



Neutrophil Extracellular Traps Formation and Aggregation Orchestrate Induction and Resolution of Sterile Crystal-Mediated Inflammation

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OPEN ACCESS

Edited by:

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INSERM U1098 Interactions
Hôte-Greffon-Tumeur
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Specialty section:

This article was submitted to
Inflammation,
a section of the journal
Frontiers in Immunology

Received: 30 March 2018

Accepted: 25 June 2018

Published: 06 July 2018

Citation:

Li Y, Cao X, Liu Y, Zhao Y and
Herrmann M (2018) Neutrophil
Extracellular Traps Formation
and Aggregation Orchestrate
Induction and Resolution of Sterile
Crystal-Mediated Inflammation.
Front. Immunol. 9:1559.
doi: 10.3389/fimmu.2018.01559

The formation of neutrophil extracellular traps (NETs) to immobilize pathogens represents a novel antimicrobial strategy of the immune system. The microcrystals related to human diseases are classified into endogenous microcrystals, including monosodium urate (MSU), calcium pyrophosphate dihydrate, calcium carbonate, calcium phosphate, calcium oxalate, cholesterol, and exogenous material like crystals from silica. Although microcrystals possess distinct compositions and shapes, they have a common characteristic: they stimulate neutrophils to release NETs. In low and high densities, neutrophils form NETs and aggregated NETs (aggNETs) that reportedly orchestrate the initiation and resolution of sterile crystal-mediated inflammation, respectively. Here, we summarize the different roles of NETs and aggNETs stimulated by the crystals mentioned above in related inflammatory reactions. The NETosis-derived products may represent a potential therapeutic target in crystal-mediated diseases.

Keywords: sterile crystal, inflammation, neutrophil, neutrophil extracellular traps, aggregated neutrophil extracellular traps

INTRODUCTION

Neutrophils are the most abundant circulated leukocytes in blood and represent the first line of innate immune system to defense against the injury or infection, including bacteria, fungi, and protozoa. In addition to their classical functions like phagocytosis and degranulation, neutrophils are endowed with a novel antimicrobial strategy. They are able to entrap and kill pathogens by extruding their nucleohistone network into the extracellular space, known as neutrophil extracellular traps (NETs) (1). NETs formation can be induced by various kinds of stimuli, such as specific microbes, sterile inflammatory mediators like lipopolysaccharides (LPS), phorbol-12-myristate-13-acetate (PMA), IL-8, ionomycin (2) or cytokines (3), immune complexes, activated platelets (4), particulate matter, and microcrystals (5). Under certain circumstances NETs formation is accompanied by a unique cell death program referred to as NETosis. The latter is distinct from apoptosis and necrosis (1, 6). During NETosis, the chromatin decondenses, the membranes of nucleus and granules disintegrate to generate chromatin blended with nuclear and granular proteins and enzymes. Following the rupture of the cellular membrane, chromatin is decorated with a plethora of internal molecules, including histones, neutrophil elastase (NE), myeloperoxidase (MPO), proteinase-3 (PR3), lactoferrin, cathepsin G,

matrix metalloprotease 9, peptidoglycan-recognition proteins, high mobility group protein B1, pentraxin, LL-37, and the bactericidal/permeability-increasing protein (1, 6). These contents are then expelled into the extracellular environment (1, 7–10). However, several aspects of NETs formation still remain elusive. In some forms of NETs formation the production of reactive oxygen species (ROS) by NADPH oxidase or mitochondria emerged to play an integral role; ROS helps to translocate the granular proteins MPO and NE into the nucleus (6, 7). Meanwhile, a number of molecules have been identified contributing to NETs formation, including peptidyl arginine deiminase 4 (PADI4), TGF- β -activated kinase 1, intracellular Ca^{2+} , and RAF/MEK/ERK (11). Due to the complicated structure and cytotoxic enzymes, NETs have been implicated in orchestrating the local immune response *via* eliminating pathogens, releasing pro-inflammatory mediators, and damaging tissue directly (12–18). In the pathogenesis of autoimmune inflammatory diseases, such as vasculitis, RA, and SLE they may serve as source of neoantigens that trigger the production of autoantibodies. A number of studies have suggested that NETs are involved in trapping microcrystals when the human body is exposed to crystals from monosodium urate (MSU), calcium pyrophosphate dihydrate (CPP), cholesterol, calcium carbonate (CaCO_3), calcium phosphate (CaP), calcium oxalate (CaOx), or silica under conditions of sterile inflammation (19). The latent role of NETs in crystal-induced inflammation is either to induce local necroinflammation or to perform a state of alleviating inflammation, just like acute or chronic gout, respectively (20). Herein, we review the current state of knowledge regarding NETs formation and aggregation in sterile inflammation induced by different sizes and shapes of microcrystals.

MONOSODIUM URATE CRYSTALS

Crystals of MSU monohydrate ($\text{NaC}_5\text{H}_3\text{N}_4\text{O}_3\cdot\text{H}_2\text{O}$) are macroscopically needle-shaped (21, 22). Their deposition in joints and soft tissues can cause an acute, inflammatory joint disease, usually referred to as gouty arthritis (23). In joints, MSU crystals induce the secretion of cytokines and chemokines by phagocytes, including interleukin (IL)-1 β , tumor necrosis factor (TNF), IL-6, and IL-8 (24). These inflammatory mediators are crucial to amplify inflammation by the recruitment into the joint synovial fluid of further neutrophils and monocytes (24). MSU crystals are supposed to activate neutrophils to release cytokines and induce infiltration of further neutrophils to form NETs (25, 26) leading to acute, extremely painful, and tissue-damaging inflammation in joints.

The NET release from neutrophils induced by MSU crystals is a complex yet highly coordinated sequence of events. To facilitate this, a lot of signaling pathways have been evolved. It was reported that NADPH oxidase-mediated ROS production is required for NETs formation evoked by MSU crystals (19, 27). Neutrophils from patients with chronic granulomatous disease (CGD) or NADPH oxidase-deficient mice displayed weak NETs formation in response to MSU (19, 27). *In vitro*, neutrophils treated with various anti-oxidants, lack ROS production and NETs formation in response to MSU crystals (19, 27). Furthermore, NETs formation induced by soluble uric acid is mediated by NF- κ B activation

is independent of ROS production (28). Unexpectedly, there is report that MSU crystals induce NETosis in an NADPH oxidase-independent fashion distinct from PMA-induced NETosis (29). Also, autophagy, necroptosis, RIPK1-RIPK3-MLKL signaling, and endosomal acidification have emerged as key regulators of MSU crystal-induced NETs formation (20, 30). The stress-related protein REDD1 which expressed in neutrophils is regulated in development and DNA damage responses. At the attack phase of familial Mediterranean fever (FMF), upregulated REDD1 promote autophagy and augment NETs formation (31). Consistently, during remission phases of FMF, the transcription of REDD1 is impaired and resistant to autophagy-mediated NETs release (31). Specifically, blocking phosphatidylinositol 3-kinase signaling or phagolysosome fusion prevents MSU crystal-induced NETs formation (26, 30). Result from RIPK3 knockout murine demonstrated that neutrophils deficient in RIPK3 are unable to release NETs in response to MSU crystal *in vitro* or *in vivo* (26). By contrast, other studies showed that PMA stimulates NETs release independent of RIPK 3 and MLKL signaling (32). Studies have described substantial effects of the purinergic receptors, P2Y and P2Y6 on MSU crystal-induced NETs formation (33). MRS2578, an inhibitor of P2Y6, reportedly restrained neutrophil migration and production of ROS as shown by live cell imaging. This suggests that purinergic receptors are involved in NETs formation (33). Interestingly, SK&F96365 inhibited MSU crystal-induced NETs formation by affecting a store-operated calcium entry channel (33). Neutrophils and phagocytes internalize small urate micro-aggregates (UMA; $<1\mu\text{m}$ in size) in the circulation and thus suppress MSU crystals formation (34). Gradually, mass of urate micro-aggregates exceeds the phagocytic ability of neutrophils leading to MSU crystals formation. Consequently, neutrophils are frustrated in phagocytosis and generate NETs (34, 35).

The NETs formation in response to MSU crystals is a complex process modulated by a plethora of factors, including inflammatory cytokines. In a MSU crystal-induced arthritis model has been shown that IL-1 inhibition is effective to control MSU-mediated inflammation (36). Neutrophil exhibit an enhanced NETs release in response to synovial fluid from gout patients, partially hindered by the IL-1 β antagonist anakinra (30) and IL-1 β accelerates NETs formation triggered by MSU crystals (37).

Interestingly, IL-1 β alone does not stimulate NETs release (37) and positive feedback loops are crucial for NETs formation. In joints NETs release dangerous neutrophilic cargoes like histones and granule proteins like myeloperoxidase (MPO) and NE (1) further amplifying MSU crystal-induced inflammation. In addition, NE cleaves pro-IL-1 β into its bioactive IL-1 β and IL-1 β is a crucial cytokine of the inflammatory response in gout. It recruits neutrophils to joints and enhances NETs formation triggered by MSU crystals (37–39). Apart from that, DAMPs released from NETs, such as DNA-activating Toll-like receptors or NLRP3 inflammasomes can augment inflammatory responses (40). MSU crystals induce NETosis through neutrophils in patients with gout. NETosis is further enhanced by impaired NETs degradation result from low DNase-1 activity in synovial fluids, in conjunction with enriched actin that is resistant to DNase degradation (29, 40). The aberrant accumulation of aggregated NETs (aggNETs) is associated with NETs clearance deficiency and formation of

extended NETs-crystal aggregates (40). In high neutrophil densities, NETs will agglomerate and form aggNETs (19).

It has been shown that aggNETs degrade pro-inflammatory chemokines and cytokines and suppress crystal-induced inflammation (19) *in vitro* and *in vivo*. Animal studies suggest an important anti-inflammatory role of aggNETs in the regulation of cytokines like TNF- α , IL-1 β and IL-6, and chemokines, such as chemokine ligand CCL2 and monocyte chemoattractant protein-1 (19). Collectively, these findings suggest that aggNETs promote the resolution of acute gouty arthritis (19). Despite its important role in the resolution of inflammation, little is known about the regulation of aggNETs formation. It has been reported that MSU crystals induced aggNETs formation depends on the production of ROS (19). *In vitro*, neutrophils of patients with CGD co-cultured with MSU crystals show reduced formation of aggNETs (19). *In vivo*, NADPH oxidase-deficient mice reduced the formation of aggNETs, when stimulated with MSU, both in an air pouch model, and in MSU crystal-induced paw inflammation (19). Accordingly, the authors also reported that ATP, lactoferrin (19), IL-1 β (37) and the P2Y6 receptor antagonist MRS2578 (33) enhance and inhibit MSU crystal-induced aggNETs formation, respectively (Figure 1).

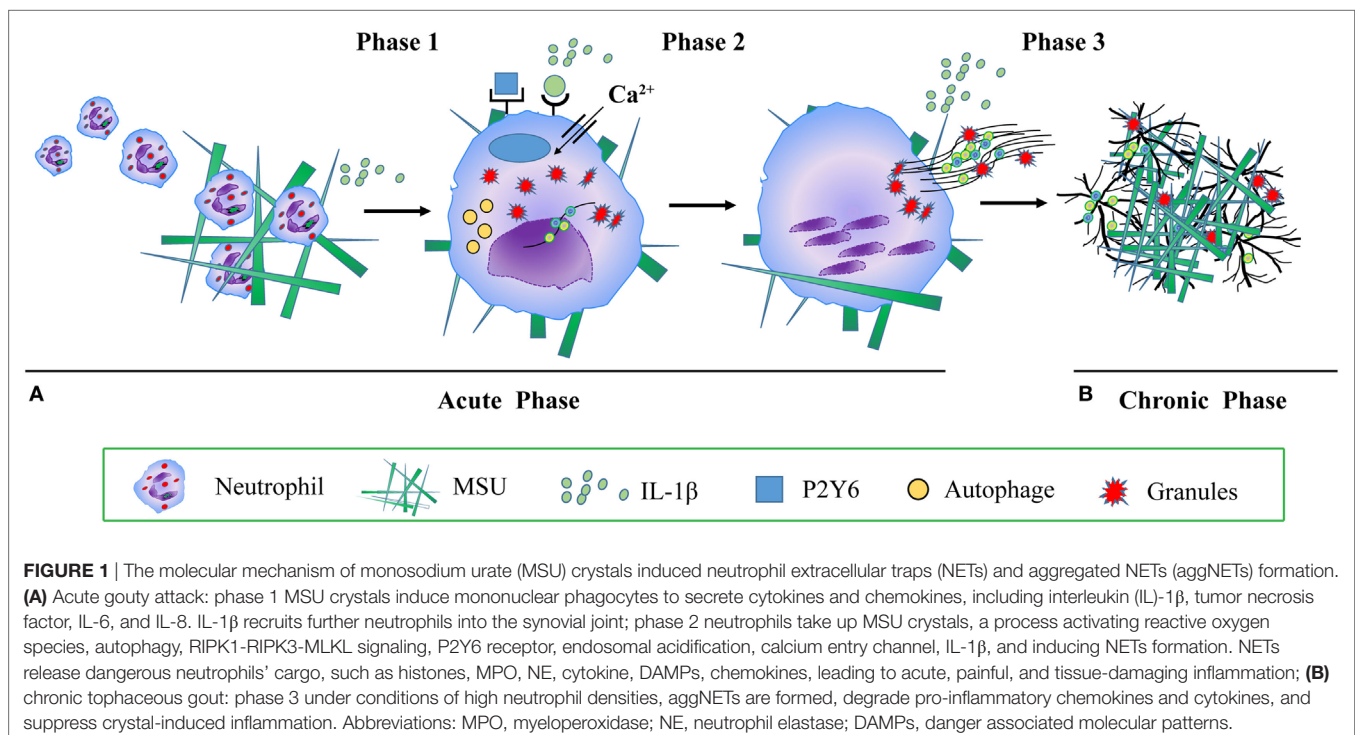
CALCIUM PYROPHOSPHATE DIHYDRATE CRYSTALS

Calcium pyrophosphate dihydrate (CCP) crystals display a rhomboid or elongated shape, and are generally shorter than 10 μm in length (41). They tend to deposit in joints and causes pseudogout, characterized by periodic acute joint flares (42). CPP crystals directly interact with and activate macrophages to release IL-1 β ,

which attract more neutrophils to the inflamed joints (43–46). Neutrophils phagocytosis of CPP crystals resulted in the release of NETs composed of extracellular DNA colocalizing with citrullinated histone H4 and myeloperoxidase (MPO) (47). CPP crystals elicited NETs faster and more efficiently than PMA (47). Going along with nuclear DNA morphological changes, neutrophils execute an NADPH oxidase-independent process of NETs release (47). The activity of the ERK/MEK signaling pathway, heat shock protein 90, and PI3K are essential for NETs formation triggered by CPP crystals (47). An intact cytoskeleton is required for CPP crystal-promoted NETs extrusion (47) and the release of IL-1 β , IL-8, TNF- α , and GM-CSF from macrophages and neutrophils are important driving forces that promote NETs formation (47). In turn, NETs release ROS, MPO, DNA, IL-1 β , and IL-8, and thus further activate inflammation, which can damage the joints (47).

CHOLESTEROL CRYSTALS

Cholesterol is a lipid of endogenous or environmental origin. When the temperature in plasma is under 37°C, circulating cholesterol will form monohydrate cholesterol crystals which foster the formation of atherosclerotic plaques (48). In addition to the crystallization of cholesterol in the circulation the release of IL-1 β may activate the IL-1 receptors (IL-1R) on the surface of neutrophils to further enhance the migration of neutrophils into the site of atherosclerotic lesions (49–53). The first observation that cholesterol crystals trigger NETs formation reported that translocation of ROS, NE, and PR3 into the nucleus are required for cholesterol crystals to induce NETs release (54). Interestingly, inhibiting peptidyl arginine deiminase enzymes did not affect NETs formation triggered by cholesterol crystals (54), whereas



NE and PR3-deficient mice do not form NETs in an atherosclerosis model and exhibited a reduced plaque size relative to controls (54). NETs augment the transcription of pro-inflammatory cytokines. In co-cultures with cholesterol crystals, NETs can enhance the cytokines released from macrophage *via* the IL-1/IL-17 loop (54). NETs and cholesterol crystals can also activate Th17 cells to sustain a chronic sterile inflammation (54). Overall, NETs release modulated the inflammation in atherosclerotic plaques.

CALCIUM CARBONATE CRYSTALS

Calcium carbonate—a white and tasteless microcrystalline powder—is generally less than 1 μm in diameter (54) and possess different shapes encompassing layered, rhombohedral, irregular, needlelike, spherulitic, or cubelike shape according to various polymorphic crystalline phases (55). As the component of pancreatic juice, calcium carbonate crystals can induce NETs and aggregate NETs formation (56, 57). Ductal occlusion is a main cause in the pathogenesis of pancreatitis; however, the mechanism of action remains elusive. Samples of histological tissue sections and pancreatic juice from patients with pancreatitis show aggNETs, as a specific occluding agent that agglomerate in the pancreatic ducts (58). Further studies confirmed that bicarbonate ions and calcium carbonate crystals can both induce aggNETs formation in the ductal tree during pancreatitis *via* a PADI4-dependent signaling pathway (58, 59).

SILICA CRYSTALS

Silica crystals, also termed crystalline silica or silicon dioxide (SiO_2), are usually harmless and non-toxic crystals; however, inhalation of silica can cause pulmonary inflammation (58). Prolonged exposure to silica crystals, especially for coal-miners and smokers, confers a great risk to develop pneumoconiosis silicosis, a chronic, progressive, irreversible, and incurable disease characterized by pulmonary fibrosis. Therefore, the understanding of the mechanisms of silica-induced pulmonary fibrosis is indispensably. After the interaction with silica, alveolar macrophages and endothelial cells (60) ingest silica, activate the NALP3 inflammasome (61), produce ROS and lead to phagolysosomal damage (60). Then massive chemokines and pro-inflammatory cytokines like IL-1 β and TNF- α are generated, neutrophils are recruited to lung tissue and assist in the clearance of the silica crystals (60). In addition to phagocytic clearance, neutrophils undergo NETosis (60, 62–64) is an important factor in the establishment of the lung disease (65).

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At onset of the diseases, NETs are scarce and release numerous pro-inflammatory cytokines and cytotoxic contents which promote the acute inflammation (20). Along with the disease progression, aggNETs are formed and showed an anti-inflammatory effect on the local microenvironment by eliminating pro-inflammatory mediators, trapping silica coated with proteases and sequester them in silicotic nodules, which resemble gouty tophi (20). The persistence of silicotic nodules is prone to sustain inflammation and may cause silicosis or even lung cancer (60).

FURTHER CRYSTALLINE MATERIALS

There is evidence that CaP and CaOx may trigger RIPK1-RIPK3-MLKL-dependent neutrophil necroptosis and promote NETs or aggNETs formation *in vitro* and *in vivo* (20). When exposed to CaP, IL-1 β derived from macrophages activates NETs formation which in turn induces the secretion of TNF- α by macrophages (66). Further studies on NETs in CaP or CaOx related to diseases like nephrolithiasis are needed.

CONCLUSION

Sterile crystal-mediated diseases, such as gout, pseudogout, atherosclerosis, and silicosis are highly prevalent worldwide. As summarized here, NETs are often involved in the progression of these kinds of diseases where they play detrimental and also beneficial roles. The activation of the NALP3 inflammasome, production of ROS, and the pro-inflammatory cytokines IL-1 β , TNF- α are critical for NETosis in crystal-induced inflammation. NETs and aggNETs govern acute and chronic phase of sterile inflammation, respectively. Finally, targeting NETosis and NETs-derived products may provide new therapeutic approaches for crystal-mediated diseases.

AUTHOR CONTRIBUTIONS

YL and XC contributed equally to this article. YL, XC, and YZ wrote the first draft of this article. YL, XC, and YZ designed the figures. YL and MH critically revised the manuscript for important intellectual content. All authors approved the final version.

FUNDING

The present work was supported by the National Natural Science Foundation of China (81771742) to YZ and the National Key Research and Development Program of China (Project No. 2016YFC0906201) to YL.

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