

## REVIEW

# Neutrophil subsets and their differential roles in viral respiratory diseases

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## Abstract

Neutrophils play significant roles in immune homeostasis and as neutralizers of microbial infections. Recent evidence further suggests heterogeneity of neutrophil developmental and activation states that exert specialized effector functions during inflammatory disease conditions. Neutrophils can play multiple roles during viral infections, secreting inflammatory mediators and cytokines that contribute significantly to host defense and pathogenicity. However, their roles in viral immunity are not well understood. In this review, we present an overview of neutrophil heterogeneity and its impact on the course and severity of viral respiratory infectious diseases. We focus on the evidence demonstrating the crucial roles neutrophils play in the immune response toward respiratory infections, using influenza as a model. We further extend the understanding of neutrophil function with the studies pertaining to COVID-19 disease and its neutrophil-associated pathologies. Finally, we discuss the relevance of these results for future therapeutic options through targeting and regulating neutrophil-specific responses.

## KEYWORDS

anti-viral functions, COVID-19, heterogeneity, Influenza A, neutrophils, tissue damage, Viral Respiratory Diseases

## 1 | INTRODUCTION

Neutrophils are known as early responders against bacterial or fungal infections, releasing their powerful antimicrobial arsenal to neutralize and contain the infection. Neutrophils were first reported by Elie Metchnikoff in 1883 as professional phagocytes.<sup>1</sup> They are capa-

ble of engulfing foreign microbes and neutralizing them with various secretory granules<sup>2</sup> and potent reactive oxygen species (ROS).<sup>3</sup> Neutrophils also have the unique ability to release neutrophil extracellular traps (NETs) to limit the spread of infections.<sup>4</sup> These NETs were shown to help neutralize large fungal hyphae together with the secretion of cytotoxic granules.<sup>5,6</sup> The effects of neutrophil-mediated immunity are

further amplified as neutrophils work in large numbers, forming a concerted effort to eliminate foreign threats. Although beneficial, these effects can often be detrimental to the host tissues and the immune response,<sup>7,8</sup> including further exacerbation of organ damage, resulting in critical and life-threatening conditions.<sup>9</sup> In viral diseases, however, the roles neutrophils play are not well understood. Furthermore, the recent evidence of a strong neutrophil presence in severe acute respiratory syndrome coronavirus (SARS-CoV2)-infected patients has garnered interest in the function these neutrophils elicit in the progression of COVID-19 disease.<sup>10,11</sup>

Neutrophils are innate immune cells that are frequently overlooked in discussions of viral immunity, in part because adaptive immune cells such as T and B cells are recognized to play essential roles in initiating cytotoxic killing and antibody generation in response to viral infection, both of which are major steps toward viral clearance. While the role of neutrophils in viral infection is still unclear, growing evidence suggests that neutrophils play a role in resolving viral infection. For instance, data from preclinical mouse models of influenza A virus (IAV) infection show depletion of neutrophils by anti-Ly6G or anti-Gr1 antibody treatment led to severe disease outcomes in infected mice.<sup>12,13</sup> Similar findings were also made in encephalitis<sup>14</sup> and HSV-1<sup>15</sup> infection models, supporting the need for neutrophils to achieve optimal viral immunity. Studies have also demonstrated that mice with deficient inflammasome signaling through the NLR3 pathway have poor neutrophil recruitment due to decreased production of neutrophil chemokines such as KC, TNF- $\alpha$ , and IL-1b, which results in severe pathology and mortality following an IAV infection.<sup>16,17</sup> Neutrophils in respiratory syncytial virus (RSV) infections, on the other hand, has been proven in several studies to have no influence on viral load or pathogenicity.<sup>18–20</sup> Although some studies suggest that neutrophils play a beneficial or redundant role in the body, clinical observation indicated that a high neutrophil count is associated with the severity of many diseases. For instance, hematologic analysis and clinical studies of COVID-19 patients' neutrophil counts showed strong association with disease severity.<sup>21–23</sup> It has been proposed that the neutrophil-to-lymphocyte ratio (NLR), which has been used to stratify cancer patients,<sup>24,25</sup> may be utilized as a predictive and prognostic marker for COVID-19 patients.<sup>10,11,26</sup> These results suggest that the presence of neutrophils in severe types of inflammation is linked to disease severity in COVID-19 patients. Here, we will review the current evidence of neutrophil heterogeneity and consolidate information on neutrophil function in viral respiratory infection. We expect to better understand the role of neutrophils in the immune response to viral respiratory infections and their participation in the pathology of the most severe cases.

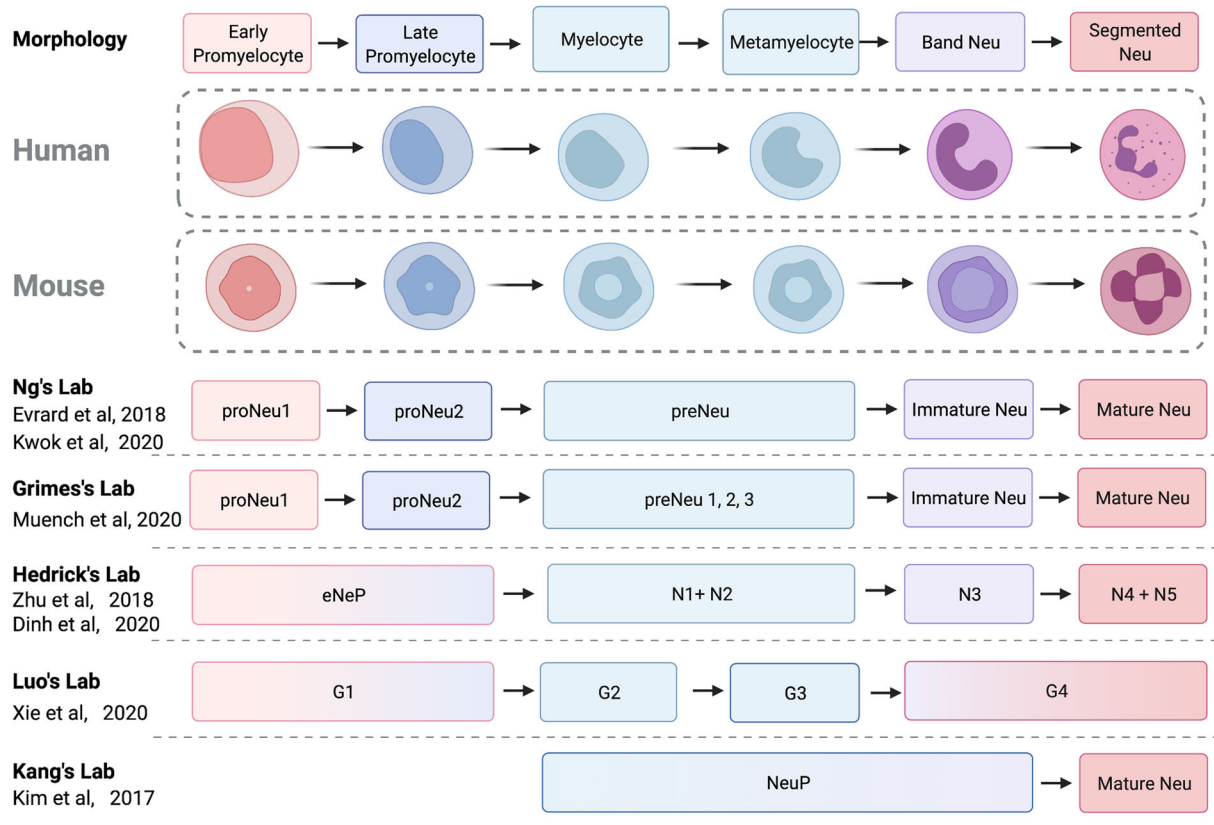
## 2 | DEVELOPMENT AND FUNCTIONAL HETEROGENEITY OF NEUTROPHILS

The daily production of billions of neutrophils takes place within the bone marrow, where committed progenitors reside in specialized niches providing growth signals and developmental cues. Due to

the short lifespan of neutrophils, this developmental process is critical in producing a steady supply to the circulation. Granulopoiesis is therefore well studied, and is described by the characterization of the various maturation stages through their granule content and morphologic attributes.<sup>27</sup> According to their granule content and nuclear shape, neutrophils are produced in a step-wise order of early myoblasts  $\rightarrow$  promyelocytes  $\rightarrow$  myelocytes  $\rightarrow$  metamyelocytes  $\rightarrow$  band cells  $\rightarrow$  segmented neutrophils.<sup>28–31</sup> Under homeostatic conditions, only the mature segmented neutrophils exit the bone marrow, performing their various roles.<sup>32</sup> However, during inflammatory conditions, hematologists typically observe immature phenotypes of neutrophils in the circulation. This is commonly known as a left shift and is used as an indicator of inflammation.<sup>33</sup>

These immature phenotypes have been widely reported in various inflammatory conditions, ranging from cancer, pregnancy, stress, cardiovascular diseases, and notably viral infections.<sup>34–38</sup> The way these immature neutrophils are characterized differs between research groups. There is no standardized approach for phenotyping neutrophil subsets, making it difficult to corroborate functional data from multiple laboratories. Some groups have proposed markers to identify these subsets, such as CD10,<sup>39</sup> CD177,<sup>40</sup> Olfactomedin-4 (OLFM4),<sup>41</sup> and CD49d.<sup>42</sup> Separating these subtypes can be difficult since they might emerge from various distinct neutrophil precursor stages with different degrees of maturity. A more profound knowledge of the ontogeny of the neutrophils will be required to overcome this issue. In recent years, several research groups have characterized neutrophil development at each step of differentiation and maturation. The advancements in single-cell technologies and sequencing techniques have led to the identification and characterization of various neutrophil progenitors and precursors in both mice and humans.<sup>32,43–46</sup> This includes the early progenitors, such as the proNeus<sup>46</sup> and eNePs,<sup>45</sup> as well as late precursors preNeus,<sup>32</sup> NePs,<sup>44</sup> and NeuPs.<sup>47</sup> In-depth single-cell analyses further reveal finer transcriptomic distinct developmental stages<sup>43,48</sup> (Figure 1). These studies demonstrate that immature neutrophils can exist in the circulation during disease states, creating a heterogeneity of developmental states that can potentially elicit various unique functions in response to the inflammatory stimulus. However, it is still unclear if these immature forms go on to become functionally distinct mature neutrophils.

Mature neutrophils undergo an ageing process when released into the circulation, decreasing their expression of L selectin (CD62L) while increasing chemokine receptor CXCR4 expression.<sup>49,50</sup> Aged neutrophils are then cleared from the circulation by specialized efferocytic macrophages present in the lung, liver, and spleen.<sup>27</sup> This physiologic process regulates granulopoiesis, providing feedback signaling to the bone marrow through an IL-23 and IL-17-mediated manner.<sup>51</sup> Clearance of aged neutrophils also prevents unwanted necrosis or spillage of their store of cytotoxic granules.<sup>52</sup> Interfering with this clearance process results in an accumulation of circulating aged neutrophils. This build-up of exhausted phagocytes has been shown to play significant roles in disease. Aged neutrophils have a much higher phagocytic activity as compared with the non-aged neutrophils.<sup>53</sup> Additionally, they respond faster toward inflammatory signals, migrating to sites of



**FIGURE 1** Characterization of neutrophil subsets in mice and humans. Neutrophil development is historically characterized by various morphologic structures and granules using bone marrow smears. Undifferentiated myeloblasts differentiate into promyelocytes, myelocytes, nonproliferating meta-myelocytes, band cells, and finally mature as segmented neutrophils. These stages are accompanied by stage-specific granules. The advent of high dimensional single-cell phenotyping technologies enabled both the transcriptome and protein expression characterization of the developmental continuum, giving rise to the identification of discrete subsets proposed by various groups. These enable the study of neutrophils in viral inflammatory conditions and discover subset-specific functions leading to disease pathology and resolution. Created with BioRender.com

infection to neutralize threats. A transgenic mouse model affecting the ageing process in neutrophils showed these distinctions in functionality between aged and fresh neutrophils. In this model, the group showed that mice with mostly aged neutrophils were able to survive better against fungal infections as compared with mice with mostly fresh neutrophils. However, aged neutrophils confer a worse disease pathology in a vascular inflammation model, resulting in larger infarction sizes and poorer survival.<sup>54</sup> Therefore, it is important to consider the heterogeneity of neutrophil age states in addition to the developmental state of the circulating neutrophils during inflammatory states such as viral infections.

Neutrophils in the circulation also comprise a subset of myeloid-derived suppressor cells that possess immune-modulating properties observed in various conditions such as cancer, pregnancy, and sepsis.<sup>35,55,56</sup> These neutrophils possess a general identity of Ly6G<sup>+</sup>CD11b<sup>+</sup> and can have a morphologic resemblance of both immature and mature neutrophil phenotypes as described by various groups.<sup>57</sup> These suppressor cells are reported to inhibit T cell proliferation and activation, dampening the immune response. They may also be akin to low-density neutrophils (LDN) found in the mononuclear

fraction after density gradient separation.<sup>58</sup> LDNs are also reported as a combination of immature and mature phenotypes observed first in SLE<sup>58</sup> and rheumatoid arthritis.<sup>59</sup> These cells have perhaps degranulated and accumulated in the bloodstream of these patients, as reported by some groups.<sup>60,61</sup> This indicates that neutrophils can exist in various states, eliciting both beneficial and detrimental functions to the immune response.

In the lung specifically, a major subset of neutrophils exists as a margined, intravascular pool, adhering to the endothelium of capillaries and postcapillary venules. These neutrophils have been shown to be activated once in the lung, acquiring higher expression of adhesion receptors such as CD11b but lower expression of CD62L.<sup>62</sup> Notably, the authors argued that this phenomenon occurs regardless of the inflammatory condition. Recent evidence in mice further proposes that neutrophils change at the transcriptome level when they infiltrate various tissues.<sup>63</sup> In the study, the group showed a lung-specific signature in the neutrophils, possessing proangiogenic genes involved in vascular growth and repair. Intravital imaging of lung neutrophils showed that they interact closely with B cells, allowing for their clearance by macrophages. A disruption of neutrophil clearance by B cell depletion

was shown to cause pathologic consequences.<sup>64</sup> This opens new questions on how tissue neutrophils, such as those in the lungs, are activated during viral respiratory diseases (VRDs) and how this might impact their function against the infection.

### 3 | NEUTROPHILS AND VRDs

Respiratory viruses are one of the greatest contributors to endemics and pandemics in the history of humankind, with a significant morbidity and mortality rate.<sup>65,66</sup> The most common ones are influenza virus, RSV, parainfluenza virus, metapneumovirus, rhinovirus (RV), coronavirus, and adenovirus. They can affect the upper respiratory tract, which presents mild to moderate symptoms such as fever, cough, sore throat, and/or running nose.<sup>67,68</sup> This infection can progress to the lower respiratory tract, which causes damage to the lungs and results in severe symptoms such as pneumonia.<sup>65,68-78</sup> In pediatric hospitalizations for lower respiratory tract illnesses (LRTIs), 40% are caused by seasonal parainfluenza virus epidemics.<sup>72</sup> RV, typically associated with upper respiratory tract illnesses (URTIs) such as the common cold, has been reported to result in severe LRTIs and is the second leading cause of pneumonia and bronchiolitis in infants and young children.<sup>74</sup> In IAV infections, clinical studies have reported pneumonia as the most frequent severe clinical manifestation, affecting an estimated one-third of the IAV-infected and hospitalized patients.<sup>79-81</sup> Severe pneumonia can progressively develop into acute respiratory distress syndrome (ARDS), the most severe form of acute respiratory failure.<sup>82</sup> A life-threatening respiratory condition with a pooled mortality rate of 43% across various evaluated studies, ARDS is characterized by pulmonary edema with large infiltration of neutrophils into the interstitial and bronchioalveolar space.<sup>83</sup>

Infiltration of neutrophils is a common trait in VRDs, reported in IAV, RSV, metapneumovirus, RV, adenovirus, and coronaviruses.<sup>79,84-88</sup> In RSV infection, an increased number of neutrophils in the lung is a hallmark for disease severity in both humans and mice.<sup>89,90</sup> Severe RSV-infected infants have neutrophils as the predominant cell type in the bronchoalveolar lavage (BAL).<sup>89</sup> An increase in lung neutrophils, markers, and genes of neutrophil function and activation has been shown in severe IAV and SARS-CoV-2 infections.<sup>91-97</sup> Additionally, neutrophil infiltration is also observed in URTIs. RV and adenovirus are typical causes of URTIs, such as the common cold. In the early course of cold from symptomatic RV-infected patients, neutrophils have been shown to infiltrate the nasal mucosa and secretion.<sup>98</sup> During the common cold of adenovirus-infected children, a high level of neutrophils, HNP-1, -3, and -4 was observed in their upper respiratory tracts.<sup>99</sup> This increased level of neutrophils observed across respiratory viral infections emphasizes the importance of studying the role of neutrophils in VRDs.

### 4 | TAKING EVIDENCE FROM IAV INFECTIONS

Before delving into the biologic significance of neutrophil subsets for viral immunity, we first integrate the current evidence of neutrophils

in viral respiratory infections to ascertain their functions, both beneficial and detrimental to the host. Among the numerous research involving neutrophils and viral infections, IAV infection investigations have made significant contributions to our understanding of the several functions neutrophils can play during a VRD. IAV is the cause of the yearly seasonal flu and the global human flu pandemics.<sup>100</sup> Since 1900, 5 influenza pandemics has hit the world, with the most recent 2009 IAV H1N1 causing over half a million deaths globally.<sup>101</sup> Moreover, a global estimate of 5 million severely diseased and 650 thousand respiratory deaths has been associated with seasonal influenza each year.<sup>102</sup> Due to its seasonal emergence and multiple subtypes, IAV is a persistent global public health concern that results in a spectrum of pathologic severity.<sup>100</sup> Due to the diversity of individual patients and the difficulties of conducting mechanistic research on human individuals, IAV infection studies are widely carried out on mice, which provides clues on the pathology and responses that neutrophils participate in.<sup>103</sup> It is important to consider murine models of viral infections as well as human studies as they, together, form a cohesive understanding of the mechanisms and underlying pathologies. When possible, we also incorporate relevant studies of other respiratory viruses to complement our comprehension of neutrophil function.

### 5 | ANTIVIRAL NEUTROPHIL FUNCTIONS IN VRDs

Neutrophils are phagocytes, and their ability to engulf viral particles suggests a possible antiviral function. Indeed, Mullarkey et al.<sup>104</sup> demonstrated that neutrophils perform antibody-dependent cellular phagocytosis (ADCP) on IAV through Hemagglutinin stalk protein-specific IgG antibodies. The opsonized viral particles are phagocytosed by neutrophils resulting in the generation of ROS and perhaps the elimination of virus. In a study using a flank model of Modified Vaccinia Ankara (MVA) infection, it was demonstrated that neutrophils harboring viruses were shown to be APCs. Duffy et al.<sup>105</sup> discovered the infected neutrophils that homed to the bone marrow activated residing CD8+ memory T cells specific to MVA. This effect was later seen to be abrogated by a disruption in neutrophil migration through CCR1 signaling. Additionally, Hufford et al.<sup>106</sup> reported de novo synthesis of viral RNA and protein of IAV in neutrophils, suggesting how they can be infected and serve as antigen-presenter cells to CD8+ T cells for antiviral immunity function. Additionally, Hufford's group showed an increase in IFN $\gamma$  production in activated CD8+ T cells, but not CD4+ T cells, and the depletion of neutrophils through anti-Ly6G antibodies showed a significant decrease of these activated CD8+ T cells in the lungs.<sup>106</sup> Notably, while being infected and act as transporters/presenters, neutrophils were incapable of supporting active IAV growth.<sup>107</sup> In an elegant study by Lim et al.,<sup>108</sup> neutrophils were shown to secrete the chemokine CXCL12, which induced the migration of influenza-specific CD8+ T cells to the infected lung. This function of T cell recruitment was lost with either neutrophil-specific knock-out of CXCL12 or blocking its receptor, CXCR4, through the inhibitor AMD3100.

Degranulation of antimicrobial peptides and mediators has been shown to greatly damage lung epithelial cells and tight junction integrity.<sup>109,110</sup> These powerful proteinases and granules serve to eliminate microbial threats and contain infections. Though it seems unnecessary in viral infections, these mediators are key players in viral clearance. In VRDs like RSV, matrix metalloproteinase 9 (MMP-9) is important for viral clearance.<sup>111</sup> Dabo et al.<sup>111</sup> demonstrated that MMP-9 decreases RSV infectivity and modulates neutrophil recruitment and cytokine generation in the lung using an MMP-9 knock-out model of RSV infection. Another study reported similar findings, showing that secretion of MMP-9 through TLR signaling was required for neutrophil migration to the lung on IAV-infected mice.<sup>112</sup> It is still unclear how MMP-9 reduces infectivity of RSV, possibly disrupting viral attachment or indirectly triggering signaling cascades of receptors MMP-9 is known to bind to.<sup>113,114</sup> Apart from MMP-9, it was demonstrated that the neutrophil-derived cathelicidin LL-37 has direct antiviral effects, interacting with the virus and decreasing its virulence, suppressing both IAV and RSV infections. Separately, antimicrobial peptides called human neutrophil peptides can neutralize IAV through a process of viral aggregation. This process was shown to promote uptake of IAV by neutrophils, preventing further infections.<sup>115,116</sup> Along with antimicrobial peptides, neutrophils produce considerable amounts of ROS via oxidative burst, which can be used to remove phagocytosed material or cause tissue damage. Oxidative burst has been detected in mice during IAV and RSV infection.<sup>117-119</sup> Excessive amounts of ROS, when inflammation is prolonged, can often lead to severe disease pathology in IAV infection.<sup>117,120</sup> Additionally, mediators like myeloperoxidase (MPO) and neutrophil elastase (NE) can either proteolyse or catalyze ROS production.<sup>121</sup> MPO was shown to be important for viral clearance but also a contributor of lung tissue damage.<sup>122</sup> Similarly, NE has been found in the respiratory tract and serum of severe RSV-infected infants, potentially contributing to disease pathology.<sup>123,124</sup> Interestingly, individuals with chronic granulomatous disease exhibit ROS-deficient neutrophils but no increased vulnerability to VRDs, indicating that ROS may be primarily engaged in disease pathogenesis as the disease progresses.<sup>125</sup>

ROS is also linked to another important neutrophil function: NET formation. NETosis has garnered much contention as an antiviral role in VRDs. Persistent NET formation in IAV-infected models was associated with lung damage and increased pathogenesis, as NETs are highly toxic.<sup>126,127</sup> ARD-related histopathologic manifestations such as diffuse alveolar damage (DAD) caused by alveolar injury were entangled with NETs. NET formation and endothelial damage were detected when alveolar epithelial cells (AECs) were infected, suggesting the potential link of NET formation to alveolar damage in IAV-infected patients. Additionally, high extracellular histones levels were found in the lungs of IAV-infected mice and were shown to exacerbate lung pathology.<sup>128</sup> RSV-infected epithelial cells were shown to be recognized by neutrophils, triggering NETosis,<sup>129-133</sup> which can capture RSV particles in vitro.<sup>133</sup> Additionally, histones have been shown to neutralize H3N2 and H1N1 IAV.<sup>134</sup> These findings highlight NETosis as potential antiviral capabilities against VRDs. On the other hand, close examinations of patients' sputum, lung biopsies, or mouse models with

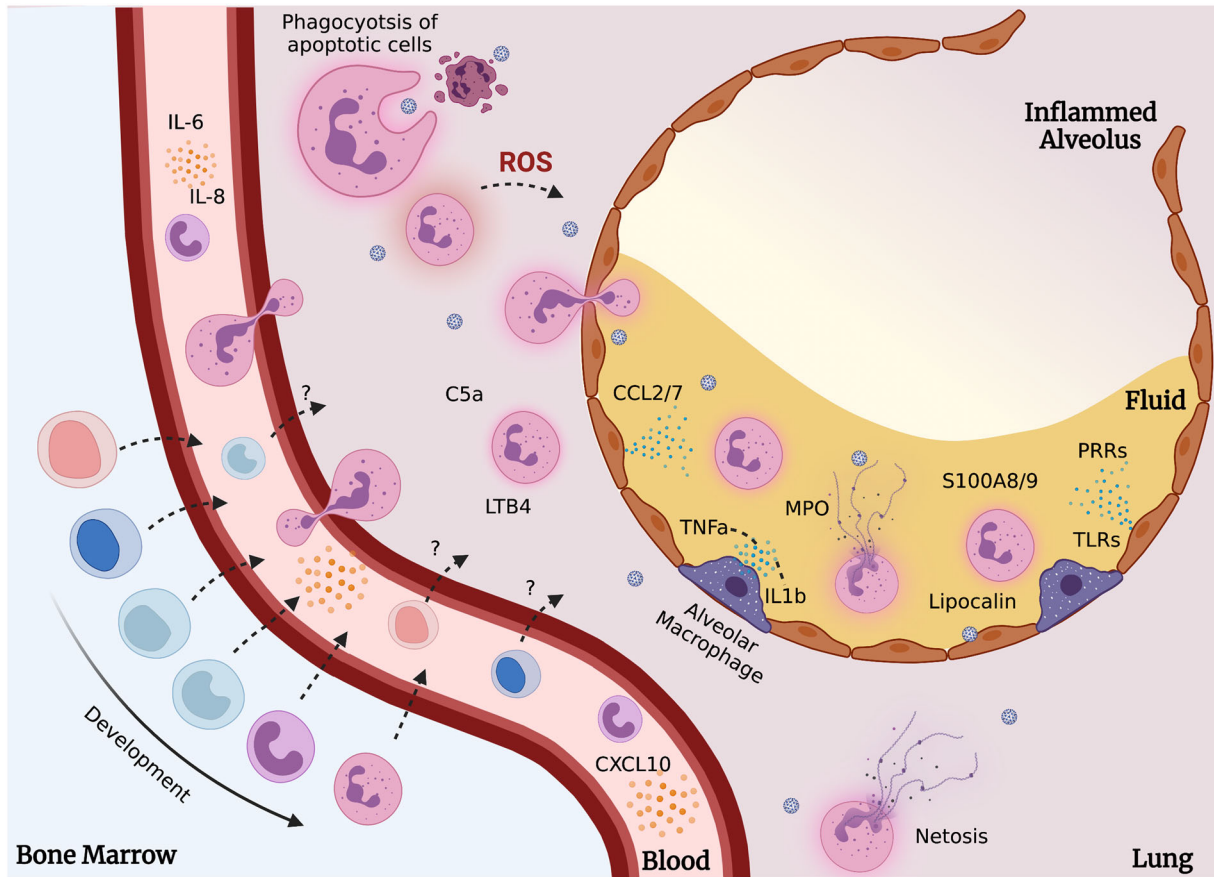
severe VRDs frequently reveal elevated NETs and neutrophil levels, suggesting neutrophils as major drivers of disease pathology and mortality in severe disease.<sup>128,135,136</sup> Most of the airway obstructions during severe RSV infections were found to have NETs plugs.<sup>133</sup> As a result of this, airway obstruction and poor prognosis may be linked to an increased neutrophil response by NETs. However, these observations are likely a consequence of uncontrolled disease progression, led by dysregulation of cellular signaling and unresolved inflammation.<sup>137,138</sup>

After executing their functions, neutrophils can perform an additional function in the lung by secreting epidermal growth factor (EGF), which has been demonstrated to be required for monocyte development into competent APCs. A recent study showed that the absence of EGF significantly reduced CD8+ T cell cytokine expression and activation, and showed a marked decrease in dendritic cell maturation markers like MHCII and CD86.<sup>139</sup> Collectively, the data suggest that neutrophils may carry out their antiviral activities through indirect processes.

## 6 | RECRUITMENT OF NEUTROPHILS TO THE INFECTED RESPIRATORY TRACT

The innate immune players in the lung include the residential lung leukocytes and the epithelial cells lining the alveolar surface and the conducting airways.<sup>140</sup> The immune cells in healthy lung tissues are ~95% alveolar macrophages, 1-4% lymphocytes, and ~1% neutrophils. Pattern recognition receptors (PRRs) such as TLRs are found on alveolar walls and epithelial cells lining the respiratory tract to sense pathogen-associated molecular patterns and damage-associated molecular patterns.<sup>141-144</sup> These epithelial cells in the airway are the first to be infected,<sup>145</sup> secreting proinflammatory cytokines and chemokines to activate and recruit leukocytes into the infected respiratory tract.<sup>146,147</sup> These mediators include MIP-1 $\alpha$ , TNF- $\alpha$ , IL-1, IL-6, CXCL1, CXCL2, CXCL10, and IL-17.<sup>148-151</sup> Enhanced levels of proinflammatory cytokines and Type I IFNs are also contributed by infected alveolar macrophages.<sup>152-156</sup> Depending on the inflammatory stimuli, massive infiltration can occur, leading to lung tissue damage and loss of lung function<sup>147,157,158</sup> (Figure 2).

Following IAV infection and activation of tissue-resident macrophages and epithelial cells, neutrophils are the first wave of immune cells recruited to the site of infection, which is approximately 2 days after the IAV infection.<sup>13,159-161</sup> During IAV, RSV, and SARS-CoV infection, MyD88/TRIF signaling is crucial for recruitment of neutrophil to the lung.<sup>162-164,165,84</sup> Following IAV infection, an increase in both local and systemic levels of neutrophils was commonly observed in clinical patients, mice, and ferrets. This neutrophilia correlates with the increased IL-6, CCL2, CXCL8, and CXCL10 levels and disease severity of IAV infections.<sup>153,166-168</sup> The release of proinflammatory cytokines (e.g., TNF- $\alpha$ , IL-1, and IL-6), chemokines (CCL2, CCL7, CXCR8), complement component C5a, and leukotriene B4 attracts neutrophils to the infected respiratory airway.<sup>83,169-172</sup> Similarly, these molecules were highly associated with increased disease severity.



**FIGURE 2** Neutrophil recruitment and action in the infected airway tract. Evidence now shows the presence of developing neutrophil precursors in the circulation during viral infections. These subsets may provide immunosuppressive or effector functions that are not fully understood. In the infected lungs, through recruitment by cytokines (such as IL-1b, LTB4, C5a, and TNF- $\alpha$ ), TLRs, and pattern recognition receptors (PRRs), neutrophils perform various functions that help control the infection. Left unchecked, these actions lead toward increased disease severity and tissue damage. Created with BioRender.com

After migration to the infected airway, neutrophils are found to confer a protective function to the host in both the early and late stages of the infection.<sup>67</sup> Cell depletion studies in mice revealed that recuperation from severe cases of IAV depended heavily on neutrophils presence and contribution.<sup>13</sup> Interestingly, mild IAV infection did not exhibit a similar dependency on neutrophils. Moreover, studies in mice using IAV strains with varying levels of virulence suggest that neutrophil response is dependent on the pathogenicity of the viral infection.<sup>173</sup>

In the lungs, the release of proinflammatory cytokine IL-6 is essential to stimulate neutrophil survival and promote viral clearance, as it can reinstate antiapoptotic factors levels (Mcl-1 and Bcl-XL) suppressed by IAV.<sup>168</sup> Low levels of IL-6, coupled with low neutrophil numbers, strongly correlated with increased disease severity. However, retention of neutrophils might not be necessarily beneficial. Retainment of lung neutrophils has been associated with IAV disease severity, and observed especially in highly pathogenic IAV strains.<sup>112,155,173,174</sup> CXCL8 (a neutrophil chemoattractant) increase in lung airspaces was correlated to elevated neutrophil numbers from recovered patients with ARDS.<sup>175,176</sup> Notably, this increase was correlated to higher disease severity and symptoms.

As mentioned, neutrophils are needed in the early stage of IAV infection for viral clearance. They are effectors in viral clearance, but the accumulation of neutrophils are signs of dysregulated inflammatory signaling and life-threatening tissue damage. In IAV, mild lung pathology can be observed in neutrophil-depleted mice, while ARDS-like pathology and excessive neutrophil infiltration were found in macrophage-depleted mice.<sup>110</sup> Hence, neutrophil responses need to be well balanced during IAV to have an adequate but not excessive inflammation response. It might also be more beneficial for neutrophils to have early apoptosis later into the IAV infection to prevent excessive neutrophil accumulation in the lung.

## 7 | SEX, AGE, AND THE ACCUMULATION OF NEUTROPHILS IN INFECTED LUNGS

IAV infection severity has been associated with multiple demographic factors such as age and sex.<sup>177</sup> For both seasonal and pandemic strains of IAV, children below the age of 10 and adults above age 65 have an increased risk of disease severity. Their immunocompromised nature leaves them more vulnerable to infections. Interestingly,

young adults (15–49 years of age) have severe outcomes during IAV pandemics as compared with seasonal outbreaks, and this increased risk is seen more in females than in males. This observation was attributed to the male reproductive hormone, testosterone, that has been shown to confer protection in male mice.<sup>178</sup> Interestingly, treatment of female mice with high doses of exogenous estradiol showed higher survival rates.<sup>179</sup> In estradiol-treated female mice, increased neutrophil recruitment was observed, and the depletion of neutrophils reverses the protective effects of the treatment.<sup>180</sup> This suggests that the protective effect of estradiol is influenced by its amount produced, which might account for the higher risk in females. Future studies are required to study the role of sex differences and the response toward IAV and other VRDs.

While other respiratory viruses such as the RSV, parainfluenza virus, metapneumovirus, RV, and adenovirus affect mainly children,<sup>68</sup> the influenza virus and coronavirus have more disease complications in elderly of 65 years old and above.<sup>65,181,182</sup> Young children can excrete the viruses earlier and over a longer period of time than adults.<sup>65</sup> A recent study conducted by Kulkarni et al.<sup>174</sup> between lung neutrophil infiltration and age demonstrated an increased level of neutrophils in aged compared with young mice. This observation might have been contributed by the elevated secretion of neutrophil-recruiting chemokines, CXCL1 and CXCL2, by AECs in infected, aged mice. The receptor of these neutrophil chemoattractants, CXCR2, is highly expressed on circulating neutrophils for chemotaxis to the site of inflammation.<sup>183</sup> CXCR2 has been shown to play a major role in neutrophil migration to the lungs during influenza infection, but the neutrophils recruited were reported as dispensable for influenza viral clearance.<sup>86,184–186</sup> This suggests that the increase in disease severity and mortality rate concerning age is related to the increased secretion of CXCL1 and CXCL2, which attract excessive numbers of neutrophils to the lungs and confer a pathogenic effect.<sup>174</sup> This corresponds to ageing studies in humans, where individuals aged above 60 have deficiencies in circulating neutrophils and reductions in neutrophil effector functions such as phagocytosis of bacteria, production of NETs, ROS, and migration.<sup>187</sup>

However, a study conducted by Lu et al.<sup>188</sup> demonstrated increased resistance to IAV infection in aged as compared with young mice. While younger mice showed a faster viral clearance, they also had a higher mortality rate and tissue damage. Due to their weakened immune system, aged mice cleared viruses more slowly without causing tissue damage. Studies by Kulkarni et al.<sup>174</sup> and Lu et al.<sup>188</sup> employed the same viral strain (H1N1; PR8) and mouse strain (female C57BL/6), however their findings were inconsistent. Notably, in the second study by Lu et al.,<sup>188</sup> the age range of the mice is significantly older. Thus, this age difference in mice may account for the observed disparity in experimental results. Hence, this suggests that stronger immune responses might not result in better survival rates, and therapies targeted at reducing excessive neutrophil levels may have to take into consideration the age group and sex of the patient.

With age, neutrophil functions such as phagocytosis and ROS production decline.<sup>189,190</sup> Numerous retrospective studies have demonstrated, severe COVID-19 disease is frequently observed in elderly

patients.<sup>22,191–194</sup> Transcriptomic and cytokine analyses of aged COVID-19 patients revealed higher degranulation signatures and IFN- $\gamma$  signaling. This was also similarly seen using a non-human primate model of SARS-CoV2 infection, including a higher level of VEGF in the lungs of old macaques.<sup>195</sup> Interestingly, the airway epithelial cells of children with SARS-CoV2 possessed augmented antiviral sensing and immune cell activation.<sup>196</sup> This, along with the higher viral sensing and IFN production in myeloid cells, prevents children from acquiring severe disease pathology.

## 8 | CORONAVIRUS (SARS, MERS, SARS-COV2): HIGH NEUTROPHIL COUNTS OBSERVED IN CORONAVIRUS-INFECTED PATIENTS

Coronaviruses are enveloped, positive-sense single-stranded RNA viruses with spike-like structures on their viral surfaces when observed under the electron microscope.<sup>197,198</sup> Similar to IAV, they can cause mild to severe respiratory infections in humans that progress toward fatal outcomes.<sup>197</sup> Since 2000, the emergence of highly pathogenic coronavirus as pandemics has been an unsolved global public health concern. SARS-CoV in 2002 and Middle East respiratory syndrome coronavirus (MERS-CoV) in 2012 have caused a high mortality rate of 9.6% and 35.5%, respectively.<sup>199</sup>

Only a few investigations have been conducted to determine the involvement of neutrophils in SARS-CoV and MERS-CoV infection. Animal models that are ideal for studying these coronaviruses are currently being investigated.<sup>200–203</sup> In the acute phase of SARS-CoV infection, a high neutrophil count and a low number of CD4+ and CD8+ T cells were observed in patients' blood, which was correlated with unfavorable consequences.<sup>204–206</sup> The excessive neutrophils may have contributed to lesion formation in SARS-CoV-infected patients via secretion of granules and ROS that give rise to necrosis in neighboring cells and recruitment of other inflammatory cells. For MERS-CoV infection, high levels of IL-8 (CXCL8), IL-1 $\alpha$ , and IL-1 $\beta$  were expressed in the lower respiratory tract of patients.<sup>207</sup> These cytokines positively correlated with case fatality rates that were reported. Notably, the high expression level of IL-8 and IL-1 $\beta$  in MERS-CoV patients can be drawn parallel to the studies conducted with IAV.<sup>175,176</sup> This suggests a similar inflammatory response observed in both VRDs, allowing us to understand deeper the mechanisms that underlie coronavirus infections through IAV infection studies.

Since December 2019, the world was hit by SARS-CoV-2. It is a novel, highly contagious and pathogenic coronavirus that has resulted in more than a million deaths worldwide. Transmitted between humans via respiratory droplets and close contact, SARS-CoV-2 causes COVID-19, which primarily presents as respiratory symptoms.<sup>208–210</sup> To this date, SARS-CoV-2 has overtaken both SARS-CoV and MERS in the total number of infected individuals and death toll.<sup>210–212</sup> Viral pneumonia caused by SARS-CoV-2 can be grouped into 2 phenotypes: a milder pneumonia phenotype and an ARDS-like phenotype. COVID-19 patients can recover from mild pneumonia or progressively develop to the more ARDS-like severe symptoms.<sup>213</sup> During this

progression, conditions such as atelectasis (blockage of the airway) and lung derecruitment (collapse of the lungs) are developed. Progression into ARDS occurs 20–30% of the time. Primary histologic manifestation of severe COVID-19 includes bilateral DAD, pulmonary edema, and hyaline membranes.<sup>214</sup> DAD is also associated with high mortality in patients. Postmortem histopathologic analysis of COVID-19 patients reveals endothelialitis, which is the inflammation of the endothelium and alveolar wall injury with the presence of neutrophils and lymphocytes.<sup>215,216</sup> This suggests that neutrophils and lymphocytes contribute to the lung tissue damage and pathogenesis of COVID-19.

Hence, the association between the NLR and COVID-19 disease severity has been studied and identified as an early indicator for severe COVID-19 disease.<sup>10,11,217–221</sup> A retrospective cohort study in Wuhan consisting of 210 COVID-19 patients (87 of them are severe cases) showed a higher NLR of 6-fold difference in severe cases compared with mild cases, with an increased level of neutrophils and a significant reduction in T cell level.<sup>218</sup> Neutrophil markers including resistin, lipocalin-2, hepatocyte growth factor, IL-8, G-CSF, and dual endothelin-1 and VEGF signal peptide-activated Receptor (DEspr) have also been identified as predictors of severe COVID-19 disease.<sup>97,222</sup> In critical cases, high NLR and neutrophilia have been associated with excessive ROS, which may contribute to COVID-19 disease severity through induction of disease manifestation such as tissue damage, atherosclerosis, thrombosis, and endothelialitis.<sup>223</sup> Additionally, excessive ROS may cause an imbalance of iron homeostasis and contribute to inefficient oxygen transport.<sup>224</sup> Furthermore, the elevation of circulating NETs was observed.<sup>225</sup> Studies in patients revealed a pathogenic role of NETs in disease progression,<sup>93,226–239</sup> contributing to immunothrombosis. In vitro experiments found SARS-CoV-2 capable of activating healthy neutrophils and inducing the release of NETs, which promotes lung epithelial apoptosis.<sup>226</sup> These suggest that both excessive ROS and NET formation from dysregulated levels of neutrophils in the lung contribute to disease pathology. Hence, NETosis is a potential therapeutic target against severe COVID-19 disease progression.<sup>240</sup> R406, an ATP-competitive SYK inhibitor and active metabolite of fostamatinib, inhibited NETosis of healthy donor neutrophils in COVID-19 patient plasma, demonstrating its potential to inhibit NETosis in COVID-19 patients.<sup>241</sup> Interestingly, these neutrophil-related pathologies closely resemble IAV infections, reinforcing how neutrophil functions can be closely studied using IAV as a model.

To better study COVID-19, animal models are actively being developed.<sup>242,243</sup> While angiotensin-converting enzyme 2 (ACE2) is the functional receptor in humans for SARS-CoV-2, ACE2 in mice does not bind to the virus effectively.<sup>244</sup> Strategies to overcome this problem are being explored. Currently, no mouse model replicates all aspects of COVID-19 displayed in humans.<sup>245</sup> However, continued refinement may result in models even for these aspects of the human disease. Recent studies using a transgenic mouse model with human ACE2 demonstrated that the neutrophil mediator S100A8/9 is highly expressed in SARS-CoV2 infection and inhibition through the drug paquinimod reduced pathogenesis.<sup>246</sup> These models will pave the way

for new insights into the mechanisms that neutrophils partake in during SARS-CoV2 and other VRDs to elucidate possible treatment regimens that can target neutrophil subsets at various stages of the disease.

## 9 | NEUTROPHIL HETEROGENEITY IN VRDS

The current literature on neutrophils in VRDs, covered in this review, provides the foundation for understanding the role of neutrophil subsets in VRDs. There are many layers of heterogeneity to consider, resulting from environmental, developmental, and activation states.<sup>27,59,247,248</sup> The first layer of heterogeneity is the neutrophil's infection status. IAV-infected neutrophils were shown to produce less ROS, cathelicidin LL-37, and lipocalin.<sup>249–251</sup> This implicates their functional capacity for viral clearance and may explain higher viral loads that are observed with high infiltrating neutrophil numbers. As discussed, these infected neutrophils can also act as antigen-presenters for CD8+ T cell immunity.<sup>106</sup>

The second layer of heterogeneity is the neutrophil's developmental status. During inflammation, the appearance of immature band cells has been observed, marked as a “left shift” of precursor neutrophils.<sup>33,39</sup> Retrospective studies of infants with various VRDs noted high frequencies of immature neutrophils, and this observation was not influenced by bacterial coinfections.<sup>252</sup> Cortjens et al.<sup>253</sup> further identified 3 blood neutrophil subsets during viral infections in infants, namely immature CD16<sup>lo</sup>CD62L<sup>hi</sup> neutrophils, mature CD16<sup>hi</sup>CD62L<sup>hi</sup> neutrophils, and a suppressive CD16<sup>hi</sup>CD62L<sup>lo</sup> subset. The group previously identified the suppressive CD16<sup>hi</sup>CD62L<sup>lo</sup> subset in LPS-treated individuals.<sup>254</sup> It is unclear if this LPS-induced CD16<sup>hi</sup>CD62L<sup>lo</sup> subset is analogous to the VRD-induced subset identified. Recent evidence with single-cell sequencing technologies has shown that neutrophils in SARS-CoV2-infected patients are frequently developmentally immature, with lower expression levels of S100a8, S100a9, CD10, and CD101.<sup>255</sup> These neutrophils were also reported to consist of proneutrophils, preneutrophils, and immature neutrophils that associate highly with severe COVID-19 disease.<sup>256</sup> Immature neutrophils, in particular, showed a strong correlation with disease severity and is suggested to perform better than the NLR.<sup>257</sup> Martinelli et al.<sup>258</sup> used microarray profiling and compared immature bone marrow neutrophils with mature blood neutrophils, showing that immature neutrophils lacked type-1 IFN signaling receptors and associated genes. They further showed the lack of STAT-1 phosphorylation during IFN $\alpha$  or IFN $\gamma$  stimulation in immature neutrophils.<sup>258</sup> These results suggest a differential ability to respond toward VRDs, leading to a lower propensity for NET formation and cytokine release. Single-cell analysis of SARS-CoV2-infected patient leukocytes confirms this, showing proNeus and preNeus in the blood have much lower expression of IFN signaling genes.<sup>256</sup> Moreover, both proNeus and preNeus have low or no expression of CD16 required for ADCP as mentioned earlier. This further differentiates the antiviral potential between mature and immature neutrophil subsets.

The role of immature neutrophils during VRDs is still unclear. It is proposed that their appearance and accumulation is a consequence



of the high inflammation present in the patients that stimulates the premature mobilization of immature neutrophils from the bone marrow to the circulation and sites of inflammation. These banded nuclei cells are perhaps less efficient in providing viral clearance and forming NETs and may trigger the recruitment of more immature granulocytes to compensate for the loss of efficiency in viral clearance. Studies on immature neutrophils during inflammatory conditions suggest a high ROS function and migration capacity, but low NETs and phagocytosis function.<sup>259,260</sup> This was also shown with in vitro human studies, demonstrating an increased immature neutrophil migration through CXCL8 signaling, but a higher propensity for NET formation correlating with severe COVID-19 disease.<sup>261</sup>

The third layer of heterogeneity is the neutrophil's activation status. Reports of LDNs in VRDs have suggested a degranulated form of neutrophils capable of immunosuppression.<sup>262,263</sup> This subset of neutrophils consisted of a mixture of immature and mature phenotypes, unlike what is commonly thought.<sup>262-264</sup> Morrissey et al.<sup>262</sup> identified a specific CD16<sup>int</sup> LDN subset that was shown to possess enhanced cytokine production upon stimulation. RNA sequencing analysis further suggests increased phagocytosis and degranulation function when compared with CD16<sup>hi</sup> subset.<sup>262</sup> However, recent evidence of LDNs questions this difference with normal density neutrophils (NDNs). Hardisty et al.<sup>265</sup> were able to generate LDNs from activating NDNs with TNF- $\alpha$ , fMLP, or LPS. These LDNs were shown to have no significant difference in ROS production or surface marker expression. Moreover, no differential effect on T cell proliferation or IFN $\gamma$  production was observed.<sup>265</sup> One key difference was the lowered ability for NET formation, possibly linked to the decrease of granules such as NE and MPO required for NETosis. Similarly, LDNs of patients with severe fever with thrombocytopenia syndrome (SFTS) was shown to be derived from NDNs after culture with SFTS media. However, Li's group<sup>266</sup> showed that LDNs could secrete higher amounts of IL-8, IL-6 and IL-17, suggesting a contributor of higher cytotoxicity to endothelial cells. In their study, Li showed that LDNs had higher viral loads, signifying possible differences in antigen-presentation potential.

## 10 | FUTURE OPPORTUNITIES AND CHALLENGES

Neutrophils can play various roles during VRDs. However, some of these activities that aid in viral clearance can become harmful to the host when dysregulated. Complicated by a spectrum of heterogeneity in neutrophil subtypes and states, identifying pathologic neutrophils remains a significant hurdle in devising biomarkers and novel treatment strategies. Current evidence of neutrophils in VRDs lack a consensus of identifying neutrophil subsets. Lung neutrophils are, at physiologic conditions, different from blood neutrophils phenotypically.<sup>63</sup> Surface markers such as CD62L and CD11b, which are used to identify suppressive and activated neutrophils, were shown to be lower in expression in lung neutrophils.<sup>253,63,267</sup> Moreover, many surface markers, used to differentiate neutrophil subsets might alter drastically depending on severity and disease progression. It is obvious that emergency granulopoiesis occurs during VRDs, mobilizing immature precursors

into the periphery.<sup>268</sup> Therefore, an inflammation-stable maturation marker, such as CD10 (human) and CD101 (mouse), could be incorporated into future studies to dissect development-specific changes in neutrophil activity during VRDs.<sup>32,39</sup> Future work also should focus on lung neutrophils from BAL samples as the role of the local environment is increasingly appreciated. We anticipate that these future findings will lead to novel interventions and therapies capable of modulating neutrophil activity to improve clinical outcomes.

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## AUTHORSHIP

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## DISCLOSURE

The authors declare no conflict of interest.

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