Paragraph section

1. Molecular and cell biological pathways of NET formation

1A. NET formation as a consequence of regulated cell death

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NET formation implies the release of chromatin decorated with cytoplasmic proteins, a process that has been documented to occur without nuclear and plasma membrane ruptures and immediate neutrophil death under certain conditions. In many cases, however, the same NET-like structure is the consequence of neutrophil necrosis involving the rupture of nuclear and plasma membranes and a release of decondensed chromatin together with cytoplasmic content into the extracellular space [1, 2, 3, 4, 5]. Distinguishing the two processes is reliably possible only with ultrastructural or live cell imaging using morphological criteria of membrane rupture and cell viability. In contrast, dissecting the type of neutrophil necrosis is not possible by morphological criteria [6]. According to the current recommendations by the cell death community the type of cell necrosis can only be defined by identifying one of the several signaling pathways leading to regulated cell necrosis such as necroptosis, pyroptosis, ferroptosis, parthanatos, etc [7]. This requires the use of selective signaling pathway antagonists or targeted deletion of critical pathway elements in neutrophils. Current evidence suggests that PMA (2-hour stimulations) and crystal-induced NET formation involves the RIPK1/RIPK3/MLKL-dependent pathway of necroptosis [8, 9]. Other triggers might involve other pathways of regulated neutrophil necrosis. NET formation may also occur as a consequence of passive neutrophil necrosis not involving any specific signaling pathways, e.g. histone-related cytotoxicity due to positive charge-dependent plasma membrane rupture [10]. As histones are released during NET formation massive NET formation likely involves both passive necrosis and regulated necrosis affecting different cells or even identical neutrophils in the same microenvironment [11].

1B. Neutrophil cytolysis vs NETs in the study of human disease

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Decades before the description of NETs, necrotic cells were recognized as a source of extracellular DNA and associated histones [12]. The extracellular release of nuclear material, once used as a marker of cytolysis [13], is now considered a hallmark of NETs. Initial efforts focused on dissecting NETs from unique forms of cell death [14, 15]. However, the lack of rigorous criteria used to define NETs has led to the practice of defining any process involving the release of nuclear and cytoplasmic material from neutrophils [16, 17, 18, 19, 20], regardless of the driving mechanism, as NETs. The limited specificity to define NETs, the shortage in the use of proper controls, and potential differences in NET inducing pathways between mice and humans, among other possible caveats, have likely overstated the role of NETs in disease. In rheumatoid arthritis (RA), for example, accumulating evidence has suggested that citrullination in NETs is a major source of citrullinated autoantigens in both humans and experimental arthritis [18, 21]. However, several studies have also questioned the need for citrullination in the formation of NETs by human neutrophils [22, 23, 24, 25]. Potential inconsistencies in the relationship between citrullination, NETs and disease in the human model may have resulted from the study of other PAD activating mechanisms of neutrophil damage or death that may have been mistaken as NETs [24]. The lack of stringent controls to define the magnitude and immunogenic consequences of citrullination in NETs may have also contributed to this paradox [26].

Cytolysis induced by host (i.e. perforin and complement) and bacterial (i.e. toxins) pore-forming proteins (PFPs) is a self-defense mechanism commonly used by immune cells and virulent bacteria, respectively, to target unwanted cells [27]. Like PFPs, the formation of discrete pores in the neutrophil plasma membrane (using electropermeabilization) promotes extracellular release of nuclear DNA decorated with MPO, demonstrating that nonspecific cytolysis induces neutrophil changes, currently indistinguishable from NETs [28]. Unlike NETs, however, PFPs induce prominent calcium influx that hyperactivates PADs generating neutrophil hypercitrullination (a process termed leukotoxic hypercitrullination, LTH), which is

likely used by virulent bacteria to inactivate neutrophils [24, 29]. The generation of the RA citrullinome is more likely explained by LTH, not by NETs [24, 29, 30]

Additional problems in the study of NETs in health and disease include the assumption that neutrophils are the only cells that release nuclear material upon death, and that NETosis is the only form of cell death in neutrophils. Thus, although all nucleated cells contain chromatin, studies have quantified free dsDNA or chromatin in serum as surrogates to measure NET production *in vivo* [17, 19, 31, 32]. Similarly, while multiple stimuli can induce histone citrullination and neutrophils are not the only cells that can citrullinate [24, 29, 33, 34], detection of citrullinated histones has also been used as specific markers to quantify NETs [31, 35]. More recently, the detection of soluble complexes of DNA and neutrophil-derived proteins, such as myeloperoxidase (MPO) and neutrophil elastase (NE), have been used to increase the specificity of NET quantification *in vivo* [32]. However, although NETs can be a source of MPO/NE-DNA complexes, the specificity of these findings in relation to other forms of neutrophil death has never been challenged. Almost any form of cell death in neutrophils (such as apoptosis, necrosis, LTH, and necroptosis, among others) can develop secondary necrosis and release intracellular material [29, 36]. Indeed, nonspecific cytolysis promotes extracellular release of nuclear DNA decorated with MPO [28]. Thus, similar to other NET detection assays, the specificity of soluble MPO/NE-DNA complexes as markers of NETs fully relies on the assumption, but not experimental evidence, that no other biological mechanism could mimic this process. The use of non-specific markers to detect and quantify NETs in vivo may explain the growing number of mechanisms and diseases that have been linked, in some cases erroneously, to NETs [24]. Defining markers of distinct mechanisms of neutrophil activation and damage should therefore be a high priority to truly understand to role of NETs in health and disease.

1C. PAD4 and NET Release

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Peptidylarginine deiminase IV (PAD4), the enzyme which converts arginine residues into citrulline residues, is relevant for two important reasons. One is that PAD4 modifies a variety of human autoantigens which, due to the newly introduced citrulline, become preferential targets of autoimmune responses. The second is the fact that PAD4 performs essential functions that mediate the classical (nuclear) form of NET release. Neeli *et al.* showed that various inflammatory stimuli induce histone deimination and identified deiminated histones as integral components of NETs [37]. Subsequent studies confirmed these observations [38] and determined that PAD4 activity is essential for the regulated release of NETs because extracellular chromatin release is impaired in PAD4-deficient neutrophils [39, 40]. These results complement the findings that PAD4 inhibitors effectively block NET release [41].

However, the mechanism whereby PAD4 contributes to NET release is not clear. One possibility is that PAD4 makes an essential contribution toward NET deployment by converting arginine residues in histones into citrulline residues. By doing so, PAD4 removes the positive charge from the amino termini of core histones and diminishes the attractive forces between histones and DNA. As result, histone deimination loosens the structure of chromatin [38]. A similar transition may provide the force that expands the nucleus and ultimately ruptures the nuclear envelope to release NETs.

Multiple stimuli that lead to NET release and the potential variety of forms of NETs make it difficult to establish the signaling pathways that participate in the activation of PAD4. Signals from Gram-negative bacteria, including lipopolysaccharide acting on the Toll-like receptor 4, may transmit signals via MyD88 and its associated catalytic subunits to IRAK1 [42]. Through the activation of distinct IKK subunits, the pro-inflammatory axis of NFkappaB is engaged, leading via MEK1 to the further activation of ERK1 and 2. Alternatively, FcgammaRIIIb, acting via TAK1, leads to the activation of ERK1/2 [43]. Additional feed-forward signals may involve activation of G-protein-coupled receptors that induce PLCgamma to form its messenger IP3, followed by calcium release from endogenous ER stores [44]. Alternatively,

a calcium-activated potassium channel may directly engage signals leading to NET formation [22]. Calcium could act as an additional signal by activating PKC subunits, which have been shown to have a direct effect on NET release. Experiments by Neeli and Radic revealed an unexpected complexity of PKC contributions to NETosis [25]. Experiments with an inhibitor of classical PKC, chelerythrine, as well as a structurally related compound, sanguinarine, demonstrated that classical PKC enzymes may block activation of PAD4, yet an atypical PKC, most likely PKCzeta, exerts an activating role upstream of PAD4 [25]. The opposing effects of two PKC isoforms argue for very precise regulation of PAD4 in neutrophils. Through as yet incompletely understood mechanisms, these enzymes contribute to the disruption of granule and nuclear membranes, chromatin relaxation and, ultimately, NET release.

1D. Externalization of mitochondrial DNA.

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Despite vast numbers of publications describing NET formation, very little is known about the molecular mechanisms underlying this function of granulocytes which is so important for microbial defense. Physiological stimulation of granulocytes by cytokines, complement, adhesion molecules, or toll-like receptors leads to formation of extracellular traps containing mainly mitochondrial DNA (mtDNA) and granule proteins [45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55]. Our studies on the molecular mechanism have shown that extracellular DNA trap formation by neutrophils [45, 53], eosinophils [50, 52, 56], and basophils [49, 54] in our hands does not require their death as previously suggested [15]. Hence, granulocytes remain viable after mtDNA release [45, 49, 50, 52, 53, 54, 57]. Furthermore, in contrast to another report [9], we found no evidence for the involvement of the RIPK3-MLKL pathway [45]. Moreover, genetic deletion of ATG5 correlated with defective autophagy, but elicited no defect in extracellular trap formation either in neutrophils or in eosinophils [58]. Recently, we reported that pharmacological inhibition of the cytoskeletal dynamics or the depletion of genes in neutrophils regulating the cytoskeleton prevents degranulation and mitochondrial

DNA release required for NET formation [59]. Furthermore, we have recently demonstrated that glycolytic ATP production is required for microtubule network assembly and NET formation [60]. While both neutrophils [45, 53] and eosinophils [50, 52] required a functional NADPH oxidase for DNA trap formation, basophils did not [49].

We demonstrated that extracellular DNA traps contain mtDNA also *in vivo* under pathological conditions. For instance, we have addressed the question whether eosinophils and neutrophils infiltrating the airways in asthmatic patients produce extracellular DNA traps consisting of mtDNA and granule proteins. This was indeed the case and was supported by the observation that the mitochondrial *ATP6* gene signal was readily detectable in extracellular DNA traps released by eosinophils infiltrating the tissue. The *GAPDH* gene signal was selectively seen only in nuclei [46]. Taken together, granulocytes are able to kill pathogens in the extracellular space by the release of mtDNA together with granule proteins.

2. Physiological and pathophysiological aspects of NETs

2.A Interplay between bacteria and NETs

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The original discovery of NETs revealed a phenomenon wherein microbial pathogens such as *Staphylococus aureus* or *Listeria monocytogenes* efficiently induce the release of DNA-based structures from human neutrophils [14]. Beginning shortly thereafter, evidence for a key innate host defense function of NETs accumulated from studies manipulating the microbial side of the host–pathogen equation. Several pathogens were shown to express virulence determinants that conferred resistance to NET-based antimicrobial killing, including nucleases that degrade NET architecture, as shown Gram-positive pathogens such as *Streptococcus pyogenes* [61], *Streptococcus pneumoniae* [62], *S. aureus* [63], and *Streptococcus suis* [64] as well as Gram-negative pathogens such as *Yersinia enterocolitica* or *Vibrio cholera* [65]. Other pathogens resistance intrinsic antimicrobial effectors of NETs such as histones and cationic defense peptides, e.g. the M1 protein of *S. pyogenes* [66, 67] or the suppression of NET production through engagement of inhibitory neutrophil receptors,

e.g. Siglec-9 by *Streptococcus agalactiae* [68] and *Pseudomonas aeruginosa* [69] or elaboration of neutrophil cytotoxins such as *Bordetella pertussis* adenylate cyclase (Ref) or *S. pyogenes* streptolysin O (SLO) [70].

The exact molecular mechanisms that drive entrapment and killing of the microbes within NETs are still not completely understood. Upon disruption of NETs with DNase or DNase/proteinase mixtures, the extracellular antimicrobial capacity of neutrophils or other ET-releasing cells is reduced. It has been postulated that electrostatic interactions between cationic components of NETs (e.g. histones) and the anionic surface of microorganisms [71] or even the DNA itself [72] play a role in this process. Specific factors such as the antimicrobial peptide cathelicidin LL-37 [73] or calprotectin [74] contribute to the antimicrobial capacity of NETs; however, since most cationic peptides or proteins lose killing capacity when bound to DNA, it may be that NETs primarily serve to ensnare pathogens near a high concentration gradient of antimicrobial effector molecules accumulating from the activated immune cells.

For proof of a protective role of NETs, in vivo data are essential. Of note, there are well documented differences between the amount and morphology of NET formation *in vitro* versus *in vivo* in response to certain pathogens. Thus, more *in vivo*-related NET data are needed using specific immunofluorescence-based NET-probes. Improvements in *in vitro* model systems for studying pathogen-NET-interaction that reflect in *vivo* physiological relevant conditions are also a priority. As an example, release of NETs by neutrophils is significantly altered under hypoxic oxygen conditions [75], a situation that predominates in tissues during infection or inflammation, aggravated by overconsumption of oxygen by pathogens and recruited immune cells.

S. aureus is one pathogen shown to be entrapped and partially killed by NETs not only in vitro but also *in vivo.* Berends *et al*. [63] showed that *S. aureus* degradation of NETs contributes to acute pneumonia in mice, and Yipp *et al*. [76] revealed anti-bacterial NETs produced by chemotactically active neutrophils during *S. aureus* skin infection.

Correspondingly, pharmacological agents that boost NET production *in vitro*, such as statins or tamoxifen, increased *S. aureus* clearance during systemic infection models [77, 78]. Conversely, another Gram-positive pathogen, *S. pneumoniae*, appears highly resistant to NET-mediated killing *in vivo*. For example, primary influenza A infection of the middle ear boosts formation of NETs by infiltrating neutrophils, and resistant *S. pneumoniae* can use those NETs to augment biofilms and persist during otitis media [79]. Branzk *et al.* [80] presented a hypothesis that bacterial particle size is a key mediator of NET versus phagocytosis-mediated killing of pathogens, such that neutrophils selectively release NETs in response to larger pathogens. Ultimately, it will depend on the pathogen, its array of immune resistance factors, and the anatomical site of infection, as to whether NETs can provide an immune defense function for the host [81].

2.B Barrier function of neutrophil extracellular traps

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Neutrophils form neutrophil extracellular traps (NETs) of decondensed DNA and histones that trap and immobilize pathogens like bacteria as well as particulate matter, which cannot be removed from the body. Examples of the latter can be natural crystals of monosodium urate formed during gout [82] or man-made nanoparticles with which the body comes in contact but can neither degrade nor remove (like nanodiamonds or polystyrene nanoparticles) [83]. During some acute inflammatory conditions, involving internal organs, like acute necrotizing pancreatitis massive tissue necrosis occurs, which is organized as pancreatic pseudocysts [84]. In contrast to regular cysts, these pseudocysts are not surrounded by epithelial layers. Recently we investigated necropsy samples of internal organs of 2 patients with acute abdominal inflammation, revealing areas of the interface between intact and necrotizing tissue. Immunohistochemical analysis has demonstrated that necrotic areas observed in necrotizing pancreatitis and peritonitis are isolated from the surrounding healthy tissues by aggregated NETs. Between the areas of viable tissues and those destroyed by necrosis we found a distinct condensed tissue layer stained positive for

extracellular DNA (PI), neutrophil elastase and citrullinated histone H3, and may, therefore, be considered NET-derived. Neutrophils undergoing different stages of NET-formation were observed between this shielding layer and viable tissue, which was also infiltrated by neutrophils [85]. A condensed layer of aggregated NETs thus spatially shields and isolates the site of necrosis, thereby limiting the spread of necrosis-associated proinflammatory mediators. We propose that necrotic debris may initiate and/or facilitate the formation of the NET-based surrogate barrier.

2C. The role of NET-formation in resolution of inflammation

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While there are numerous studies showing that NETs contribute to auto-immune inflammation and cause bystander tissue injury, an impairment of NET-formation can also be associated with exacerbation and/or chronification of autoimmunity and inflammation. Hence in a mouse model of gouty arthritis, depletion or genetic deficiency of neutrophils or impairment of NET-formation led to chronification of joint inflammation [82, 86]. Also in models of SLE and drug-induced lupus, mouse strains that cannot form NETs exhibited exacerbation of autoimmunity [87, 88]. The outcome of a deficiency of NET-formation in humans can be observed in individuals with chronic granulomatous disease (CGD) and Papillon-Lefèvre syndrome (PLS). In CGD ROS-dependent NET-formation is impaired due to mutations in the NADPH oxidase complex 2 [89]. Individuals with CGD suffer not only from recurring bacterial and fungal infections, but are also prone to develop autoimmune syndromes [90]. In PLS NET-formation is compromised by a mutated Cathepsin C that renders neutrophil serine proteases (NSP) inactive. Subjects with PLS are characterized by hyperactivation of neutrophils resulting in exaggerated and non-resolving inflammation, especially in the oral cavity and the skin [91]. Since these are not caused by an increased susceptibility towards bacterial infections [92, 93], other functions of NSPs than their antimicrobial action must promote regulation of inflammation [94].

As a mechanism for the anti-inflammatory effects of NETs, degradation of inflammatory mediators by NET-inherent proteinases was suggested [82, 86, 87, 95]. Thus, while in low neutrophil densities (e.g., in peripheral blood) the pro-inflammatory roles of NETs predominate, in high neutrophil densities (e.g., inflammation sites) the local removal of cytokines and chemokines by aggregated NETs works as a built-in safeguard to interrupt the self-amplifying loop of cell- activation and recruitment in neutrophilic inflammation [95, 96, 97].

2D. Neutrophil-derived proteases and cytokine processing

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Neutrophil serine proteases cathepsin G, elastase and proteinase-3 have classically been viewed as antimicrobial enzymes, eliminating invading pathogens during phagocytosis through degradation of the latter within phagolysosomes. Activated neutrophils are also well known to release their granule proteases extracellularly via degranulation and the formation of NETs. However, it is unclear what beneficial physiological role neutrophil proteases play in the extracellular space, as the excessive release of these enzymes has been linked with local tissue damage and inflammation in surrounding healthy tissue [98, 99, 100, 101]. However, accumulating evidence now suggests that neutrophil proteases play an important role in the processing of cytokines and chemokines to modulate and amplify inflammatory responses. Indeed, mice deficient in cathepsin G, elastase and proteinase-3 display robust protection against a range of inflammatory insults, including endotoxic shock [102, 103]. Neutrophils are rapidly mobilized to sterile inflammatory sites by endogenous factors released from damaged cells known as damage-associated molecular patterns (DAMPs). Although multiple putative DAMPs have been identified, the IL-1 cytokine family have been proposed to serve as the canonical DAMPs due to their ability to promote robust inflammatory responses from a wide range of cell types [104]. A key feature of IL-1 family cytokines is their requirement for N-terminal proteolytic processing to achieve their fully active state. Multiple studies have now demonstrated that neutrophil serine proteases,

released extracellularly at sites of infection or injury, modulate the activity state of multiple IL-1 cytokines and robustly enhance the activity of IL-1α, IL-33, IL-36α, IL-36β and IL-36γ [98, 105, 106, 107, 108]. NETs act a source of active proteases, increasing their local concentration by preventing their diffusion into surrounding tissues. Cathepsin G, elastase and proteinase-3 are externalised on NETs and can process and activate IL-1α and IL-36 cytokines, suggesting that NETs can serve as platforms for extracellular cytokine activation [109]. In addition, neutrophil-derived proteases modulate chemotaxis, inflammation and adaptive immunity by regulating the activity of other pro-inflammatory cytokines and chemokines including IL-8, CCL15, RANTES and TNFα, thereby exquisitely fine-tuning inflammatory responses [110, 111]. Thus, in addition to their classical role as antimicrobial phagocytes, neutrophils play a key role in amplifying inflammation through deployment of their granule proteases. Consequently, neutrophil proteases represent attractive therapeutic targets in autoimmune and inflammatory diseases, particularly those driven by IL-1 family cytokines.

2E. NETs in rheumatologic diseases

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In patients with rheumatologic diseases, there is evidence that NETs are responsive to key environmental triggers, serve as sources of autoantigen, perpetuate and amplify autoimmunity, and mediate organ damage. The role of NETs has been best characterized in systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), anti-neutrophil cytoplasmic antibody-associated vasculitis (AAV), and antiphospholipid syndrome (APS), all of which will be briefly discussed here.

Environmental exposures linked to rheumatologic diseases have been found to induce NET formation [112, 113]. Similarly, several drugs that are important inducers of autoimmunity cause robust NET formation and autoantibody responses to NET components [114, 115]. Proteins found within NETs represent some of the most important autoantigenic targets in rheumatologic diseases. These include double-stranded DNA and histones in SLE [116];

316 citrullinated vimentin, α-enolase, and histones in RA [18, 117]; and myeloperoxidase and
317 proteinase 3 in AAV [118]. While beta-2 glycoprotein I (APS) has yet to be demonstrated in
318 NETs, it is present on the surface of neutrophils and a well-established DNA-binding protein
319 [119, 120]. Several mechanisms contribute to perpetuation of autoimmunity by NETs. NET-
320 specific autoantibodies protect NETs from degradation and recruit complement components
321 to NETs [116, 121, 122, 123, 124], potentially amplifying their immunostimulatory potential.
322 NETs [20, 125, 126], and especially oxidized mitochondrial DNA [47], trigger cytokines such
323 as type I interferons that predispose to autoimmunity [127]. NETs also trigger inflammasome
324 activation [128]. While disease-specific autoantibodies directly trigger NET release [18, 47,
325 118, 120, 125, 126], rheumatologic diseases, and especially SLE, favor the emergence of an
326 inflammatory subset of neutrophils known as low-density granulocytes (LDGs) [20, 129].
327 LDGs have a significantly diminished threshold for NET release, with those NETs containing
328 abundant oxidized DNA [47]. Beyond SLE, LDGs have also been described in AAV and APS
329 [47, 130, 131].
330 A neutrophil signature predicts disease flares in SLE and AAV [130, 132]. Armed with
331 histones and granular enzymes, NETs have significant potential for toxicity. Evidence of *in*
332 *vivo* NET formation in humans has been documented in circulation [18, 47, 118, 120], and in
333 tissues such as skin (SLE) [20], kidneys (SLE, AAV) [20, 118], synovium (RA) [18], sputum
334 (RA and first-degree relatives of RA patients) [133], and thrombi (AAV) [134]. The impact of
335 NETs on the vasculature may be especially important. Examples include the MMP-
336 dependent toxicity toward endothelial cells by SLE NETs [20, 135], and the thrombin-
337 activating potential of AAV and APS NETs [120, 136]. In parallel, NETs may also modify
338 plasma lipids to make them proatherogenic [137].
339 Rheumatologic disease develops at the nexus of genetic predisposition and environmental
340 exposure, a complexity that is absent from available mouse models. The issue is further
341 complicated when neutrophils are the focus of study. Mouse neutrophils differ in quantity
342 (reduced numbers in peripheral blood) and quality (reduced myeloperoxidase and

defensins), as compared to their human counterparts [138, 139]. Activated neutrophil subsets such as LDGs have not been defined (and may not be present) in mice. In contrast, the role of suppressive subsets including myeloid-derived suppressor cells have been much easier to reveal (and could play a more important role) in mice [140]. Caution must therefore be exercised when interpreting mouse studies. In some SLE models, inhibition of PADs [141, 142], mitochondrial reactive oxygen species [47], HMGB1 [143], and CXCR2 [144] are protective, while deletion of NADPH oxidase and myeloperoxidase exacerbate SLE [88, 145, 146]; furthermore, PAD inhibition has not been protective in all SLE models [87, 147]. A better understanding of the factors required for SLE-specific NET release, as well as the role of neutrophils subsets in SLE, are required to resolve these discrepancies. In models of RA and AAV, NET-loaded synovial fibroblasts (RA) [21], and dendritic cells (AAV) [148], can trigger disease when transferred into mice. Pharmacologic blockade of PADs and PI3K-gamma interfere with NET release and kidney damage in models of AAV [149, 150]. In APS, transfer of human antibodies into mice triggers NET release and large-vein thrombosis [151], a phenotype that is dependent on neutrophil adhesion [152]. Going forward, mouse studies will surely remain an important part of the field, but we would emphasize the need for continued guidance by work with patient neutrophils.

2F. What we have learned about NETs from in vivo studies

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Data obtained *in vitro* (in a test tube) or *ex vivo* (in body fluids or unfixed tissue) often differ from results acquired from *in vivo* settings (within the living organism) due to simplification of the mimicked condition. These shortcomings include, but are not limited to, a lack of appropriate substrata to activate signaling pathways through specific adhesion molecules, a lack of intercellular contact of different cell populations, a lack of the full spectrum of released factors, anoxia/oxygen levels, a lack of plasma and all its constituents including DNAses and complement and a lack of shear due to blood flow [153]. In the particular case of studies on

NETs a significant amount of data differ between *in vivo* and *in vitro* results [153]. These limitations include the fact that NETs are three-dimensional complex structures which can cover significant areas of tissue or vasculature under flow conditions. Using intravital microscopy (IVM) to visualize biological processes (such as NET release) in blood and tissue accurately represents the actual *in vivo* situation. Despite its power, IVM is not widely used due to challenging surgery procedures and the necessity of high-tech microscopes. Nevertheless, whenever possible, intravital imaging of NETs should be applied to verify the significance of *in vitro* data in the complex, living organism. Importantly, the converse is also true, researchers utilizing IVM in mice should verify their *in vivo* observations to ensure events occur in human systems. In addition, *in vitro* allows for more effective examination of cellular events at super-resolution.

Among the parameters related to NETs which have been confirmed *in vitro* and *in vivo* are (i) the dependency on neutrophil elastase and PAD4 [14, 154], (ii) the ability of NETs to immobilize pathogens [14, 155], thus reducing dissemination to limit sepsis [155, 156], and (iii) the induction of NETs by bacteria, viruses and fungi and their immobilization by web-like structures [157, 158]. Despite these commonalities, a number of parameters related to NET formation and function lack agreement. Among these observations are (j) the ability of the neutrophil to stay alive versus dying after NET release with PMA [76], (jj) the requirement of NADPH oxidase activity and oxidants for NET release [154], (jjj) the amount of time (min vs. hours) required for NET release [154, 159, 160]. While (jj) and (jjj) are discussed in detail elsewhere, we will focus here on *in vivo* release of NETs, and the concept that neutrophils remain alive and continue to phagocytose pathogens while maintaining directional cell movement (chemotaxis) [76]. These cells were demonstrated to be intact and alive by their ability to exclude cell viability dyes *in vivo* [76, 159]. It makes intuitive sense that live neutrophils would make NETs in an organized manner without releasing their contents including bacteria and danger signals. Analyses of SEM images of single cells showed that neutrophils release these structures by a vesicular transport in that way preserving the

integrity of the plasma membrane [76, 160]. In contrast, most of the *in vitro* studies use PMA and report cell death, or even rupture of neutrophils during NET production. It is conceivable that neutrophils outside their natural environment are always perturbed, no matter how careful and gentle the handler is and no matter how much care is taken to reduce *in vitro* environmental artefact. Indeed, isolation of neutrophils on a coated coverslip in HBSS is quite different from a neutrophil in the vasculature adherent to endothelium under shear conditions in the presence of whole blood. One role for neutrophils is to detect environmental perturbations and as such it is not surprising that even under the most gentle of conditions, control neutrophils will take up some sytox green *in vitro*, an event never seen under control conditions *in vivo*. Importantly, some recent reports over the years suggest that some neutrophils release NETs *in vitro* but remain viable [161]. For example *S.aureus in vitro* seems to cause NET release independent of cell death [76, 160]. Moreover, NET release from mitochondria by neutrophils has been reported and is also a viable process [53]. These examples clearly demonstrate that when possible, the tandem *in vitro* and *in vivo* approach should be employed for NET studies.

Another phenomenon which could not be detected without intravital imaging *in vivo* is the actual impact of DNase on NETs. It is worth mentioning that *in vivo*, NETs under shear conditions anchor to the vessel wall and become immobilized [154]. In videos recorded in real time, one can observe that DNase very efficiently removes the DNA scaffold of anchored NETs, but not the protein components of NETs (elastase or histones) [154]. This inability to clear many of the protein components of NETs is a consequence of secondary anchoring of these proteins to endothelial von Willebrand factor (VWF). This finding contrasts *in vitro* observations in which DNase dissolves the NET structure [14], and without anchoring of NET proteins, the entire NET seems to disappear. It is worth noting though that DNases may unveil NET-associated proteases such as elastase or cathepsin G to their endogenous inhibitors. The proteases are normally sheltered and protected by the DNA itself and thus DNases might reduce tissue damage [162].

2G. NETs in ductal structures

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Neutrophils have the capacity to actively trespass epithelial layers from the basolateral to the apical side [163]. This may lead to the accumulation of neutrophils on epithelial surfaces including alveolar and bronchiolar lumen in the lung, the nasal sinuses, the gastrointestinal lumen, ducts of exocrine glands including the lactating breast, the prostate, the bladder, salivary, sebaceous and lacrimal glands and the biliary and pancreatic ducts. Cavities of the body can also be infiltrated by large amounts of neutrophils including the pleural, peritoneal, synovial or meningeal cavity. There is no circulation of blood in the cavities and ductal lumina of the body. Special conditions with regard to oxygenation, pH, bicarbonate-pCO2 levels, ionic and osmotic constitutions may, therefore, exist at various anatomic sites. These conditions strongly influence the function of neutrophil granulocytes [164]. On the other hand, the presence and function of neutrophils strongly alter the environmental conditions and impact neighboring epithelium: metabolic needs of neutrophils may lead to a reduction of glucose, an increase in lactate and a decrease in pH, while neutrophil oxidative burst may further reduce local oxygen saturation and induce hypoxic signaling in epithelial cells [165]. Epithelial cells are equipped to functionally interact with trespassing neutrophils and may upregulate adhesion molecules (e.g. ICAM-1) on the apical surface of the cells to guide neutrophil adhesion and function [163]. In samples derived from both mice and men, we have observed the presence of neutrophil aggregates inside the lumina of ductal structures in acute inflammatory attacks [166]. These neutrophil aggregates display intact neutrophils with segmented nuclei which are surrounded by amorphous material including extracellular DNA. Both nuclei and the adjacent extracellular DNA display citrullinated histones and granular proteins typical of NETs (MPO, neutrophil elastase). In our view, NET formation strongly contributes to the formation of these intraductal aggregates. Neutrophil aggregates on epithelial surfaces preferentially showed citrullination of extracellular and intracellular histones, while neutrophils inside the parenchymal tissues were rather H3cit-negative. These findings point to specific conditions within ductal structures, which facilitate PADI4 activity

and NET formation. Factors which contribute to NET formation may include activation of homotypic aggregation between neutrophils and neutrophil-epithelial adhesion molecule activation, as well as environmental factors within the specific epithelial lumen including the pH-bicarbonate-pCO_2-axis [167].

2H. Activated platelets entice neutrophils to generate NETs.

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A paroxysmal burst of interaction with formation of neutrophil/platelet circulating heterotypic aggregates contributes to intense vascular inflammation, including that occurring in sepsis, systemic autoimmune diseases, acute coronary syndromes and some neoplasms [168]. The mechanisms by which interacting platelets and neutrophils damage vessels are only partially known. However, activation of the platelet toll-like receptor 4 by LPS eventually results in neutrophils releasing NETs, which might amplify and sustain the vascular injury [14, 159]. Sterile stimuli causing platelet activation also commit neutrophils to generate NETs in static and flowing conditions, in the presence or absence of plasma and independently of the platelet agonist. Moreover NETs recruit and activate platelets [169, 170] possibly enforcing a self-sustaining vicious circle sustaining inflammation and tissue injury. NET formation induced by activated platelets abates in the presence of competitive antagonists of the prototypic alarmin HMGB1 or by using $Hmgb1^{-/-}$ platelets [171, 172]. Platelets indeed release HMGB1 upon activation [171, 173, 174] and RAGE, a well-characterized neutrophil receptor for HMGB1, mediates NETs formation caused by platelet-derived signals [171, 172]. The ability of platelet-derived HMGB1 to prompt neutrophil autophagy might be important to sustain the metabolic requirement associated to the process [171, 175]. HMGB1-expressing platelets are detectable along the NETs of human coronary thrombi, while deletion of platelet HMGB1 reduces/prevents deep vein thrombosis [172]. Platelet-derived disulfide HMGB1 facilitates the formation of NETs via RAGE eventually leading to obstructive venous thrombosis in the mouse [176] while the phagocytosis of activated platelets and of apoptotic cells dramatically reduce the ability of neutrophils to generate NETs [177, 178]. Thus

platelets might represent in physiological and pathological conditions a critical player tuning the sensitivity of neutrophils to inflammatory and thrombogenic stimuli and an interesting novel target for molecular intervention.

2I. The role of NETs in SLE

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SLE is a multisystem autoimmune disease in which loss of tolerance to nucleic acids and nucleoproteins results in rampant immune activation and end-organ damage[179]. NETs are postulated to be a primary and non-redundant source of antigenic nucleic acids in lupus [20, 57, 122, 125, 126, 180, 181]. This paradigm is challenged by murine studies in which classical NETs were abolished by genetically deleting essential components of the NADPH oxidase complex in the spontaneous MRL.Fas^{lpr} [88] and NZM.2328 [182] lupus mouse models or the pristane-induced lupus (PIL) system [87] NADPH oxidase-deficiency exacerbated multiple manifestations of SLE and immune activation [87, 88, 182], a finding that extends to human patients [145, 183, 184, 185, 186]. However, it is possible that global regulatory properties of NADPH oxidase and NET formation of nuclear or mitochondrial origin independent of NADPH oxidase confound these findings. Furthermore, inhibitors of PAD4, a distal mediator of NET formation [39, 41, 187], are reported to improve lupus and proliferative nephritis in murine models [142, 188, 189]. In contrast to these inhibitor studies, genetic deletion of PAD4 in the MRL.Fas^{lpr} model does not ameliorate any aspect of nephritis, loss of tolerance, or immune activation [147] Paralleling these observations, a pharmacological approach to inhibit PAD4 in both the anti-GBM model of proliferative nephritis and a human serum transfer model of SLE nephritis [190] had no effect on end-organ damage [147]. Intriguingly, PAD4-deficient mice subjected to the PIL model had elevated titers of antinuclear autoantibodies, inflammatory mediators, and exacerbated glomerulonephritis [87]. Collectively, these findings do not support a dominant role for NETs in SLE pathogenesis and should prompt a reevaluation of the concept that NETs promote autoimmunity.

2J. NETs in glomerulonephritis

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Several studies showed a deleterious role of NETs in glomerulonephritis. Murine models as well as human renal biopsies revealed the presence of NETs in multiple renal pathologies, including lupus nephritis [116, 191], anti-GBM nephritis [192], thrombotic microangiopathies [193] and ANCA-associated vasculitis [118]. An overlapping feature in these diseases appears to be the failure of circulating endonucleases to adequately degrade NETs [116, 122, 191, 194, 195]. In particular, the capacity of serum to degrade NETs *ex vivo* seems to correlates with renal function and disease activity in the aforementioned diseases [116, 122, 191]. In addition to an impaired degradation, the formation of NETs seems to be enhanced [122, 194, 195, 196]. Together, the imbalance between NET formation and NET degradation leads to a prolonged exposure of NETs to glomerular endothelial cells, thereby jeopardizing the integrity of the glomerular filtration barrier. Four mechanisms through which NETs could inflict glomerular damage have been proposed. The first mechanism is mediated by histones, the main constituents of NETs, which appear to be direct mediators of cell death of both podocytes and glomerular endothelial cells [197, 198]. Indeed, cytotoxic effects of extracellular histones have long been acknowledged [199]. The second mechanism involves neutrophil elastase, the main proteolytic enzyme within NETs [191]. Neutrophil elastase specifically cleaves the intercellular junction protein VE-cadherin, which impairs endothelial monolayer integrity and causes transendothelial albumin leakage. The third mechanism also involves neutrophil elastase, since the elastase-mediated cleavage of VE-cadherin induces β-catenin signaling to facilitate a process known as endothelial-to-mesenchymal transition. This endothelial-to-mesenchymal transition has previously been linked to (renal) fibrogenesis and may therefore explain observations that the inhibition of NET formation protects against age-related organ fibrosis [170]. A fourth mechanism of NET-mediated glomerular injury is mediated by the complement system, as NETs can activate both the classical and alternative pathway of the complement system [122, 124, 200]. Regardless the precise mechanism

through which NETs compromise glomerular integrity, restoring the balance between NET formation and NET degradation may hold the key to prevent NETs from damaging these pivotal organs.

2K. Integrin mediated NET formation and platelets

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In addition to being important elements in thrombosis and hemostasis, platelets have an important role in the inflammatory response. As platelets express toll-like receptors, they can also detect pathogens and can be activated by them. Activation of platelets leads to the secretion of chemokines, various cytokines, and growth factors stored within their granules, and the expression and activation of cell adhesion molecules that allows interaction with other immune cells, mainly neutrophils and monocytes. The interaction of activated platelets with neutrophils might induce the formation of neutrophil extracellular traps (NETs). NETs are formed by proteases, chromatin, and antimicrobial proteins, and their main function is to trap and kill fungi, virus, and bacteria, avoiding their dissemination. Besides microorganisms, NET formation might be triggered by pro-inflammatory molecules and platelets. During the interaction with platelets, neutrophils have to be simultaneously activated by integrin-mediated outside-in- and G-protein-coupled receptor (GPCR) signaling to induce NET formation [201]. Targeting NET components by DNAse1 application or neutrophil elastase-deficient mice protected mice from tissue damage, whereas DNase1-deficient mice had aggravated tissue damage. Therefore, the uncontrolled formation of NETs might exert tissue damage and has been involved in the pathophysiology of different diseases.

2L. NETs and regulation of inflammation in familial Mediterranean fever

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Familial Mediterranean fever (FMF) is a prototype IL-1β-mediated autoinflammatory disorder associated with mutations in the MEFV gene encoding the protein pyrin and characterized by inflammatory, self-limited, attacks often triggered by various stress factors [202]. The crucial role of neutrophils in FMF attacks through the release of NETs has been recently

571 demonstrated [203, 204]. Neutrophils during FMF attack spontaneously release NETs
572 decorated with bioactive IL-1β. These structures are able to stimulate the expression of IL-1β
573 by mononuclear cells, resulting in further propagation of IL-1β-mediated inflammation [203].
574 Concomitantly, NETs themselves can inhibit further NET generation, providing a homeostatic
575 regulatory mechanism that might be associated with the resolution of inflammation in FMF
576 [203]. Current studies have linked autophagy with pyrin function and NET-associated IL-1β
577 responses [203, 204, 205, 206, 207, 208]. Neutrophils from FMF patients in remission are
578 resistant to NET formation, which is correlated with low basal autophagy levels, while the
579 induction of autophagy primes neutrophils to release NETs [203, 204, 209]. In this context,
580 the "two-hit" model proposes that the inflammatory environment of FMF initially induces the
581 expression of IL-1β, while an additional autophagy-related stimulus enables NETs formation
582 and extracellular exposure of IL-1β via NETs [203, 204, 210]. To this end, transcriptome
583 analysis of neutrophils derived from FMF patients revealed the role of mTOR repressor
584 REDD1 as a key regulator of FMF attack, linking environmental stress with autophagy-
585 mediated NET formation and NET-driven IL-1β inflammation [204]. REDD1/NET
586 formation/IL-1β axis is also involved in the pathogenesis of other autoinflammatory disorders,
587 such as Still's disease and ulcerative colitis, promising novel diagnostic and therapeutic
588 options in autoinflammation [204, 205, 208, 210].

589
590 ***2M. NETs in periodontitis***
591 *Ljubomir Vitkov, Salzburg, Austria and Homburg, Germany*
592
593 Periodontitis is a bacterial inflammatory disease of the tooth supporting tissues characterised
594 by alveolar bone resorption. The disease progression culminates in tooth loosening and
595 subsequent tooth loss. Periodontitis develops on the basis of untreated gingivitis [211], which
596 is completely reversible. As in other mucosal infections, the host response to the bacteria in
597 periodontitis is characterised by the mucosal efflux of PMNs [212, 213]. The PMNs influx into
598 the crevice appears to be the first line of defence against dental biofilm bacteria [214]. The
599 crevicular PMNs barely phagocytose [215, 216, 217, 218], but abundantly form NETs [214,

218]. The main function of crevicular NETs appears to be the gingiva shielding and the evacuation of dental plaque pathogen-associated molecular patterns (PAMPs) out of the crevice. The inability to produce NETs, which occurs in the Papillon–Lefèvre syndrome and ELANE mutations, is concomitant with aggressive periodontitis and early tooth loss [93, 219, 220, 221]. Periodontitis is further sustained by the deepening of the crevice and the formation of gingival pockets obstructing the evacuation of PAMPs and damage-associated molecular patterns, which are responsible for the self-perpetuation of the inflammation. In cases with exaggerated NET production, NETs are unable to maintain periodontal health and bystander damages occur [222]. Lipopolysaccharide (LPS) injections into the rodent gum are sufficient to cause experimental periodontitis without additional bacterial challenge. Similarly, the excess of LPS and other PAMPs produced by the dental biofilm might contribute to exaggerated NET formation [223]. Additionally, the increased PMN responsiveness may underlie NET overproduction. The exaggerated crevicular NET production might be a consequence of the PMN hyperactivity in patients with periodontitis [224, 225, 226, 227]. Interestingly, PMN hyperactivity persists even following successful periodontal therapy [228]. These findings support the idea that NET dysregulation might be a key factor responsible for periodontitis.

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