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Neutrophil Extracellular Trap Formation after Sterile Insults in Lung, Liver, Heart and Kidney: A Mini-Review

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Abstract

The formation of neutrophil extracellular traps (NETs) has been an important topic in biomedical research. Once released into the vasculature, NETs ensnare microbes from the bloodstream and prevent dissemination. NET formation is now known to occur not only in infectious diseases, but also in sterile inflammation. This mini-review aimed to discuss recent studies on the pathophysiology of NET formation induced by sterile insults, i.e., mechanical and hypoxic injury, and inflammatory mechanisms, with a focus on acute lung, liver, heart and kidney failure. A number of studies on various organs have shown that such sterile insults lead to NET formation, but the underlying mechanisms remain poorly understood. Understanding cellular and molecular mechanisms that drive NET formation will provide the opportunity to selectively target pathways involved in sterile diseases of organs. Notably, recent arguments in this area increasingly focus on the roles of extracellular mitochondrial DNA in sterile inflammation induced, for example, by ischemia/reperfusion injury. As our current understanding regarding mechanisms of NET formation after sterile insults is insufficient, further analyses on the time course of release of damage-associated molecular patterns, mediators and NET constituents and on their *in vivo* effects are warranted for better management of post-injury inflammation.

NET formation in noninfectious conditions

Formation of neutrophil extracellular traps (NETs), a new effector function of neutrophils, was first described in 2004 [1]. Since then, the formation of NETs has widely been studied. NETs have a web-like structure mainly made of decondensed chromatin fibers, whose expulsion from the cell is aided by enzymatic citrullination of histones and which are decorated with granule proteins, such as elastase, myeloperoxidase (MPO) and histones [2,3]. There is ample evidence supporting the idea that formation of NETs plays an important role in immune responses, allowing neutrophils to capture, neutralize, and degrade a variety of invading microorganisms. NETosis, the type of programmed cell death resulting in NET formation, also occurs during sterile inflammation resulting in thrombosis [4,5], autoimmunity [6], and NET-mediated cytotoxicity [7,8], and therefore can be harmful. DNA and damage-associated molecular patterns (DAMPs) embedded within NETs act as alarmins, augmenting inflammation, and exerting cytotoxic effects. The prothrombotic role of NETs has been well documented [9-11]. This feature of NETs may become problematic, especially in cases of sterile insults. Thus, while NETs may provide evolutionary advantages of trapping and killing bacteria in infectious diseases, they appear to do more harm in sterile inflammation and thrombosis, as dysregulated NET formation leads to vascular inflammation, thrombosis, and atherogenesis,

Let us provide some examples of sterile insults causing NET formation. As shown by many of the studies we discuss below, ischemia/reperfusion (IR) injury is a clinically relevant cause for NET formation. IR injury is a major sterile insult resulting from hemoragic, traumatic or septic shock, burns, and surgical procedures, including organ transplantation. The response to IR injury is comprised of a diverse network ranging from, innate to adaptive immune responses. [12]. Restoration of the blood supply, paradoxically, causes cell damage and exacerbation of inflammatory responses through reactive oxygen species (ROS) and other reactive molecules, worsening organ injury [13]. However, the specific pathways that link IR to NETs are only partially understood.

Besides IR injury, several milder insults are known to induce NET formation. Beiter et al. showed that NET formation increases in response to exhaustive treadmill or cycling exercise in healthy individuals [14]. This study proved that NETosis is the main cause for increased cell-free DNA in the circulation, as reported in an earlier study [e.g., 15]. Jain and coworkers analyzed NETosis associated with dry eye disease, in which hyperosmolar stress is considered the main factor [16]. They further showed that hyperosmotic stress promotes NET formation by neutrophils [17].

NETs have been found in venous and arterial thrombosis, trauma-induced coagulopathy, and disseminated intravascular coagulation [18-20]. NETs promote clot formation; in addition to the interaction of NET components with the coagulation pathway, NETs provide scaffolds for clot growth by catching platelets, fibrin, von Willebrand factor (vWF), and other cells/molecules [21]. It is notable that fluid shear stress is a critical factor for rapid and intense NETosis [22]. Intriguingly, in sterile occlusive clots, fibrin suppresses NET generation, and the absence of fibrin promotes NETs. Shear-induced NETosis is strongly inversely correlated with fibrin in sterile occlusive clots.

It has not been fully understood as yet what initiates NETosis in noninfectious conditions. Unlike bacterial infections, in which neutrophils are likely stimulated by a variety of components, stimulation of neutrophils with a single component such as lipopolysaccharides (LPS) is known to be less efficient in NET production. While a number of *in vitro* studies confirmed that activated platelets, LPS, calcium ionophores, and phorbolester (PMA) induce NETosis, relatively few *in vivo* studies focused on the mechanisms underlying NET formation in noninfectious conditions [23].

Although the mechanisms for NET formation after sterile insults are only partially understood, an emerging consensus postulates that DAMPs play an important role in NET formation in noninfectious conditions [e.g., 7,24]. It is generally accepted that DAMPs includes histones, high-mobility group box 1 proteins (HMGB1), heparan sulfate fragments, and extracellular DNA, including both genomic and mitochondrial DNA (mtDNA) [25,26]. We refer readers to recent excellent review articles regarding the immunothrombotic activity of DAMPs [7,27] and on neutrophils [28].

It should be noted that involvement of mtDNA in NET formation has not fully been examined as yet. An increasing number of studies have demonstrated that mtDNA can serve as DAMPs and exert proinflammatory effects [29,30]. mtDNA has been observed to participate in NET formation after trauma [31]. Specifically, using neutrophils isolated from eight patients requiring orthopedic surgery, McIloy et al. showed that NETs are present immediately after the operation and at all time points up to 5 days post-operatively. Strikingly, the NETs were made of mtDNA, without no detectable nuclear DNA components [31]. This study highlights the importance of separate measurement of mtDNA and nuclear DNA in circulation. As the combination of severe shock and severe trauma has been shown to result in a substantial rise in circulating mtDNA [32], it is likely that mtDNA plays a key role in cases of severe traumatic injury.

It has long been known that mitochondria can produce N-formyl peptides that attract and activate neutrophils via receptors including formyl peptide receptor 1 (FRP1) [33,34]. Further, recent researches have elucidated that mitochondria-derived ROS and mtDNA assist activation of NLRP3 inflammasome, which mediates maturation of IL-1β and IL-18 [35]. It is therefore unsurprising that recent studies have increasingly suggested that mtDNA is a highly important regulator of innate immune responses and an inducer of NET formation by nuclear DNA [29,36]. Of note, while NETosis is a type of programmed cell death, viable neutrophils have been shown to able to produce NETs made up only of mtDNA [37]. This seems to implicate mtDNA at an early phase after cellular injury. In most studies involving NETs, mtDNA and nuclear DNA were not measured separately. So, while citrullinated histones are taken as a marker of NETosis, citrullination can occur in the extracellular environment and cannot exclude the possibility of non-nuclear DNA [36]. Moreover, as mitochondria are considered to have evolved through endosymbiosis, and that mtDNA contains CpG motifs [26,33,38], it is plausible that mtDNA serves as a more efficacious DAMP than nuclear DNA. mtDNA has been shown to stimulate formation of NETs [26]. Hence, it is possible that the role of NETs as an alarmin may mainly be accounted for by mtDNA and other components of mitochondria [36,39]. In support of this view, numerous studies have reported associations between mtDNA and post-injury complications such as multiple organ failure [26,40]. For current views concerning mtDNA, a number of recent articles are suggested [26,29,30,41].

In the following sections, we first briefly discuss the effects of platelets and recent studies on signaling pathways leading to NET formation. Subsequently, we focus on mechanisms underlying NET formation in acute lung, liver, heart, and kidney injuries, mainly based on findings reported after 2014.

Platelets and other stimuli — *in vitro* analysis

Activated platelets are highly effective in inducing NETosis. This is not surprising as platelets can promote neutrophil migration in various inflammatory models [42,43]. The *in vivo* effect of platelets to induce NETosis is well documented for NET formation mediated by gram-negative bacteria [44-46], but platelets also seem to be relevant mediators of NETs in sterile inflammatory conditions as well, including in different experimental models of organ injury. However, while platelet activation with classic agonists such as thrombin receptor-activating peptide (TRAP) and ADP can trigger NET production in *in vitro* systems, molecules mediating platelet-mediated NET generation in organ injury remain only partially understood [47].

The molecules underlying platelet-mediated neutrophil activation can be divided into two types; adhesion molecules mediating cellular interactions, and platelet-derived secretory products [48]. The well-studied adhesion molecules in this regard include GPIb/neutrophil Mac-1 (integrin $\alpha_M\beta_2$, CD11b/CD18), platelet P-selectin/neutrophil PSGL-1 (P-selectin GP ligand 1), and fibrinogen-mediated binding of platelet GPIIbIIIa ($\alpha_{IIb}\beta_3$ integrin)/neutrophil Mac-1 [49]. For platelet-derived secretory products, molecules including CCL5 (RANTES), CXCL4 (platelet factor 4, PF4), CXCL7 (neutrophil-activating peptide-2, NAP-2), and P-selectin, which are stored in alpha granules of platelets, are released or expressed at the platelet surface upon activation [50]. Chemokines such as CCL5 and CXCL4 are important attractants of neutrophils and monocytes. CXCL7 has been implicated in the complex formation of neutrophils and platelets under inflammatory conditions, and promotes chemotaxis of neutrophils [51]. More comprehensive discussion regarding factors produced by platelets has been published [52,53].

It has been shown that context-dependency exists in the molecular mechanisms underlying platelet-mediated neutrophil infiltration. For example, P-selectin is of minor importance in platelet-mediated neutrophil infiltration in LPS-induced acute lung injury (ALI) models [48], whereas, in contrast, in an acid-induced lung injury model, selectins have been shown to play important roles [49]. Moreover, another layer of complexity is added when the mechanisms for platelet-induced NET formation are considered, not only

neutrophil recruitment. Several studies support the importance of β_2 integrin in platelet-induced NETosis. For example, using mice deficient in $\alpha_L\beta_2$-integrin LFA-1 (CD11a/CD18), McDonald et al. showed that LFA-1-dependent platelet-neutrophil interactions within sinusoids regulate intravascular NET production in the liver in response to LPS or *E. coli* sepsis [45]. The relevance of β_2-integrin is further discussed in the next section.

Using both a mouse model of transfusion-related acute lung injury (TRALI) and an *in vitro* system of platelet-induced NET formation, Caudrillier et al. showed that platelets play an essential role in NET formation. They further showed important roles for thromboxane A2 (TXA_2) produced by platelets, and the Raf/MEK/extracellular signal-regulated kinase (ERK) pathway in neutrophils [47].

Carestia et al. focused on the mechanistic basis of platelet-neutrophil interaction effects on human NET formation [46-54]. Platelets prestimulated with gram-positive bacterial component Pam3CSK4 (TLR2 ligand) enhance neutrophil NET formation. Among the classic agonists, arachidonic acid (AA) exhibits more pronounced effects than ADP, collagen, and thrombin, in platelet prestimulation efficacy for neutrophil NET formation. Among leukotrienes (LTs) generated from AA, leukotriene B4 (LTB_4) is a potent inducer of NETs, and LTB_4 receptor was found to mediate the effects of AA. Furthermore, blocking analysis revealed important roles for vWF and CXCL4, which are secreted from platelets in a manner dependent on TXA_2 in platelet-induced NET formation. One interesting finding of this study was obtained in analysis using flow chambers; under flow conditions, compared to non-flow conditions, far fewer platelets were sufficient for induction of NET formation. Further analyses and discussions may deepen our understanding surrounding the reasons why NET formation exhibits such responsiveness to mechanical stress.

Diverse pathways leading to NET production

Molecular pathways inducing NET formation have been studied mainly with *in vitro* systems. Generally accepted as classical NETosis is that, after triggering, generation of ROS by NADPH oxidase (Nox), translocation of neutrophil elastase (NE) and MPO to the nucleus, and peptidylarginine deiminase (PAD)4-dependent citrullination of histones take place, acting in concert to promote chromatin decondensation [55]. However, recent evidence strongly suggests that the pathway leading to NETosis varies depending on the stimuli used [56]. This is an interesting feature of NET formation, although most upstream molecular pathways have been studied using *in vitro* systems. For example, it is known that ROS- and MPO-dependence are stimulus-dependent [57,58]. It is well known that PMA-induced NETosis is mediated by ROS production by Nox2. However, calcium ionophores such as A23187 can induce NETosis independently of Nox2-derived ROS, but depend on PAD4 for NETosis [59].

Exhausting the molecular pathways leading to NETosis is beyond the scope of this review, so we only discuss a few examples here. As Fonseca et al. discuss, PMA-induced NET formation involves the Raf/ERK pathway [60] as well as ROS produced by Nox [61]. NET formation induced by activation of the FcγRIIIb receptor employs a distinct set of signaling molecules; pathways upstream of NET formation involve Nox, and, along with the ERK and Syk/TAK1 pathways [62,63].

Tatsiy and McDonald analyzed pathways upstream of NETosis in human neutrophils triggered by formyl-methionyl-leucyl-phenylalanine (fMLP), PMA, TNF-α and GM-CSF [57]. Of note, fMLP is produced by bacteria and mitochondria [64]. Inhibitor analysis showed that the TAK1, p38 MAPK, and MEK pathways are involved in early events of NETosis induced by fMLP, GM-CSF, or TNF-α. Syk and PI3K were found to be involved in late events in NET formation induced by these agonists. Consistent with other reports, the Nox pathway is involved in PMA-induced NETosis, but is unnecessary for NETosis induced by these agonists. PAD4 importance has been shown in many mouse models, but this study showed that PAD4 was essential in neutrophil NETosis induced by fMLP, GM-CSF, TNF-α or PMA in humans.

β_2 integrin is strongly expressed on human neutrophils. Of note, β_2 integrin has been shown to be important for NET formation induced by viruses that use β_2 integrin as an entry receptor [65]. β_2 integrin is important for PMA-induced NETs; neutrophils from β_2 integrin null mice barely respond to PMA, whereas the response to exogenously added H_2O_2 is normal [65]. It may be that β_2 integrin signaling is necessary for cytoskeletal rearrangement, which is necessary for extruding NETs [65]. However, context-dependency can also be seen for β_2 integrin. In the aforementioned study of hyperosmotic stress-induced NET formation [17], both of the two proresolution formyl peptide receptor 2 (FPR2) agonists, annexin/lipocortin-1 mimetic peptide and 15-epi-lipoxin A4, exhibit partial therapeutic potential. In this system, anti-β_2 integrin blocking antibody shows no effect.

These *in vitro* studies have delineated diverse molecular mechanisms for NET formation, but how these pictures can be translated to *in vivo* situations is largely unclear. Importantly, in the case of platelet-triggered NETosis, Carestia et al. have shown that signaling through ERK, PI3K, and Src kinases, but not P38 or Nox, is important [46]. Thus it is likely that platelet-neutrophil interactions have much influence on the signaling pathways relevant to NET formation.

Although NET formation associated with infectious disease is beyond our scope here, we will briefly cover a few notable examples. NETosis induced by interactions between human neutrophils and *Entamoeba histolytica* trophozoites is a recent *in vivo* example that shows the diversity of pathways leading to NETosis. Diaz-Godinez et al. showed that *E. histolytica* amoebic trophozoites trigger NETosis by a rapid non-classical mechanism in human neutrophils; it was independent of the Nox2-ROS pathway, but mediated by extracellular calcium, although partial activity remained in the presence of calcium [66]. The requirement for calcium in their system appears to arise from the necessity of calcium for neutrophil adhesion. In this setting, contact with the trophozoites induced NETosis in just a few minutes. Involvement of PAD4 was not significant in this case. As the authors discuss, mechanosensitive stimulation could be important [66]. It should be noted that in many settings, NET

formation occurs without PAD4 activity. In several studies we discuss below, a partial degree of NET formation was observed in PAD4 knockout mice. Notably, Claushuis et al. observed that, even in PAD4$^{-/-}$ mice, *Klebsiella* infection caused NET structures containing other markers [67]. Moreover, Guiducci et al. showed that *Candida albicans*-induced NETosis did not require PAD4 [68]. Thus, PAD4 is not required for the formation of all NETs.

The same group further showed that *E. histolytica* triggers signaling pathways to induce NET formation that involve Raf/MEK/ERK, but not, protein kinase C (PKC), ROS, Syk and TAK1 [61,69]. Although specific receptors mediating this signaling are under investigation, it is quite likely that multiple Toll-like receptors (TLRs) mediate this effect.

Lung

Acute respiratory distress syndrome (ARDS) is a life-threatening disease that poses a serious burden to public health. Among many organ systems, lung injury and resultant ARDS is a common cause of severe morbidity associated with sepsis and systemic inflammatory response syndrome (SIRS). Recent evidence shows that NETs contribute to the pathogenesis of acute lung injury, and that platelet-neutrophil interactions play an important role in NET formation in acute lung injury [70]. Mechanical ventilation is used in the course of therapies for patients with ARDS, but such ventilation may further exacerbate the original lung injury, which is termed ventilator-induced lung injury (VILI).

Rossaint et al. focused on NET formation in a VILI mouse model [71]. In this system, the induction of NETs occurred in lung microcirculation in a platelet-dependent manner. Their *in vitro* system, in which thrombin receptor activator peptide (TRAP)-stimulated platelets induced NET formation, showed the essential role of Mac1 and but not of LFA-1. Analysis using pertussis toxin showed the necessity of simultaneous activation of GPCR- and integrin-signaling pathways for NET formation. The *in vivo* analyses showed consistent results. Of therapeutic importance, MKEY peptide that disrupts CXCL4/CCL5 heteromer formation was effective in preventing NET formation in both *in vivo* and *in vitro* systems. These two chemokines have been shown to form heteromers in human and murine acute lung injury samples, and the concentration of the heteromers shows correlation with leukocyte influx into the lung [48].

Li et al. analyzed the role of TLR4 in NET formation in a VILI mouse model [72]. After demonstrating NET formation in the lungs of their VILI animal model, they observed that TLR4 knockout mice show lower levels of in citrullinated histone H3 (citH3) in lung homogenate, and DNA in bronchoalveolar lavage fluid (BALF), compared with TLR4 WT mice. It is noted that TLR4 knockout did not abolish NET formation; in TLR4 knockout mice, citH3 and DNA in BALF were clearly increased by high-tidal volume ventilation, and citH3 reached to approximately 50% of the level of citH3 observed in TLR4 WT mice. Thus, NET formation in this model is partially dependent on TLR4.

Moreland and coworkers observed a protective anti-inflammatory function of Nox2. In their system of SIRS induction by intraperitoneal injection of zymosan (a ligand for TLR-2 and dectin-1), it was suggested that Nox2-derived ROS serve to repress inflammatory responses in the lung [73]. This observation corroborates with findings in chronic granulomatous disease that suggested a role for Nox2 in limiting and resolving inflammation. As an extension of this study, Hook et al. showed that Nox2-derived ROS has an active role in repressing platelet chemokine secretion in the lung under resting and stimulated conditions [74]. They also revealed that Nox2 downregulates PAD4 activity in neutrophils and represses NET formation in the lung following systemic inflammation. Reflecting the multifaceted roles for Nox2, they observed anti-inflammatory effects of Nox2 in multiple aspects, including chemokine production from alveolar macrophages, platelet-neutrophil interactions, and NET formation.

Besides VILI, TRALI is a model system in which the relevance of NET formation has been addressed. Caudrillier et al. observed NET formation in the lungs of both experimental and clinical TRALI [47]. Prevention of platelet activation or interference with NET components showed protective effects in TRALI. Inhibition of TXA_2 signaling reduced NET formation in an *in vitro* system of activated platelet-mediated NET formation. The clinical relevance of this finding was supported by an analysis using aspirin in a mouse model of TRALI.

In general, lung is an organ in which the contribution of NETs to the pathogenesis of tissue injury is clear. Lung is also important in terms of its vulnerability to systemic inflammation. It is hoped that analyses of blocking chemokine activity, platelet-neutrophil interactions, or NET formation lead to clinical benefits in a wide range of cases of lung injury.

Liver

Neutrophils are actively targeted to the vasculature of the liver. Neutrophils recruited to the liver are known to enhance the clearance of pathogens from the circulation [44,45]. As such, liver is an important organ for sepsis. On the other hand, sterile inflammation plays an important role in alcoholic and nonalcoholic steatohepatitis, drug-induced hepatotoxicity, and IR injury. What triggers NETosis in such liver injury has been under intensive study. We suggest a review article by Woolbright and Jaeschke for a broad view on sterile liver inflammation [75], and an article by Yang et al. focusing on HMGB1 and histones as DAMPs that contribute to liver injury and subsequent multiple organ failure [76].

NET formation in liver IR injury models have been studied by several groups. Earlier studies reported that, following liver IR injury, DAMPs such as HMGB1 and histones are released and exacerbate hepatic injury through activation of TLRs [77,78]. This leads to recruitment of neutrophils and NET formation. Huang et al. showed that histones and HMGB1 might activate NET formation, and that NETs could contribute to inflammation and damage during liver IR injury [79]. Inhibition of NET formation by PAD4

inhibitor or DNase I reduces liver damage. CitH3 dramatically increases to a much higher level after intraperitoneal injection of histone or HMGB1 after liver IR injury, compared with the level seen in TLR4-deficient mice [79]. TLR9 knockout mice exposed to these conditions also express less citH3 and exhibit insignificant increases compared to WT. Neutrophil transfer analysis from TLR4 or TLR9 knockout mice to neutrophil-depleted mice demonstrated that NET formation after liver IR injury is a result of TLR4 and TLR9 activation within neutrophils. Thus, HMGB1 and histone have considerable ability to induce NETosis through TLR4 and TLR9. It is likely that some NET formation is not mediated by PAD4, as DNase I treatment is more effective in reducing NET formation than are PAD4 inhibitors.

Roles of chemokines and cytokines in neutrophil-mediated liver injury have been well studied, but only a limited number of such studies have addressed NETs. IL-33 is a cytokine that has drawn much interest in recent studies of sterile liver injury. IL-33, a new member of the IL-1 family expressed in endothelial cells, epithelial cells, and fibroblast-like cells, is known to function as an alarmin released upon cell injury/tissue damage [80]. IL-33 is normally located in the nucleus and associates with chromatin. IL-33 is released from necrotic cells as an alarmin, and activates a variety of immune cells expressing the IL-33 receptor, namely, suppression of tumorigenicity 2 (ST2). Interrelationships among NLRP3 inflammasomes and IL-33 in sterile liver inflammation and associated NET formation have been discussed [81].

In Yazdani et al., the role of IL-33 and its receptor ST2 in NET formation was analyzed in a partial liver IR injury model [82]. *In vitro* analysis showed that IL-33 is released from liver sinusoidal endothelial cells. After liver IR insult, NET formation and liver injury were less pronounced in mice deficient in IL-33 or ST2. Adoptive transfer of ST2 knockout neutrophils to neutrophil-depleted WT mice decreased NET formation. Thus, IL-33 directly induces neutrophils through ST2 to form NETs, and this pathway is important in IR injury.

Recently, Zhang et al. showed an essential role for CCL2-CCR2 signaling in a liver IR injury model [83]. Other studies on cytokines and chemokines in liver injury include Tohme et al., which uniquely combined bioinformatic and experimental approaches [84]. The authors applied computational network analysis to the inflammatory mediator networks which emerge within a few hours of liver IR injury and contribute to NET formation. IL-17A was shown to play an early critical role in orchestrating immune responses to hepatic IR injury. Administration of anti-IL-17 antibody decreased neutrophil infiltration as well as MPO-DNA complexes and citH3 levels. They also showed by *in vitro* analysis that IL-17 can induce NET formation by peripheral neutrophils, bone marrow-derived neutrophils, and human peripheral blood neutrophils.

Recent interesting studies include those by Hilscher et al., who addressed the question of how mechanical stretching of sinusoidal endothelial cells leads to portal hypertension [85]. Analysis with microarray and RNA-sequencing techniques showed that mechanical stretching of primary liver sinusoidal endothelial cells induces upregulation of CXCL1. They also performed partial ligation of the suprahepatic inferior vena cava, which can eventually induce portal hypertension in mice. Intriguingly, this procedure led to formation of neutrophil-platelet complexes and NETs. Analysis of NE- and PAD4-deficient mice suggested that NET-mediated formation of sinusoid microthrombi might increase portal pressure. They elucidated the signaling pathway that links stretching to increased expression of CXCL1, but involvement of key signaling pathways leading to NET formation in this setting itself requires further analysis. As we discuss in a section below regarding kidney, it would be interesting to envisage that such NET formation may form a vicious cycle involving NET formation, portal hypertension, and CXCL1 expression.

The roles of mtDNA in sterile liver injury may not have been fully studied as yet. Due to their high requirement for oxidative phosphorylation, hepatocytes have high mitochondrial content. So, it is conceivable that mtDNA plays an important role in sterile liver injury. Indeed, in APAP (N-acetyl-p-aminophenol)-induced liver injury, necrotic hepatocytes release mtDNA, which stimulates TLR9 and induces neutrophil infiltration, exacerbating hepatocellular damage [86,87]. Although NET formation was not the focus of these studies, such findings implicate mtDNA as an important alarmin in drug-induced acute liver injury. It would be interesting to compare multiple liver injury models with an interest in relative importance of mtDNA as opposed to nuclear DNA. Although it has been shown that ROS function as DAMPs and activate the NLRP3 inflammasome [88], this linkage may be mediated by mtDNA, given that mtDNA has been shown to play an important role in NLRP3 inflammasome activation [89]. Al-Khafaji et al. showed that, in a model of liver IR injury, extracellular superoxide can induce NETs through a process initiated by neutrophil TLR4 and propagated by intracellular Nox [90]. Further analyses of this system focusing on IL-33, IL-17, mtDNA and other DAMPs may afford more comprehensive understanding of molecular mechanisms of NET formation.

Heart

In most cases of cardiac injury, ischemic insult comprises a major cause. After the initial lack of blood supply that causes the death of cardiomyocytes and upregulation of inflammatory cytokines, repeated IR injury results in necrosis and release of DAMPs [91]. In myocardial infarction (MI), neutrophils and pro-inflammatory monocytes are recruited to the site of infarction shortly after ischemia occurs. These cells exert proinflammatory functions that are deleterious to post-ischemic tissues. During this period, NET formation occurs [92]. Innate immune responses in ischemic heart injury have been discussed in recent excellent articles [93,94]. Here we focus on a number of studies regarding NET formation in heart injury.

Savchenko et al. showed that extracellular chromatin released through NETosis exacerbates myocardial IR injury [92]. Both DNaseI and ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type-1 motif, member 13) showed protective effects in their myocardial IR

injury model, with decreased numbers of neutrophils and citH3-positive cells. After 24 h of myocardial IR, PAD4$^{-/-}$ mice had significantly smaller infarction areas and lower levels of circulating nucleosomes as compared with WT mice. PAD4 deficiency reduced leukocyte recruitment to the infarcted myocardium. This report is based on the concept that PAD4 is required for NET formation and, as the authors expected, the WT and PAD4$^{-/-}$ difference was evident. Intriguingly, however, partial levels of NETs were formed in PAD4$^{-/-}$ mice, implying the presence of a PAD4-independent pathway in this study.

In general, in several animal models of MI, chemokine system antagonism was beneficial, although immune cells are also important for tissue repair [95]. As we have seen above (in the section concerning lung), CCL5/CXCL4 heteromers are potent chemokines and induce NET release by neutrophils contacting platelets [71]. Notably, heteromers have been implicated in pathological changes in vascular disease. Administration of MKEY peptide in mice attenuates vascular remodeling processes i.e. atherosclerosis and aortic aneurysm [96,97]. Inhibition of CCL5 also exhibits cardioprotective effects [97,98]. Vajen et al. demonstrated that MKEY showed efficacy in the treatment in a myocardial IR injury mouse model [91]. In their model, MKEY treatment (from 1 day before and until up to 7 days after IR insult) reduced neutrophil infiltration and preserved heart function as compared with control mice. Thus, disruption of the CCL5/CXCL4 interaction attenuates post-ischemic inflammatory reactions. Moreover, MKEY treatment almost completely abrogated NET formation, based on the number of citH3-positive cells in the infarct tissue.

Midkine is a heparin-binding growth/differentiation factor expressed in small intestine and thyroid and, at modest levels, in the lung, colon, stomach, kidney and spleen [99]. The midkine level in plasma increases upon various traumatic conditions, but the function of midkine is still controversial, showing beneficial and detrimental effects in cardiac pathology [100]. Wechbach et al. showed that midkine plays a critical role in polymorphonuclear neutrophil (PMN) adhesion and extravasation during acute inflammation [101]. It was also suggested that midkine mediates PMN adhesion by binding to low-density lipoprotein receptor-related protein 1 (LRP1), a member of the LDL receptor family important for endocytosis [101,102]. More recently Weckbach et al. showed that midkine-LRP1 interaction is important for β_2 integrin-dependent PMN recruitment and NET formation [103]. Specifically, after revealing NETs in endomyocardial biopsies of patients with acute myocarditis, they used a mouse model to show that NETs are an important factor promoting cardiac inflammation during experimental autoimmune myocarditis. Blocking of midkine caused reduction of the proportion of NET-positive PMNs among all extravasated PMNs. They further demonstrated that midkine promotes NET formation via LRP1.

Kidney

NET formation has also been implicated in acute kidney injury (AKI). AKI is classified into prerenal, intrinsic or postrenal. Besides sepsis and nephrotoxicity, IR injury accounts for a major proportion of intrinsic (i.e., non-prerenal and non-postrenal) AKI. IR injury and resultant AKI are two of the major complications of renal transplantation, and trigger inflammatory response, including the recruitment of various immune cells, including neutrophils. The mechanisms for recruitment of immune cells to kidney has been discussed [104]. NET formation has been reported in animal models of ischemic AKI, as well as in human renal allograft biopsies [105-107]. The roles of DAMPs in IR injury have been discussed [108]. In this section we focus on recent studies involving ischemic AKI and NETosis.

Nakazawa et al. provided important insights into NET formation through analyses of the relationship between tubular cell necrosis and NETosis in an IR injury mouse model [105]. They found that tubular cell necrosis is an early event that leads to release of DAMPs after IR injury, and which is followed by NET formation by neutrophils. Intriguingly, their *in vitro* analysis suggested the presence of a vicious cycle; NET components further induce additional tubular injury, which in turn promotes NET formation. They focused on histone as a factor mediating this cycle; extracellular histone induced NET formation and, on the other hand, mediated NET toxicity toward tubular epithelial cells. Although verification of the vicious cycle *in vivo* awaits further analyses, this is a notable result.

It would also be of significance to examine the relative importance of the molecules that act as DAMPs in the same experimental system. Of note, Jansen et al. showed that in ischemic AKI, DNA released from necrotic renal tubular cells is likely to activate platelets, resulting in platelet-neutrophil interactions and NET formation [107]. The relative importance of DNA (possibly involving mtDNA) and histones in NET induction in ischemic AKI is not presently clear. Doi et al. showed that, in bilateral IR injured murine kidney, HMGB1 induced ALI independent from TLR4 [109]. This may be explained in that IR injury can induce necroinflammation that leads to NET formation and histone and HMGB1 release, thereby stimulating various TLRs [105].

Raup-Konsavage et al. showed the importance of neutrophil PAD4 in IR-induced AKI [106]. Transfer of PAD4-expressing neutrophils to PAD-deficient mice restored NET formation and resensitized the animals to ischemic AKI. Overall this study showed a pivotal role of neutrophil PAD4 in NET formation in ischemic AKI.

Rhabdomyolysis-induced myoglobinuric acute renal failure (ARF) is of clinical importance and accounts for approximately 10–40% of all cases of ARF [e.g., 110,111]. Recent notable studies on AKI include Okubo et al. who focused on macrophage extracellular traps (METs) in rhabdomyolysis-induced AKI [112]. Heme released from damaged muscle activates platelets, which in turn enhances MET formation through a mechanism mediated by ROS and histone citrullination. It was suggested that heme leads to platelet activation and accumulation, and macrophages interact with platelets, leading to MET formation in renal extravascular space. Neutrophils are unimportant in this setting. In their glycerol injection-induced injury model, increased circulating DNA levels were partly dependent on

PAD4. METs and METosis represent new areas in which further analyses are warranted [113].

Several questions remain regarding the initial factors mediating NET formation in IR injury and other types of AKI. While IL-33 release from peritubular and periglomerular endothelial cells upon kidney IR injury has been reported [108], to our knowledge, the relative contribution of IL-33 in NET formation upon AKI has not well been addressed.

Finally, it should be stressed that a number of recent studies have implicated mtDNA as a potentially useful biomarker that reflects progression to AKI after traumatic injury. To examine any possible causative role for mtDNA in AKI, Jansen et al. conducted systemic intravenous injection of mtDNA. This experiment did not show any significant effects, and the authors concluded that the elevation in mtDNA levels cannot be regarded as a key causative factor in AKI development in SIRS [114]. Nonetheless, further analyses may elucidate certain roles of mtDNA as a mediator of AKI [25,115]. We also discuss in the next section additional examples of *in vivo* analyses that showed proinflammatory effects of mtDNA.

Concluding Discussion

Molecular mechanisms for neutrophil recruitment are known to be different among organ/tissues [104]. Nonetheless, with respect to the mechanisms involved in NET formation, key commonalities among findings from sterile injury models are emerging. First, DAMPs, such as histones and HMGB1 and possibly mtDNA, are released from damaged tissue and activate neutrophils. As Huang et al. [79] demonstrated, TLR4 and 9 of neutrophils mediate DAMP-induced NETosis. Second, it is unarguable that platelets play crucial roles in recruiting and activating neutrophils, and such platelet functions are important in NET formation. The participation of these molecules and cells appear to be common denominators. Besides, as studies utilizing MKEY peptides demonstrate, platelet chemokines play crucial roles in organ injury, including in IR injury. Furthermore, mechanical stress may become important in many cases. As Hilscher et al. showed, liver sinusoidal endothelial cells are sensitive to mechanical stress, which forms a vicious cycle with NETs. The finding of the requirement for calcium in many settings leading to NET formation supports the importance of mechanical stress in endothelial cells and neutrophils [85]. Thus, the current consensus on NETosis is that NET formation is promoted by the combination of the presence of macrophage-derived cytokines, activated platelets, NET-inducing chemokines, and the upregulation of adhesion molecules [73]. However, the complex of mediators and cellular responses to IR injury in various organs has been difficult to unravel [116].

One of the outstanding questions is to what extent mtDNA is involved in the extracellular DNA in cases in which the extracellular DNA was assumed to be nuclear DNA expelled through NETosis. Importantly, an *in vitro* analysis by Itagaki et al. demonstrated that mtDNA induces NET formation through TLR9-dependent and NADPH oxidase-independent pathways [117]. They also showed that after trauma, mtDNA levels increase in the circulation, and this change is more pronounced in elderly patients. Intriguingly, however, NETs were detected in PMNs from elderly trauma patients to a lesser extent relative to younger patients. This study is important in demonstrating the NET-inducing ability of mtDNA, implying a role for mtDNA in an early phase of NETosis. More recently, Aswani et al. demonstrated that release of mtDNA is sufficient for the development of multiple organ injury [32]. Their analysis using blood sampled at 2 h time points after trauma showed prognostic value of mtDNA for multiple organ dysfunction syndrome. They further showed that injection of pure mtDNA into healthy animals caused moderately severe acute lung injury in rats. A small but significant rise in plasma and lung IL-6 was observed after mtDNA injection.

It should also be noted that ROS-mediated mtDNA damage during IR injury can worsen tissue damage through a vicious cycle; oxidized DNA causes dysfunction in mitochondria, which in turn causes increased generation of ROS [29,118]. Lood et al. recently focused on NETs rich in oxidized mtDNA. They showed that oxidized mtDNA is proinflammatory in vitro, and that upon injection of this DNA into mice, type I interferon (IFN) signaling through cGAS (cyclic GMP-AMP synthase)-STING pathway was stimulated rather than the TLR9 pathway [119]. It is quite possible that in the near future, more analyses will demonstrate release of mtDNA from injured or stressed cells in infectious and non-infectious insults, exacerbating immune responses through amplifying the secretion of type I interferon and pro-inflammatory cytokines. Also notable is the recent findings on the roles of cytoplasmic mtDNA. Even without being released to extracellular environment, mtDNA plays important roles; mtDNA released into cytoplasm promotes apoptosis as shown by McArthur et al. [120]. Recently, both PTEN-induced putative kinase 1 (PINK1), a ubiquitin ligase, and parkin, an E3 ubiquitin ligase, have been shown to act to remove damaged mitochondria via mitochondria-specific autophagy (mitophagy) [121]. Sliter et al. demonstrated that parkin and PINK1 act to suppress innate immune response and this suppression is mediated by mitophagy, which prevents the increase of cytoplasmic mtDNA and therefore the IFN-β production through cGAS-STING pathway [122].

It is likely that some alarmins have not been fully studied as yet, despite their potential significance. So, the roles of IL-33 and midkine, for example, may well be analyzed in a much wider range of tissue injury models and patients. Regarding the signaling pathways leading to NET formation, it is well known that distinct pathways induce NETosis under different conditions, but some pathways (or combination of promoting factors) may eventuate to have higher efficacy in promoting NETosis. In this context it is interesting that in both *in vivo* and *in vitro* analyses, Yotsumoto et al. showed that sulfasalazine (SSZ), a drug that is commonly used to treat inflammatory bowel disease or rheumatoid arthritis, greatly sensitizes neutrophils toward NETosis. Intriguingly, SSZ did not enhance ROS in neutrophils, but accelerated lipid oxidation was essential for SSZ-induced NETosis [123]. They further identified the importance of oxidization of ether-linked

phospholipids. Studies addressing neutrophil sensitization of neutrophils toward NETosis may become important.

Interestingly, the overall benefits of NETosis have been under recurrent question from an evolutionary perspective [36,124]. In the absence of infection, the collateral damage caused by the initiation of the inflammatory response triggered by DAMPs can be detrimental; the proinflammatory and prothrombogenic activities of NETs can be problematic, as shown by the fact that prevention of NET formation is beneficial in most cases of sterile insults in animal models. Although we did not cover the topic in this article, NETs have been implicated in deep vein thrombosis by promoting the coagulation process [125]. Thus, compared with apoptosis and programmed necrosis, neutrophil death through NETosis appears harmful. It may be conceived that, for young humans, NET formation may provide more advantages arising from protective effects in infectious diseases than disadvantages under non-infectious conditions. For older humans, however, such demerits may outweigh the merits.

Conflict of Interest

Authors declare that they have no conflict of interest.

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