J Mol Med (2015) 93:727–734
DOI 10.1007/s00109-015-1295-x

JMolMed

REVIEW

How neutrophil extracellular traps orchestrate the local immune response in gout

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Received: 24 March 2015 / Revised: 22 April 2015 / Accepted: 13 May 2015 / Published online: 24 May 2015

Abstract Neutrophil granulocytes possess a large arsenal of pro-inflammatory substances and mechanisms that empower them to drive local acute immune reactions to invading microorganisms or endogenous inflammatory triggers. The use of this armory needs to be tightly controlled to avoid chronic inflammation and collateral tissue damage. In gout, inflammation arises from precipitation of uric acid in the form of needle-shaped monosodium urate crystals. Inflammasome activation by these crystals in local immune cells results in a rapid and dramatic recruitment of neutrophils. This neutrophil influx is accompanied by the infamously intense clinical symptoms of inflammation during an acute gout attack. Neutrophilic inflammation however is equipped with a built-in safeguard; activated neutrophils form neutrophil extracellular traps (NETs). At the very high neutrophil densities that occur at the site of inflammation, NETs build aggregates that densely pack the monosodium urate (MSU) crystals and trap and degrade pro-inflammatory mediators by inherent proteases. Local removal of cytokines and chemokines by aggregated NETs explains how acute inflammation can stop in the consistent presence of the inflammatory trigger. Aggregated NETs resemble early stages of the typical large MSU deposits that constitute the pathognomonic structures of gout, tophi. Although tophi contribute to muscosceletal damage and mortality in patients with chronic gout, they can therefore be considered as a payoff that is necessary to silence the intense inflammatory response during acute gout.

Keywords Neutrophil extracellular traps · Gout · Resolution of inflammation · Tophus

Introduction

Neutrophil granulocytes constitute the most abundant leukocytes in the blood and are recruited in large numbers to entry sites of invading microorganisms or in response to other inflammatory stimuli. Neutrophils kill pathogens by either phagocytic uptake or degradation in intracellular vesicles or by the release of anti-microbial cytotoxic molecules from secretory granules. In 1996, a novel form of neutrophil suicide was described that takes several hours and comprises a stepwise progression of chromatin decondensation, nuclear swelling, spilling of the nucleoplasm in the cytoplasm, and membrane perforation [1]. In 2004, this phenomenon was identified as other anti-microbial mechanism neutrophils that are capable of the formation of neutrophil extracellular traps (NETs) [2]. In the process called NETosis, neutrophils release nuclear DNA into the extracellular milieu. During externalization, the DNA is decorated with anti-microbial peptides, granular enzymes, and DNA-associated proteins such as histones.

Citrullination of histones and the presence on chromatin of proteases normally confined to intracellular granules distinguishes NETosis from other forms of cell death. Citrullination and chromatin decondensation are mediated by the enzyme peptidylarginine deiminase 4 (PAD4) that is highly expressed in neutrophils. PAD4 removes positive charges from core histones by converting arginine residues to citrullines, thereby weakening the interaction between histones and DNA. Inhibition of PAD4 was reported to decrease NET formation and PAD4-deficient mice to display impaired NETosis [3, 4]. The neutrophil-specific serine proteases neutrophil elastase (NE)

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and myeloperoxidase (MPO) are involved in the later stages of NETosis. NE migrates from azurophilic granules to the nucleus where it degrades histones. This contributes to chromatin decondensation which is further enhanced by the binding of MPO to chromatin. Both enzymes seem to be essential for NETosis since NE knockout mice and MPO-deficient humans exhibit a reduced ability to produce NETs after stimulation with phorbol myristate acetate [5, 6]. The canonical NETosis pathway also depends on the production of reactive oxygen species (ROS) during the oxidative burst [7]. In particular, ROS are essential for the release of NE and MPO from azurophilic granules [5, 8].

More recently, a non-suicidal pathway of NET formation was described, where the cell remains intact and normal cellular functions of neutrophils, such as chemotaxis and phagocytosis, are still carried out [9, 10]. During this so-called vital NETosis, DNA is released by vesicular trafficking of the DNA from the nucleus to the extracellular space. This process occurs more quickly than an oxidative burst can be mounted and is therefore independent of ROS production [11].

Pro-inflammatory role of NETs in autoimmune diseases

Apart from invading microorganisms, also endogenous molecules trigger formation of NETs; pro-inflammatory cytokines and chemokines, activated platelets and endothelial cells, nitric oxide, monosodium urate (MSU) crystals, anti-neutrophil cytoplasmic antibodies, and immune complexes were all reported to induce NETosis [2, 12–17]. Since the discovery of NETs, there has been a renewed interest in the neutrophil as a potential driver of systemic autoimmune diseases. Despite the beneficial effects for host defense, NETs occur at the expense of tissue injury to the host, since NET constituents, especially histones, can cause bystander injury on endothelia [14, 18]. The tissue-damaging properties of NETs have particularly been observed in the lungs and the circulation in severe sepsis [19, 20]. In addition, excessive NETs in the vasculature system provide a scaffold and stimulus for deep vein thrombosis [21].

Apart from that, NETs provide a source of autoantigens in SLE, small vessel vasculitis, and other autoimmune diseases and are prime candidates for the initiation or enhancement of autoimmunity. For example, SLE patients produce autoantibodies against a range of nuclear antigens associated with NETs, including dsDNA, histones, and anti-microbial peptides [22, 23], and patients with defective NET degradation tend to have higher levels of anti-NET and anti-dsDNA autoantibodies and suffer renal damage [24]. Tight control of the formation and clearance of NETs is therefore crucial to prevent an autoimmune reaction and host damage.

Somewhat confusingly, however, it has recently been shown that decreased production of ROS is associated with a higher risk to develop lupus in humans and mice [25] [8]. Furthermore, human individuals that cannot produce ROS due to mutations in subunits of the NADPH oxidase 2 complex and are therefore unable to form NETs develop the clinical picture of chronic granulomatous disease which, apart from persistent infections, often also includes autoimmune disorders resembling SLE, Crohn's disease, and inflammatory arthritis [26–29]. Thus, the role of NETosis in the pathogenesis of autoimmune diseases is more complex than what meets the eye at first glance.

Aggregation of NETs for resolution of inflammation

Activated neutrophils at the site of inflammation release large amounts of pro-inflammatory mediators that attract and activate further granulocytes and other immune cells. To avoid a positive feedback loop of cell activation and mediator release, stringent control of these processes is required. This control was thought to be exerted mainly by induction of neutrophil apoptosis [30, 31] followed by phagocytosis by macrophages, leading to neutrophil clearance and release of anti-inflammatory cytokines such as TGF-β. However, the concept of the mere "fizzling out" of an ongoing neutrophilic inflammation was recently undermined by reports providing evidence for an active role of neutrophils in the resolution of inflammation; in a murine model of gout, we observed that the depletion of neutrophils led to chronification of joint inflammation [32]. In this study, we further noted that under conditions of high neutrophil densities, NETs induced by MSU crystals form larger aggregates. The size of these so-called aggNETs depends on the density of neutrophils in the culture and the amount of added MSU crystals but was not influenced by the addition of peripheral blood mononuclear cells. AggNETs trap and efficiently degrade neutrophil-derived cytokines and chemokines by inherent serine proteases.

NETosis and the aggregation of NETs after exposure to MSU crystals are dependent on the oxidative burst [32]. In the traditional view, ROS have been connected with promotion of inflammation and tissue damage, but in recent years, ROS have also been implicated in regulation of inflammation and protection from autoimmunity [25, 33–35]. Employing mice that are unable to mount an oxidative burst in phagocytes, we showed that lack of ROS results in exacerbation and chronification of MSU-triggered joint inflammation, suggesting that ROS production is crucial for the termination of inflammation elicited by MSU. We observed that the anti-inflammatory action of ROS is mediated by enabling formation of aggNETs that trap and degrade inflammatory mediators. In line with that, DNase I treatment, which leads to disassembly of aggNETs, also restored impaired neutrophil chemokine release.

In conclusion, aggNET formation helps to explain the spontaneous remission of acute inflammatory attacks in gout and might also be of high relevance for other forms of neutrophilic inflammation.

Local situation in gout

In gout, inflammatory flares are evoked by oversaturated concentrations of urate that precipitates in the tissue as needle-shaped MSU crystals. A dramatic neutrophil influx to the synovial fluid and synovium is the hallmark of acute gout, yet neutrophils are absent in the healthy joint. MSU crystals that are newly precipitated or shed from pre-existing MSU pools are therefore first encountered by cells of the synovial lining (type A synoviocytes, i.e., cells of the monocyte-macrophage lineage and fibroblast-like type B synoviocytes), dendritic cells (DC), possibly endothelial cells, and mast cells.

The negatively charged MSU crystals directly interact via electrostatic and hydrogen bonding with lipid membranes and signaling proteins (integrins, FcyR, CD16, CD14, phospholipases, or chemokine receptors) on the cell surface. MSU crystals are also taken up by these cells via phagocytosis, followed by lysosomal fusion, oxidative burst and synthesis of nitric oxide, and the release of inflammatory mediators [36, 37]. Crystal uptake disrupts the phagolysosome acidic compartment and leads to the release of cathepsin B [38]. Activation of the inflammasome is triggered by volume gain of the cells by water influx after massive release of sodium from acidified phagolysosomes. Water influx dilutes intracellular K^+ which turns on the inflammasome [39].

Inflammasome activation via MSU crystals may have evolved as a danger signal; upon cell injury, urate and ATP are released into the environment [40]. Locally, concentrations can exceed the solubility limits and crystals may form. Resident cells are then stimulated, with DCs providing a link to the adaptive immune response. The DC-activating properties of MSU depend on the interaction between crystals and membrane cholesterol on DC [41].

As a consequence of inflammasome activation, IL-1β is produced. In macrophages and neutrophils, neither inflammasome activation by MSU nor IL-1β processing are dependent on ROS in vivo, with ROS even negatively regulating caspase-1 activity and IL-1β secretion [42–45]. IL-1β production by macrophages, monocytes, and DCs is a key regulator of gout and stands at the beginning of the inflammatory cascade [46]. During later stages of inflammation, also neutrophils importantly contribute to IL-1β production [44, 47]. Interestingly, the release of IL-1β from neutrophils requires ROS. A recent report has shown that NADPH oxidase complexes are not necessarily the only source of ROS production during inflammasome activation. The NLRP3 inflammasome/IL-1β secretion axis is also susceptible to changes in mitochondrial and intracellular ROS, as well as to overall mitochondrial dysfunction [48].

Processing and release of IL-1β do not have to occur simultaneously, thus giving room to its release during the process of NETosis. Indeed, bioactive IL-1β has been detected on NETs released during inflammatory attacks of familial Mediterranean fever [49]. Moreover, IL-1β can also be created outside of the cell via mechanisms involving caspase-1 or independently of the inflammasome by cleavage of pro-IL-1β into its bioactive form by neutrophil serine protease 3 [50–52]. Results from mice suggest that this extracellular IL-1β production crucially contributes to the development of inflammatory arthritis.

Since IL-1β is the main trigger for neutrophil influx, the inflammasome is a crucial link between the causal stimulus of gout (MSU crystals) and the subsequent pathological hallmark of the acute attack.

Recruitment of neutrophils is further mediated by CXCR2, CXCL-8, CXCL-1, CXCL-2, and CXCL-3 [53], while CCL-2 recruits monocytes [54]. Blood neutrophils attach to the synovial vascular endothelium through selectins, exit into the synovial capillaries, and migrate through the synovial matrix to the joint cavity, guided by the chemokine concentration gradient.

Pro-inflammatory mediators at the inflammation site prime the neutrophil for an elevated oxidative burst and a higher degranulative response and prolong their survival by inhibiting apoptosis. At the site of inflammation, neutrophils also ingest MSU crystals which trigger NETosis and the release of inflammatory mediators, including TNFα and IL-6, as well as neutrophil attractants (e.g., CXCL-8) and activators (e.g., CCL3 and CSCL10) [32, 55, 56]. Due to the intense local inflammation, cytokines are produced in large quantities and also enter the circulation, resulting in an acute phase response that can trigger fever and leukocytosis.

Continuous recruitment of neutrophils to the site of inflammation results in very high neutrophil densities [57]. After the neutrophil concentration in the tissue exceeds a certain threshold, NETs begin to aggregate and build aggNETs in which the crystals are embedded in a mesh of DNA and proteins from neutrophil granules (Fig. 1). As such, these aggNETs resemble the crystalline core of tophi in patients during established gout [32].

MSU crystal-induced aggNET formation is augmented by release of ATP and lactoferrin from activated neutrophils. The release of ATP during NET formation is of high importance since extracellular nucleotides initiate anti-inflammatory clearance of dead cells by mononuclear phagocytes [58]. In addition, lactoferrin on NETs abrogates further recruitment of neutrophils and thus contributes to the anti-inflammatory action of NETosis in highly infiltrated tissues [59]. Both ATP and lactoferrin have an additional function in NET aggregation; with more and more neutrophils infiltrating to the site of inflammation, the local concentration of these mediators will

increase, gradually fostering aggNET formation. Indeed, addition of both mediators promoted aggNET formation also in low density cultures [32].

The tophus—good, bad, or just ugly?

Upon prolonged hyperuricemia, micro-aggregates of MSU crystals occur in all patients with gout, but in some, also large aggregates are present. In these tophi, the pathognomonic structures of gout, the MSU crystals are embedded in an amorphous whitish matrix surrounded by connective tissue and inflammatory cells such as mononuclear phagocytes, osteoclasts, mast cells, and even B and T cells [60]. Tophi are typically a late feature of gout but may also be present in early phases of the disease [61, 62]. Since only subcutaneous tophi are visually apparent, the dark figure of tophi is likely to be higher, and non-invasive detection methods such as dual energy computer tomography (DECT) may reveal additional tophi that cannot be picked up by physical examination.

Tophi can be regarded as a kind of granuloma in which the body tries to wall-off MSU crystals that it cannot eliminate. Deposition of crystals in tophi may continue for months or years without causing clinical symptoms. This silent buildup is however interrupted by occasional acute attacks that are accompanied by the typical signs of strong inflammation, swelling, redness, and pain. Subtle differences in the physical properties, including surface charge and size, coating

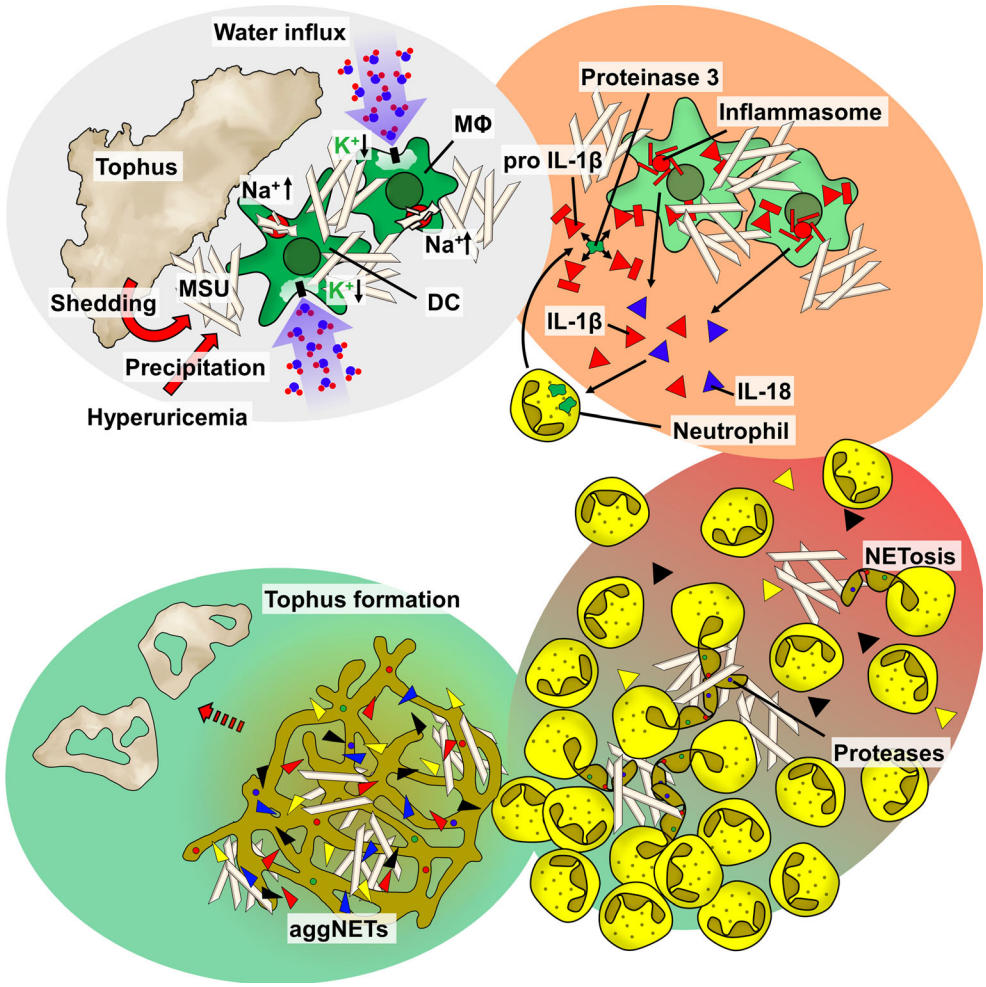

Fig. 1 Development and resolution of an acute gout flare. MSU crystals newly formed in situ or shed from pre-existing MSU deposits are taken up by mononuclear phagocytes. Sodium set free in large amounts from acidic phagolysosomes disturbs the osmotic balance of the cells. As a reaction, cells enhance the entry of water through aquaporins. Aside from the dilution of sodium in the cell, also potassium is diluted, which falls below the threshold for the activation of the inflammasome. As a consequence, large amounts of IL-1β are produced. IL-1β can also be produced in the extracellular milieu by the serine proteinase 3 that is released from activated neutrophils and cleaves pro-IL-1β. Bioactive IL-1β recruits neutrophils to the joint space that ingest MSU crystals and undergo a specific form of cell death known as NETosis. During low concentrations of neutrophils, this process releases pro-inflammatory mediators that recruit further cells. Recruitment proceeds until a critical concentration of neutrophils is reached at the inflammation site, and aggregation of NETs sets in. AggNETs then interrupt the inflammatory circle by degrading chemokines and pro-inflammatory cytokines by associated proteases, thus bringing NETosis to a halt and resolving inflammation. AggNETs also densely pack and wall-off MSU crystals and can mature into larger tophi

molecules, the local cytokine milieu, and the cells encountered first are important in determining the magnitude of the inflammatory response to MSU crystals. Crystals exceeding 20 μm may be too large to trigger an inflammatory response, and therefore, microcrystals need to be released from larger crystals or tophi. In support of that, gout flares are often associated with quick changes in urate concentrations (e.g., upon anti-hyperuricemic therapy [63]) and a rapid reduction might release microcrystals from the margins of tophi.

Crystals located within synovial tophi are usually coated with several proteins; IgG attaches through charge interaction and hydrogen bonding and encourages phagocytosis via FcyR. Opsonization of crystals with complement components either promotes inflammation directly or via FcyR signaling. As shown in rabbits, lack of complement components results in a reduced neutrophil inflammatory response to MSU [64]. However, crystals can also be coated with anti-inflammatory proteins, e.g., lysosomal enzymes, the anti-inflammatory cytokine TGF-β, and apolipoprotein E, a component of the LDL fraction in serum that inhibits the stimulation of neutrophils [65]. Progressive dissolution of tophi may thus expose uncoated crystal surfaces which further fuel inflammation.

Less differentiated monocytes show a higher pro-inflammatory mediator response to MSU crystals than macrophages despite equally efficient internalization [66]. Some mature phagocytes residing in the human synovium may be able to ingest MSU crystals without triggering inflammation, whereas the entry of fresh monocytes and neutrophils into the joint is likely eliciting an acute attack.

The observation that acute gout attacks often occur after abundant meals or alcohol consumption can be explained by the release of free fatty acids synergizing with MSU crystals to induce IL-1β release and to enhance inflammation [51, 67].

Aside from the different inflammatory potential of existing MSU crystals, their formation itself is influenced by the presence of particulate seed nuclei (e.g., cartilage debris, collagen, chondroitin, or hyaluronate released by joint trauma or arthritis), local cation concentrations, pH, temperature, and hydration. Damaged joints could thus serve as nidus for crystal formation.

The aggregated structures that form in MSU-stimulated neutrophil cultures at high cell densities show striking resemblance to the crystalline core of tophi; colocalization of extracellular DNA with material from neutrophil granules (neutrophil elastase, myeloperoxidase, anti-microbial peptides) shows that these structures are NETs interspersed with MSU crystals [32]. It is feasible that these aggNETs represent the initial stage of tophus formation and might ripe to the complex multilayered structure that can be found in patients with chronic gout. Tophus formation could therefore be a mechanism to intervene with the self-reinforcing loop of inflammation in gout and stop an acute flare that would otherwise result in dramatic tissue damage and bone remodeling. In line with that, tophi are a way to store MSU crystals in a comparably immunologically silent way. However, in the long run, also tophi cannot completely eliminate the burden of ongoing hyperuricemia.

Although tophi can be clinically silent for a long time, they are associated with radiographic findings [68]. Furthermore, an enhanced Doppler signal is present also in asymptomatic gout patients, indicating hidden or non-clinically apparent inflammation [69]. Tophi contribute to muscosceletal disability through direct contact of the bone with the crystals and through the action of osteoclasts in the peripheral corona zone of the tophus. In vitro, MSU crystals have catabolic effects on stromal cells of the joint, such as chondrocytes and osteoblasts [70, 71]. Typical for the damage caused by tophi are punched-out extramarginal lesions with preservation of the joint space and bone density [72]. Although tophi do not overtly correlate with serum urate levels, they contribute to the total urate pool and total urate pool correlates with flare frequency in long-standing tophaceous gout [61, 73, 74].

Established and novel treatment options of acute and chronic gout

Gout affects 1–2 % of adults in developed countries. Despite the high prevalence, treatment options have remained mainly static for half a century. Long-term treatment relies on reducing the serum urate levels well below the saturation point of MSU (6.8 mg/dl or 408 μM at 37 °C, [75, 76]), so that new crystals cannot form. Serial joint aspiration and DECT studies also confirmed the disappearance of already existing crystals with effective urate-lowering therapy [77, 78]. The xanthine oxidase inhibitor allopurinol has been the standard first-line drug for more than 50 years. During the last years, another xanthine oxidase inhibitor, febuxostat, has become a valuable alternative [79]. However, urate-lowering therapy often suffers from poor adherence. Only 25 % of patients attending general practice keep urate levels on target during follow-up [80], and the maintenance rate of therapy is low [81]. In addition, urate-lowering therapy involves the danger of "mobilization flares" in the first few months, resulting from shedding of microcrystals from pre-existing MSU deposits. An especially high incidence of acute attacks has been observed after initiation of treatment with the polyethylenglycol-coupled recombinant uricase pegloticase, which is used in severe cases of refractory gout and causes a very rapid fall in serum urate [82]. Prophylactic low-dose colchicine or NSAIDs is currently recommended to prevent such flares [83]. Oral colchicine, NSAIDs, and glucocorticoids are also used for the treatment of acute flares.

In the last years, anti-IL therapy has shown its potential for the treatment of acute gout [84]. Treatment with the monoclonal anti-IL-1β antibody canakinumab, the soluble decoy receptor rilonacept, or the IL-1 receptor antagonist anakinra can be important alternatives when NSAIDs, colchicine, or

glucocorticoids are contraindicated, which is frequently a problem in gout patients with renal and metabolic comorbidities [84, 85]. Moreover, blocking the IL-1 pathway can also result in the decrease of inflammasome activation in ROS-deficient individuals and therefore has important implications for the treatment of patients with chronic granulomatous disease [86].

Therapeutic intervention exploiting the mechanism of aggNET formation might involve shifting the balance towards resolution of inflammation by promoting aggregation of NETs during acute inflammation. This could be theoretically accomplished by local induction of the oxidative burst or application of the aggNET-accelerator lactoferrin, which triggered aggNET formation even in low cell densities in vitro. If this concept is feasible and how it can be implemented in vivo will be the subject of future studies.

Acknowledgements This work was supported by the doctoral training program GRK1660 and the priority program SPP 1468 Osteoimmunology IMMUNOBONE of the German Research Foundation (DFG) and the IMI funded research project BTCure.

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