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Neutrophils as Invigorated Targets in Rheumatic Diseases

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NEUTROPHIL BIOLOGY

Neutrophils are terminally differentiated innate immune cells with fundamental roles in host defense against microbes. The bone marrow (BM) is the primary site of neutrophil production and, under conditions of homeostasis, these cells can also be found in the lung, spleen and liver. Characteristically, neutrophils have a short half-life and constant production in the BM is needed to maintain a steady state(1). However, there is still significant uncertainty regarding neutrophil turnover in bone marrow and circulation under homeostatic and non-homeostatic conditions (reviewed in (2)). Neutrophil production is tightly controlled through synthesis of granulocyte-colony stimulating factor (G-CSF), in response to interleukin-17. Neutrophils express four categories of intracytoplasmic granules: primary (azurophilic, containing among many proteins myeloperoxidase (MPO), defensins, neutrophil elastase and membrane-bound-CD63); secondary (containing various molecules such as lactoferrin, LL-37 and membrane-bound CD66b); tertiary (loaded with gelatinase, cathepsin and various toxic proteins), and secretory vesicles that are considered an important store of membrane-bound receptors and express alkaline phosphatase and CD35 on their membranes(3) (Figure 1).

In circulation, neutrophils remain in resting state, ensuring that their toxic intracellular material is not accidentally released. Significant decreases in neutrophil numbers or in their function can lead to severe infections, while a very tight regulation of these cells is crucial to prevent tissue injury(4). Under inflammatory conditions, circulating neutrophils are recruited to tissues following a gradient, and their half-life may increase to support proper response to noxious stimuli(1, 3). Inflammatory tissue neutrophils share many of the functions of macrophages, including synthesis of cytokines and chemokines, phagocytosis and MHC class II-dependent antigen presentation. In tissues, neutrophils employ different mechanisms to destroy pathogens, including phagocytosis, degranulation of granular proteins, and a distinct form of cell death characterized by the extracellular extrusion of

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structures formed by intracellular proteins bound to a meshwork of chromatin and other nuclear material, named neutrophil extracellular traps (NETs)(5) (Figure 1).

Neutrophils are now recognized as major orchestrators of inflammation and as deeply influencing the phenotype and function of other innate and adaptive immune cells (Figure 1) (4). Uptake of apoptotic neutrophils by conventional myeloid dendritic cells (DCs) can enhance their antigen-presentation capabilities(6). Neutrophils synthesize cytokines fundamental for B-cell ontogeny such as B-cell activating factor (BAFF)(7) and a proliferation-inducing ligand (APRIL)(8). Splenic neutrophils can display T-cell independent B cell helper capabilities(9). Furthermore, neutrophils can both suppress and stimulate T cell responses, cross-prime CD8+ T cells in a MHC-class I-dependent manner and activate γδ T cells(10). Neutrophils promote T cell apoptosis through program-death ligand-1 (PD-L1)/PD-1 interactions(11), while their proteases can inactivate T-cell stimulating cytokines including IL-6 and IL-2(12). Neutrophils can downregulate the T cell receptor ζ chain, thereby promoting cell cycle arrest, and this appears to be triggered by ROS production and arginase synthesis (13). Recent evidence suggests that neutrophils can regulate humoral autoimmunity through a pathway that involves ROS generation and modulation of NK cells, leading to restriction of interferon (IFN)-γ production (14).

Neutrophils are much less understood than other immune cells with regards to development, plasticity, phenotypical and functional characteristics. This is due in part to inherent challenges in studying these cells. Their terminal differentiation status renders them resistant to genetic manipulation, and their very short half-life poses a survival challenge during freezing-thawing methods. As such, the study of neutrophils requires use of freshly isolated samples for accurate immune-phenotyping and functional assays. In addition, there are very few neutrophil cell lines and how well they represent primary cells is questionable. Importantly, mouse neutrophils differ in many important biological aspects from their human counterparts. This also limits the utility of mouse models of disease to understand neutrophil biology. Nevertheless, in part due to important technological advances, the last decade has seen a revival of interest in neutrophil biology in the context of autoimmunity and inflammatory diseases. This review will explore the role of neutrophil as important shapers of the immunologic landscape in systemic autoimmune and chronic inflammatory diseases.

a) NEUTROPHIL SUBSETS

Various attempts at characterizing neutrophil subsets based on phenotypic or functional analyses are being pursued (Table I). Clarification on whether phenotypic differences among neutrophils are genetically driven or secondary to transmigration, microbiome, or senescence is required(15). Similar to macrophages, some groups have proposed a N1 (inflammatory neutrophil) versus N2 (anti-inflammatory neutrophil) phenotype in models of cancer or injury(16, 17); however, the inducers and implications of polarized neutrophils remain superficially characterized. While olfactomedin 4 (OLFM4), a specific granular protein, is present in approximately 25% of neutrophils, the role of $OLFM4^{+}$ neutrophils remains unclear(18). CD177, a GPI-anchored neutrophil antigen that binds endothelial PECAM-1, is present in variable numbers of neutrophils and may be associated with

enhanced capacity to migrate across the endothelium; however, the specific role of this cell subset in homeostasis and disease is unknown(19). In sepsis models, $CD16^{dim}$ neutrophils have reduced ability to opsonize bacteria and generate ROS, while $CD16^{bright}$ neutrophils display enhanced antimicrobial and ROS activity(20). Also in sepsis, levels of CD62L in neutrophils correlate with suppressive capabilities (21). Another neutrophil subset that has received significant recent attention in autoimmunity is low-density granulocytes (LDGs) (22). These cells will be mentioned later in the text with regards to their potential role in autoimmune diseases. Importantly, a comprehensive algorithm to distinguish between suppressive neutrophil subsets and myeloid suppressor cells (MSCs) is needed, given that various cell surface markers are shared between these cells.

b) ROS GENERATION

ROS are key signaling molecules with important roles in the progression of inflammatory disorders in immunity-based pathways. ROS can function as anti-microbial effectors and as signaling molecules that regulate processes like nuclear factor-kB transcriptional activity, autophagy, and NET production. The widely studied ROS members are superoxide anion, hydrogen peroxide, hydroxyl radical, and hypochlorous acid. The main sources of ROS are mitochondria and nicotinamide adenine dinucleotide phosphate-oxidase (NADPH-oxidase, NOX). There are seven homologs of NOX (NOX1-5, Duox1 and 2) and this complex consists of a dimeric, enzymatic part (p22-phox, gp91-phox) and of three regulatory subunits: neutrophil cytosolic factor (Ncf, p47-phox), Ncf-2 (p67-phox), and Ncf-4 (p40-phox). Phosphorylation of Ncf-1 induces assembly of regulatory and catalytic subunits and activates NOX(23), which can mainly be found in plasma and phagosome membranes. In neutrophils, NOX2 has reported to play important roles in bacterial killing and NETosis. The absence of phagocytic NOX-dependent ROS production leads to the development of chronic granulomatous disease (CGD), an immunodeficiency characterized by increased susceptibility to bacterial and fungal infections but also a heightened inflammatory and pro-autoimmune state. This highlights that, while production of ROS has been connected with promotion of inflammation and tissue damage, it is also implicated in the regulation of inflammation and protection from autoimmunity. Evidence for the latter comes from the discovery that decreased ROS-production promotes severe chronic inflammation in animal models of arthritis and multiple sclerosis(24) and that lupus-prone mice that lack NOX2 have worsening autoimmunity, while genes that promote decreased NOX activity confer SLE susceptibility (23, 25, 26). Furthermore, the source of ROS may determine immunomodulatory versus immunostimulatory responses in neutrophils in lupus, as discussed below.

c) PHAGOCYTOSIS

Phagocytosis is essential for the innate immune response and a complex process crucial for pathogen control. Invading microorganisms are engulfed by neutrophils and macrophages, and subsequently killed. Phagocytosis is mediated by pattern recognition receptors (PRRs) including FcγRs and C-type lectins that recognize conserved microbial structures, such as LPS or fungal β-glucans, known as pathogen associated molecular patterns (PAMPs)(1). Neutrophil phagocytosis is very rapid, and is followed by fusion of the phagocytic vacuole with preformed neutrophil granules to form the phagolysosome, in which enzymes and toxic

peroxides digest the pathogen. These granules contain NOX subunits and hydrolytic enzymes(27). Neutrophils can phagocytose IgG-opsonized particles in less than 20 seconds, while macrophage phagocytosis is much slower. In contrast to macrophages, the phagosomal maturation in neutrophils depends on cytosolic calcium. However, the mechanism of phagocytosis is not a perfect process, as granules may fuse with the phagosome before it is completely incorporated. Consequently, cytolytic contents and oxidative products may get released and cause damage to adjacent cells and tissue injury.

d) NETosis

NET formation was originally described as a mechanism by which neutrophils trap, immobilize and subsequently kill microbes. During this process, activated neutrophils release their nuclear material loaded with granular enzymes, forming an extracellular web-like structure (Figure 2)(5). The formation of NETs leads in many instances to the death of the neutrophil through a distinct program called NETosis. Significant research in this area has indicated that, in addition to microbial stimuli, “sterile” inflammatory conditions such as platelets, immune complexes, cytokines, cholesterol and uric acid crystals and autoantibodies, can also induce NETs(28). NETosis requires ROS production via NOX and other sources. Recent evidence indicates that mitochondrial ROS is sufficient to induce NETs in the absence of functional NOX(23, 29). ROS synthesis leads to the activation of peptidylarginine deiminase-4 (PAD4) that, given its intranuclear localization, converts histone arginine residues to citrullines, thereby promoting nuclear histone decondensation and gene transcription(30, 31). During NETosis, neutrophil elastase acquires nuclear localization and plays an additional role in histone modifications and chromatin unfolding, with significant expansion of the nuclear diameter. In later stages, nuclear membrane disintegration leads to mixing of nuclear and cytoplasmic contents, followed by NET extrusion through cell membrane disruption and eventually lysis. Since the initial description of NETs, it has become apparent that this biological process has implications beyond response to microbes. The externalization of autoantigens during NETosis may have immunostimulatory roles in predisposed hosts, while NETs are now implicated in diverse disease processes such as cancer, fibrosis, thrombosis, atherosclerosis and wound healing (Table II)(32)((33).

NEUTROPHILS IN SYSTEMIC AUTOIMMUNITY

1. Systemic lupus erythematosus (SLE)

While a potential association between lupus and neutrophil biology was suggested decades ago, it has been only within the last ten years that a resurgence in the interest of these cells as shapers of immune dysregulation and inducers of organ damage has become evident(22). In the lupus BM, neutrophils are the main source of type I IFNs and may promote abnormalities in B cell ontogeny through enhanced synthesis of BLyS/BAFF(35). Significant evidence supports a plethora of phenotypic and functional neutrophil abnormalities in lupus, including neutropenia, impaired phagocytic capabilities and dysregulated oxidative activity(23).

SLE is characterized by the presence of LDGs that are typically isolated from the mononuclear cell layer following a peripheral blood density separation. While to this date there are no distinctive cell surface or epigenetic markers that differentiate LDGs from normal-dense neutrophils (NDGs)(22, 36), there are various transcriptomic and functional differences that suggest a potentially pathogenic role for LDGs. These cells are observed at higher levels in lupus subjects with blood vessel or cutaneous involvement, and longitudinal studies are needed to assess if levels of these cells predict any distinct outcome in patients affected by this disease(22). Upregulation of mRNAs encoding for granular proteins has been observed in LDGs, potentially indicating a more immature phenotype(37). Functionally, LDGs have a higher capacity to synthesize inflammatory cytokines and type I IFNs, a phenomenon that may contribute to lupus pathogenesis(22). *Ex vivo* analysis of LDGs indicates a significantly enhanced propensity to form NETs in the absence of added stimulation(37). Through enhanced NETosis and externalization of matrix-metalloproteinase-9 in these structures, LDGs have an increased capacity to induce endothelial cell apoptosis with deleterious effects to the vasculature(38). NETs can activate the NLRP3 inflammasome machinery in macrophages in a P2X7-dependent manner, leading to enhanced synthesis of active IL-1β and IL-18, and potential amplification of inflammatory systemic and organ-specific responses(39). The cathelicidin LL37 (externalized in NETs) triggers adaptive immune responses, and immune complexes (ICs) formed of LL37-anti-LL37 can activate plasmacytoid DCs to synthesize IFN-α, further promoting immune stimulation(40). Enhanced NETosis is not only observed in circulating LDGs, but also in various lupus organs including skin and kidneys(37).

Recent evidence indicates that NETosis in lupus LDGs is dependent on mitochondrial ROS, rather than other sources of ROS. Indeed, enhanced mitochondrial ROS production is observed in these cells and NETosis can be significantly inhibited in LDGs with mitochondrial ROS scavengers. Mitochondrial ROS lead to the generation of NETs that are more immunostimulatory than NETs generated by other stimuli, when induction of type I IFNs and inflammatory cytokines in target monocytic cells is used as outcome. An important factor that may enhance LDG NETs immunogenicity is the enhanced oxidation of nucleic acids(23),(41). Genomic or mitochondrial DNA oxidation decreases nucleic acid degradation and makes it more likely to be sensed by intracellular sensors such as STING, thereby promoting enhanced type I IFN responses. Mitochondrial ROS inhibition in lupus-prone mice promotes significant improvement in lupus phenotype, decreases in type I-IFN responses and immune dysregulation(23). Other strategies to decrease NETosis *in vivo* in lupus models have recently been investigated. As PAD-4 activity has been reported to play important role in NET formation, chemical inhibitors of PADs have been recently tested in murine lupus. These inhibitors significantly improved organ damage, decreased type I-IFN responses and ameliorated vasculopathy and prothrombotic phenotype, implicating that abrogation of NET formation through PAD inhibition should be further explored as a potential therapeutic strategy in this disease(32, 42, 43).

SLE sera displays a decreased ability to degrade NETs, either due to low DNase-I activity, the presence of DNase-I inhibitors or autoantibodies protecting NETs against degradation. Impaired NET clearance is associated with disease activity, complement activation, increased anti-dsDNA titers and a higher frequency of nephritis (44). This imbalance of

NET formation and degradation may then create a propensity for modified autoantigen persistence that, in a predisposed host, may stimulate autoimmune responses and tissue damage.

2. Rheumatoid Arthritis (RA)

Responses to citrullinated autoantigens are considered to be key steps in the pathogenesis of RA(45). While neutrophils are considered main effector cells in joint destruction in RA, their role in modified autoantigen generation and as local and systemic activators of innate and adaptive immune responses had been less well characterized. More recently, however, evidence indicates that NETosis may serve as an important source of citrullinated autoantigens in individuals predisposed to develop this disease. NETosis is enhanced in neutrophils present in RA blood, synovium and other tissues(46). Through PAD activation, NETosis may lead to citrullination not only of intracellular antigens such as histones, vimentin and enolase(46) but also to the release of active PAD isoforms that can citrullinate extracellular autoantigens(47). The RA microenvironment, including autoantibodies directed to citrullinated antigens, rheumatoid factor and cytokines (TNF, IL-17) is highly conducive to enhanced NETosis, generating a vicious cycle of enhanced modified autoantibody generation leading to inflammatory responses to further fuel NET formation(46). NETs are strong activators of inflammatory responses in RA synovial fibroblasts, and this may stimulate joint damage(46). Citrullinated histones generated during NETosis have been described as important autoantigens in RA and Felty's syndrome(48–50). Protein tyrosine phosphatase, non-receptor type 22 (lymphoid) (PTPN22) was described as an inhibitor of PAD-4 and therefore of protein citrullination(51). This observation adds a novel potential mechanism to understand the association between *PTPN22* polymorphisms and RA risk through enhancement of protein citrullination and spontaneous NETosis. Neutrophil PAD activation during complement and perforin activity may also play important roles in autoAg generation in RA(52). Conversely, neutrophil-derived microvesicles can enter cartilage and project the joint in inflammatory arthritis(53).

3. Primary APS

Thrombosis and pregnancy loss are key features in APS. Neutrophil effector functions are activated by antiphospholipid (APL) Abs and further boosted by microbial products(54). Mitochondrial dysfunction has been reported in APS neutrophils (55). A signaling cascade of tissue factor/factor VIIa/PAR2 promotes neutrophil activation and fetal death in experimental APS(56). An interaction between complement and neutrophils has been reported to play key roles in fetal demise in APS models(57). A LDG population was recently described in patients with primary APS(58). APS-related autoantibodies can enhance NETosis through ROS- and TLR4-dependent mechanisms(59). Impaired NET clearance has also been described in primary APS(60). The therapeutic implications of NETs and LDGs in thrombosis generation and pregnancy loss in this syndrome remain to be determined.

4. Anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV)

The discovery of ANCAs directed against specific neutrophil granular proteins and subsequent clinical association with AAV has generated several decades of research focused

upon the role neutrophils may play in the pathogenesis of small vessel vasculitis(61). A body of evidence suggests that ANCA are pathogenic and have an established diagnostic role in AAV. ANCA directed against neutrophil granular proteins MPO or proteinase-3 (PR3) are detectable in the majority of patients with AAV. *In vitro*, neutrophils primed by pro-inflammatory stimuli undergo activation of the p38 MAPK pathway and upregulation of MPO/PR3 to the surface membrane where they can directly interact with ANCA(62). Binding of ANCA to the neutrophil surface is achieved via Fc receptor stimulation or antigen cross-linking(63). This engagement triggers neutrophil activation and downstream pro-inflammatory cytokine synthesis, NETs' release, and degranulation, with subsequent toxicity to the local environment, including organ-specific damage to the microvasculature.

Dysregulated NETosis represents a relatively recent advancement in the understanding of disease pathogenesis in AAV. NETs are detected in active vasculitic kidney lesions and disease-associated thrombi in AAV and may contribute to vascular toxicity and the increased thrombo-embolic risk observed in these diseases(64). NETs may provide a source of modified autoantigens with causal immunogenic roles in ANCA formation. AAV patients display evidence of enhanced NETosis and delayed NET clearance; proteins commonly found within NET cargo include PR3 and MPO(65). ANCA antibodies can trigger NETosis(64), and immunization of NET-loaded DCs promotes ANCAs and vasculitis in mice(66). Alternative pathway complement activation has recently been implicated in AAV pathogenesis, and AAV serum can induce NETs that contain activating components of the alternative pathway(67). In a novel rodent model of drug-induced vasculitis, and impairment in NET clearance triggered by propylthiouracil-induced is associated with MPO-ANCA formation and pauci-immune glomerulonephritis(68).

A small proportion of AAV patients do not have detectable ANCA at any point during disease and ANCA is of questionable utility as a longitudinal biomarker to predict relapse, and can be detected in some disease-free individuals. Naturally occurring MPO-autoantibodies that occur in healthy individuals have epitope specificities that differ from those detected in AAV. (69). Indeed, pathogenic MPO-ANCA directed against immunodominant epitopes more strongly correlates with disease activity than total titers of MPO-ANCA. Some subjects with vasculitis previously labeled as ANCA-negative were found to have occult MPO-ANCA that is blocked from detection by a fragment of ceruloplasmin circulating in serum.

As such, despite the established role of ANCA as a diagnostic biomarker in AAV, a few lines of evidence call into question their direct role in disease pathogenesis and there is unmet need for biomarkers that reliably predict AAV clinical outcomes. Recently, mRNA levels of neutrophil granular proteins(70) and a LDG population present in AAV patients have been proposed as predictors of decreased response to standard of care treatment in AAV. A subset of resting neutrophils that express PR3 on the surface membrane(71), and assessment of epigenetic modifications in promoter regions of genes for MPO/PR3(72) have been proposed as potential neutrophil-related biomarker candidates in AAV.

NEUTROPHILS IN AUTOINFLAMMATION

The discovery and characterization of monogenic diseases that can affect neutrophil function has increased understanding of the relevance of innate immunity in the context of human disease. Given the known differences between human and rodent neutrophil biology, characterization of the genes and molecular pathways involved in human monogenic diseases represents a significant opportunity to discover novel aspects of neutrophil biology, especially since animal models of these diseases often do not replicate the human phenotype.

1. Monogenic Autoinflammatory Diseases

These are immunological disorders characterized by seemingly unprovoked episodes of fever and inflammation without evidence of autoantibodies or antigen-specific T cells. Implicit in this definition is the concept that these diseases are disorders of innate rather adaptive immunity. Like other myeloid cells, neutrophils possess a broad repertoire of pattern recognition receptors (PRRs) that can active innate immune responses. Given the inherent challenges of performing *in vitro* experiments using neutrophils, a comprehensive understanding of the nuances of PRRs in human neutrophils is lacking, and functional studies in the various autoinflammatory diseases are typically conducted in monocyte/macrophage cell lines. An activated neutrophil phenotype has been described in Familial Mediterranean Fever even during attack-free periods(73), and NETs can regulate IL-1β-mediated inflammation in this disease(74). Point mutations in inflammasome-related molecules NLRP3, NLRC4, and NLRP12 define a spectrum of autoinflammatory diseases responsive to IL-1 blocking therapies. While inflammation in these conditions is frequently attributed to IL-1β production by monocytes, neutrophils possess the functional machinery to potentially contribute to disease. Neutrophilic urticarial dermatosis is a defining histologic characteristic of neonatal-onset multisystem inflammatory disorder (NOMID). Chronic atypical neutrophilic dermatitis with lipodystrophy and elevated temperatures (CANDLE), a disease of immunoproteosome dysfunction, in in part characterized by immature neutrophils that infiltrate the dermal layer of affected skin(75). The functional significance of these neutrophils is currently unknown. Continued investigation into the specific roles of neutrophils in autoinflammatory diseases is likely to yield broad insight into neutrophil function.

2. Neutrophils and gout

The innate immune system plays crucial roles in the pathogenesis of gouty arthritis. Gout is triggered when uric acid, the final product of human purine metabolism, crystalizes in sodium-rich fluids into needle-shaped, pro-inflammatory monosodium-urate crystals (MSU). Activating the NLRP3-inflammasome, MSU induces inflammation in joints and tissues; following phagocytosis of MSU by monocytes, the endosomes fuse intracellularly with acidic lysosomes. The low pH in the phagolysosomes causes a massive release of sodium and consequently raises intracellular osmolarity, which is balanced by passive water influx through aquaporins. This process dilutes intracellular sodium and potassium concentrations. Low potassium reportedly activates NLRP3-inflammasomes that cleave pro- IL-1β to form active IL-1β (76). Inflammasome activation and cytokine release by MSU in resident

immune cells results in a rapid recruitment of neutrophils. To avoid a positive feedback loop of cell activation and mediator release, stringent control of these processes is required. An acute gouty attack is a self-limiting process, despite the presence of residual crystals during remission. In contrast to other forms of inflammatory diseases affecting the musculoskeletal system, gouty attacks symptoms decrease after few days. This indicates the presence of effective mechanisms to stop MSU-induced inflammation. Anti-inflammatory mediators like IL-10 or transforming growth factors-β or the switch from pro-inflammatory to anti-inflammatory macrophages have been discussed as possible reasons for the resolution of gouty inflammation(77). A recent study has further clarified the mechanisms implicated in beginning and the resolution of inflammation in gout (Figure 3). At the site of inflammation, attracted neutrophils ingest MSU, which triggers the formation of single NETs. During low concentrations of neutrophils, this process releases pro-inflammatory mediators, including IL-6 and tumor necrosis factor, as well as neutrophil attractants (e.g. CXCL-8) and activators (e.g. CCL3 and CXCL10). Consequently, more neutrophils are attracted. Recruitment proceeds until a critical concentration of neutrophils at the site of inflammation leads to the aggregation of the solitaire NETs and the formation of aggregated NETs (aggNETs). This happens at late stages of the gouty attack, when sufficient amounts of neutrophils have already been recruited. In aggNETs, the crystals are embedded in a microenvironment comprised of DNA and granular proteins. These aggregates resemble the crystalline core of tophi, commonly found in patients suffering from chronic gout. The aggNETs can trap, degrade and inactivate inflammatory mediators by serine proteases (e.g. neutrophil elastase, PR3), and thus orchestrate the resolution of the inflammatory process(78). Mice deficient in the oxidative burst develop chronic arthritis after injection of MSU into their footpads or air pouches. This is in striking contrast to the short self-limiting inflammation observed in wild-type mice, implicating ROS production as an important regulator of inflammation (78).

NEUTROPHILS AS THERAPEUTIC TARGETS IN AUTOIMMUNITY AND AUTOINFLAMMATION

Many disease-modifying antirheumatic drugs and biologics currently used to treat various rheumatologic diseases target primarily the adaptive immune system. While these treatments have dramatically improved clinical outcomes for patients, the high relapse rates that characterize most systemic rheumatologic illnesses highlight the need for medications that induce more durable remission. Dysregulation of the innate immune system is an increasingly recognized aspect of many systemic rheumatologic diseases including RA, SLE, and AAV. Improved understanding of the critical mechanisms that govern the roles of neutrophils across a range inflammatory responses may facilitate the development of novel therapeutics that target innate immune responses. Neutrophils play a crucial role in host defense; therefore, development of therapeutics that target neutrophils in autoimmune diseases may seem potentially unsafe. Identification of pathogenic subsets of neutrophils, such as LDGs, may facilitate the development of treatments that target selective neutrophil populations while preserving critical aspects of neutrophil-mediated host defense. The successful treatment of certain autoinflammatory diseases with therapies directed against specific aspects of the innate immune system has shown that such approaches can be feasible, safe, and efficacious. Increased understanding about neutrophil development and

function will be essential if neutrophil-targeted therapies are to become a viable therapeutic option.

Many medications commonly used to treat various inflammatory diseases have known effects on neutrophil function. Various studies have reported an anti-apoptotic effect of glucocorticoids on human neutrophils, promoting an increase in their demargination after steroid therapy and potential accentuation of neutrophilic inflammation(79) Various mechanisms for glucocorticoid mediated inhibition of neutrophil apoptosis have been proposed including upregulation of anti-apoptotic Bcl-2 family members, activation of NF-κB, suppression of components of the extrinsic pathway of apoptosis, and induction of inhibitor of apoptosis proteins (IAPs)(80) Colchicine has anti-inflammatory effects particularly targeting neutrophils. One of the mechanisms by which colchicine is anti-inflammatory is through its capacity to block microtubule assembly in neutrophils and other inflammatory cells, leading to diminished phagocytosis and decreased transport of pathogens to the lysosome(81). The anti-inflammatory influence of dapsone has been related to suppression of leukocyte chemotactic and cytotoxic functions. Dapsone suppresses the migration of neutrophils to extravascular sites through inhibition of adherence functions required for their recruitment(82). Neutropenia is a well-known side effect of cyclophosphamide but how this drug modulates granulocyte function and the weight on the efficacy of this medication related to neutrophil modulation remains unclear. Furthermore, how biologics that target IL-6, IL-17A, TNF, IL-1 and other proinflammatory pathways do so, in part, by modulation of aberrant neutrophil biology, remains to be better characterized.

Drugs that target key subcellular events in NET formation or clearance may have therapeutic benefit in specific diseases associated with enhanced NETosis. Several medications currently in clinical use, including hydroxychloroquine and cyclosporine A, are known to inhibit NETosis at physiologic concentrations(83, 84). Targeting critical steps in the pathway of NET formation, such as inhibition of PAD enzymes, or modulation of specific sources of ROS, can ameliorate disease in animal models of lupus(23, 42). Whether similar approaches would be efficacious and safe, in the treatment of human diseases remains to be determined. Of note, human KOs of PADs have been identified and do not appear to have significantly enhanced susceptibility to infections(85).

CONCLUSIONS

Neutrophils have emerged as primordial players in the pathogenesis of various systemic autoimmune diseases and autoinflammatory syndromes. Many groups have highlighted their role as sources of autoantigens, drivers of innate and adaptive immune responses and inducers of tissue damage and chronic complications of inflammatory diseases. Technological advances in systems biology, genomics, pharmacogenomics, live imaging, and many others will hopefully advance the field of neutrophil biology to better understand the heterogeneity of these cells and the precise roles that they plan in chronic inflammatory diseases and in maintenance of homeostasis so targeted effective approaches can be designed for the treatment of these devastating conditions.

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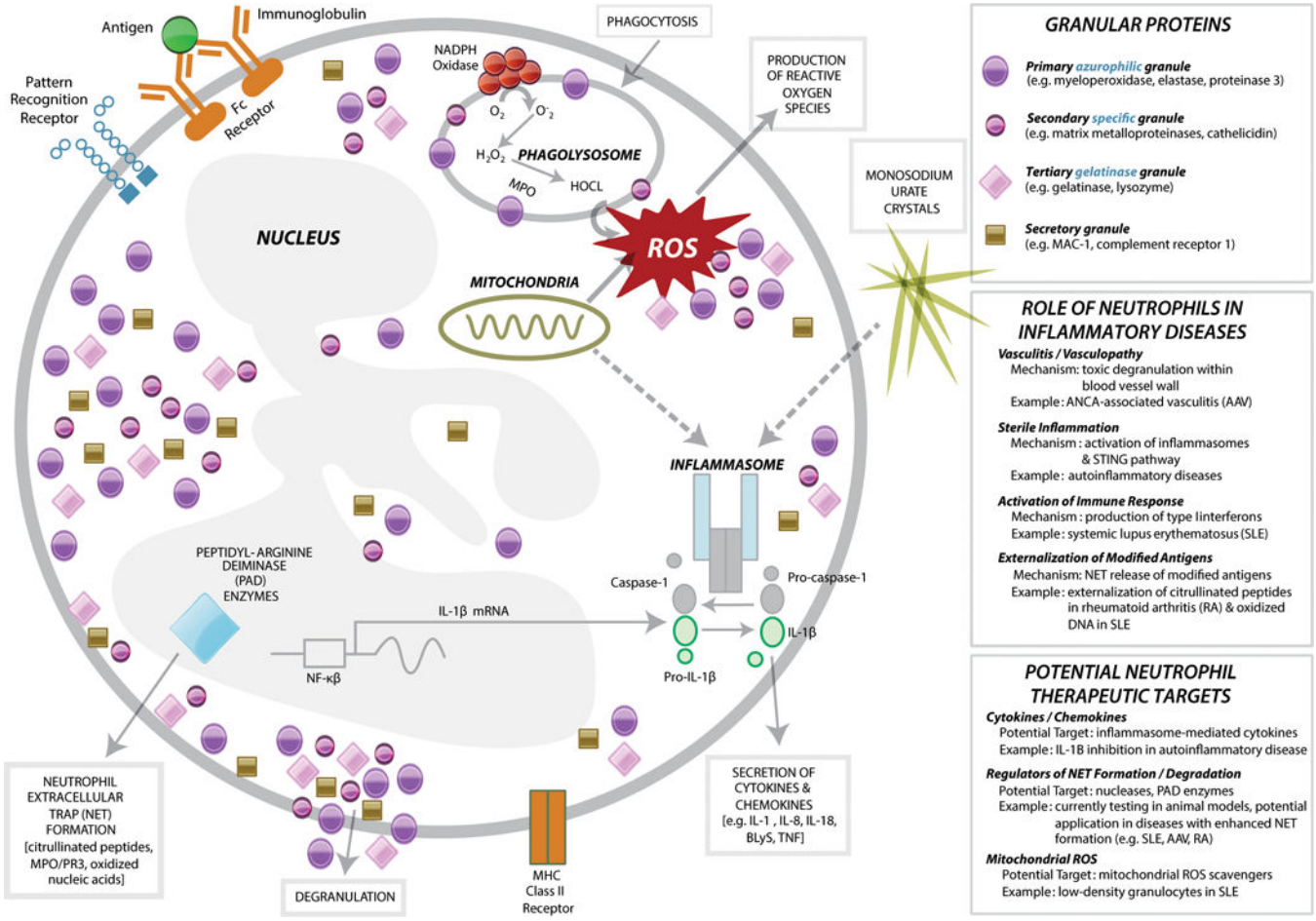

Figure 1. Characteristics of neutrophils

Neutrophils are multifunctional granulocytes that contribute to pathology across a spectrum of inflammatory diseases. Activation of neutrophils can occur through a variety of specific receptors including pattern recognition receptors and Fc receptors. Activation of the inflammasome by pathogens or danger signals, such as intracellular monosodium urate crystals, can lead to production of inflammatory cytokines including IL-1β. Secretion of cytokines/chemokines and antigen presentation by MHC Class II receptors can coordinate broader immune responses. Peptidylarginine deiminase enzymes contribute to the formation of neutrophil extracellular traps (NETs). NETs serve a variety of immunogenic and immunosuppressive functions in human disease, including externalization of modified antigens. Reactive oxygen species (ROS) are produced intracellularly through multiple mechanisms including the NADPH complex and mitochondria. Degranulation of the antimicrobial, cytoxic contents within various types of neutrophil granules can be toxic to the local tissue environment. Key regulators of neutrophil function may constitute novel therapeutic targets. Abbreviations: NF-KB= nuclear factor kappa-light-chain-enhancer of activated B cells; ASC = apoptosis-associated speck-like protein containing CARD; NLRP3 also known as cryopyrin or NALP3; MPO = myeloperoxidase; H202 = hydrogen peroxide.

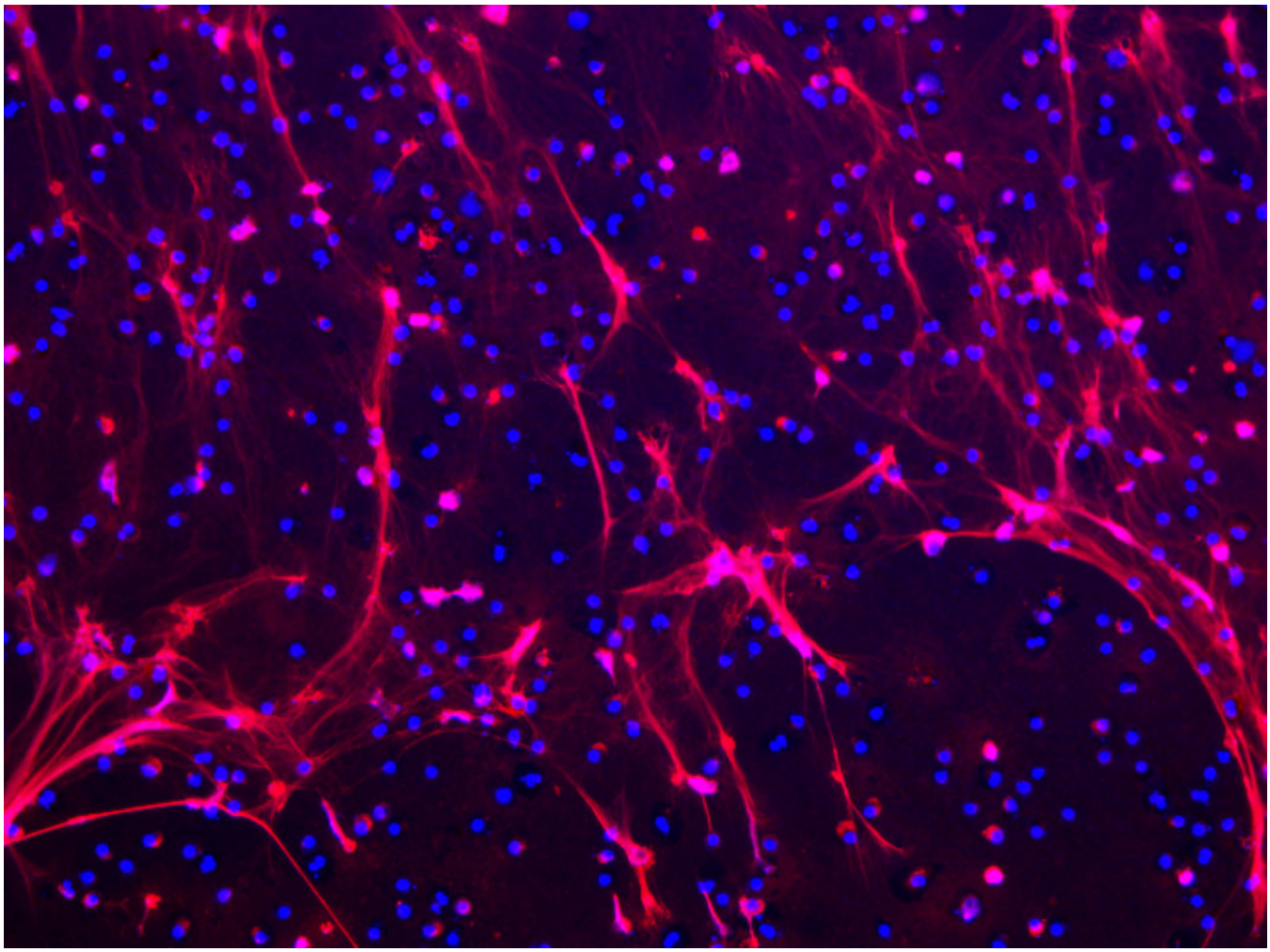

Figure 2. Neutrophil extracellular traps

Human control neutrophils were induced to undergo NET formation with LPS stimulation. Red represents MPO and blue represents DNA. Magnification is 40X. Photograph obtained by Dr. Carolyne Smith, Systemic Autoimmunity Branch, NIAMS/NIH.

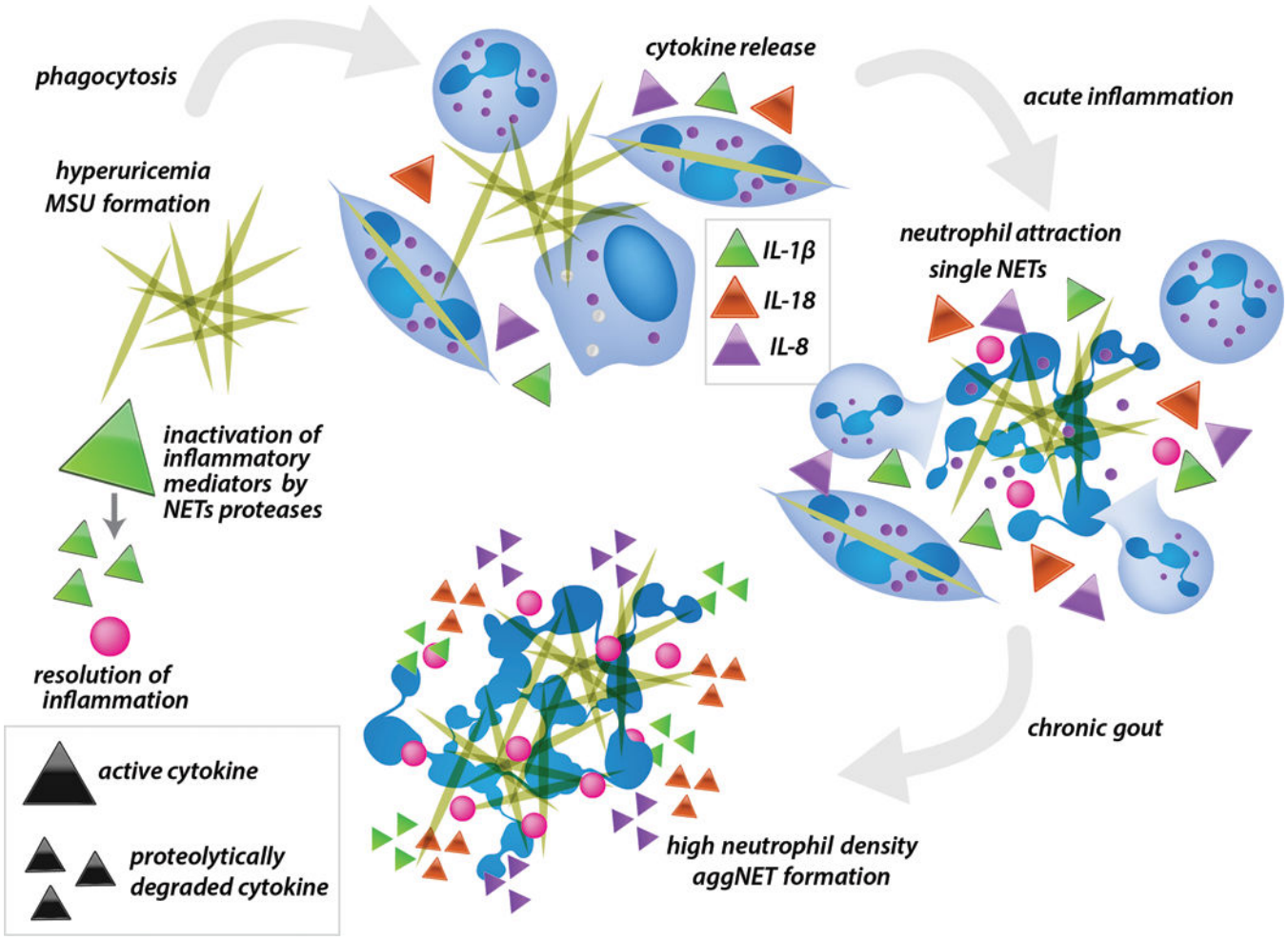

Figure 3. NETs in gouty arthritis

In areas of low neutrophil densities, individual netting neutrophils trap MSU-crystals in an inflammatory manner, releasing their load of pro-inflammatory mediators (acute inflammation). After the release of chemokines (e.g. IL-8) further neutrophils are attracted and the density of neutrophils rises. The NETting neutrophils clump together and form aggregates (aggNETs). These structures initially trap and finally degrade the inflammatory mediators and thus initiate the resolution of inflammation.

Table I

Neutrophil Subsets

Subset	Description	Functional Significance
N1	Tumor associated, hypersegmented neutrophils identified in association with factors in the tumor microenvironment	Pro-inflammatory neutrophil subset with antitumor cytotoxic effects.
N2	Tumor associated neutrophils seen in the majority of untreated tumors.	Anti-inflammatory neutrophil subset with tumorigenic effects.
OLFM4^{+}	Found in 25% of human peripheral blood neutrophils within specific granules.	Unknown
CD177^{+}	Present in the membrane of specific granules and the cell surface in 0–100% of neutrophils. The fraction of CD177^{+} neutrophils appears to be constant in each individual.	Binds to endothelial cell adhesion molecules and may facilitate migration across the endothelium. Predictor of relapse in ANCA-associated vasculitis.
CD16^{dim}/CD62L^{bright}	Immature, banded neutrophil identified in human experimental models of endotoxemia.	Reduced ability to opsonize bacteria and generate ROS.
CD16^{bright}/CD62L^{dim}	Hypersegmented neutrophil identified in human experimental models of endotoxemia.	Increased capacity to produce ROS and suppress T cell proliferation.
Low density granulocytes (LDGs)	Colocalize with mononuclear cells upon density gradient separation. Identified and characterized in various infectious and autoimmune diseases, notably systemic lupus erythematosus.	Pro-inflammatory neutrophil with enhanced propensity to form NETs

Table II

Relevance of Neutrophil Extracellular Traps in Clinical Disease

	SLE	RA	APS	AAV	Athero	Gout
Enhanced NET Formation	Yes	Yes	Yes	Yes	Yes	Yes
Delayed NET Clearance	Yes	?	Yes	Yes	?	?
Low-Density Granulocytes	Yes	?	Yes	Yes	?	?
Key Proteins Externalized During NETosis	dsDNA histones LL-37	Citrullinated peptides	DNA	MPO PR3	DNA LL37	AggNETs
Disease-Relevant Stimuli of NETosis	ICs AutoAbs	ACPA	aPL	ANCA	Cholesterol crystals	MSU crystals
Clinical Consequences of NETosis	Vasculopathy Immune dysregulation	Joint inflammation Immune dysregulation	Prothrombotic phenotype	Vasculitis	Vasculopathy Immune dysregulation	Promotes & resolves joint inflammation
Key References	22,23,37–44	46–51	58–60	64–68	32,34	78

NET = neutrophil extracellular trap; SLE = systemic lupus erythematosus; RA = rheumatoid arthritis; APS = antiphospholipid syndrome; AAV = ANCA-associated vasculitis; Athero = atherosclerosis; dsDNA = double stranded DNA; MPO = myeloperoxidase; PR3 = proteinase 3; AggNETs = aggregated NETs; IL = interleukin, IFNa = interferon alpha; ICs = immune complexes; ACPA = anti-citrullinated protein antibody; aPL = antiphospholipid antibody; ANCA = antineutrophil cytoplasmic antibody; MSU = monosodium urate.