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Molecular Aspects of Medicine 81 (2021) 100996

Contents lists available at ScienceDirect

Molecular Aspects of Medicine

journal homepage: www.elsevier.com/locate/mam

Neutrophils at the crossroads of acute viral infections and severity

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ARTICLE INFO

Keywords:
Neutrophil extracellular trap
Neutrophil heterogeneity
Emergency granulopoiesis
COVID-19
Dengue

ABSTRACT

Neutrophils are versatile immune effector cells essential for mounting a first-line defense against invading pathogens. However, uncontrolled activation can lead to severe life-threatening complications. Neutrophils exist as a heterogeneous population, and their interaction with pathogens and other immune cells may shape the outcome of the host immune response. Diverse classes of viruses, including the recently identified novel SARS-CoV-2, have shown to alter the various aspects of neutrophil biology, offering possibilities for selective intervention. Here, we review heterogeneity within the neutrophil population, highlighting the functional consequences of circulating phenotypes and their critical involvement in exaggerating protective and pathological immune responses against the viruses. We discuss the recent findings of neutrophil extracellular traps (NETs) in COVID-19 pathology and cover other viruses, where neutrophil biology and NETs are crucial for developing disease severity. In the end, we have also pointed out the areas where neutrophil-mediated responses can be finely tuned to outline opportunities for therapeutic manipulation in controlling inflammation against viral infection.

1. Introduction

Neutrophils are polymorphonuclear leukocytes that comprise the first line of defense of the host immune responses against invading pathogens. While adequate neutrophil activation is necessary to clear pathogens, inappropriate or uncontrolled activation causes severe pathological consequences in infected patients. After sensing the microbial invasion in our body, neutrophils immediately move to the infection sites and restrict them by their armamentarium of effector functions. The primary means of defense is phagocytosis, by which the cells engulf and destroys the invading pathogens. Neutrophils also produce reactive oxygen species (ROS) by a process called "respiratory burst," which helps in killing the engulfed pathogen (Robinson, 2008). Their interaction with pathogens sometimes degranulates them, releasing their cytoplasmic and nuclear contents in the circulation, forming a web-like structure known as Neutrophil Extracellular Traps (NETs) (Brinkmann et al., 2004). Diverse classes of viruses, including newly identified novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (Arcanjo et al., 2020; J.Wang et al., 2020), Dengue virus (DENV) (Opasawatchai et al., 2019; Sung et al., 2019), Respiratory syncytial virus (RSV) (Funchal et al., 2015; Muraro et al., 2018), influenza virus (Narasaraju et al., 2011; Zhu et al., 2018) have shown to induce NET formation by various mechanisms. These viruses are also reported to alter the various aspects of neutrophil biology, leading to their activation (Opasawatchai et al., 2019), development of different subtypes (Cortjens et al., 2017), delay in the apoptosis (Parnell et al., 2011), and an increased propensity for the formation of NETs (Veras et al., 2020). Both the beneficial (Hiroki et al., 2020; Saitoh et al., 2012; Tate et al., 2011) and detrimental roles (Cortjens et al., 2016; Dicker et al., 2018; Narasaraju et al., 2011; J.Wang et al., 2020) of different subsets of neutrophils and NETs are implicated with the pathological outcome of many viral infections. Despite being the primary responders against viral infections, several clinical studies have correlated multiple factors associated with neutrophil biology, leading to a severe outcome. From their biogenesis to their death and clearance, various aspects of neutrophil biology have been associated with the severe form of viral

Abbreviations: NET, Neutrophil Extracellular Trap; $\alpha_M\beta_2$, alpha subunits for integrins Mac-1; VLA4, Very Late Antigen 4; TLR4, Toll-Like Receptor 4; ICAM-1, Intercellular Adhesion Molecule-1; ROS, Reactive Oxygen Species; NOX, NADPH Oxidase; G-CSF, Granulocyte Colony-Stimulating Factor; DENV, Dengue virus; CMP, Common Myeloid Progenitors; C/EBP, CCAAT/enhancer-binding protein; TPO, Thrombopoietin; LDN, Low-Density Neutrophil; PKC, protein kinase C; PAD4, peptidyl arginase deaminase 4; PD-L1, Programmed death-ligand 1.

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https://doi.org/10.1016/j.mam.2021.100996
Received 4 February 2021; Received in revised form 14 July 2021; Accepted 15 July 2021
Available online 18 July 2021

diseases. Thus, neutrophils can be considered a double-edged sword (Smith, 1994). A detailed understanding of the factors that influence neutrophil biology will provide new insights into their heterogeneous roles in disease conditions.

2. Neutrophil biogenesis and life cycle: steady-state vs. emergency granulopoiesis

Neutrophils constitute about 60% of white blood cells in human blood and are the significant sentinels of the innate and adaptive immune system (Nicolás-Ávila et al., 2017). They are considered to be short-lived with an estimated half-life of about 13–19 h (Lahoz-Beneytez et al., 2016; Tak et al., 2013), but in-vivo labeling with $^{2}H_2O$ in humans revealed a lifespan of up to 5.4 days (Pillay et al., 2010), suggesting neutrophil lifespan may vary with environmental or experimental conditions. In humans, the neutrophil pools need to be replenished by approximately 10^{11} cells per day at a steady state to maintain their physiological roles (Dancey et al., 1976; Hidalgo et al., 2019). This homeostasis is maintained by an intricate balance between the regulation of neutrophil production, storage, and egress from the bone marrow into the circulation (Anel et al., 2019). Additionally, the life span, apoptosis, and clearance of aged and senescent neutrophils from circulation by specialized macrophages are also crucial for maintaining homeostasis (Lawrence et al., 2020). The production of neutrophils in the bone marrow is achieved through a finely regulated process called granulopoiesis. Granulopoiesis leads to the production of multipotent and lineage-committed common myeloid progenitors (CMP) that are transformed into lineage-committed granulocyte progenitor cells (GMP) (Akashi et al., 2000). These cells then later differentiate into unipotent neutrophil progenitors. The entire process can be divided into different stages based on traditional methods of density gradient centrifugation and histological examination of the different fractions using Giemsa staining. These stages include myeloblast, promyelocyte, myelocyte, metamyelocyte, band cell, and polymorphonuclear neutrophils (Lawrence et al., 2018). The neutrophil production and maturation process in the bone marrow may vary under healthy and disease conditions (Manz and Boettcher, 2014). As a result of systemic viral and bacterial infections or inflammatory conditions, the circulation demand for neutrophils increases. Therefore, to meet the higher demand for neutrophils, granulopoiesis starts operating in emergency mode. Emergency granulopoiesis represents a regulatory loop that involves increased de-novo production of neutrophils in the bone marrow, which results in an accelerated cellular turnover and the release of immature and mature neutrophils from the bone marrow into the peripheral blood (Manz and Boettcher, 2014). Emergency granulopoiesis may lead to the production and release of immature and dysfunctional neutrophils with impaired oxidative burst response, high propensity to form NETs, and suppressive capacity on T-cell functions, indicating its failure in pathogen defense and imparting an overactive immune response, which can contribute to disease severity (Lemaitre et al., 2020; Manz and Boettcher, 2014; Schulte-Schrepping et al., 2020). Pathogen sensing mainly occurs in non-hematopoietic cells through PRAP, leading to the release of granulopoiesis cytokines like granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-3 (IL-3), and IL-6, that may also initiate signals for emergency granulopoiesis (Manz and Boettcher, 2014; Presneill et al., 2000). In addition, the direct interaction of pathogens with hematopoietic progenitor stem cells can trigger emergency granulopoiesis (Nagai et al., 2006). G-CSF stimulates the proliferation and differentiation of granulocytic precursor cells in steady-state as well as in emergency granulopoiesis (Zhang et al., 1997). Transcriptionally, CCAAT/enhancer-binding protein β (C/EBPβ) is the master regulator of emergency granulopoiesis (Hirai et al, 2006, 2015), whereas steady-state granulopoiesis is driven by C/EBPα, which is necessary for its initiation (Zhang et al., 1997). The other key transcription factors whose coordinated expression is required for early neutrophil differentiation are CEBPε and Gfi1 (Zhuang et al., 2006) while terminal differentiation is carried out by CEBPδ and PU.1 (Borregaard, 2010; Olson et al., 1995). After being terminally differentiated under resting conditions, neutrophils remain in the bone marrow for the next few days before being released into circulation. Bone marrow retention of neutrophils is achieved by the constitutive expression of CXCL12 by bone marrow stromal cells, which interacts with CXCR4 present on neutrophils (Eash et al., 2009). G-CSF also plays a vital role in neutrophil retention and trafficking from the bone marrow (Semerad et al., 2002). However, after sensing microbial invasion, neutrophils start mobilizing from the bone marrow to the infection site through chemotactic guidance. The release of neutrophils under both steady-state and emergency mode occurs by inhibiting the CXCL12-CXCR4 axis (Eash et al., 2009). The other mechanisms for their release include CXCR2-mediated mobilization (Devi et al., 2013; Wareing et al., 2007) and the release of thrombopoietin (TPO) by G–CSF–induced cells leading to neutrophil egress (Köhler et al., 2011). The number of neutrophils to be released is tightly regulated to ensure adequate protection at the infection sites and diminish collateral damage (Day and Link, 2012). Excessive neutrophil infiltration and activation tend to damage tissues at the infection site during inflammatory conditions (Narasaraju et al., 2011; J. Wang et al., 2020). Pathogenicity and viral load can influence the amount of neutrophil egress from the bone marrow to infected tissues (Bradley et al., 2012; Perrone et al., 2008).

After being released into circulation, neutrophils can also be retained in lung microvasculature, spleen, and liver, forming a reservoir defined as a marginated pool. The total blood granulocyte pool is estimated to be $9–79 \times 10^{7}$ cells/kg, with 51% of the cells in the marginated pool and the rest of the cells in circulation (Athens et al., 1961). This marginated pool probably serves as a pool for triggering rapid mobilization and imparting a protective role by provoking a local antimicrobial response. A large number of neutrophils are retained in the lungs, both as adhered to vascular lumen via CXCR4-CXCL12 interaction or in interstitial spaces (Devi et al., 2013). On reaching the infection site, circulatory and tissue-resident neutrophils exert their primary functions in host innate defenses by phagocyting the invading pathogen, release a plethora of antimicrobial granular proteins, and aid in the prevention of the disease (Amulic et al., 2012; Borregaard, 2010). CXCR4 plays dual roles in the retention of newly synthesized neutrophils and homing back of senescent or aged neutrophils to the bone marrow (Casanova-Acebes et al., 2013; Eash et al., 2009; Nagase et al., 2002). Neutrophils die by spontaneous apoptosis after aging and are removed through efferocytosis by specialized stromal macrophages (Lawrence et al., 2020). During infection and inflammation, neutrophils can survive for a longer time due to inhibition in their apoptosis, triggered by the cytokine milieu, pathogen-associated molecular patterns (PAMPs), and damage-associated molecular pattern molecules (DAMPs) (Geering et al., 2013). The extended neutrophil lifespan in chronic inflammatory conditions like asthma and acute coronary syndrome results in increased severity of the disease (Garlichs et al., 2004; Uddin et al., 2010). The extension in the lifespan of neutrophils under inflammatory conditions may enhance their capacity to exhibit heterogeneous phenotypes that may also shape the heterogeneity in their functions and thus the outcome of the infection (Silvestre-Roig et al, 2016, 2019).

3. Phenotypic heterogeneity and functional versatility of neutrophils

Neutrophils were traditionally considered as a homogenous population of terminally differentiated cells. But with the advancement of technologies, recent studies over the last decade have evidenced unexpected diversity in their phenotypic heterogeneity and functional versatility. As shreds of evidence begin to accumulate, it is now well known that neutrophils can have multiple phenotypes throughout their lifetime, i.e., during differentiation, maturation, and even after maturation (Hidalgo et al., 2019; Hong, 2017; Silvestre-Roig et al., 2019) but

the mechanisms that drive heterogeneity among neutrophils remains unknown. The neutrophil subpopulations have been characterized by their buoyant density, expression of particular surface markers (Fig. 1), interaction with other immune cells, and unconventional effector functions (Ng et al., 2019).

During their differentiation and maturation, neutrophils undergo a tremendous amount of changes in their nuclear morphology, mRNA expression of the granular proteins, and surface markers on the cells (Evrard et al., 2018; Kwok et al., 2020). According to the new model proposed for development of bone marrow neutrophils (Ng et al., 2019), GMPs ($Lin^{-}cKit^{+}Sca\text{-}1^{-}CD34^{hi}CD16/32^{hi}$) (Akashi et al., 2000) are revealed to be a heterogeneous pool of lineage-committed progenitors, comprising of an earliest neutrophil progenitor - eNeP ($Lin^{-}CD34^{+}CD66b^{+}CD117^{+}CD17^{+}$) (Dinh et al., 2020), unipotent neutrophil progenitors called pro-neutrophils (proNeu) ($Lin\text{-}CD34^{+}CD66b^{+}CD106^{+/-}CD49d^{high}CD11b^{low}CD71^{+}$), preNeu ($Lin\text{-}CD34^{low}CD66b^{+}CD15^{+}CD33^{mid}CD49d^{mid}CD101^{-}CD10^{-}$), non-proliferative terminally differentiated immature ($CD11b^{+}CD66b^{+}CD101^{+/-}CD10^{-}CD16^{low}$) and mature neutrophils ($CD11b^{+}CD66b^{+}CD101^{mid}CD10^{+}CD16^{high}$) (Evrard et al., 2018; Kwok et al., 2020) (Fig. 1A). Kwok et al. (2020), have dissected the proNeu subset in two phenotypically distinct subsets of $CD34^{hi}CD106^{-}CD11b^{low}$ proNeu1 and $CD34^{low}CD106^{-}CCD11b^{hi}$ proNeu2 subset and revealed that the proNeu1 subset is released at the expense of monocytes during emergency granulopoiesis in case of sepsis (Kwok et al., 2020).

After maturation, neutrophils display various phenotypes upon activation by various environmental cues, including aging, crosstalk with immune cells in different tissues. Pathological or inflammatory conditions can contribute to their phenotypic and functional heterogeneity (Ng et al., 2019). The activated neutrophils ($CD11b^{high}/CD16^{high}/CD62L^{low}$) can be differentiated from inactivated mature ($CD11b^{+}/CD16^{high}/CD62L^{high}$) and immature ($CD11b^{low}/CD16^{low}/CD62L^{high}$) cells after stimulation with LPS, G-CSF or PMA (Lakschevitz et al., 2016; Molloy et al., 2005). As neutrophils age and in the absence of an extracellular signal, they increase the expression of CXCR4, which increases migration of these senescent neutrophils back to the bone marrow, lungs, or spleen (Casanova-Acebes et al., 2013; Martin et al., 2003; Nagase et al., 2002).

Based on the localization of neutrophils in these tissues (Fig. 1B), they can display different phenotypes. The $CXCR4^{+}$ lung resident neutrophils have poor migratory capacity unless they are attracted by cytokines released by foreign invaders or inflammatory stimulus (Kreisel et al., 2010). These senescent neutrophils have increased surface marker expression pattern that includes CD11b (αM), CD49d (α4), VLA4, TLR4, ICAM1, CD11c, CD24, and CD45 (Adrover et al., 2016; Uhl et al., 2016). The neutrophils present in the spleen display the phenotype $CD62L^{low}/CD11b^{high}/ICAM1^{high}$ and tend to produce NETs (Cerutti et al., 2013). Neutrophils present in the marginal zone of the spleen have the $ICAM1^{high}/CD11b^{high}/CXCR1^{low}$ phenotype and can produce cytokines (BAFF, APRIL, and IL21) that promote immunoglobulin class switching and antibody production by splenic B cells (Puga et al., 2012).

Neutrophils can also change their phenotype while migrating back into the vasculature after their infiltration in the inflammatory tissues by a phenomenon termed reverse transendothelial migration (rTM) (Buckley et al., 2006). Based on the expression of ICAM1 and CXCR1, neutrophils are regarded as circulating neutrophils ($ICAM1^{low}/CXCR1^{high}$); tissue-resident neutrophils ($ICAM1^{low}/CXCR1^{low}$), and reverse transmigrated neutrophils ($ICAM1^{high}/CXCR1^{low}$) (Buckley et al., 2006). The $ICAM1^{high}/CXCR1^{low}$ are long-lived with increased ROS levels and increased propensity to produce NETs (Cerutti et al., 2013; Villanueva et al., 2011). Although the role of these rTM neutrophils can be both protective and tissue-damaging, but current studies suggest their detrimental roles in case of sepsis (Ji and Fan, 2021), trauma, ischemia-reperfusion, and other chronic inflammatory diseases (Hirano et al., 2016). Further studies need to be done to identify these rTM neutrophils in case of severe viral infections.

Based on their buoyant density, neutrophil subpopulations were further characterized. Neutrophils found in circulation typically are of normal density (NDNs), but low-density neutrophils or granulocytes (LDNs/LDGs) have also been reported in the case of systemic inflammation (Carmona-Rivera and Kaplan, 2013) and severe cases of sepsis (Drifte et al., 2013) where the majority of LDNs are found in the PBMC fraction (Hassani et al., 2020). LDGs have been poorly defined and comprise a wide variety of neutrophils with both pro-inflammatory and anti-inflammatory properties. They have markedly altered neutrophil chemotaxis, oxidative burst, lactoferrin content. These aspects were associated with an increased risk of death after septic shock (Drifte et al., 2013).

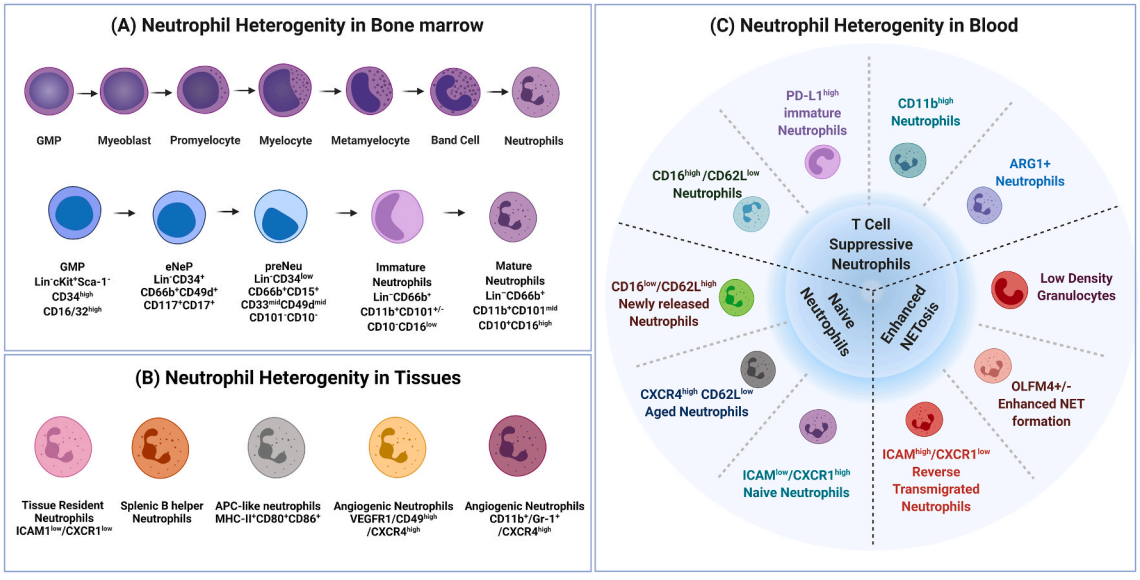

Fig. 1. (A) Heterogeneity of neutrophils in Bone marrow shows different stages of neutrophil development according to traditional methods (top panel), and neutrophil maturational subsets identified based on advanced methodologies (bottom panel). (B) Heterogeneity of neutrophils in Tissues – different neutrophil phenotypes with unconventional functions present in different tissues. (C) Heterogeneity of neutrophils in Blood – various phenotypes of neutrophils found in circulation can be classified into (i) naïve neutrophils, (ii)T cell suppressive subpopulations, and (iii) subsets showing the enhanced capacity to undergo NETosis.

Apart from this, various subsets of neutrophils can also activate and suppress other immune cells. A subset of neutrophils has also been reported to activate CD8 T cells. In the context of viral infection, neutrophils transport antigens from the dermis to the bone marrow, where they induced CD8 T cell priming and proliferation (Duffy et al., 2012). Based on the unique and unconventional functions of neutrophils, some subsets of neutrophils have been identified, like the pro-angiogenic subsets. These tissue infiltrating subsets characterized by VEGFR1/CD49-^{high}/CXCR4^{high} and CD11b^{+}/Gr-1^{+}/CXCR4^{high} neutrophils can promote angiogenesis and are associated with the growth of tumors (Christoffersson et al., 2012; Massena et al., 2015).

3.1. *Different phenotypes and functional modulation of neutrophils in viral infections*

Accumulating data showed that neutrophils could switch phenotypes and display distinctive subpopulations with unique and versatile functions (Hong, 2017) (Fig. 1C). The LDG phenotypes of neutrophils can specifically be altered during the viral invasion and have been reported in various types of viral diseases like dengue (Banerjee et al., 2017), HIV (Cloke et al., 2012), and even SARS-CoV-2 infection (Schulte-Schrepping et al., 2020). A subpopulation of aberrant LDGs is mainly reported to have enhanced capacity to form NETs in autoimmune diseases (Carmona-Rivera and Kaplan, 2013). LDGs detected in HIV-infected patients have increased cell surface expression of CD66b, CD63, and CD11b and decreased intracellular arginase 1 (ARG1) expression, suggesting that LDGs are probably activated neutrophils and primed to degranulates (Cloke et al., 2012). As observed in bacterial sepsis, LDGs from patients with severe COVID-19 also showed an impaired oxidative burst response, while their phagocytic capacity remained preserved (Schulte-Schrepping et al., 2020). Viruses also induce neutrophil heterogeneity to suppress the immune response elicited by the host. HIV directly influenced the expression of programmed death-ligand 1 (PD-L1) under in-vitro conditions, which can inhibit the proliferation of lymphocytes (Bowers et al., 2014). Neutrophils from HIV-infected patients also had increased levels of PD-L1, which correlated with markers of T cell exhaustion and increased neutrophil degranulation products (Bowers et al., 2014). High numbers of ARG1+ immature and PD-L1+ mature neutrophils were found in COVID-19 patients as distinct non-overlapping subpopulations (Schulte-Schrepping et al., 2020). Along with PD-L1, these neutrophils had increased CD64 and decreased CD62L expression, which suggests their activated and immunosuppressive phenotypes as observed with Myeloid-derived suppressor cells (MDSCs) (Schulte-Schrepping et al., 2020). Subsequent studies identified MDSC-like neutrophils from COVID-19 patients and showed their ability to inhibit T cell proliferation and IFNg production (Agrati et al., 2020; Sacchi et al., 2020). In the case of RSV infection in infants, blood neutrophils displayed heterogeneous phenotypes, with suppressive subtype (CD16^{high}/CD62L^{low}) and immature subtype (CD16^{low}/CD62L^{low}), which showed increased degranulation markers and decreased adhesion markers (Cortjens et al., 2017). This suppressive subtype (CD16^{high}/CD62L^{low}) has been reported to suppress T cell function (Pillay et al., 2012). The suppressive subset population increased upon bacterial co-infections later in the course of disease severity (Cortjens et al., 2017). Neutrophil heterogeneity during viral infections can have miscellaneous fates, which can be attributed to their modulated functions.

Apart from their heterogeneity, viruses can also modulate the function of neutrophils that helps them establish infection. Viruses, e.g., SARS-CoV-2, West Nile virus (WNV), and influenza virus, hijack neutrophils for replication and further dissemination in the host (Bai et al., 2010; Veras et al., 2020; Zhao et al., 2008). Although it is still unclear if RSV can replicate in neutrophils, but the presence of RSV protein and mRNA transcripts in neutrophils isolated from blood and BAL of severely infected infants show signs of active transcription in neutrophils (Halfhide et al., 2011). Many viruses activate neutrophils via PRRs to produce proinflammatory cytokines, chemokines, granular enzymes, and ROS (Brandes et al., 2013; Jaovisidha et al., 1999; Speth et al., 2013). RSV and human immunodeficiency virus (HIV) stimulated neutrophils to release neutrophil degranulation products like Neutrophil elastase (NE) and myeloperoxidase (MPO), chemokines (Abu-Harb et al., 1999; Jaovisidha et al., 1999; Speth et al., 2013), and IL-8 (König et al., 1996). Some viruses like DENV and SARS-CoV-2 trigger the release of ROS from neutrophils which can have virucidal effects (Arcanjo et al., 2020; Opasawatchai et al., 2019).

Viruses can also delay apoptosis of neutrophils which has also been reported to be associated with severe diseases (Parnell et al., 2011). Viruses like RSV and HCMV can delay apoptosis of neutrophils, thus increasing their lifespan and expansion of proinflammatory conditions (Lindemans et al., 2006; M. Coleman et al., 2011; Pocock et al., 2017). The interaction of RSV with neutrophils delays their apoptosis via Phosphatidylinositol 3-kinase, NF-κB pathway, TLR dependent pathways, and circulating IL-6 (Lindemans et al., 2006), and by a monocyte-derived soluble factor (M. Coleman et al., 2011). However, the exact mechanism of delay in apoptosis is unknown in the case of HCMV. Its genome encodes for a novel apoptosis inhibitor gene called UL36, which inhibits Fas-mediated apoptosis by inactivating caspase 8 in fibroblasts (Skaletskaya et al., 2001). Also, in the case of influenza A virus, the proto-oncogene B cell lymphoma 6 (Bcl6) deficiency in neutrophils promoted apoptosis of neutrophils in the lung tissues but not of circulatory or bone marrow neutrophils, which was associated with attenuated host inflammation and morbidity following IAV infection without decreasing viral load (Zhu et al., 2019).

4. Role of neutrophils in lungs pathology in respiratory viral infections

The infiltration of neutrophils in the lungs can help in clearing the infection or generating an antiviral immune response in acute viral infections (Tate et al., 2009). In some cases, excessive neutrophil infiltration can be pathogenic as viral replication in the host and infiltration of neutrophils in lung airways can cause increased mucus production and edema leading to blockage of airways and severe clinical outcomes (Cortjens et al., 2016; N. Wang et al., 2019). Neutrophils recruited to lungs two days post-infection by the influenza virus played protective roles by virus clearance (Tate et al, 2009, 2011), recruitment of effector CD8^{+} T cells (Lim et al., 2015), thereby limiting the severity of the disease. Interestingly, the lung infiltration of neutrophils may vary in response to the influenza virus as the infiltration was higher in viruses with higher pathogenicity as compared to that of viruses of lower pathogenicity (Perrone et al., 2008; Tate et al., 2011) and also in case of high doses of infection than the lower doses (Bradley et al., 2012). Infiltration of neutrophils in lungs can be critical in determining the severity of the infection since it can be both protective and pathological as the histopathological findings of lungs of two fatal human infections of avian influenza A (H7N9) virus (Feng et al., 2015), H5N1 virus (Perrone et al., 2008) revealed excessive neutrophil infiltration. Increased number of neutrophils in the lung is also a hallmark of severe RSV disease in both mouse and human, where neutrophils can constitute >90% of the cellular compartment of the bronchoalveolar lavage (BAL) during RSV-induced bronchiolitis in infants (Goritzka et al., 2015; McNamara et al., 2003). A systemic neutrophil influx from the bone marrow in infants with severe RSV bronchiolitis enhanced robust CD8^{+} T cell response leading to severe clinical manifestations (Lukens et al., 2010). RSV infection in epithelial cells increased the adherence capacity of neutrophils in *in vitro* conditions by increasing the expression of adhesion molecules like ICAM-1 on epithelial cells (Stark et al., 1996) and Mac-1 (CD11b/CD18) and LFA-1 (CD11a/CD18) on neutrophils (Wang et al., 1998). This increased interaction between neutrophils and epithelial cells significantly led to detachment of epithelial cells, thus augmenting the damage caused by the virus, which can contribute to airway injury and obstruction (Wang et al., 1998). Despite such

evidence, a recent study from Kirsebom et al. (2020) has reported that neither antibody-mediated (α-Ly6G) neutrophil depletion in mice model nor the enhancement neutrophils in lungs by administration of the chemoattractant CXCL1 during RSV infection affected disease severity in terms of (a) viral load, (b) induction of proinflammatory antiviral response or (c) weight loss (Kirsebom et al., 2020).

The airways of many infected patients have dense neutrophil infiltration because of the production of high levels of proinflammatory cytokines and chemokines (Rzepka et al., 2012). H3N1 viral RNA binds to TLR7/TLR8 to produce inflammatory cytokines (Wang et al., 2008). Human cytomegalovirus (HCMV) encodes a viral chemokine, vCXCL-1, that attracts neutrophils (Heo et al., 2015) and disseminates. In influenza infection, CXCL12 derived from neutrophil trials triggers the migration of protective virus-specific $CD8^+$ T cells in the lung airways (Lim et al., 2015). WNV infection increases the expression of neutrophils attracting chemokine osteopontin in both mice and humans, which could trigger an influx of WNV-infected neutrophils into the brain, making the infection more severe (Paul et al., 2017).

5. NETosis: underlying molecular mechanisms

The response of neutrophils against viral and bacterial infection may be multifaceted. One of the most important weapons in their arsenal is the formation of NETs through a process called NETosis. The NETosis seems to function as an alternative defense mechanism that is deployed when a cell's phagocytic capacity is overwhelmed (Branzk et al., 2014). NETosis is a special type of cell death, different from apoptosis and necrosis (Fuchs et al., 2007). NETs were first discovered in 2004, but they have recently gained massive attention in the scientific community. The antimicrobial activity of NETs is exerted through direct contact of the entrapped pathogens with antimicrobial peptides present in the azurophil granules (Borregaard and Cowland, 1997). Depending on the pathogenic nature or chemical inducer, NET formation pathways may vary. Classical NET formation pathways include the activation of integrins and Toll-like receptors (TLR) in response to bacterial-associated pathogen-associated molecular patterns (PAMPs) (Brinkmann, 2018; Sollberger et al., 2018). To date, two major types of NETosis have been described based on the origin of ROS (Vorobjeva and Chernyak, 2020); (1) NADPH oxidase (NOX) dependent and (2) NOX independent. NOX gets increased after neutrophil activation via the protein kinase C (PKC) pathway and increases ROS level in the cytoplasm. In NOX-independent NETosis, ROS are primarily produced by the mitochondria (Douda et al., 2015). ROS causes the rupture of granules and the nuclear envelope. This results in cytosolic calcium intake, peptidyl arginase deaminase 4 (PAD4) activation, and chromatin decondensation. NE and MPO are released from the granules and relocate into the nucleus to aid in DNA condensation (Papayannopoulos et al., 2010). Then chromatin spreads throughout the cytoplasm together with cytoplasmic and granule proteins, and finally, NETs are released out of the cell (White et al., 2017). The pathway of NETosis varies in the bloodstream and tissues (Pieterse et al., 2016). Under serum and platelets free conditions, mimicking tissue circumstances, lipopolysaccharide (LPS) from different bacterial sources induced ROS-dependent suicidal NETosis (Pieterse et al., 2016). Neutrophils die after NET formation in suicidal NETosis (Brinkmann et al., 2004). In bloodstream, in the presence of serum and platelets, LPS from different bacteria can induce ROS-independent vital NETosis. This form of NETosis is integrins and TLRs mediated, where the neutrophils remain viable and capable of chasing and engulfing the pathogen even after NET formation (Yipp et al., 2012). Another form of NETosis was observed by Azzouz et al., in 2018, where high exposure to ultraviolet light simultaneously induced NETosis and apoptosis in the same cell. This form was called apoNETosis and was NOX independent and different from calcium-induced NO independent NETosis (Azzouz et al., 2018). In 2015, Mohanty et al. reported another novel mechanism of NET formation in the oral mucosa induced by saliva and was independent of NADPH, NE, and integrins (Mohanty et al., 2015). It is important to note that only a proportion of responding cells within the pool undergo NETosis, suggesting that only a unique neutrophil subtype or maturation stage is susceptible to NETosis induction (Goldmann and Medina, 2012). A subset of neutrophils (~20–25%) expressing glycoprotein olfactomedin 4 (OLFM4) in healthy subjects. However, the frequency of $OLFM4^+$ neutrophils is increased in sepsis relative to healthy conditions (Alder et al., 2017). Proteomic analysis of NETs induced by different stimuli is heterogeneous in terms of both protein composition and post-translational modifications, suggesting that NET induced in other conditions may have different biological effects (Chapman et al., 2019). This is further supported by the fact that influenza A virus-induced NETs do not protect against secondary bacterial infection, suggesting virus-induced NETs may be structurally and functionally different from bacterial-induced NETs (Moorthy et al., 2013).

5.1. Molecular triggers for NETosis in viral infection

Virus-induced NETosis is mainly triggered by PRRs expressed on the surface or in endosomes of neutrophils, e.g., HIV-1 by endosomal PRRs - TLR7 and TLR8, hantavirus by β-Integrins, and DENV by C-type lectin receptor 5 (CLEC5) (Table 1). Both the RSV virion and RSV fusion protein has been reported to induce NETosis triggered by classical ROS dependent pathway via mechanisms dependent on PAD4 (Muraro et al., 2018) and via TLR-4, respectively (Funchal et al., 2015). Interestingly, NETs formed by RSV trapped the virion but could not kill the virus but caused airway obstruction in the case of severe lower respiratory tract disease in calves (Cortjens et al., 2016). SARS-CoV-2 was itself able to induce NET formation in a PAD-dependent manner and required angiotensin-converting enzyme 2 (ACE2) receptor, viral replication, and serine proteases (Veras et al., 2020). The PAD-independent NET formation has also been reported in the case of Enterovirus 71 (EV71), which induces NET by its capsid protein VP1 and causes lung pathology in mice (N. Wang et al., 2019). In addition, platelet activation is frequently observed during viral infections. Activated platelets form aggregates with neutrophils and, in this process, stimulate NETosis (Carestia et al., 2016; Sung et al., 2019). The other factor that can contribute to the formation of NETs by viruses includes proinflammatory cytokines and extracellular vesicles released by virus-infected cells. Virus-induced inflammation milieu and type I interferon (IFN) may prime neutrophils for NET formation (Martinelli et al., 2004). Extracellular vesicles DENV infected cells have been reported to induce NETs via CLEC5a and TLR2 (Sung et al., 2019).

Neutrophil activation and NET formation during viral infection may have beneficial and detrimental fate to the host. The significant beneficial effect reported is the prevention of viral spread. This is achieved mainly by the induction of NET formation by several viruses (Jenne and Kubes, 2015; Leppkes et al., 2020; Opasawatchai et al., 2019; Raftery et al., 2014; Toussaint et al., 2017) (Table 1). The negative charge and sticky nature of NETs help to immobilize or inactivate free virions via MPO and α-defensin (Saitoh et al., 2012), thereby preventing viral spread (Cortjens et al., 2016), and can potentiate the type I interferon release to increase the resistance of local cells to further infection (Souza et al., 2018) (Tillack et al., 2012). Similar advantageous roles of neutrophils and NETs in the case of HIV (Saitoh et al., 2012), poxvirus (Jenne et al., 2013), chikungunya virus (Hiroki et al., 2020), and RSV (Souza et al., 2018) have been reported.

Virus-induced NET can cause severe tissue damage. An example of NET-induced tissue damage was observed in patients having hemorrhagic fever, where NETs promote thrombosis by aggregating platelets and increasing the expression of tissue factor levels (Nicolai et al., 2020; Sivanandham et al., 2018). Increased NETs deposit on the blood vessel and increase capillary damage, hemorrhagic lesions, causing severe damage to the host (Villanueva et al., 2011). This phenomenon is most important in dengue patients with hemorrhagic fever where platelet count decreased insignificant proportion. In hantavirus, NETs generate

Table-1
Mechanism of NET formation by different viruses and their effect on neutrophil biology.

S. No.	Virus	Mechanism of NET formation	Effect of the virus on neutrophils	Reference
1.	SARS-CoV-2	- via SARS-CoV-2 through ACE2 - requires serine protease, PAD4, and viral replication	- An increased propensity for NET formation - Presence of immature neutrophils in severe cases - An increased expression of PD-L1	(Schulte-Schrepping et al., 2020; Veras et al., 2020)
2.	Dengue Virus	- via DENV through CLEC5a - via CLEC2 on platelets - via Extracellular vesicles	- Enhanced NET formation in the presence of DENV activated platelets	Sung et al. (2019)
3.	Influenza Virus	- via H1N1 in NADPH independent manner - no NET formation by H5N1	- Replication of H5N1 - Production of TNFα, IFN-β, CXCL10, MIP-1α, and IL-8	Chan et al. (2020)
4.	Respiratory Syncytial Virus	- via RSV Virion – ROS dependent and PAD4 mediated - via RSV fusion protein – TLR mediated	- Suppressive neutrophils are present in RSV infected infants - Shedding of L-Selectin and PAECAM-1 -Upregulation of Mac-1 and ICAM-1	(Cortjens et al., 2017; Funchal et al., 2015; Muraro et al., 2018; Wang et al., 1998)
5.	Hantavirus	- via Hantavirus through β-integrins	- Production of autoantibodies to nuclear antigens	Raftery et al. (2014)
6.	Chikungunya Virus	- via CHIKV through TLR7 and ROS dependent	- NETosis control CHIKV infection	Hiroki et al. (2020)
7.	HIV	- via HIV-1 through endosomal TLR7/TLR8	- Immune suppression via PDL-1/PD1 pathway	(Bowers et al., 2014; Saitoh et al., 2012)
8.	Simian immunodeficiency viruses (SIV)	- via unknown mechanisms in pigtailed macaques	- NETs trapped CD4, CD8, B cells, and monocytes -Presence of immature neutrophils - Decreased phagocytosis capacity	(Lemaitre et al., 2020; Sivanandham et al., 2018)
10.	Enterovirus V71	- via VP1 capsid protein in a PAD4 dependent pathway	- NETs downregulated vimentin on epithelial cells	(N. Wang et al., 2019)
11.	Poxvirus	- via poxvirus through TLR mediated pathway	-Presence of neutrophil-platelet aggregates -Enhanced NET formation by NPA	Jenne et al. (2013)

autoantibodies to nuclear antigens, leading to severe disease (Raftery et al., 2014). The NET-induced tissue damage is most prominent in lung pathology. NETs induced by Simian immunodeficiency virus (SIV) have been shown to capture immune cells like $CD4^{+}$ and CD8+T-cells, B cells, and monocytes, which may further contribute to a characteristic feature of immune cell depletion in HIV and SIV infections (Sivanandham et al., 2018), thus help in establishing the infection. The detailed detrimental roles of NETs in the case of SARS-CoV-2 and DENV have been discussed in the following sections.

6. Neutrophils as drivers of severity in COVID-19

COVID-19 is an ongoing pandemic caused by the newly discovered SARS-CoV-2. Nearly 18–80% of the individuals infected by the virus remain asymptomatic (Mizumoto et al., 2020; Nikolai et al., 2020), while some develop common symptoms like fever, cough, myalgia, and fatigue. Approximately 15% of patients may suffer severe outcomes (Huang et al., 2020; Z. Wang et al., 2020). The patients with severe symptoms eventually develop acute respiratory distress syndrome (ARDS), septic shock, or multiple organ failure (Huang et al., 2020; Zhou et al., 2020). A hyperactivated, immune-thrombotic response triggered by SARS-CoV-2 has been considered to cause the severity of disease and even death in many cases (Blanco-Melo et al., 2020; McKechnie and Blish, 2020; Schulte-Schrepping et al., 2020; J. Wang et al., 2020). A series of reports are now available, supporting the fact that neutrophil-mediated dysregulated responses as one of the critical drivers

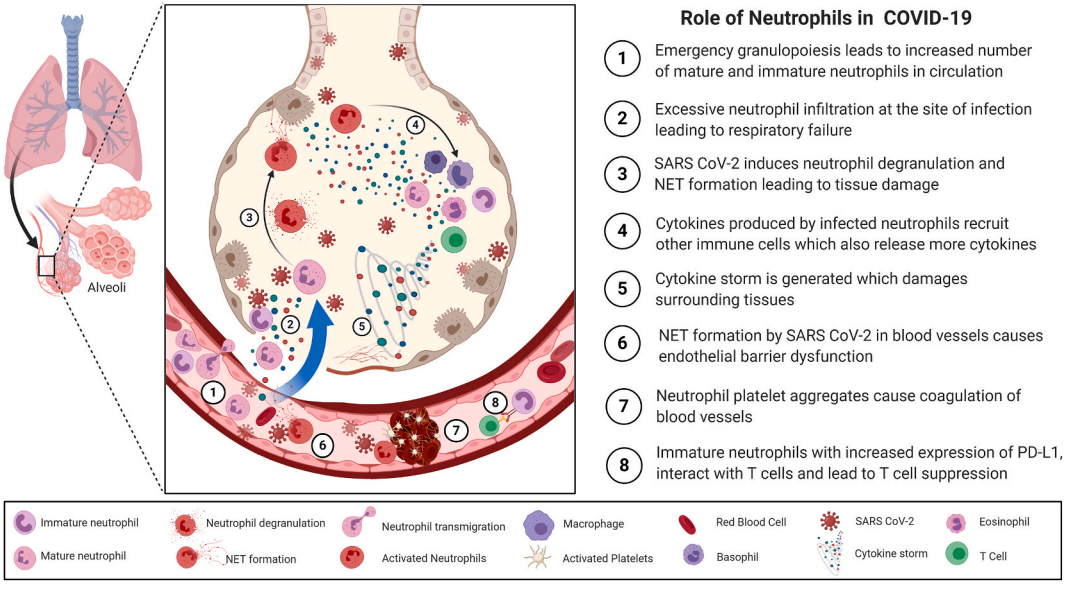

Fig. 2. Involvement of neutrophils in SARS-CoV-2 pathogenesis - Overview of the different roles of neutrophils that contribute to the pathogenesis of COVID-19.

of severity in COVID-19 (Fig. 2) (Qin et al., 2020; Radermecker et al., 2020; J. Wang et al., 2020). Earlier studies reported that the neutrophil to lymphocyte ratio (NLR) is the predictor of COVID-19 severity (J.Liu et al., 2020; Mutinelli-Szymanski et al., 2020) and an independent risk factor for severe disease (Y. Liu et al., 2020). Evidence of infiltration of an excessive number of neutrophils in many organs was reported from autopsies studies of severely infected patients who eventually succumbed to death (Li et al., 2020; J. Wang et al., 2020). These neutrophils are recruited to the sites of viral infection in response to the pro-inflammatory mediators (J. Wang et al., 2020) (Fig. 2). Increased infiltration of neutrophils and macrophages in the lungs contributed to the cytokine storms leading to fatal outcomes (Tang et al., 2020; Wichmann et al., 2020). A severe form of COVID-19 is driven by proinflammatory cytokine storm as evident by increased plasma levels of many mediators like IL2, IL6, IL8, IL7, IL10, G-CSF, IP10, MCP1, MIP1A, TNFα, and chemokines like CCL2 and CCL3 in ICU patients (Huang et al., 2020; Xiong et al., 2020). The levels of IL-6 and IL-8 directly correlated with the severity of the disease (Z. Liu et al., 2020; Ma et al., 2021) and are the major chemotactic factors that recruit neutrophils to the lungs (Hierholzer et al., 1998; Kunkel et al., 1991). The RNA-seq data from lung epithelial cells and bronchoalveolar lavage fluid of COVID-19 patients also highlighted the elevated levels of neutrophil attracting chemokines and several neutrophils associated genes (Blanco-Melo et al., 2020; Xiong et al., 2020). Apart from that, plasma TPO levels were significantly elevated in all COVID-19 patients (Manne et al., 2020), which increases neutrophil egress from bone marrow (Köhler et al., 2011). An increased number of immature neutrophils, particularly in severe cases of COVID-19 as compared to mild COVID-19 or patients with other flu-like illness, indicated towards emergency granulopoiesis in the bone marrow (Parackova et al., 2021; Schulte-Schrepping et al., 2020), which can be due to the elevated levels of G-CSF and GM-CSF in COVID-19 patients with severe disease (Huang et al., 2020; Zhao et al., 2021). Mass cytometry and single-cell RNA-seq studies revealed that severe COVID-19 patients consisted of transcriptionally distinct subclusters of proNeu and preNeu in later stages of the disease (Schulte-Schrepping et al., 2020). These LDNs were dysfunctional, primed to produced proinflammatory cytokines, exhibited an increased interferon response, degranulated, and showed an impaired oxidative burst response (Parackova et al., 2021; Schulte-Schrepping et al., 2020). The proNeu expressed genes like MPO, NE, and PRTN3, which are involved with NET formation (Schulte-Schrepping et al., 2020). Neutrophil infiltration in lungs and NETs have been reported to be involved in the inflammatory responses that contribute to the pathogenic manifestations of ARDS (Li et al., 2018; Mikacenic et al., 2018). In severe COVID-19 cases, the development of ARDS, thick mucus secretions in the lung airways, and blood clots were observed, similar to the symptoms of diseases being caused by NETs (Twaddell et al., 2019). The serum samples from severe COVID-19 patients had elevated levels of free ds DNA, MPO, and citrullinated histone H3 suggesting the release of NETs in severe cases (Arcanjo et al., 2020; Veras et al., 2020; J. Wang et al., 2020). Both the cell-free ds DNA and MPO levels in serum of COVID-19 patients correlated with the severity (Zuo et al., 2020). Serum isolated from COVID-19 patients and the SARS-CoV-2 virus-induced NET formation under in-vitro conditions (Middleton et al., 2020; Veras et al., 2020; Zuo et al., 2020). Excessive infiltration of neutrophils in the lungs of hospitalized COVID-19 patients has been reported (Barnes et al., 2020; N. Chen et al., 2020). Interestingly, the NET formation was comparatively higher in the bronchoalveolar lavage (BAL) than in blood in ARDS patients, suggesting the potential induction of NETs in the marginated pool in lungs either by the virus itself or by the inflammatory mediators (Bendib et al., 2019). Examination of immunofluorescently stained postmortem lung specimens of COVID-19 patients revealed that MPO + Cit-H3+ NETs were also present in lung airways, interstitial, vascular compartments (Radermecker et al., 2020), and vascular spaces of patients with ARDS (Mikacenic et al., 2018). These NETs were found to be associated with fibrin which completely occluded some alveoli or bronchioles (Radermecker et al., 2020). Severe COVID-19 patients also exhibited a highly pronounced formation of NETs inside the micro-vessels. Intravascular aggregation of NETs causes occlusion of the affected vessels, leading to organ damage (Leppkes et al., 2020; Radermecker et al., 2020). Another pathological mechanism that can lead to the enhanced propensity of NET formation in lungs is their interaction with activated platelets (Carestia et al., 2016). Activated platelets were present in COVID-19 patients (Nicolai et al., 2020; Petito et al., 2021) and increased neutrophil-platelet aggregates correlated with inflammation and severity in COVID-19 patients (Le Joncour et al., 2020). The activation pattern of platelets varied with the severity of COVID-19 as patients with intermediate severity showed exhausted phenotype while severe patients showed hyperactive phenotype (Nicolai et al., 2020). Platelets also aid in the recruitment of neutrophils in the lungs by directly interacting with P-selectin expressed on platelets (Margraf et al., 2019; Rossaint and Zarbock, 2013; Zarbock et al., 2006) and indirectly by release of proinflammatory mediators (Huang et al., 2004). This interaction can lead to the enhanced NET formation in the lungs and can contribute to inflammatory microvascular thrombi containing NETs associated with platelets and fibrin in the lungs, heart, and kidney of severe COVID-19 patients (Le Joncour et al., 2020; Nicolai et al., 2020). NETs are reported to contribute to microthrombi through platelet-neutrophil interactions in COVID-19 ARDS (Le Joncour et al., 2020; Middleton et al., 2020).

Apart from platelets, another reason for the enhanced capacity of NET formation by neutrophils isolated from COVID-19 patients is their modulated phenotype. In severe COVID-19, neutrophil granulocytes adopt a so-called low-density phenotype, prone to form NETs spontaneously (Radermecker et al., 2020). COVID-19 neutrophils ex-vivo displayed an increased propensity to form NETs at baseline (Middleton et al., 2020; Veras et al., 2020).

Blood transcriptomics study of COVID-19 patients revealed an enhanced expression of the CD177 gene and other neutrophil-associated genes (Manne et al., 2020). Importantly, CD177 is a cell surface antigen that may help mediate neutrophil adherence to the endothelium and thus impair human neutrophil migration (Bai et al., 2017). Other reports also suggested that the phenotype of neutrophils was different in severe cases from that of healthy donors or patients with moderate severity as they had hyporeactive phenotype as compared to overactivated phenotype in severe cases. (Leppkes et al., 2020; Nicolai et al., 2020). The SARS-CoV-2 infected neutrophils have enhanced expression of CD11b, CD66b, and CD64, indicating that the virus can modulate their phenotype (Peruzzi et al., 2020). Importantly, activated neutrophil release NE, a significant protease produced in the lungs during inflammation, may help the SARS-CoV-1 virus to infect cells efficiently (Belouzard et al., 2010) and plays a vital role in the exacerbation of SARS viruses (Matsuyama et al., 2010). Considering the similarity in sequence between the spike proteins of SARS-CoV-1 and SARS-CoV-2, we may speculate that in response to SARS-CoV-2 infection, in addition of high binding affinity to ACE2 receptor, neutrophils degranulation can release a proteolytic enzyme that helps to facilitate viral entry into the cells. All this accumulating evidence pointed towards the critical role of neutrophils and NET formation in the development of COVID-19 severity.

Apart from their inflammatory and tissue-damaging roles, neutrophils also have the propensity and ability to modulate the adaptive immune response. Interestingly, neutrophil subpopulations can also inhibit T cell proliferation and activation (Cathelijn E.M. Aarts et al., 2019; Pillay et al., 2012) and induce T-cell apoptosis (Michaeli et al., 2017). Transcriptomics data of blood from severe COVID-19 patients revealed an enhanced expression of genes with suppressive functionality, such as ARG1 or PD-L1 (Manne et al., 2020). Distinct populations of ARG+ and PD-L1+ neutrophils were present in COVID patients, which directly correlated with increasing severity (Schulte-Schrepping et al., 2020). Lymphopenia is observed in SARS-CoV-2 infected patients (Chen et al., 2020; J. jin Zhang et al., 2020) and the severe group had

decreased lymphocyte count than the non-severe group (Diao et al., 2020; Schulte-Schrepping et al., 2020). However, the mechanisms of lymphocyte reduction in severe patients remain unclear. On the other hand, neutrophils also influence lung resident γδ T cells mediated responses as NE potentiates the activation and proliferation of γδ T cells (Minns et al., 2019; Towstyka et al., 2018). γδ T cells tissue-resident immune cells are enriched in lung mucosal and epithelial sites (Cheng and Hu, 2017). Lung-resident γδ T cells play vital roles in antiviral immune responses and are involved in virus-induced lung inflammation and injury (Cheng and Hu, 2017). It is now known that elderly individuals have a lower number and slower kinetics changes of activated and proliferating γδ T cells than young men (Clark and Thomas, 2020; Stervbo et al., 2017). Therefore, age serves as an essential factor to affect the efficiency of T cell response. This is now supported by the recent study on COVID-19 patients that the percentages of CD4 γδ T cells within the γδ T cell population increased dramatically (Lei et al., 2020). Simultaneously, CD8 γδT remained unchanged in COVID-19 patients (Lei et al., 2020). The increase of CD4 γδ T cells indicated that in response to SARS-CoV-2 infection, this particular subset of γδ T cells might play roles in antigen presentation and facilitation of activation of adaptive immune cells. It is important to note that older people are most vulnerable to SARS-CoV-2 infection, and the morbidity rate is higher in older patients. Neutrophils isolated from aged people are phenotypically and functionally different from the younger population and may impact disease outcomes (Butcher et al., 2001; De Martinis et al., 2004; Stout-Delgado et al., 2009). Therefore, neutrophil biogenesis, phenotypes, and their interaction with other immune cells play a vital role in determining COVID-19 outcomes.

7. Neutrophils in severe dengue and hemorrhagic fever

Dengue is a mosquito-borne flavivirus that is spreading explosively in many parts of the world. Globally ~400 million people are infected with dengue each year, with the case fatality rate of about 5–20%. Clinically, dengue can present as a febrile illness or a severe, life-threatening disease known as Dengue Hemorrhagic fever (DHF). The hallmark of DHF is increased vascular permeability, leading to rash, bleeding, circulatory collapse, and shock. The molecular mechanisms of vascular leakage syndrome during dengue disease progression are ill-understood. Increased vascular permeability is associated with plasma leakage. It mainly occurs due to the malfunction of the vascular endothelium layer induced by either DENV alone (Glasner et al., 2017; Puerta-Guardo et al., 2016), or by cytokine storm (Bethell et al., 1998) and NET release by neutrophils (Sung et al., 2019) (Fig. 3). Previous reports have demonstrated transcriptomic changes associated with neutrophil activation in severe dengue patients (Banerjee et al., 2017; Long Truong Hoang et al., 2010). Activated neutrophils or their secretary product can interact with endothelial cells (Gupta et al., 2010; Saffarzadeh et al., 2012; Villanueva et al., 2011) and can shape immune function (Carmona-Rivera et al., 2015; Gupta et al., 2010; Villanueva et al., 2011). There is a lot of evidence that indirectly relates neutrophils and their products to the severity of the infection. The circulating levels of neutrophil enzymes (e.g., levels of free circulating DNA, MPO, NE) were higher in case of severe dengue as compared to uncomplicated dengue (Banerjee et al., 2017; L. T. Hoang et al., 2010; Juffrie et al., 2000; Opasawatchai et al., 2019), indicating an association of NET formation with dengue severity (Banerjee et al., 2017; Ha et al., 2011).

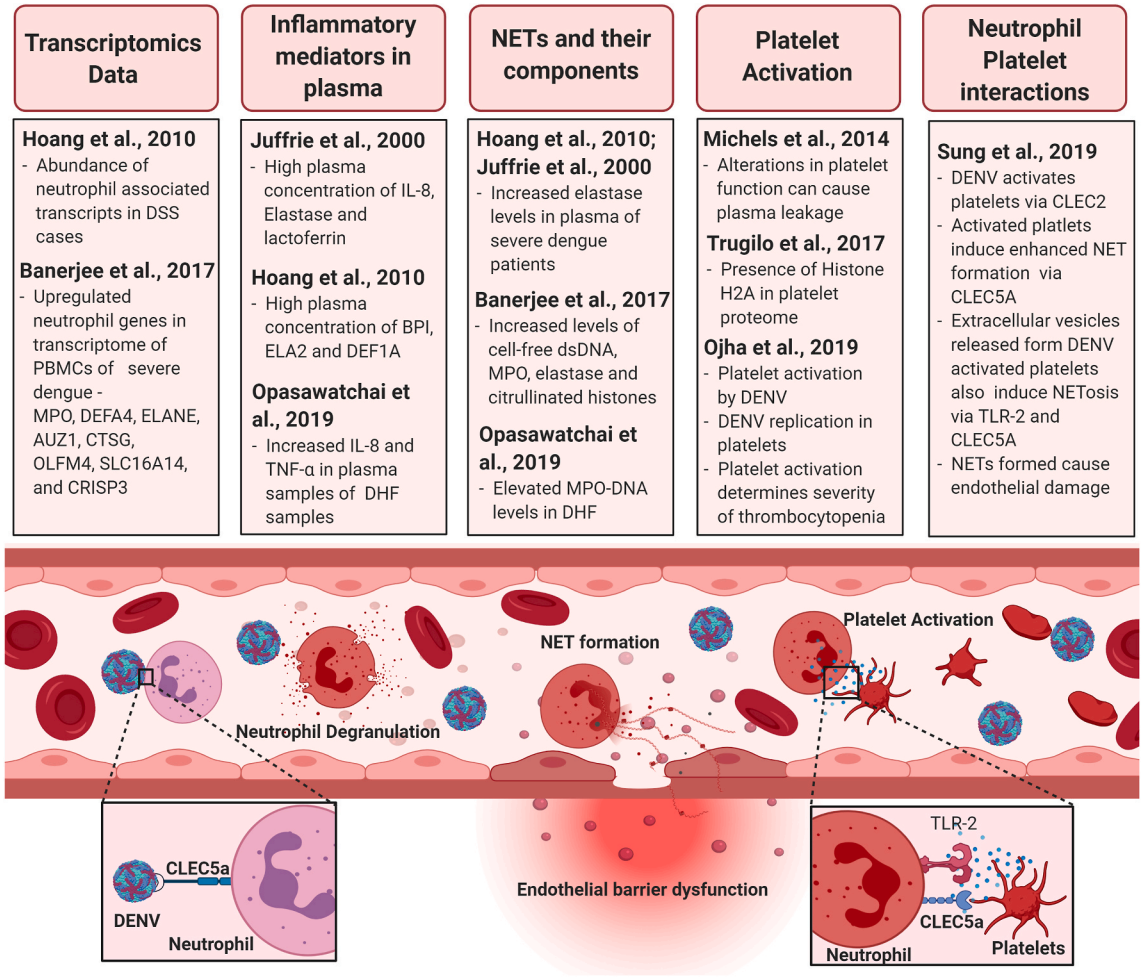

Fig. 3. Evidences for the involvement of neutrophils and platelets in Dengue pathogenesis.

Moreover, a higher concentration of Histone H2A in plasma of severe dengue than other arboviral infections suggests a specific association of NETs with dengue disease progression (Trugilho et al., 2017). However, it is not clear which conditions trigger a higher level of NET formation in severe dengue. The interaction of neutrophils with activated platelets triggered NET formation. This is supported by the proteomics study of dengue-infected platelets, where an increased abundance of cell-free Histone H2A was found to associate with platelet protein profile (Trugilho et al., 2017). It was speculated that the association of Histone H2A probably resulted from activated platelet and neutrophil interaction. It should be noted that in healthy conditions, platelet and neutrophils never interact in circulation. However, the dengue virus can activate platelet (Sung et al., 2019) and replicate inside them (Ojha et al., 2019). The activated platelets express P-selectin, which is stored on the membrane of α-granules under basal conditions. Its upregulation onto the platelet surface is one of the earliest events during neutrophil recruitment and is required for platelet-neutrophil interactions (Polanowska--grabowska et al., 2011). The extent of plasma leakage has been shown to correlate with alterations in platelet function (Hottz et al., 2013; Michels et al., 2014). Sung et al. (2019) reported that DENV alone could activate neutrophils to induce NET formation via CLEC5a. DENV also activated platelets via CLEC2, and their interaction with neutrophils enhanced NET release by almost two folds (Sung et al., 2019). DENV activated platelets also released extracellular vesicles that can also induce neutrophils to undergo NETosis (Sung et al., 2019). The NET release through the extracellular vesicles by DENV activated platelets also contributed to enhanced systemic vascular permeability in *in-vivo* mice models leading to severe dengue (Sung et al., 2019) (Fig. 3). Despite evidence of the detrimental role of neutrophils in dengue infection, several questions remain unanswered. Is dengue virus infection sufficient to induce a phenotypic change of neutrophils that accelerates NET formation? Do all dengue serotypes activate neutrophils to an equal extent? Are NETs in the dengue patients immunogenic? Does NET formation, in turn, accelerate platelet activation and apoptosis? Dengue virus can infect bone marrow (Albuquerque et al., 2009; La Russa and Innis, 1995). Bone marrow is the site for neutrophil biogenesis. Several reports suggested that viral infection may trigger emergency granulopoiesis to meet the excess demand of neutrophils in circulation (Hirai et al., 2006; Manz and Boettcher, 2014). On the one hand, emergency granulopoiesis can be beneficial: it helps boost the body's cellular response to resolve the viral infection. But on the other hand, when the virus and the resulting antiviral response persist, the inflammatory feedback to the hematopoietic system will become chronic and detrimental to a balanced bone marrow output. A point to be noted that dengue-infected patients develop severity after 5–7 days of post-infection. It is unknown whether the dengue virus can trigger emergency granulopoiesis during this period, and the resulting neutrophils may have different phenotypes? If yes, how do phenotypic differences of neutrophils affect other immune cell functions and develop severity? Therefore, future studies in these areas will be interesting to understand the molecular mechanisms of dengue pathogenesis at multiple levels.

8. Neutrophils as potential target: promise and limitations

The involvement of neutrophils in the pathogenesis of a wide range of infections makes them attractive therapeutic targets. Several aspects of neutrophil biology can be therapeutically targeted, ranging from the maturation process to effector functions. Blocking neutrophil activation can be achieved by using antibodies or inhibitors against the receptors. However, the restoration of normal neutrophil function is also essential to consider. As evidence accumulates, several viruses may able to induce NETosis. The excessive NET formation can trigger a cascade of inflammatory reactions that destroy surrounding tissues, facilitate micro thrombosis, and results in organ impairment and even death (Middleton et al., 2020). Due to the similarities between the clinical presentation of severe COVID-19 and known NETopathies, blocking excess NET formation at an early stage may be beneficial to patients.

It should be noted that treatments targeting NETs would not directly target the SARS-CoV-2 virus. Still, they could improve host response, reducing the number of patients who need ventilation, and importantly, reducing mortality. They were often overlooked after their identification in 2004 (Brinkmann et al., 2004). People have recently started reporting NETs association with diverse clinical pathology and hold neutrophils as critical therapeutic targets (Mutua and Gershwin, 2020). The clinical development of inhibitors drugs that target NETs is mainly in the developing stage. Most of the drugs are targeting two important neutrophil granular enzymes, i.e., NE and MPO. A new generation of potent NE inhibitors, including lonodelestat (POL6014) (ClinicalTrials.gov Identifier: NCT03748199), alvelestat (ClinicalTrials.gov Identifier: NCT04539795), CHF6333 (ClinicalTrials.gov Identifier: NCT03056326), and elafin (ClinicalTrials.gov Identifier: NCT03522935), have undergone Phase I testing. Another option for reducing NET-related complications is to dissolve a primary constituent of NET structure, i.e., DNA—using DNase-I. DNase I treatment may help resolve aggregations develop due to excessive NET formation, which may be an appropriate strategy for preventing NET-related pathogenesis in SARS-CoV-2 patients. Recombinant human DNase enzyme, Dornase alfa, has undergone a Phase II clinical trial on COVID-19 patients (ClinicalTrials.gov Identifier: NCT04359654). DNase-I-coated melanin-like nanospheres were developed and showed promising results in reducing NETs related complications and cytokine storm in the animal model (Park et al., 2020).

Another possibility is to specifically block IL-1β/NET feedback loop signaling (Yaqinuddin and Kashir, 2020). The elevated levels of IL-1β, as observed in severe COVID-19 patients (McElvaney et al., 2020; Wilson et al., 2020), activate a higher number of neutrophils, resulting in elevated levels of NET production. Ankinara can block IL-1β, potentially disrupting the IL-1β/NET feedback loop (Mitroulis et al., 2011). In a retrospective cohort study, high doses of ankinara have shown to be safe and are associated with the improvement of severe COVID-19 patients (Cavalli et al., 2020). While Dornase alfa and ankinara are FDA-approved drugs, specific clinical trials are still required to evaluate the efficacy of these drugs against COVID-19.

It is important to note that the final product of NET seems to be quite common, i.e., DNA entrapped with toxic proteases. However, depending on the nature of stimuli, NETs may follow different mechanisms and functions. Therefore, a detailed study is required to understand the mechanisms of NET formation, the extent of NETosis concerning a particular virus, and the impact of NETs in the disease pathology. Further investigations are required to study which part of neutrophil biology should be targeted for combating the infection. Because the same drugs may have a different outcome as the mechanisms by which neutrophils contribute to the pathogenesis may vary from virus to virus. For example, DNase treatments in case of age-associated mortality in influenza infections do not affect mice survival (Kulkarni et al., 2019), but opposite results have been observed in DENV infections (Sung et al., 2019). Therefore, it is necessary to carefully consider the type of stimulus and the biological function imparted by NETs to target the disease more specifically.

Based on evidence from literature, another option that can be carefully considered is to explore the G-CSF pathway. G-CSF is a myelopoietic growth factor that plays a vital role in steady-state granulopoiesis and the maturation, activation, and functions of neutrophils (Semerad et al., 2002). Inhibition of the G-CSF receptor (G-CSFR) with monoclonal antibodies and administration of recombinant G-CSF (rhG-CSF) are being therapeutically tested in different cases of influenza A and COVID-19, respectively. G-CSFR is mainly expressed on neutrophils and bone marrow precursor cells. Blocking of the G-CSFR may affect excessive neutrophil production as well as trafficking (Campbell et al., 2016). Therapeutic targetting of G-CSFR with monoclonal antibodies reduced the neutrophil number, their trafficking in the BAL compartment, and neutrophil-mediated inflammation without affecting

pathogen clearance in the case of *Streptococcus pneumonia* and influenza infection in mice (H. Wang et al., 2019). Although selective inhibition of G-CSFR was safe and effective for pathogen clearance, the complete depletion of circulating and tissue-resident neutrophils with IA8 Anti-Ly6G antibody led to increased severity of viral and bacterial infection (H. Wang et al., 2019). On the other hand, administration of G-CSF increases the number of peripheral blood lymphocytes. Preliminary findings from a randomized clinical trial (Chinese Clinical Trial Registry: ChiCTR2000030007) on COVID-19 patients with lymphopenia showed that the administration of rhG-CSF did not accelerate clinical improvement but was effective in reducing the number of patients developing critical illness or dying (Cheng et al., 2020). On a contrasting note, giving G-CSF can potentially danger in COVID-19 patients with ARDS (Lazarus and Gale, 2020). The NLR ratio increased to 5 within 72 h of G-CSF administration in three COVID-19 patients with ARDS, which further led to severe disease (Nawar et al., 2020). Similar results were observed in the case of a COVID-19 patient with neutropenia whose condition deteriorated within 24 h after receiving G-CSF (Taha et al., 2020). Also, five people receiving chemotherapy or bone marrow transplantation who were administered G-CSF developed ARDS with elevated IL-8 and TNF-α (Takatsuka et al., 2002). These studies suggested that G-CSF administration can worsen lung function by increasing neutrophil infiltration and raises concerns regarding G-CSF therapy. Also, it is hard to speculate the effect of G-CSF therapy in COVID-19 patients with comorbidities due to the small sample size of these studies. Therefore, careful and critical monitoring is needed before targeting the G-CSF pathway in viral infections, particularly for severe patients.

9. Future perspectives

Neutrophils mediated immune response is an essential arm of our defense mechanisms against invading pathogens. In recent times, neutrophil biology has been extensively studied in the context of viral infections. Neutrophils, on the one hand, are essential in inducing a balanced pro-inflammatory antiviral response and help in clearing the viruses either by engulfing them or through the release of NETs, thus limiting the viral spread and helping to resolve acute infections. On the other hand, an uncontrolled trigger for neutrophil activation and deposition of NET in tissue and blood vessels can cause bystander damage, leading to exacerbation of the disease. The current shreds of evidence do not support an association between specific neutrophil functions and the type of virus involved (e.g., RNA vs. DNA virus, enveloped vs. nonenveloped virus) or the route of viral entry (e.g., airborne vs. bloodstream). Depending on the virus, the primary infection site, and the host immune response, neutrophil activation and NET-related complications may vary. Therefore, careful decisions should be taken while considering neutrophil-based interventions. It is now evident that neutrophil phenotype perhaps is crucial for dictating their functions but still, there is a long way to go to understand the crucial factors that determine the outcome of the neutrophil-mediated responses during viral infections. Parallelly more focussed in studies in context to viral infections are required in areas that include (1) the effect of the virus on neutrophil biogenesis and development process; (2) release of neutrophils from bone marrow (3) phenotypes of the circulating neutrophils and trafficking ability of neutrophils from circulation to the site of infection; (4) the interaction of the neutrophils with other immune cells; (5) the extent of NET formation and (6) termination of inflammation after pathogen clearance. Therefore, more research on these aspects of neutrophil biology and multitargeted interventions are required to deal with mortality and morbidity associated with severe viral infections. Such studies are likely to help develop more efficacious therapeutic interventions to cure these devastating, widely spreading, and life-threatening viral illnesses.

Declaration of competing interest

All authors declare that they have no conflicts of interest.

Acknowledgments

This work was supported by the Regional Centre for Biotechnology (RCB). SR is supported by the Department of Biotechnology, India (Fellowship award number - DBT/JRF/BET-18/1/2018/AL/21). The figures in the paper were generated using Biorender App (BioRender.com).

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