Neutrophils, NETs, NETosis and their paradoxical roles in COVID-19

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Abstract

The pandemic of COVID-19 has adversely affected the world in many aspects. The health and economic sectors suffer most of the repercussions of this disease. The search for a cure for this rapidly spreading virus which is causing massive life losses worldwide requires clear understanding of the immunopathogenesis of this virus so as to develop pinpointed targeted therapies rather than relying mainly on supportive care measures and drug repurposing to fight this life-threatening virus infection.

Neutrophils, neutrophil extracellular traps (NETs), and NETosis are not well studied not only in COVID-19, but also in coroviruses in general. The review will shed lights on the functions of neutrophils, NETs, and NETosis in various infectious complications as well as in sepsis and acute lung conditions in an attempt to understand their actual roles and in order to help in designing targeted therapies in the near future.

Introduction

In late December 2019, an unprecedented outbreak of pneumonia emerged in Wuhan City, Hubei Province in China. The infection was caused by a novel beta coronavirus which was initially called severe acute respiratory syndrome (SARS-CoV-2), then it was named coronavirus disease-2019 (COVID-19) by the world health organization (WHO) [1-4]. Due to the continuous and rapid rise in the incidence of COVID-19 worldwide and due to the massive human life losses as well as the huge impact of the infection on world economy, the WHO declared COVID-19 a pandemic on March 11th, 2020 [3-5].

The incubation period ranges between 2 and 14 days [3,4]. The clinical manifestations of COVID-19 include: fever, cough, shortness of breath, sore throat, fatigue, nausea, vomiting, and diarrhea. However, the illness may be complicated by: severe pneumonia, acute respiratory distress syndrome (ARDS), and respiratory failure; acute cardiac decompensation, arrhythmias, and heart failure; secondary bacterial infection; acute renal and liver dysfunction followed by multiorgan failure; sepsis and septic shock; and death [1,3-5]. Although the detailed immunopathogenetic mechanisms have not been fully elucidated, the main pathological findings that have been described include: diffuse alveolar damage in the lungs manifested by severe pneumonia; immune dysregulation; infection of the cells expressing the surface markers angiotensins converting enzyme (ACE)-2 and TMPRSS2 protein; recruitment of several inflammatory and immune cells including monocytes, macrophages, T-lymphocytes, neutrophils, and B-lymphocytes; and massive production of inflammatory cytokines and chemokines [2,6].

Unfortunately, no specific antiviral treatment is recommended and there is no available vaccine so far [1,5,7]. The available therapeutic interventions include: (1) symptomatic measures and supportive care; (2) administration of oxygen via mask, non-invasive ventilation, endotracheal intubation and mechanical ventilation; (3) management of septic shock and secondary bacterial infections; (4) several antiviral and anti-inflammatory drugs have been repurposed and these include: corticosteroids, interferons, chloroquine, ribavirin, lopinavir, ritonavir, remdesivir, and arbidol; (5) monoclonal antibodies such as tocilizumab which is used in the treatment of cytokine release syndrome to inhibit interleukin (IL)-6; (6) ACE inhibitors; (7) Chinese traditional medicines; (8) convalescent serum or plasma containing virus antibodies; (9) auxiliary blood purification therapy; and (10)
cellular therapies including the use of mesenchymal stem cells (MSCs) [1,2,5-7]. However, studies have shown that combination of several therapeutic modalities appear to be more successful than using single agents [1,5,7].

**Neutrophils**

Neutrophils are classically considered as essential players in host defense against invading pathogens [8]. They are: the most abundant leukocytes in the peripheral circulation, the key components of the effector and regulatory mechanisms of both the innate and adaptive immune responses, and the first cells to migrate to the sites of infection and sterile inflammation in order to exhibit a wide range of sophisticated functions including NETosis, which is the release of neutrophil extracellular traps (NETs), and killing microorganisms by phagocytosis [9-14]. The short half-life of neutrophils in the circulation, which is approximately 4 hours, is balanced by their continuous and tightly controlled release from the bone marrow [12]. Neutrophils reaching the circulation are: equipped with the proteins that are required to kill microorganisms and directed by cytokines into the infected cells [15]. In response to infection, polymorphonuclear leukocytes (PMNLs) are recruited to the sites of infection and they employ the following three major strategies to fight various microbes: phagocytosis, degranulation, and NETosis [16-18].

Neutrophils have various types of granules that contain hundreds of proteins, enzymes and other substances with important effects on innate and adaptive immune responses and these include: α-defensins, lactoferrin, neutrophil elastase, myeloperoxidase, citrullinated histone H3, human cathelicidin antimicrobial peptide LL-37, and human cathelicidin cationic antimicrobial protein-18 (hCAP18) [9,11]. Neutrophils target pathogens by diverse mechanisms that include: phagocytosis, pinocytosis, cytolysis, cytotoxicity, NETosis with the extrusion on an extracellular chromatin meshwork, generation of reactive oxygen species (ROS), and release of microbicidal molecules from cytoplasmic granules [12,15,19-22]. During infection, neutrophils can undergo beneficial suicide resulting in the production or release of NETs in order to combat invasion by pathogens [13].

Several studies have shown that, during overwhelming infections and severe sepsis, not only that neutrophils become dysfunctional or even paralyzed but also that their antimicrobial arsenal may contribute to further tissue damage and organ failure so that the host becomes unable to contain or eliminate the infection [8,23-26]. In patients with severe inflammation, infectious complications may develop even in the presence of neutrophilia as dysfunction of neutrophils will ultimately result in inability of the host to clear the existing infection [26]. Hence, in immunocompromised patients having neutropenia with severe sepsis and overwhelming infections, host immunity can be boosted further by donor granulocyte transfusions and intravenous immunoglobulins [27-30].

**New concepts on neutrophils and their functions:** Traditionally, neutrophils have been considered as short-lived, relatively homogeneous population and as terminally differentiated cells that do not recirculate [31]. However, recent studies have shown that neutrophils may differentiate into distinct subsets defined by: specific phenotype and functional profile with well-defined genomic and molecular markers under certain physiological as well as pathological circumstances that include: cancer, sepsis, trauma, ischemic reperfusion injury in addition to ageing and transformation of neutrophils [31-34]. As early as the year 1920, it was realized that circulating neutrophils could show significant differences in parameters such as phagocytosis, protein synthesis, and oxidative metabolism [31]. Additionally, neutrophils can exhibit reverse transmigration and reenter the circulation after shifting their phenotype towards a proinflammatory state with longer life span of about 5.4 days and this may ultimately lead to dissemination of systemic inflammation [31].

**Studies have shown that neutrophils are involved in:**

1. activation and maturation of macrophages, monocytes and dendritic cells (DCs),
2. regulation of T-cell immune responses against various pathogens and tumor antigens, and
3. complex bidirectional interaction or crosstalk with macrophages, T-lymphocytes, natural killer cells, MSCs, platelets, and B-lymphocytes [33-35]. Many of the effector functions of neutrophils are regulated by a series of immunoreceptors on the plasma membranes [36]. Progress in understanding the heterogeneity and plasticity of neutrophils, determination of specific neutrophil subtypes, and the identification of the interactions mediated by the immunoreceptors of neutrophils may be helpful in the diagnosis of specific diseases and in the development of novel therapeutic interventions [31,32,36].

**Neutrophils in viral infections:** Neutrophils are capable of recognizing viruses via viral pathogen-associated molecular patterns (PAMPs) and they respond to viruses with specific effector functions [39]. The number of neutrophils in the lower respiratory tract in patients with severe pneumonia correlates with disease activity but may contribute to ALI and other detrimental effects to the host [37,38]. In a mouse model, systemic administration of virus analogs or poxvirus infection may induce recruitment of neutrophils to the sites of infection in order to release NETs that can protect host cells from virus infection [39]. Mechanisms by which neutrophils contribute to clearance of viral pathogens include: virus internalization and killing; interaction with other immune cell populations; release of cytokines, chemokines, and antimicrobial components; viral sensing by cytosolic RNA helicases; and NET formation which may further mediate antiviral defense by trapping and inactivating viruses [38].

The cytokines induced by PAMPs and produced by leukocytes are predominantly inflammatory and they include: tumor necrosis factor (TNF)-α, IL-6, and IL-1 components [38,40]. Production or release of inflammatory cytokines and
Chemokines is the siren of neutrophil recruitment to the sites of infection and inflammation. However, in patients having severe pneumonia or sepsis, accumulation of neutrophils in the microcirculation leads to excessive cytokine release or cytokine storm that can lead to deleterious complications and poor clinical outcome [41-43]. Therefore, neutrophils may be a keystone species in determining the outcome of viral disease [37,38].

Recently, it has been shown that viruses act as triggers of the process of NETosis [44-47]. However, virus-induced NETosis can act as a double-edged sword: on one hand making mechanical entrapment of the virus while on the other hand causing harm by the release of NETs triggered by the inflammatory and immunological reactions. Additionally, virus-induced NETs can circulate in an uncontrolled manner leading to an extreme systemic response manifested by production of cytokines, chemokines, and immune complexes that favor inflammation [44]. Neutrophils as well as NET formation play important roles in Dengue virus infections in humans [48,49]. In addition to the induction of thrombosis, NETs may acquire proinflammatory roles and cause damage to the activated human endothelial cells [50].

**Immunological and hematological changes in COVID-19**

Several studies on COVID-19 have shown the following abnormalities: (1) dysregulation of immune responses; (2) functional exhaustion of cytotoxic lymphocytes; and (3) abnormal peripheral blood picture including: low blood counts of lymphocytes, monocytes, eosinophils, and basophils; low hemoglobin level; low platelet count; leukocytosis or leukopenia; high neutrophil: lymphocyte ratio (NLR); and high monocyte: lymphocyte ration [51-55]. Also studies have shown that high NLR and lymphopenia are independent risk factors for: disease severity, poor clinical outcome, and mortality [51,53,54].

**NETs**

NETs are threads or web-like structures of unique extracellular DNA framework, decorated with antimicrobial peptides or proteins released from cell death of activated neutrophils to trap, degrade, fight, and kill pathogens [14,56-59]. So, NETs are extracellular structures composed of chromatin and granule proteins that bind and kill microorganisms [15,19]. NETs arise from neutrophils that have activated a cell death program called NETosis or NET formation and nuclease activity are reduced [20].

It is unknown whether NET formation takes place in bloodstream or in body tissues [56]. Pathogens are trapped, immobilized within viscous web-like structures and influenced by high concentrations of antimicrobial compounds such as: neutrophil elastase, histones, and myeloperoxidase [57]. NETs; which are composed of degraded chromatin and granule of neutrophil origin; may play important roles in innate immunity against microbial infections [9,58]. Factors that have been demonstrated to influence NET pathways include: (1) internal factors such as production of ROS and activation of transcription factor, and (2) external factors such as alkaline PH and hypertonic conditions [56]. During sepsis, NETs promote pyroptosis or regulated cell death of macrophages [16]. In patients with septic shock, unstimulated NET formation and nuclease activity are reduced [20].

**The formation of NETs can be influenced by:** (1) microorganisms such as bacteria, viruses, fungi, and parasites; (2) cytokines such as IL-8 and TNF-α; (3) antimicrobials such as amoxicillin; and (4) chemicals such as calcium ionophore A23187 and phorbol myristate acetate (PMA) [10,13,57]. The key enzymes that are involved in NET formation include: (1) neutrophil elastase which degrades intracellular proteins and triggers nuclear disintegration; (2) peptidyl arginine deiminase type 4 (PAD-4) which citrullinates histones to facilitate the decompensation and release of chromosomal DNA; (3) gaseidermin D which generates pores in the membranes of neutrophils thereby facilitating cell membrane rupture and expulsion of DNA and associated molecules; and (4) myeloperoxidase granule enzyme [21,65,66].

**Antimicrobial activity of NETs can be measured by different methods including:** (1) induction of the formation of NETs then addition of microbe and finally assessment of the number of surviving bacteria after an incubation period, and (2) measuring microbial killing by blocking NET components with antibodies or cation chelators such as zinc [18]. Statins have been found to enhance the formation of phagocyte extracellular traps [67].

NETs can inactivate virulence factors or microbial proteins that modify the function of host cells [18,61]. Fully hydrated NETs have a cloud-like appearance and they occupy a space which is 10 to 15 times larger than the volume of the cells they originate from [18]. Identification or detection of NETs might serve as a biomarker that could help in identifying individuals at high risk of developing consequences of acute lung injury (ALI) and acute kidney injury (AKI) [68,69]. Methods that can be used to quantify or visualize NETs include: high resolution scanning by electron microscopy; intravital photon microscopy; flow cytometry; fluorescent labelling of microorganisms by direct visualization; immunostaining and automated microscopy using computer-assisted analysis.
to quantify NETs from fluorescence images and machine learning using conventional neural networks [10,17,18,61-64].

The dark side of NETs: In addition to their antimicrobial actions, NETs have a dark side reflected by their involvement in certain diseases or complications such as: (1) autoimmunity and autoimmune disorders such as systemic lupus erythematosus, rheumatoid arthritis, and psoriasis; (2) pregnancy associated disorders such as pre eclampsia; (3) cystic fibrosis; (4) coagulopathy and thrombosis; (5) periodontitis; and (6) tissue injuries [10,13,14,18,50,65,75-79]. NETs have prothrombotic properties by stimulation of fibrin deposition and increased NET levels correlate with larger infarct size and predict major cardiovascular complications [78]. Additionally, excessive NET formation can trigger a cascade of inflammatory reactions that destroy surrounding tissue, facilitate microthrombi and result in permanent damage to the pulmonary, cardiovascular and renal systems [21]. NETs can exert direct cytotoxic effects on lung epithelium and endothelium and excessive production of NETs has been found in patients with ALI and pneumonia. Thus, NET formation can exert positive as well as negative influences on multiple lung pathologies [80,81].

In patients with diabetes mellitus, hyperglycemia induces or boosts NET formation and this may cause direct damage to endothelial cells and may predispose to complications such as diabetic retinopathy and diabetic wounds [10,78]. In patients with severe influenza A virus infection, high levels of NETs contribute to lung injury, correlate with disease severity and imply poor prognosis [82]. In a model of influenza A virus (H1N1) infection, excessive neutrophils and NETs might lead to: ALI, ARDS, and pneumonitis with alveolar-capillary damage [83]. Nicotine has been found to induce NETs which may contribute to smoking-related lung diseases [84].

NETosis

NETosis, a recently described neutrophil function, leads to the release of NETs in response to various stimuli and it represents the most dramatic stage in the process of cell death [11,14]. During NETosis, PMNLs undergo specific morphological changes that include: chromatin condensation leading to loss of the lobulated nucleus, disintegration of intracellular membranes that allows chromatin and extracellular protein to mix, and the release of chromatin filaments decorated with PMNL proteins derived from several cell compartments into the extracellular medium [14].

In NETosis, the following enzymes, chemicals, and signaling pathways are involved: (1) neutrophil elastase, (2) myeloperoxidase, (3) PAD-4, (4) PMA, (5) nicotinamide adenine dinucleotide phosphate (NADPH) which generates ROS, (6) mitogen activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathway, (7) Toll-like receptors (TLRs), and (8) autophagy pathway. Also, the following steps or changes take place during NETOSIS: activated neutrophils flatten and lose lobes of their nuclei, chromatin becomes condensed, nuclear detachment of the inner and outer membranes, separation of granules, the nuclear envelope breaks into pieces, and the cells roundup until the cell membrane ruptures and ejects the inner contents into the extracellular space forming NETs [15,17,18,74].

There are 2 types of NETosis [17,74]. The first type is suicidal NETosis which is slow, takes hours, and is induced by chemical stimuli such as PMA. In this type of NETosis, the following take place: occurrence of morphological changes in activated neutrophils, the release of NETs results in neutrophil death through a different pathway than apoptosis or necrosis, and the intracellular NET formation is followed by rupture of plasma membrane releasing the contents into the extracellular space thus forming NETs. The second type is vital NETosis which is rapid, takes minutes, and is induced by bacteria and other pathogens. In this type of NETosis: stimulated neutrophils remain active and functional following NET formation, the process results in blebbing of the nucleus to produce a DNA-filled vesicle that is exocytosed thus leaving the plasma membrane intact, and neutrophils can continue to phagocytose and kill microbes after NETosis [17,74].

Neutrophils, NETs, and NETosis in COVID-19

The pathological consequences of SARS-CoV infection in the lung include: (1) the virus applies several mechanisms to overcome the immune response including: inhibition of the rapid expression on type 1 interferon (IFN-1), intervention with IFN-signaling through inhibition of STAT 1 phosphorylation, and immune exhaustion through exaggerated and prolonged IFN-1 production by plasmacytoid DCs; and (2) influx of activated neutrophils and inflammatory monocytes/macrophages resulting in ARDS and cytokine storm thus weakening the immune system through IFN-1 mediated T-cell apoptosis. SARS-CoV2 causing COVID-19 is expected to have the same or at least similar consequences on the immune system as SARS-CoV due to similarities between the 2 coronaviruses [85]. So, it is possible that: (1) excessive recruitment of various immune cells such as neutrophils, macrophages, monocytes, DCs, and T-lymphocytes; (2) NETs; and (3) NETosis may be responsible for many of the serious complications of COVID-19 such as: ARDS, cytokine storm, thromboembolic complications, acute organ dysfunction, and multiorgan failure [44,85,86]. In patients with COVID-19, high levels of NETs have been documented and it has been found that NETs may contribute to: cytokine release, ARDS, respiratory failure, as well as disseminated inflammation and microvascular thrombosis [87].

Possible therapies and therapeutic targets for COVID-19

Targeting upregulation or downregulation of NETs with destruction or protection of already formed NETs may become a valuable therapeutic intervention in patients.
having severe pneumonia or ARDS [80,88]. As circulating NETs may be directly responsible for orchestrating ALI and ARDS, inhibition of NETosis may become valuable in reducing inflammation and organ damage [68]. In patients with respiratory syncytial virus infection, neutrophils could limit viral replication and spread by stimulating antiviral adaptive and immune responses [89]. Immune mediators such as: (1) GTS-21; the selective α7Ach receptor agonist that has the same inflammatory modulation effects as nicotine but without the risk of addiction and other side effects; and (2) platelet-derived acting factor-acylhydroxylase (RAF-AH) may become widely available for the treatment of cytokine storm associated with viral pneumonia [40].

Prostaglandin-E2 has been found to inhibit NET formation [90]. Probiotic Lactobacillus rhamnosus strain BB has potent antioxidant activity and can reduce the phagocytic function of neutrophil and inhibit NET formation [62]. The combination of zinc and pyrithione has been found to inhibit the replication of SARS-CoV (SARS coronavirus) [91]. In patients with COVID-19, the ability to form NETs may contribute to organ damage and increased mortality. Hence, targeting NETs directly or indirectly with the existing drugs may reduce the clinical severity of COVID-19 infection [21]. Also, treatments that inhibit viral replication or target regulation of the dysfunctional immune reactions may offer synergistic effects in order to block viral pathologies at multiple levels [6].

Conclusion and future directions

Many of the complications of COVID-19 such as: respiratory distress and failure; multiorgan dysfunction including cardiac decompensation; thromboembolic phenomena, the associated cytokine storm as well as the poor outcome encountered in cigarette smokers and in patients with diabetes mellitus can well be explained by the dysfunctional neutrophils and their products. Apparently the functions of neutrophils, NETs, and NETosis are not well characterized in COVID-19 due to the relative lack of studies on this aspect of the disease. As depicted from other viral infections that involve the lungs and cause serious complications, the roles of neutrophils, NETs, and NETosis seem to be paradoxical under certain circumstances. Therefore, further studies are needed in this field. These studies should focus not only on the numbers of neutrophils but also on their functions, subsets, life span of each subset, as well as migration to tissues and organs affected by infection, inflammation and other injuries. Such studies are likely to help in developing more efficacious therapeutic interventions that can bring cure to this devastating, widely spreading and life-threatening viral illness.

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