Review

Mechanism of neutrophil extracellular traps in the pathogenesis of gout

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ABSTRACT

Gout is a self-limited inflammatory disease caused by the deposition of monosodium urate (MSU) crystals in joints and surrounding tissues due to abnormal purine metabolism. Neutrophil extracellular traps (NETs) are formed by neutrophils in response to pathogen attack. During gout, NETs induced by MSU crystals exacerbate inflammation, and aggregated NETs (aggNETs) promote the resolution of gout-associated inflammation by encapsulating MSU crystals, degrading cytokines and chemokines, and blocking the recruitment and activation of neutrophils. With disease progression, NETs participate in the formation of tophi. Therefore, aggNETs are a possible mechanism of spontaneous gout regression. Studying the specific mechanism by which NETs affect inflammatory bursts and spontaneous regression in gout patients is important. This review summarises the role of NETs in different stages of gout and the specific pathogenesis of NETs in gout to provide new ideas for the diagnosis and treatment of gout.

Introduction

Gout is a crystal-associated arthropathy caused by the deposition of MSU crystals in joints. Gout is directly related to hyperuricaemia caused by purine metabolism disorders and/or decreased excretion of uric acid. In addition to joint damage, gout can also be accompanied by renal lesions and other manifestations of metabolic syndrome, such as hyperlipidaemia, hypertension, diabetes, and coronary heart disease (1). Hyperuricaemia is a major risk factor for gout. MSU crystals precipitate when the blood uric acid concentration exceeds the dissolution limit (70 μg/ml), and MSU crystals become an endogenous danger signal, stimulating the innate immune response and causing acute inflammatory attacks (1, 2). Gout is a global disease, and its prevalence varies greatly depending on ethnicity, region, dietary habits, sex, age, genetics, drugs (such as diuretics), and body mass index (3). Epidemiological studies have shown that the global incidence of gout is 1–4%. In Western developed countries, the prevalence of gout in recent years has been 3–6% in men and 1–2% in women, which is significantly higher than that in previous decades. In the Asian population, the male-to-female ratio is 8:1 (4), and the incidence of gout is increased in postmenopausal women (5). Hyperuricaemia is the most important risk factor for gout. Clinically, 5–15% of patients with hyperuricaemia will develop gout. Neutrophils are the most abundant white blood cells in the circulation and serve as the first line of defence of the innate immune system against injury or infection by bacteria, fungi, and protozoa. In addition to classical functions such as phagocytosis and degranulation, neutrophils have novel antimicrobial strategies that enable them to capture and kill pathogens by extruding nucleotide networks into the extracellular space (6). In the process of gout inflammation, MSU crystals deposited in joints or local tissues are recognised by macrophages, which can activate the Nod-like receptor protein 3 (NLRP3) inflammasome and release interleukin-1β (IL-1β). Neutrophils are recruited to the inflammatory site to release cytokines and mediators, amplify the inflammatory response, undergo oxidative burst and release DNA to produce NETs (7). NETs can degrade cytokines and reduce inflammatory response. However, when NETs are overproduced or degraded, they will expand inflammation and damage the body (8).

Structure and function of NETs

NETs are a network of DNA fibres released by activated neutrophils. The active release of NETs, which is known as NETosis, is a unique form of neutrophil death that differs from the suicidal modes of necrosis and apoptosis and consists of nuclear and granular components that cluster together to form large threads with a diameter of 50 nm and no capsule component (6, 9). In 1996, Takei *et al*. (10) described a novel mode of neutrophil suicide that takes hours and involves stepwise progression of chromatin decondensation, nuclear swelling, nucleoplasmic spillage into the cytoplasm, and membrane perforation. In 2004, Brinkmann *et al*. (6) first discovered NETs by immunofluorescence analysis and DNA dye staining. The nuclear component of NETs mainly includes histones (H) and highly depolymerised chromatin fibres with diameters ranging from 15 nm to 17 nm (6, 11), and the core histone components are H2A, H2B, H3 and H4. The granular component accounts for 70% of the protein components associated with NETs, is globular and 25 nm in diameter and is typically stored in unique neutrophil granules located on the DNA backbone structure of NETs. NETs can exert antibacterial effects through enzymes such as neutrophil elastase (NE), myeloperoxidase (MPO), cathepsin G, leukocyte proteinase 3, lactoferrin, gelatinase, lysozyme C and calprotectin (6, 12). NETs are a double-edged sword that are produced in response to bacterial (13), fungal (14), viral (15), parasitic (16) and other microbial infections. NETs capture and limit pathogens at the inflammatory sites and can promote the production of related antimicrobial proteins and phagocytes to kill pathogens (17). NETs can also be produced during noninfectious diseases. When NETs are overproduced, degraded or dysregulated, they participate in the development of diseases, reduce resistance to diseases, exacerbate inflammation, produce inflammatory storms, and damage tissues or organs, leading to pathogenic inflammation and abnormal autoimmunity. These diseases include systemic lupus erythematosus (18), rheumatoid arthritis (19), gout (8), psoriasis (20), and tumours (21). At present, the specific mechanism and related pathways of NETs in diseases are still unclear, but their related characteristics have attracted the attention of researchers. The mechanism of NET formation and key regulatory factors can be further explored to identify new therapeutic targets and research directions for the treatment of related diseases.

Gout and NETs

The natural history of gout can be divided into 4 stages. During asymptomatic hyperuricaemia, acute gouty arthritis, gout attack, gout attack interphase and chronic gouty arthritis (1), NETs play different roles. After MSU crystals, which are endogenous danger signals, are generated, the innate immune system is activated, leading to acute gout attack, bone and joint erosion and other related injuries (22).

NETs and acute gouty arthritis

- MSU-induced NETs

During an acute gout attack, MSU crystals accumulate or are deposited in local tissues, joints and other parts of the body and are recognised and phagocytosed by mononuclear macrophages. After activation of the NLRP3 inflammasome and the release of IL-1β (23), MSU crystals can activate the caspase-1-dependent classical pathway and the caspase-11-dependent nonclassical pathway, resulting in the cleavage of gasdermin-D (GSDMD) (24) and leading to plasma membrane perforation, granular membrane lysis, chromatin decondensation, and protease and DNA expulsion (7). This process is known as pyroptosis, which is a lytic form of programmed necrosis. This process promotes the extrusion of NETs (25). Inflammasome activation depends on two signals. The first signal is recognised by Toll-like receptors (TLRs), including TLR4 and TLR2, which excessively activates NF-κB and induces the synthesis of pro-interleukin-1β (pro-IL-1β) and inflammasome components (26). MSU crystals serve as the second signal. Due to their high sodium content, MSU crystals lead to high sodium concentrations in phagocytic cells after phagocytosis. To maintain the balance of osmotic pressure between the inside and outside the cell, water enters the cell and causes cell oedema, which reduces intracellular potassium concentrations and is a key signal for the induction of inflammasomes (27). The adaptor protein apoptosis-associated speck-like protein containing caspase recruitment domain (PYCARD) links the NLRP3 inflammasome to the precursor caspase-1 to form an active heterodimer known as caspase-1. Activated caspase-1 hydrolyses inactive IL-18 and pro-IL-1β to form mature IL-18 and IL-1β, which are released from the cell (28) (Fig. 1). IL-1β binds to its receptor on endothelial cells to activate the adaptor protein Recombinant myeloid differentiation factor 88 (MyD88), which activates nuclear factor kappa B (NF-κB) and further promotes the production of interleukin- 6 (IL-6), interleukin- 8 (IL-8), tumour necrosis factor (TNF) and other proinflammatory factors to recruit neutrophils and monocytes to the synovial fluid, thereby triggering the production of neutrophil attractants, activators and E-selectin. The exacerbation of neutrophil infiltration drives the local acute immune response and induces neutrophils to form NETs (29). In addition, activated IL-1β attracts neutrophils, which undergo oxidative bursts and release DNA to produce NETs. NETs can defend against foreign pathogens and control sterile inflammation, but some components of NETs can cause tissue damage. For example, histones can damage endothelial cells, large amounts of ATP and uric acid are released into the synovial cavity, and the local uric acid concentration is increased. A strong inflammatory cascade is induced, leading to extreme pain in the affected joints (1, 30, 31). These studies suggest that inflammasomes play a key role in NET formation.

- Formation of NETs

Mitroulis *et al*. (32) first showed that MSU crystals could stimulate neutrophils to form NETs. Depending on the cause of death, NETosis can be divided into two forms: suicidal and vital NETosis (33). At present, it is still controversial whether MSU crystal-induced

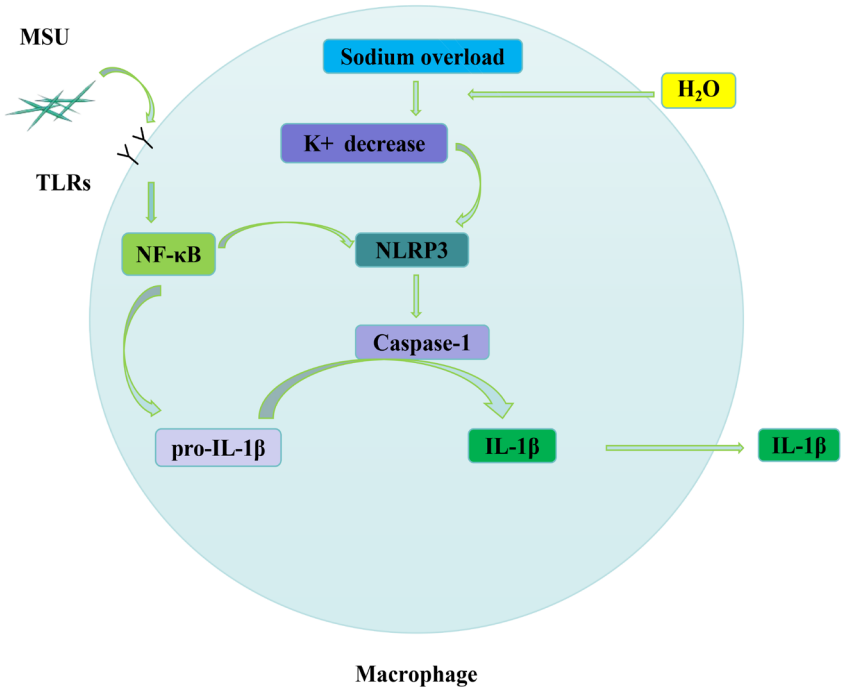

Fig. 1. Activation and formation of inflammasome.
MSU: monosodium urate; TLRs: Toll-like receptors; NF-κB: nuclear factor kappa B; NLRP3: nod-like receptor protein 3; pro-IL-1β: pro-interleukin-1β; IL-1β: interleukin-1β; H_2O: hydrogen dioxide; K^+: potassium ion.
The first signal involves TLRs, which are recognised and excessively activate NF-κB, thereby inducing the synthesis of pro-IL-1β and inflammasome components. The second signal is the MSU crystal, which leads to a high intracellular sodium concentration after being phagocytosed, and the entry of water into the cell reduces the potassium concentration, which represents a key signal for the induction of inflammasomes, and activates NLRP3 and caspase-1, which hydrolyses inactive pro-IL-1β to form mature IL-1β and releases it outside the cell.

NETosis is accompanied by neutrophil death.

During suicidal NETosis, calcium ions may be released from the endoplasmic reticulum when neutrophils are activated by MSU crystals. Protein kinase C (PKC) is activated as a result of the increase in calcium concentration. Then, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complexes (NOX) are activated and reactive oxygen species (ROS) are produced as a consequence (34, 35). NOX catalyse the respiratory burst to activate peptidylarginine deiminase 4 (PAD4). Activated NOX and ROS convert oxygen molecules to superoxide, which is decomposed into hydrogen peroxide, which is converted to an acid by MPO. ROS act as second messengers to stimulate the degradation of cytoplasmic granules containing MPO and NE and induce the disintegration of the nuclear membrane into small vesicles (36). Chromatin is translocated from granules to the nucleus in an NE- and MPO-dependent manner (37). Then, NE begin to cleave histones, resulting in chromatin decondensation. Meanwhile, PAD4 catalyses the conversion of arginine residues in histones to citrulline (38), weakens the strong positive charge of histones, binds histones and DNA, and wraps nucleosomes to promote the decondensation of chromatin, the nuclear leaflet shape is lost, chromatin and granule proteins are released to the outside of the cell through membrane pores and plasma membrane cleavage and the formation of the NETs. Neutrophils death occurs via an NADPH oxidase-dependent pathway (39-42) (Fig. 2). Activation of PAD4 during NETosis is required for the rupture of cytoplasmic granules, chromatin decondensation, and the release of nuclear DNA into the cytoplasm (41). Studies have shown that the occurrence of suicidal NETosis in gout patients is caused by an increase in nuclear DNA rather than mitochondrial DNA (43). The formation of NETs induced by MSU crystals depends on ROS, and antioxidants significantly reduce the formation of NETs induced by MSU crystals (44). Mutations in neutrophil cytoplasmic factor 1 (Ncf1) completely abolish ROS production, which in turn can reduce NETosis and increase inflammation in mice with gout (45). Other studies have shown that PAD4 inhibitors can prevent MSU crystal-induced NETosis (46), but the specific mechanism needs to be further studied. The receptor-interacting protein kinase 3/mixed lineage kinase domain-like protein (RIPK3/MLKL) signalling pathway is the main mechanism mediating necroptosis (47), the central mechanism of the cell death response (48) and is thought to be downstream of NADPH oxidase and ROS (49). Blockade or deficiency of the RIPK3/MLKL signalling pathway robustly suppresses NET formation, which includes chromatin decondensation, DNA release, and plasma membrane disruption triggered by phosphorylated MLKL, but death receptors for necroptosis are not involved in MSU crystal-induced NETosis (50). However, the correlation between neutrophil death and necroptosis remains controversial.

During vital NETosis, TLR and C3 complement proteins on the cell membrane surface are recognised and activated by stimuli or damp-associated molecular patterns (DAMPs), then calcium ions enter neutrophils (51-53). The influx of Ca2+ activates PAD4 to aid in H3 citrullination. The electrostatic bond between histone and DNA weakens, causing chromatin decondensation (54, 55). After nuclear envelope destruction and NETs release through small vesicles, neutrophils still undergo phagocytosis and chemotaxis, and their life span is not affected by DNA loss, which is mediated by an NADPH oxidase-independent pathway (39) (Fig. 2). This pathway is independent of ROS produced by NADPH oxidase to activate PAD4 and occurs independently of the ROS and Raf/MERK/ERK pathways. In addition, the interaction between glycoprotein Ib in platelets and β2 integrin (CD18) in neutrophils may induce NET formation through the activation of the kinases ERK, PI3K and SRC (56). Yousefi *et al*. (5) described anoth-

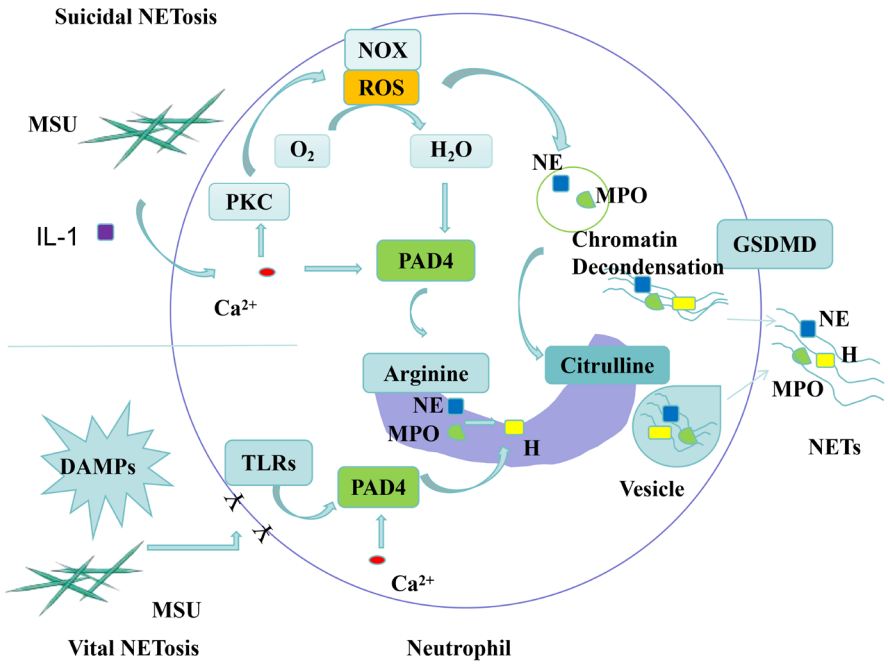

Fig. 2. The formation of NETosis.
NADPH: nicotinamide adenine dinucleotide phosphate; ROS: reactive oxygen species; O_2: oxygen; MSU: monosodium urate; TLRs: Toll-like receptors; NOX: oxidase-dependent; NE: neutrophil elastase; MPO: myeloperoxidase; PAD4: peptidylarginine deiminase 4; H: histones; DAMPs: damage-associated molecular patterns; GSDMD: gasdermin-D; NETs: neutrophil extracellular traps.
Two pathways for the formation of NETs (NETosis): "suicidal" and "vital". Suicidal NETosis: Calcium ions can be released, leading to PKC activation when neutrophils are activated by MSU crystals. The NADPH oxidase complex activates ROS produced during the "respiratory burst", which activates PAD4. ROS can promote the degradation of cytoplasmic granules containing NE and MPO. PAD4 activated by calcium and NOX, together with NE and MPO, induce the citrullination of histone, further leading to chromatin decondensation. Finally, NETs are formed and released outside the cell. Vital NETosis: After TRL is activated on the neutrophil membrane by stimuli or DAMPs, the increased Calcium ions activates PAD4, leading to the citrullination of histone and chromatin decondensation. Finally, NETs are formed and then sent out of the neutrophils as vesicles.

er ROS dependent, nonlethal NETosis pathway that does not require neutrophil death and releases mitochondrial DNA rather than nuclear DNA, and this process leads to the formation of NETs in 5% of neutrophils within 15 minutes through the recognition of C80a or lipopolysaccharide (LPS).

Uric acid is a purine metabolite that causes gout (1). Purine decomposition leads to the formation and accumulation of adenosine triphosphate (ATP), uridine triphosphate (UTP) or uridine diphosphate (UDP). These factors are recognised as danger signals by P2Y6 purinergic receptors in THP macrophages and human keratinocytes (57). P2Y6 receptors have a high affinity for UDP and are involved in IL-8-mediated leukocyte chemotaxis (58). MRS2578 is a potent and specific P2Y6 antagonist that inhibits defensin-induced IL-8 and IL-1 release from neutrophils (59-62), thereby inhibiting MSU crystal-induced neutrophil activation and the formation of aggNETs (62).

NETs and self-limiting gout

Despite the persistence of MSU crystals, acute gouty arthritis resolves within 7 to 10 days (63), and the self-limiting nature of inflammation is a prominent feature of gout (1); however, the mechanism underlying the rapid resolution of inflammation remains unclear.

Since the activity of DNase-1 in the synovial fluid of gout patients is low, and synovial fluid that is rich in actin is resistant to DNase degradation, the degradation of NETs is impaired, and NETosis is further enhanced. In the case of low- and high-density neutrophils, NETs and aggNETs, respectively, form and can coordinate the initiation and resolution of sterile crystal-mediated inflammation; moreover, it has been suggested that the number of NETs produced depends on the number of MSU crystals and is independent of the density of neutrophils (64). Mitroulis *et al*. (32) showed that NETs were closely related to the resolution of inflammation in gout patients and that treatment with IL-1 inhibitors (such as anaculin) significantly reduced the number of NETs formed by neutrophils, indicating that IL-1 was closely related to the formation of NETs. Moreover, IL-1β receptor blockers can block the formation of NETs (65). Jeong *et al*. (66) showed that $CD14^+$ macrophages significantly phagocytosed MSU crystal-induced NETs in the synovial fluid of patients with acute gout and played an important role in alleviating acute inflammation without causing any significant immune response. Christine Schauer et al. demonstrated that MSU crystal-induced NETs aggregated to form aggNETs via serine proteases in the presence of high densities of neutrophils *in vitro* and *in vivo*. This process promoted the resolution of neutrophilic inflammation by engulfing apoptotic cells, releasing TGF-β1, degrading cytokines and chemokines, and subverting neutrophil recruitment and activation. Ncf1 knockout NETosis-deficient mice exhibit more severe inflammation in response to MSU crystal stimulation, which can be alleviated by the adoptive transfer of aggNETs (45). Animal studies have shown that aggNETs have important anti-inflammatory effects and regulate cytokines such as TNF-α, IL-1β and IL-6, as well as the chemokine ligand 2 (CCL2) and monocyte chemoattractant protein-1. These studies suggest that aggNETs promote the resolution of acute gout (45). MSU crystal-induced aggNET formation was reported to depend on ROS production, and *in vitro,* neutrophils from chronic granuloma patients that were cocultured with MSU crystals exhibited reduced formation of aggNETs. *In vivo*, NADPH oxidase-deficient mice exhibited reduced formation of aggNETs when stimulated with MSU crystals in an air bag model and in claw inflammation induced by MSU crystals (45). ATP, lactoferrin, IL-1β and the P2Y6 receptor antagonist MRS2578 have

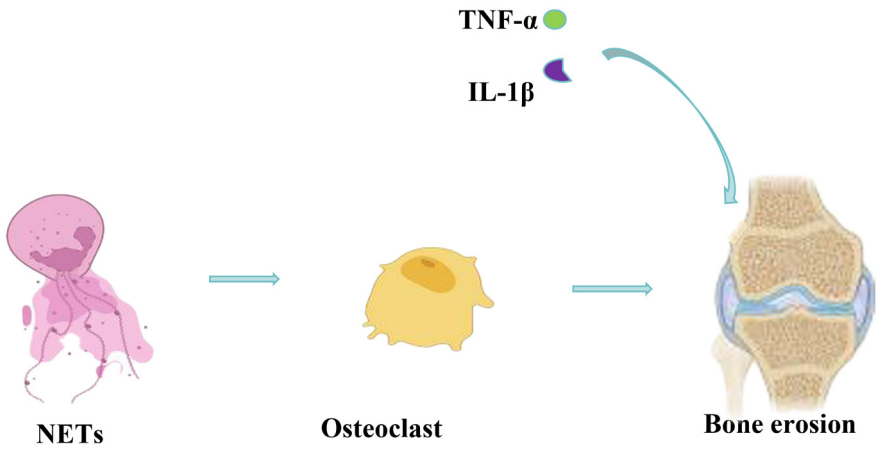

Fig. 3. Bone erosion.
NETs can enhance the effects of osteoclasts and lead to bone erosion. In addition, the inflammatory factors IL-1β and TNF-α play important roles in bone erosion.

been reported to enhance or inhibit MSU crystal-induced aggNET formation (45, 62, 67). Despite the important role of aggNETs in alleviating inflammation, little is known about the regulatory mechanisms of aggNET formation. Transforming growth factor-β1 (TGF-β1) is currently considered the main mediator of the positive resolution of gouty inflammation. During acute inflammation, the recognition and phagocytosis of apoptotic neutrophils by macrophages leads to TGFβ production (68). Liote *et al*. (69) demonstrated that exogenous TGF-β1 significantly attenuated cell recruitment and suppressed acute inflammation *in vivo* in a rat air bag model of gout. Chang *et al*. (70) also demonstrated that the TGF-β1 polymorphism 869T/C was significantly associated with the development of tophi in gout patients, suggesting that TGF-β1 was present in more advanced disease states. It has been shown that macrophages that mature *in vitro* from healthy human blood monocytes actively produce TGF-β1 in response to MSU crystal stimulation (71). *In vitro* studies have shown that TGF-β1 decreases Il-1β production and IL-1 receptor expression and plays an important role in the initiation of gouty inflammation and the subsequent recruitment of inflammatory cells (72-74). Therefore, TGF-β1 may play a role in controlling inflammation in gout through this mechanism.

Another possible mechanism for the resolution of inflammation is that MSU crystals can control inflammation by binding to proteins such as immunoglobulins and complement (75-77). The binding of apolipoproteins to MSU crystals prevents permanent activation of infiltrating and resident inflammatory cells (78).

NETs and the formation of gouty tophus

Gouty tophus is one of the characteristic manifestations of advanced gout. Studies have confirmed that NETs are involved in the formation of gouty tophus (79). Gouty tophus is characterised by chronic granulomatous lesions with MSU crystals at the core that are surrounded by mononuclear and multinucleated macrophages and wrapped by a fibrous layer of dense connective tissue (80). NETs are present in the crystals in acute inflammatory episodes of gout and in uninflamed tophi. The presence of actin in MSU-NETs and their relative resistance to DNase indicate that MSU-NETs may sequester crystals in DNA-coated aggregates within tissue and contain proteases that capture and degrade inflammatory factors. This may limit the ongoing inflammatory response (79). This hypothesis warrants further investigation but could explain how acute gout spontaneously resolves and how tophi store large amounts of crystalline MSU without causing inflammation. Although tophi cause muscle damage and bone destruction in patients with chronic gout, they can inhibit the intense inflammatory response during acute gout, which is inseparable from the presence of NETs.

NETs and the destruction of bone

NETs are resistant to DNase degradation, indicating that tophi are difficult to degrade and that degradation takes a very long time. The longer the tophi are present, the more likely they are to cause joint damage, such as periosteal reactions, bone erosion, cartilage damage and osteophyte formation (79). Several studies have shown that IL-1β and TNF-α play important roles in bone erosion during gout (Fig. 3) (81, 82), and several studies have shown that NETs triggered by MSU crystals can cause local cartilage damage, peripheral tissue damage and remodelling (Fig. 3) (83, 84). Receptor activator of nuclear factor-κb ligand (RANKL) and osteoprotective factor (OPG) are independent factors associated with joint destruction in gout patients (85), suggesting that an imbalance in osteoblast-like cells (OBs) and osteoclast-like cells (OCs) may cause joint damage through the OPG/RANKL/RANK pathway in gout patients (86). OCs are key cells involved in local bone loss during gout (87). NETs induced by MSU crystals can inhibit the viability of OBs and enhance the activity of OCs (Fig. 3); in addition, neutrophil elastin (NE) may cause an imbalance between RANKL and OPG (88). However, the mediators by which tophi act on OBs and OCs are unknown and need to be further explored.

Outlook

In summary, our review found the following points: 1. NETs not only accelerate the cascade of inflammation and promote the spread of inflammation but also form aggNETs that cover the surface of MSU crystals, form tophi and inhibit the inflammatory response. 2. NETosis includes suicidal and vital forms. Suicidal NETosis depends on NADPH oxidase,causing neutrophil death, while vital NETosis is NADPH-independent, maintaining cell viability. Both depend crucially on PAD4. 3. The self-limiting of gout may be linked to aggNETs, TGF-β1, and immunoglobu-

lins, while bone erosion is potentially associated with NETs, IL-1β, TNF-α, and OCs, reflecting a complex interplay in its pathogenesis.With improvements in living standards, the prevalence of gout is increasing. How to generate and resolve NETs to control inflammation and dissolve tophi is a great challenge. Further research is needed to explore new treatment directions.

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