETs *in vivo* are needed. Establishment of elements that unambiguously distinguish NETs from the remnants of necrotic cells is necessary.
- Identification of the tissue and cell source of DNA found in DNA-containing aggregates is critical. It should not be assumed that all DNA identified in tissue must be derived from neutrophils and therefore represents a NET.
- Understanding the mechanism of NET formation *in vivo* with emphasis on non-cytolytic versus cytolytic NET formation is needed. Distinction between vital and lytic NET formation and documentation that they both occur *in vivo* are absolutely essential. Understanding of the contribution of platelets to these processes is critical.
- Investigators in the field should establish, accept, and apply New nomenclature that identifies the variants of NETs *in vivo* and distinguishes them from *in vitro* phenomenon. Studying a disorder by isolating neutrophils from an animal challenged with a particular pathogen, inducing NETs *in vitro* with PMA, and then concluding that NET formation is important in the animal model does not, in our opinion, demonstrate with sufficient scientific rigor that a particular infectious agent would provoke NET formation during infection. We think that extrapolations such as this fuel more confusion and should be discontinued. If a pathogen cannot make NETs independent of PMA, we see no conceivable argument to continue to investigate the relationship between that pathogen and NET formation.
- Characterization of the clinical profiles of human disorders with deficiencies in NET production, independent of those with deficient oxidant production, would certainly go a long way to convincing us all that NETs are important.

Acknowledgments

The WMN lab is supported by National Institute of Health grants AI70958 and AI044642, a Merit Review award from the Veterans Affairs, and use of facilities at the Iowa City Department of Veterans Affairs Medical Center, Iowa City, IA. The PK lab is supported by Canadian Institute of Health Research and Alberta Innovates Health Solutions.

LITERATURE CITED

1. Amini P, Stojkov D, Wang X, Wicki S, Kaufmann T, Wong WW, et al. NET formation can occur independently of RIPK3 and MLKL signaling. *Eur J Immunol*. 2015
2. Belorgey D, Bieth JG. Effect of polynucleotides on the inhibition of neutrophil elastase by mucus proteinase inhibitor and alpha 1-proteinase inhibitor. *Biochemistry*. 1998; 37:16416–16422. [PubMed: 9819234]
3. Bennett RM, Gabor GT, Merritt MM. DNA binding to human leukocytes. Evidence for a receptor-mediated association, internalization, and degradation of DNA. *J Clin Invest*. 1985; 76:2182–2190. [PubMed: 3001145]
4. Branzk N, Lubojemska A, Hardison SE, Wang Q, Gutierrez MG, Brown GD, Papayannopoulos V. Neutrophils sense microbe size and selectively release neutrophil extracellular traps in response to large pathogens. *Nat Immunol*. 2014; 15:1017–1025. [PubMed: 25217981]
5. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, et al. Neutrophil extracellular traps kill bacteria. *Science*. 2004; 303:1532–1535. [PubMed: 15001782]
6. Brinkmann V, Zychlinsky A. Neutrophil extracellular traps: is immunity the second function of chromatin? *J Cell Biol*. 2012; 198:773–783. [PubMed: 22945932]
7. Caudrillier A, Kessenbrock K, Gilliss BM, Nguyen JX, Marques MB, Monestier M, et al. Platelets induce neutrophil extracellular traps in transfusion-related acute lung injury. *J Clin Invest*. 2012; 122:2661–2671. [PubMed: 22684106]
8. Chan AK, Chiu RW, Lo YM. Cell-free nucleic acids in plasma, serum and urine: a new tool in molecular diagnosis. *Ann Clin Biochem*. 2003; 40:122–130. [PubMed: 12662399]
9. Chapman AL, Mocatta TJ, Shiva S, Seidel A, Chen B, Khalilova I, et al. Ceruloplasmin is an endogenous inhibitor of myeloperoxidase. *J Biol Chem*. 2013; 288:6465–6477. [PubMed: 23306200]
10. Clark SR, Ma AC, Tavener SA, McDonald B, Goodarzi Z, Kelly MM, et al. Platelet TLR4 activates neutrophil extracellular traps to ensnare bacteria in septic blood. *Nat Med*. 2007; 13:463–469. [PubMed: 17384648]
11. Clejan L, Menahem H. Binding of deoxyribonucleic acid to the surface of human platelets. *Acta Haematol*. 1977; 58:84–88. [PubMed: 409023]
12. Davies MJ, Hawkins CL, Pattison DI, Rees MD. Mammalian heme peroxidases: from molecular mechanisms to health implications. *Antioxidants & Redox Signaling*. 2008; 10:1199–1234. [PubMed: 18331199]
13. Desai J, Vr SK, Mulay SR, Konrad L, Romoli S, Schauer C, et al. Neutrophil extracellular trap formation can involve RIPK1-RIPK3-MLKL signalling. *Eur J Immunol*. 2015
14. Fiedel BA, Schoenberger JS, Gewurz H. Modulation of platelet activation by native DNA. *J Immunol*. 1979; 123:2479–2483. [PubMed: 501081]
15. Frasch SC, Henson PM, Nagaosa K, Fessler MB, Borregaard N, Bratton DL. Phospholipid flip-flop and phospholipid scramblase 1 (PLSCR1) co-localize to uropod rafts in formylated met-leu-phe-stimulated neutrophils. *J Biol Chem*. 2004; 279:17625–17633. [PubMed: 14766753]
16. Fuchs TA, Abed U, Goosmann C, Hurwitz R, Schulze I, Wahn V, et al. Novel cell death program leads to neutrophil extracellular traps. *J Cell Biol*. 2007; 176:231–241. [PubMed: 17210947]
17. Galluzzi L, Bravo-San Pedro JM, Vitale I, Aaronson SA, Abrams JM, Adam D, et al. Essential versus accessory aspects of cell death: recommendations of the NCCD 2015. *Cell Death Differ*. 2015; 22:58–73. [PubMed: 25236395]

18. Gerber CE, Bruchelt G, Falk UB, Kimpfner A, Hauschild O, Kuci S, Bächli T. Reconstitution of bactericidal activity in chronic granulomatous disease cells by glucose-oxidase-containing liposomes. *Blood*. 2001; 98:3097–3105. [PubMed: 11698296]
19. Guglietta S, Chiavelli A, Zagato E, Krieg C, Gandini S, Ravenda PS, et al. Coagulation induced by C3aR-dependent NETosis drives protumorigenic neutrophils during small intestinal tumorigenesis. *Nature communications*. 2016; 7:11037.
20. Hakkim A, Fuchs TA, Martinez NE, Hess S, Prinz H, Zychlinsky A, Waldmann H. Activation of the Raf-MEK-ERK pathway is required for neutrophil extracellular trap formation. *Nat Chem Biol*. 2011; 7:75–77. [PubMed: 21170021]
21. Hamaguchi S, Akeda Y, Yamamoto N, Seki M, Yamamoto K, Oishi K, Tomono K. Origin of Circulating Free DNA in Sepsis: Analysis of the CLP Mouse Model. *Mediators Inflamm*. 2015; 2015:614518. [PubMed: 26273139]
22. Hemmers S, Teijaro JR, Arandjelovic S, Mowen KA. PAD4-mediated neutrophil extracellular trap formation is not required for immunity against influenza infection. *PLoS One*. 2011; 6:e22043. [PubMed: 21779371]
23. Horn RG, Collins RD. Fragmentation of granulocytes in pulmonary capillaries during development of the generalized Shwartzman reaction. *Lab Invest*. 1968; 19:451–459. [PubMed: 5698728]
24. Kolaczowska E, Jenne CN, Surewaard BG, Thanabalasuriar A, Lee WY, Sanz MJ, et al. Molecular mechanisms of NET formation and degradation revealed by intravital imaging in the liver vasculature. *Nature communications*. 2015; 6:6673.
25. Korchak HM, Roos D, Giedd KN, Wynkoop EM, Vienne K, Rutherford LE, et al. Granulocytes without degranulation: neutrophil function in granule-depleted cytoplasts. *Proc Natl Acad Sci U S A*. 1983; 80:4968–4972. [PubMed: 6308664]
26. Kral JB, Schrottmaier WC, Salzmann M, Assinger A. Platelet Interaction with Innate Immune Cells. *Transfus Med Hemother*. 2016; 43:78–88. [PubMed: 27226790]
27. Krautgartner WD, Klappacher M, Hannig M, Obermayer A, Hartl D, Marcos V, Vitkov L. Fibrin mimics neutrophil extracellular traps in SEM. *Ultrastruct Pathol*. 2010; 34:226–231. [PubMed: 20594043]
28. Li P, Li M, Lindberg MR, Kennett MJ, Xiong N, Wang Y. PAD4 is essential for antibacterial innate immunity mediated by neutrophil extracellular traps. *J Exp Med*. 2010; 207:1853–1862. [PubMed: 20733033]
29. Linkermann A, Green DR. Necroptosis. *N Engl J Med*. 2014; 370:455–465. [PubMed: 24476434]
30. Malachowa N, Kobayashi SD, Freedman B, Dorward DW, DeLeo FR. Staphylococcus aureus Leukotoxin GH Promotes Formation of Neutrophil Extracellular Traps. *J Immunol*. 2013; 191:6022–6029. [PubMed: 24190656]
31. Malachowa N, Kobayashi SD, Quinn MT, DeLeo FR. NET Confusion. *Front Immunol*. 2016; 7:1–10. [PubMed: 26834743]
32. Malawista SE, Van Blaricom G. Cytoplasts made from human blood polymorphonuclear leukocytes with or without heat: preservation of both motile function and respiratory burst oxidase activity. *Proc Natl Acad Sci U S A*. 1987; 84:454–458. [PubMed: 3025874]
33. Malawista SE, Van Blaricom G, Breitenstein MG. Cryopreservable neutrophil surrogates. Stored cytoplasts from human polymorphonuclear leukocytes retain chemotactic, phagocytic, and microbicidal function. *J Clin Invest*. 1989; 83:728–732. [PubMed: 2536406]
34. Massberg S, Grahl L, von Bruehl ML, Manukyan D, Pfeiler S, Goosmann C, et al. Reciprocal coupling of coagulation and innate immunity via neutrophil serine proteases. *Nat Med*. 2010; 16:887–896. [PubMed: 20676107]
35. Masuda S, Nakazawa D, Shida H, Miyoshi A, Kusunoki Y, Tomaru U, Ishizu A. NETosis markers: Quest for specific, objective, and quantitative markers. *Clin Chim Acta*. 2016; 459:89–93. [PubMed: 27259468]
36. Maugeri N, Rovere-Querini P, Manfredi AA. Disruption of a Regulatory Network Consisting of Neutrophils and Platelets Fosters Persisting Inflammation in Rheumatic Diseases. *Front Immunol*. 2016; 7:182. [PubMed: 27242789]

37. McDonald B, Urrutia R, Yipp BG, Jenne CN, Kubes P. Intravascular neutrophil extracellular traps capture bacteria from the bloodstream during sepsis. *Cell Host Microbe*. 2012; 12:324–333. [PubMed: 22980329]
38. McKee LC Jr, Collins RD. Intravascular leukocyte thrombi and aggregates as a cause of morbidity and mortality in leukemia. *Medicine (Baltimore)*. 1974; 53:463–478. [PubMed: 4530890]
39. Meuwese MC, Stroes ESG, Hazen SL, van Miert JN, Kuivenhoven JA, Schaub RG, et al. Serum myeloperoxidase levels are associated with the future risk of coronary artery disease in apparently healthy individuals. *J Amer Col Cardiol*. 2007; 50:159–165.
40. Mohanty T, Sjogren J, Kahn F, Abu-Humaidan AH, Fisker N, Assing K, et al. A novel mechanism for NETosis provides antimicrobial defense at the oral mucosa. *Blood*. 2015; 126:2128–2137. [PubMed: 26243777]
41. Mulay SR, Desai J, Kumar SV, Eberhard JN, Thomasova D, Romoli S, et al. Cytotoxicity of crystals involves RIPK3-MLKL-mediated necroptosis. *Nature communications*. 2016; 7:10274.
42. Naccache P, Fernandes MJ. Challenges in the characterization of neutrophil extracellular traps: the truth is in the details. *Eur J Immunol*. 2015
43. Neeli I, Khan SN, Radic M. Histone deimination as a response to inflammatory stimuli in neutrophils. *J Immunol*. 2008; 180:1895–1902. [PubMed: 18209087]
44. Nicholls SJ, Hazen SL. Myeloperoxidase, modified lipoproteins, and atherogenesis. *J Lipid Res*. 2009; 50:S346–S351. [PubMed: 19091698]
45. Olins AL, Hoang TV, Zwerger M, Herrmann H, Zentgraf H, Noegel AA, et al. The LINC-less granulocyte nucleus. *Eur J Cell Biol*. 2009; 88:203–214. [PubMed: 19019491]
46. Pilsczek FH, Salina D, Poon KKH, Fahey C, Yipp BG, Sibley CD, et al. A novel mechanism of rapid nuclear neutrophil extracellular trap formation in response to *Staphylococcus aureus*. *J Immunol*. 2010; 185:7413–7425. [PubMed: 21098229]
47. Remijsen Q, Kuijpers TW, Wirawan E, Lippens S, Vandenabelle P, Vanden Berghe T. Dying for a cause: NETosis, mechanisms behind an antimicrobial cell death modality. *Cell Death Differ*. 2011; 18:581–588. [PubMed: 21293492]
48. Roberts H, White P, Dias I, McKaig S, Veeramachaneni R, Thakker N, et al. Characterization of neutrophil function in Papillon-Lefevre syndrome. *J Leukoc Biol*. 2016
49. Roos D, Voetman AA, Meerhof LJ. Functional activity of enucleated human polymorphonuclear leukocytes. *J Cell Biol*. 1983; 97:368–377. [PubMed: 6309859]
50. Snyder MW, Kircher M, Hill AJ, Daza RM, Shendure J. Cell-free DNA Comprises an In Vivo Nucleosome Footprint that Informs Its Tissues-Of-Origin. *Cell*. 2016; 164:57–68. [PubMed: 26771485]
51. Sokolov AV, Ageeva KV, Cherkalina OS, Pulina MO, Zakharova ET, Prozorovskii VN, et al. Identification and properties of complexes formed by myeloperoxidase with lipoproteins and ceruloplasmin. *Chem Phys Lipids*. 2010; 163:347–355. [PubMed: 20167214]
52. Sollberger G, Amulic B, Zychlinsky A. Neutrophil Extracellular Trap Formation Is Independent of De Novo Gene Expression. *PLoS One*. 2016; 11:e0157454. [PubMed: 27310721]
53. Sorensen OE, Borregaard N. Neutrophil extracellular traps - the dark side of neutrophils. *J Clin Invest*. 2016; 126:1612–1620. [PubMed: 27135878]
54. Sorensen OE, Clemmensen SN, Dahl SL, Ostergaard O, Heegaard NH, Glenthøj A, et al. Papillon-Lefevre syndrome patient reveals species-dependent requirements for neutrophil defenses. *J Clin Invest*. 2014; 124:4539–4548. [PubMed: 25244098]
55. Sun K, Jiang P, Chan KC, Wong J, Cheng YK, Liang RH, et al. Plasma DNA tissue mapping by genome-wide methylation sequencing for noninvasive prenatal, cancer, and transplantation assessments. *Proc Natl Acad Sci U S A*. 2015; 112:E5503–5512. [PubMed: 26392541]
56. Urban CF, Ermert D, Schmid M, Abu-Abed U, Goosmann C, Nacken W, et al. Neutrophil extracellular traps contain calprotectin, a cytosolic protein complex involved in host defense against *Candida albicans*. *PLoS Pathog*. 2009; 5:e1000639. [PubMed: 19876394]
57. Van Avondt K, van der Linden M, Naccache PH, Egan DA, Meygaard L. Signal Inhibitory Receptor on Leukocytes-1 Limits the Formation of Neutrophil Extracellular Traps, but Preserves Intracellular Bacterial Killing. *J Immunol*. 2016; 196:3686–3694. [PubMed: 27016607]

58. Wallach D, Kang TB, Dillon CP, Green DR. Programmed necrosis in inflammation: Toward identification of the effector molecules. *Science*. 2016; 352:aaf2154. [PubMed: 27034377]
59. Wang J, Kubes P. A Reservoir of Mature Cavity Macrophages that Can Rapidly Invade Visceral Organs to Affect Tissue Repair. *Cell*. 2016; 165:668–678. [PubMed: 27062926]
60. Wang Y, Li M, Stadler S, Correll S, Li P, Wang D, et al. Histone hypercitrullination mediates chromatin decondensation and neutrophil extracellular trap formation. *J Cell Biol*. 2009; 184:205–213. [PubMed: 19153223]
61. Yipp BG, Kubes P. NETosis: how vital is it? *Blood*. 2013; 122:2784–2794. [PubMed: 24009232]
62. Yipp BG, Petri B, Salina D, Jenne CN, Scott BN, Zbytnuik LD, et al. Infection-induced NETosis is a dynamic process involving neutrophil multitasking in vivo. *Nat Med*. 2012; 18:1386–1393. [PubMed: 22922410]
63. Yousefi S, Mihalache C, Kozlowski E, Schmid I, Simon H. Viable neutrophils release mitochondrial DNA to form neutrophil extracellular traps. *Cell Death Differ*. 2009; 16:1438–1444. [PubMed: 19609275]
64. Zhang D, Chen G, Manwani D, Mortha A, Xu C, Faith JJ, et al. Neutrophil ageing is regulated by the microbiome. *Nature*. 2015; 525:528–532. [PubMed: 26374999]