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Review Article

A Comprehensive Review on the Formation of Neutrophil Extracellular Traps in Animals

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Abstract

The formation of neutrophil extracellular traps (NETs) is a complex and fascinating phenomena that is crucial for understanding the way the innate immune system reacts to infections. Activated neutrophils release chromatin fibres and antimicrobial compounds during this process, forming a web-like structure that traps and immobilizes invasive microbes. NETs are a double-edged sword in host defense because their ability to entrap pathogens is balanced by the risk of tissue injury and autoimmune reactions. This article gives an overview of the molecular mechanisms behind NET formation, including the crucial role of reactive oxygen species (ROS), specific signalling pathways with emphasis on NET formation in various pathological conditions of animals and anti-TNF-α therapy.

Keywords: NETs; ROS; Anti-TNF-α; NETosis

Introduction

The most abundant leukocyte in blood, neutrophils, are crucial components of the host's innate immune system. After identifying pathogens, neutrophils release granules and create neutrophil extracellular traps (NETs), which they use to entrap and destroy invading microorganisms [1] and this process is known as NETosis. It is an active type of cell death that happens in response to mostly pathogen-induced activation of neutrophils and resulting in the release of decondensed chromatin into the extracellular environment. According to Kaplan., *et al*. [2], cytokines, microcrystals, immune complexes, various illnesses, antibodies, and other physiological stresses can all contribute to NETosis. The induction of NETosis requires reactive oxygen species (ROS), the main source of which is NADPH oxidase. NADPH oxidase activation necessitates both the production of ROS in the mitochondria and an increase in the amount of Ca^{2+} in the cytoplasm. According to Vorobjeva., *et al*. [3], NETosis involves the release of granule components into the cytosol, histone modification, and chromatin decondensation.

The early/rapid ROS-independent method and the conventional ROS-dependent NET-formation pathway are the two main NET release techniques that have been described [4,5]. Myeloperoxidase is necessary for neutrophil elastase to be released from the membrane-associated complex known as the azurosome and activated as a result of an oxidative burst [6]. From the cytoplasm to the nucleus, neutrophil elastase translocates [6]. Furthermore, neutrophil elastase degrades histones and helps chromatin decondense. Peptidyl arginine deiminase 4 (PAD4), which is found in NETs, is the main enzyme responsible for the primary post-translational modification known as histone deimination [7].

This review focuses on the ROS-dependent and independent pathways of NETosis, their antimicrobial activity, role in autoimmune diseases with a focus on the formation of NETs in different pathological conditions of animals and anti-TNF-α therapy.

ROS-dependent NETosis

The substance most commonly used to induce NETosis is the phorbol ester, phorbol-12-myristate-13-acetate (PMA), a synthetic activator of the 19 enzymes in the protein kinase C (PKC) family. PKC is directly responsible for both the production of ROS and the activation of NADPH oxidase. Patients with chronic granulomatous illness do not have neutrophils that can produce NETs when PMA is present [8,9].

Since NADPH oxidase inhibitors such as diphenylene iodonium (DPI) and ROS-scavengers blocked NETosis caused by PMA and *S. aureus*, ROS production is necessary [8,10]. Exogenous hydrogen peroxide, however, has the potential for producing NETs since it is membrane permeable and stimulates MPO downstream of NADPH oxidase. Although superoxide by itself is not necessary for NET release, it is crucial for its conversion to hydrogen peroxide and perchloric acid.

ROS-independent NETosis

Occasionally, ROS-independent processes can also cause NETosis. In adult cells with inactivated NADPH oxidase, ROS synthesis was able to make up for the neonatal neutrophils' deficiency in NET formation, but this is not the case in neonatal neutrophils [11]. This indicates that neonatal neutrophils lack the downstream signalling capacity that ROS provides. NADPH oxidase-independent NETosis can be induced by a variety of stimuli, including calcium ionophores [12]. Additionally, a unique and extraordinarily rapid technique of NETosis in response to *S. aureus* demonstrated that the first 5 - 60 minutes of NETosis are ROS-independent [4]. The NETosis pathway that is not based on NOX, however, has its own set of mechanisms that are not well understood.

Anti-microbial activity of NETs

Through ROS-dependent and ROS-independent methods, neutrophils can get rid of invasive infections [13]. Granule proteins and anti-microbial enzymes are released by neutrophils, and these substances work with chromatin to kill bacteria and eliminate their virulence factors [1]. Exogenous or secreted microbial deoxyribonuclease (DNases) protect bacteria against NET death, and extracellular DNA has rapid membrane-damaging antibacterial activity [14]. Investigations into several NET components revealed that they are necessary for the microbial eradication. Histones are the most common proteins that are NET-bound and have antibacterial action that acts directly on the membrane [15,16]. Chromatin associated histones play a significant role in the destruction of various pathogens such as *Shigella* and *Staphylococcus*. While MPO found in NETs provide the bactericidal action required to kill these pathogens in the presence of hydrogen peroxide, purified NET chromatin alone is not very effective at killing *S. aureus* [17]. The removal of *Neisseria* by NETs requires the granular serine protease cathepsin G [14]. LL-37, lactoferrin, neutrophil elastase, and proteinase-3 (PR3) are other NET components associated with antibacterial activities [2]. Defensins, LL 37, MPO, and cationic proteases such neutrophil elastase, cathepsin G, and proteinase-3 are examples of positively charged peptides and proteins that can damage microbial membranes to kill the pathogens or prevent microbial development [18].

NETosis associated-autoimmune diseases

Recent studies have connected autoimmune responses to the emergence of NET. For instance, neutrophils of patients with rheumatoid arthritis demonstrated increased spontaneous NET production when compared to healthy individuals [19]. According to Garcia-Romo., *et al*. [20] and Kessenbrock., *et al*. [21] the majority of autoantibodies found in patients with rheumatoid arthritis, systemic lupus erythematosus, and vasculitis are directed against NETs. The molecular and clinical similarities between rheumatoid arthritis, systemic lupus erythematosus, or between systemic lupus erythematosus and vasculitis suggest that NETosis may be a significant trigger for the onset of chronic inflammatory diseases [22].

The only rheumatoid arthritis autoantibodies that have been discovered so far are those that are anti-citrullinated protein antibodies, which are regarded as separate disease markers with sufficient specificity and sensitivity to be utilised in rheumatoid arthritis diagnostic tests [23]. Peptidyl arginine deiminase IV changes the histones found in NETs from arginines to citrullines (PAD4). The deiminated chromatin might be useful for bacterial pathogen capture. Host phagocytes may ingest the mixture of bacterial antigens and deiminated chromatin. The uptake and

processing of deiminated chromatin by phagocytes in conjunction with bacterial adjuvants may result in the display of modified histone epitopes and co-stimulation, resulting in a powerful stimulus to break tolerance. Patients with rheumatoid arthritis and systemic lupus erythematosus frequently have autoantibodies against deiminated histones. These findings clearly imply that histone deamination can act as a trigger for autoantibody production [24]. In autoimmune disorders including systemic lupus erythematosus and psoriasis, type I interferons exert potent immunostimulatory effects in addition to their antibacterial functions [10].

Formation of NETs in animals

NETs have been characterized in several mammals including cows, sheep and goats, although not as extensively as in humans and mice [25]. Lippolis., *et al*. [26] showed that NETs are produced by bovine blood neutrophils stimulated with PMA/ionomycin. Importantly, neutrophils can form NETs when they have been cultured in milk for up to 6 hours before being stimulated. They observed that NETs are produced in milk as a result of neutrophil stimulation with bacteria that are common to infections of the mammary gland suggesting that milk does not inhibit the ability of neutrophils to form NETs. However, incubation in milk inhibits neutrophil transmigration and bacterial phagocytosis and oxidative burst. NETs may therefore play a crucial role in the defense against mastitis as evidenced by the fact that they are unaffected by incubation in milk. Grinberg., *et al*. [27] discovered that the mammary pathogenic *E. coli* strain P4 stimulates normal bovine neutrophils, causing them to produce antibacterial NETs. When these neutrophils were pre-incubated with increasing concentrations of beta-hydroxy-butyric acid (0.1 to 8 mmol/liter), *E. coli* P4 phagocytosis was reduced by fivefold, but intracellular killing was unaffected. Further, beta-hydroxy-butyric acid also reduced the number of NETs generated by *E. coli* P4-activated neutrophils by a factor of ten, as well as the bactericidal activity of NETs against this organism.

M. haemolytica leukotoxin (LKT) promotes NET formation in bovine neutrophils in a CD18-dependent pathway using an unacylated, noncytotoxic pro-LKT generated by a lktC mutant of *M. haemolytica* [28]. Confocal microscopy, scanning and transmission electron microscopy were used to confirm NET development. They showed that binding of unacylated pro-LKT induces NET formation despite the lack of cytotoxicity. Further, blocking LKT binding to the CD18 chain of lymphocyte function-associated antigen 1 (LFA-1) reduced NET formation in response to LKT or *M. haemolytica* cells. They observed that NETs generated in response to *M. haemolytica* could trap and destroy a percentage of the bacterial cells. Jerjomiceva., *et al*. [29] reported that the antibiotic enrofloxacin, a fluoroquinolone, increases the production of NETs in the bovine neutrophils. Enrofloxacin-induced NETs formation was significantly decreased by pharmacologically inactivated NADPH oxidase or peptidyl-arginine deiminase-4. Further, enrofloxacin-mediated NET induction was also inhibited when cells were treated with cytochalasin D or nocodazole. Taken together, they concluded that actin and microtubule polymerization are involved in the formation of enrofloxacin-induced NETs in addition to oxidative burst and histone citrullination. Swain., *et al.* in 2014 [30] investigated the variations in neutrophil activity in cows with subclinical and clinical mastitis. They observed that milk neutrophils from clinical mastitis cows formed NETs, whereas milk neutrophils from subclinical mastitis and healthy cows did not show any sign of NETs formation. They reported a link between decreased neutrophil apoptosis and increased TLR2 and TLR4 expression and the creation of NETs and changes in the surface architecture of neutrophils. They concluded that the formation of NET-like structures by neutrophils in clinical mastitis cows is an efficient strategy of resistance.

In 2017, Villagra-Blanco., *et al*. [31] studied the *in-vitro* interactions of bovine polymorphonuclear leukocyte with *N. caninum* at various ratios and time durations. Through antibody-based immunofluorescence investigations, extracellular DNA staining was employed to illustrate the characteristic molecules of NETs (i.e. histones (H3), neutrophil elastase (NE), myeloperoxidase (MPO), and pentraxin). They observed that bovine polymorphonuclear leukocyte generated NET structures that entrapped tachyzoites, according to scanning electron microscope analysis. Further, NOX, NE, MPO, PAD4, ERK1/2, and p38 MAP kinase suppression resulted in a small decrease in NET formation, indicating that *N. caninum*-induced NET formation was not a NOX, NE, MPO, PAD4, ERK1/2, and p38 MAP kinase-dependent process. They also observed that CD11b was also discovered to be a neutrophil receptor that played a role in NETosis. Mendez., *et al*. [25] investigated the processes by which *O. ostertagi* influences the development of bovine NETs. Co-localization of extracellular DNA with typical NET related proteins like histone

and neutrophil elastase confirmed that *O. ostertagi* larval soluble extract could create typical NETs in purified neutrophils *in vitro*. Further, inhibition studies confirmed that these *O. ostertagi* larval soluble extract-induced NETs were dependent on the enzymes NADPH oxidase and myeloperoxidase. They also observed that neutrophils were activated by live *O. ostertagi* larval stage 4 larvae to generate NETs identical to those induced by *O. ostertagi* larval soluble extract. They concluded that bovine neutrophils also produced NETs in response to *Caenorhabditis elegans*, a free-living soil worm, showing that NET formation is a conserved mechanism for nematode defence.

Karakurt., *et al*. [32] reported that *Toxoplasma gondii*, a protozoan parasite that causes toxoplasmosis in warm-blooded mammals, generates NETs in neutrophils of humans, mice, sheep, and cattle. *Toxoplasma gondii*-induced NETosis in sheep and cattle was affected by tachyzoite concentrations and incubation period. They concluded that NET structures formed by sheep neutrophils can only entrap *Toxoplasma gondii* tachyzoites, whereas cattle neutrophils have lethal effects *in vitro*. Ciliberti., *et al*. [33] studied the *in-vitro* NETosis of peripheral neutrophils isolated from dairy cows supplemented with olive pomace. NETosis was examined by immunofluorescence microscopy using the particular antibodies against MPO and citrullination of Histone-H1. They reported that the addition of olive pomace extract had an impact on the MPO activity of neutrophil as well as the neutrophil elastase-DNA complexes created during NETosis. Additionally, the olive pomace diet produced a greater response from neutrophils to PMA stimulation than the control diet, which did not support these responses. The results showed that olive pomace supplementation can improve neutrophil activity in dairy cows, leading to udder defense and inflammatory response, especially during immunosuppressive state.

Zhu., *et al*. [34] investigated NETs and ROS production capacity of polymorphonuclear neutrophils in different lactational stages of Holstein cows. They demonstrated that peripartum and nursing cows had greater basal NETs and ROS levels of polymorphonuclear neutrophils than nulliparous heifers. Further, ROS and NET production were enhanced when polymorphonuclear neutrophils from nulliparous heifers were stimulated *in vitro* using inflammatory agents. They concluded that polymorphonuclear neutrophils isolated from peripartum and lactating cows are primed to create NETs and ROS, and it is possible that this negatively impacted the cows' inflammatory and immunological condition during their lactation cycle.

Neutrophils and anti-TNF-α therapy

Marini., *et al*. [35] demonstrated that anti-TNF-therapeutic therapy's benefits in Crohn's disease are mediated by a mechanism involving the suppression of intestinal epithelial cell apoptosis. They reported that a single injection of a chimeric anti-murine TNF-α antibody into SAMP1/YitFc mice led to a significant suppression of intestinal inflammation and epithelial cell damage when compared to mice injected with an isotype control antibody, which is similar to the effectiveness of monoclonal anti-TNF-α antibodies in human Crohn's disease. Propidium iodide staining and DNA laddering measurements of the newly isolated intestinal epithelial cell revealed that these effects were associated with a considerable decrease in apoptosis. In contrast, animals treated with anti-TNF-α showed increased lamina propria mononuclear cell death as compared to control mice. These results showed a unique anti-TNF-therapy mechanism of action that involves homeostatic control of mucosal cell death, which 50 leads to a net reduction in the chronic inflammation generally present in Crohn's disease. According to Capsoni., *et al*. [36], neutrophils are among the targets of antiTNF-α activity in rheumatoid arthritis, and may provide insight into a novel and intriguing mechanism of action of anti-TNF-α monoclonal antibodies in the treatment of inflammatory arthritis. Etanercept, infliximab, adalimumab, and certolizumab pegol are the anti-TNF-α medications. Fossati., *et al*. [37] compared their effects for the direct *in vitro* induction of apoptosis in healthy activated lymphocytes and monocytes. It was observed that the activated monocytes and lymphocytes were equally susceptible to the apoptotic effects of infliximab, adalimumab, and etanercept contrary to other reports. However, no leukocyte under the influence of certolizumab pegol underwent apoptosis. Notebert., *et al*. [38] reported that freshly isolated bovine blood neutrophils express p65 and p50, the two most significant NF-kB family members. Both the RNA and protein levels confirmed the presence of both p65 and p50. Then gliotoxin, a strong and focused NF-kB inhibitor, was tested to see if it affected the apoptosis of bovine neutrophils. Inhibition of NF-kB was observed to significantly increased the apoptosis. Additionally, the crucial inflammatory mediator TNF-α, which only had a modest pro-apoptotic effect,

was greatly enhanced by gliotoxin. The results of this experiment were confirmed by additional measurements of caspase-3/7 activity and analysis of morphological criteria. Finally, NF-kB activity was evaluated in these circumstances. They concluded that gliotoxin was found to dramatically reduce active p65 values but only slightly influenced the activity of p50. Mukhopadhyay., *et al*. [39] reported that anti-TNF-α treatment caused overall varying degrees of efficacy in treating inflammatory diseases like inflammatory bowel disease, psoriasis, arthritis, and others. However, no concrete evidence demonstrating the effectiveness of anti-TNF-α medication for severe pulmonary diseases has yet been found. Pattacini., *et al*. [40] investigated the fibroblast-like synoviocytes apoptosis in relation to the effects of several anti-TNF-α agents like etanercept, infliximab and adalimumab. All of the treatments examined triggered fibroblast-like synoviocytes apoptosis in the presence of peripheral blood mononuclear cells from the same patient 51 only when the two cell populations were in immediate communication via activating the PTEN-FAK pathway and elevating Bax levels. This impact was not brought on by antibody dependent cell-mediated cytotoxicity. They concluded that only two antibodies infliximab and adalimumab were able to upregulate expression of Bcl-2. Atreya., *et al*. [41] also investigated anti-TNF-α antibodies impact on apoptosis in inflammatory bowel disease. They reported that clinically effective antiTNF-α antibodies are able to induce T-cell apoptosis in inflammatory bowel disease only when mucosal TNFR2+ T cells are cocultured with mTNF-expressing CD14+ macrophages. The discovery that anti-TNF-α antibodies indirectly cause apoptosis by targeting the mTNF/TNFR2 pathway may have significant ramifications for the creation of novel therapeutic approaches for inflammatory bowel disease. Billmeier., *et al*. [42] also investigated the effect of anti-TNF-α antibody induced apoptosis and reported that a secondary effect of TNF signalling is the potential direct or indirect activation of apoptosis. Apoptosis may also result from the activation of TNF itself by antibody binding or may be caused by the Fc region of anti-TNF antibodies that involve the complement or natural killer cells. Zhang., *et al*. [43] examined neutrophil migration in inflammatory bowel disease patients before and after anti-TNF-α monoclonal antibody therapy and evaluated the regulating function of anti-TNF-α in the production of proinflammatory mediators. Further, in Crohn's disease patients who responded to IFX, anti-TNF-α treatment markedly reduced neutrophil infiltration in inflamed mucosa. Further, antiTNF-α monoclonal antibody significantly reduced the production of proinflammatory mediators like MPO, calprotectin, IL-8, IL-6, and TNF-α. Furthermore, in Crohn's disease patients, inhibiting TNF-α may substantially cause neutrophil apoptosis. These findings suggest that anti-TNF-α therapy reduces neutrophil activation and migration in the gut mucosa, which reduces mucosal inflammation in inflammatory bowel disease patients. Vomero., *et al*. [44] studied the *in vitro* effects of TNF and anti-TNF-α on cell fate as well as *ex vivo* spontaneous autophagy and apoptosis in rheumatoid arthritis patients treated with anti-TNF drugs. After 4 months of anti-TNF-α medication therapy, peripheral blood mononuclear cells from rheumatoid arthritis patients responded to treatment and displayed a significant decrease in LC3-II levels 52 along with an increase in apoptotic activation. Additionally, there was a correlation between DAS28 and LC3-II expression. They observed that after 24 hours of culture, TNF-α was able to trigger autophagy in rheumatoid arthritis peripheral blood mononuclear cells in a dose-dependent manner. They concluded that etanercept also significantly decreased levels of citrullinated proteins and autophagy. Neuenfeldt., *et al*. [45] studied that increased intracellular neutrophil elastase abundance and NETosis are caused by autocrine and paracrine TNF signaling. Further, CCR5 activation accelerates NETosis. *In vivo*, they discovered that ulcerative colitis patients had inflamed lamina propria and more CCR5+ polymorphonuclear neutrophils in their peripheral blood. They concluded that increased frequencies of CCR5+ polymorphonuclear neutrophils were linked to anti-TNF-α therapeutic failure.

Conclusion

In conclusion, neutrophil extracellular trap (NET) formation is an important component of the immune response to infection and inflammation. By releasing chromatin fibers decorated with antimicrobial proteins, neutrophils can immobilize and neutralize invading pathogens, making NETs a powerful defense strategy. However, NETs also exhibit a dual nature, as excessive or uncontrolled formation can contribute to tissue damage and worsen inflammatory diseases.

Research in cattle, sheep, and goats demonstrates that NETosis is a conserved innate immune mechanism across animal species. Animal neutrophils generate NETs in response to pathogens, toxins, and inflammatory signals, and their formation is influenced by metabolic status, receptor engagement, and intracellular

pathways such as NADPH oxidase, MPO activity, histone citrullination, and cytoskeletal changes. While NETs enhance host protection-particularly in conditions like mastitis-their levels can vary with physiological states such as stress, lactation, or metabolic imbalance.

Overall, the study of NET formation in animals offers valuable insights into innate immunity. As research progresses, understanding the fine balance between NET-mediated protection and tissue injury will be key to harnessing this mechanism for improved disease management and therapeutic strategies in veterinary health.

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