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The Role of Neutrophil Extracellular Traps in Infectious Diseases

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Abstract

Neutrophils were recently thought as a key player of the innate immune response, and one of the defense mechanisms of these cells: Neutrophil extracellular traps (NETs) whereby they capture pathogens by actively releasing their nuclear contents into the extracellular space, were found in various infectious diseases. NETs are one of the major antimicrobial strategies, but extent and excessive activation of neutrophils may make collateral tissue damage.

Keywords: Neutrophils; Innate immunity; Inflammation; Influenza; Sepsis

Introduction

In the 1990s, studies of host immune responses to infection focused predominantly on elucidating the mechanisms of adaptive immunity that include T cells and B cells as primary components. Consequently, there was little progress in understanding the details of how the innate immune system functions. However, upon entering the 2000s, a family of innate immune receptors called Toll-like receptors were identified as a point of first contact between the host and invading pathogens. This finding greatly advanced knowledge of how the host recognizes many different foreign materials [1]. We have fresh memories of scientists in this research field being awarded the 2011 Nobel Prize in Physiology or Medicine.

Additionally, a previously unknown function was suggested for neutrophils, a key component of the innate immune response [2]. This novel function, called a neutrophil extracellular trap (NET), is one of the innate immune defense mechanisms of these cells whereby they capture pathogens by actively releasing their nuclear contents into the extracellular space. Currently, NETs are drawing increasing attention as one of the major antimicrobial strategies as immune mechanism employed by neutrophils.

In this article, we briefly review the significance of neutrophil activation in the field of infectious diseases, with particular emphasis on the involvement of NETs in their pathogenesis.

Definition of NETs

Neutrophils comprise approximately half of the white blood cells in the circulation and are known to be one of the main innate immune cells. In 2004, neutrophils were first reported to release meshwork structures, called NETs, which are composed of decondensed chromatin, histones, and enzymes (such as neutrophil elastase) [2]. NETs are novel innate immune defense machinery that can physically capture bacteria and kill them using antibacterial proteins associated with chromatin fibers.

Immunostaining visualized the major components of NETs. DNA, histone H3, and neutrophil elastase were actually included, and enabling us to confirm that these fibrous structures comprised mainly NETs. They contain smooth stretches of 15-17 nm in diameter, thought to consist of naked DNA and globular domains of around 25 nm where chromatin is present [3]. Moreover, citrullinated histone H3 has been reported as a characteristic molecule involved in NET formation *in vitro*, and circulating free DNA (cf-DNA), which has been suggested as a potential predictive biomarker for sepsis, and also associated with NETs.

NETs are released from neutrophils in response to various stimuli, including bacteria, fungi, and proinflammatory cytokines. Their principal function is almost unique [4]. They literally "trap" foreign microorganisms (such as invading pathogens) and efficiently kill them using antimicrobial proteins (such as neutrophil elastase) that exist at high concentrations on NET fibers.

The NETs may possibly facilitate killing of microbes in two ways: (i) concentrate the antimicrobial arsenal to the site of infection and (ii) prevent the spread of microbes from the initial site of infection [3]. NETs are presumably formed after phagocytic killing mechanisms are exhausted because NET-formation is initiated after in vitro infection whereas neutrophils phagocytose microbes within the first minutes of contact.

Upon releasing NETs, neutrophils lose nuclear DNA that is essential for cell survival, resulting in cell death. This form of active cell death is fundamentally distinct from necrosis and apoptosis, and represents a novel, third type of programmed cell death that is termed NETosis [5]. Citrullination of histone H3 by peptidylarginine deiminase 4 (PAD-4) playing a pivotal role in chromatin de-condensation during this NETosis.

Involvement of Nets in Infectious Diseases

The results of *ex vivo* experiments clearly demonstrated that NETs can trap and kill various bacteria (including both gram-positive and gram-negative bacteria), leading to decreases in their cell numbers. Furthermore, a variety of chemical and biological substances, such as phorbol 12-myristate 13-acetate (PMA), lipopolysaccharides (LPS),

and interleukin-8 (IL-8), reportedly act as stimuli that induce NET formation by human neutrophils [6,7].

Pathogenic bacteria and fungi have evolved efficient strategies to outfox the weaponry of neutrophils. Urban et al. reported that the main strategies to evade from NETs could be divided into six categories: Launching a general survival response, avoiding contact, preventing phagocytosis, surviving inside the neutrophil, inducing cell death and, avoiding killing in NETs [3].

The role of neutrophils in controlling viral infections is often overlooked. However, there is a report describing how human immunodeficiency virus-1 can trigger NET formation in mouse neutrophils [8]. Furthermore, emerging evidence obtained with animal models suggests, albeit indirectly, the involvement of NETs in the defense against infections with feline leukemia virus and influenza A virus [5].

To date, fungal pathogens such as *Candida albicans* and *Aspergillus fumigatus* have also been reported to stimulate the formation of NETs that can capture and kill these microorganisms [6]. Bianchi et al. have reported that NET formation and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase contributed to fungal infection in a patient with chronic granulomatous disease and impaired phagocyte NADPH oxidase function [9]. They also used gene therapy in this patient to restore production of NETS and the ability of neutrophils to eliminate *Aspergillus nidulans* conidia and hyphae, leading to a rapid cure of invasive pulmonary aspergillosis [10]. These data suggested that reactive oxygen species (ROS) play an important role in the formation of NET.

As for parasitic protozoans, it is known that *Plasmodium* and *Leishmania* can trigger the release of NETs that, in turn, trap these infectious agents [11].

However, these reports all presented the results of ex vivo studies. Currently, there are only a limited number of publications that demonstrate a direct involvement of NETs in infectious diseases in humans. One of the reasons for the lack of human studies is that there have been no established assay methods for NETs, which makes it difficult to quantitatively analyze these extracellular structures in human clinical samples.

Quantitation and Significance of NETs in Human Samples

We first analyzed the fluorescence microscopic images of NETs that were present in sputum aspirated from patients requiring tracheal intubation. We reported that NET levels in the sputum correlate with the severity of respiratory infection [12].

We also showed that Gram-staining can be used to detect NETs in sputum collected from a patient with severe pneumonia [13]. This patient had rapidly progressive severe pneumonia caused by *Haemophilus influenzae* (non-typeable, NTHi) and ultimately died of multiple organ failure associated with bacteremia. Most patients infected with NTHi have relatively mild bronchopneumonia. However, this was a rare case where the patient had lobar pneumonia-like shadows that are typically observed when the lungs are infected with *Streptococcus pneumoniae* or *Legionella pneumophila*. It is possible that the introduction of the *H. influenzae* type b vaccine for children caused a pathogen shift (serotype conversion), resulting in a marked increase in the incidence and severity of NTHi infection [14]. In this clinical case, we observed severe inflammation that rarely occurs in elderly people with pneumonia associated with NTHi infection.

We also detected a large number of fibrous structures (i.e., NETs) in sputum, and they were mostly colocalized with the bacteria. The patient also presented with bacteremia, suggesting the bacteria's remarkably high pathogenicity, from a microbiological viewpoint. We concluded that the primary reason for the severity of this infection was pathological conditions created by the synergistic interaction between the host and the microorganisms. This case also suggested the multifaceted functions of NETs.

The combined results described above strongly indicate the involvement of NET formation in the pathogenesis of acute respiratory infection in humans. Using accumulated clinical data, we further conducted a quantitative analysis of NETs and demonstrated their correlation with various inflammatory markers, such as cytokines and C-reactive protein [15].

In the future, in addition to pneumonia cases, we will also further investigate cases of bacteremia [16,17] and skin and soft tissue infections [15)]. We plan to evaluate them from the perspective of both the host and the microbe, and to publish a series of reports that will address the same questions as those addressed in our studies of pneumonia patients.

Autoimmunity and NETs

The inherent function of a NET is to provide a defense against microorganisms. However, its excessive production could be somewhat detrimental to host tissues. Moreover, we began to see an upward trend in the incidence of autoimmune diseases caused by autoantibodies against the extracellular DNA in the NET.

An example of self-tissue damages caused by NET overproduction is its involvement in the pathogenesis of disseminated intravascular coagulation [18,19]. Histones, a component of NETs, are also reportedly associated with the rapid progression of acute respiratory distress syndrome [20].

To understand the pathogenesis of severe lung injury caused by influenza viruses, we have been studying severe pneumonia using a model of bacterial (primarily *Streptococcus pneumoniae*) co-infection [21]. We have also been analyzing pneumonia caused by viral infection alone of genetically engineered knockout mice [22].

Using the co-infection model, we found an increase in the enzymatic activity of the neutrophil-related protein alpha-antitrypsin and a greatly enhanced expression of other neutrophil-associated proteins such as lysozyme [23]. We also reported that an inhibitor of neutrophil elastase can ameliorate the pathological changes in pneumonia [24]. Furthermore, we have been trying to determine proteins and molecules that are implicated in the pathogenesis of pneumonia, and may succeed in identifying candidate molecules in the near future [25]. We expect these molecules to be associated with neutrophils or platelets. Using genetically engineered mice, we showed that influenza virus-induced excessive immune responses could cause severe lung injury (=pneumonia< pulmonary edema?) and that these responses could be down-regulated by inhibiting neutrophil reactions [22].

In these studies, we observed a series of excessive inflammatory reactions (known as the “cytokine storm”) that ultimately led to a robust activation of neutrophils. We hypothesize that this neutrophil

activation is identical to or substantially indistinguishable from NET formation. Further studies will be conducted to verify this hypothesis.

Concluding Remarks

In this review, we focused on the merit and importance of neutrophil activation during infection, and specifically outlined the functions of the novel innate immune defense mechanism NET. Although NETs were first reported as an innate immune mechanism of neutrophils, they have been vigorously studied worldwide by both basic and clinical investigators in a wide variety of medical research areas, including not only infectious diseases, but also allergic and autoimmune diseases. However, the fundamental roles of NETs in the pathogenesis of these diseases are not yet fully understood.

In the future, results of NET research will be integrated with the accumulated knowledge of infection and immunity. This integration would help to unravel a set of biological principles that could explain how to control microbial invasions, while simultaneously preventing host tissue damage caused by excessive immune responses. We expect that these principles will play an important role in developing new treatment strategies that are directly applicable to clinical practice.

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