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REVIEW
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A Role for Neutrophils in Viral Respiratory Disease

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Neutrophils are immune cells that are well known to be present during many types of lung diseases associated with acute respiratory distress syndrome (ARDS) and may contribute to acute lung injury. Neutrophils are poorly studied with respect to viral infection, and specifically to respiratory viral disease. Influenza A virus (IAV) infection is the cause of a respiratory disease that poses a significant global public health concern. Influenza disease presents as a relatively mild and self-limiting although highly pathogenic forms exist. Neutrophils increase in the respiratory tract during infection with mild seasonal IAV, moderate and severe epidemic IAV infection, and emerging highly pathogenic avian influenza (HPAI). During severe influenza pneumonia and HPAI infection, the number of neutrophils in the lower respiratory tract is correlated with disease severity. Thus, comparative analyses of the relationship between IAV infection and neutrophils provide insights into the relative contribution of host and viral factors that contribute to disease severity. Herein, we review the contribution of neutrophils to IAV disease pathogenesis and to other respiratory virus infections.

Keywords: neutrophil, influenza, acute respiratory distress syndrome, respiratory virus, viral microenvironment

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INTRODUCTION

Neutrophils are a type of polymorphonuclear granulocyte that differentiate from myeloblasts in the bone marrow to comprise approximately 60% of the circulating blood leukocytes (1). The formation of intracellular granules (azurophilic granules, specific granules, gelatinase granules, and secretory vesicles) and the morphologically characteristic segmentation of nuclei occur during the terminal differentiation process into neutrophils (1). Neutrophils are often considered professional bacteria-responsive immune cells: they express bacteria-specific receptors (e.g., formylated peptide receptors or certain toll-like receptors, "TLRs") and their granules have anti-bacterial or bacteriostatic properties. Currently, their role in viral infection has received very little scientific attention (2).

Neutrophils are present during many types of lung diseases associated with acute respiratory distress syndrome (ARDS) and may contribute to acute lung injury (3–12). The lung has a global inflammatory response to infection regardless of etiology, and this response includes the infiltration of neutrophils and macrophages in response to chemotactic signaling which originates in the lung (3, 5, 12–20). These phagocytic cells leave circulation and hone to sites within the infected airways where they may deploy potent effector functions to control disease (**Figure 1**) in response to pathogen associated molecular patterns (PAMPs) and inflammatory cytokines and chemokines (21, 22). In the case of viral infections, the type I interferons (IFN) and IFN-stimulated genes (ISGs) signal an appropriate immune response (23–26). Lethal infections may result from insufficient information or incorrect information about the specific cause(s) of

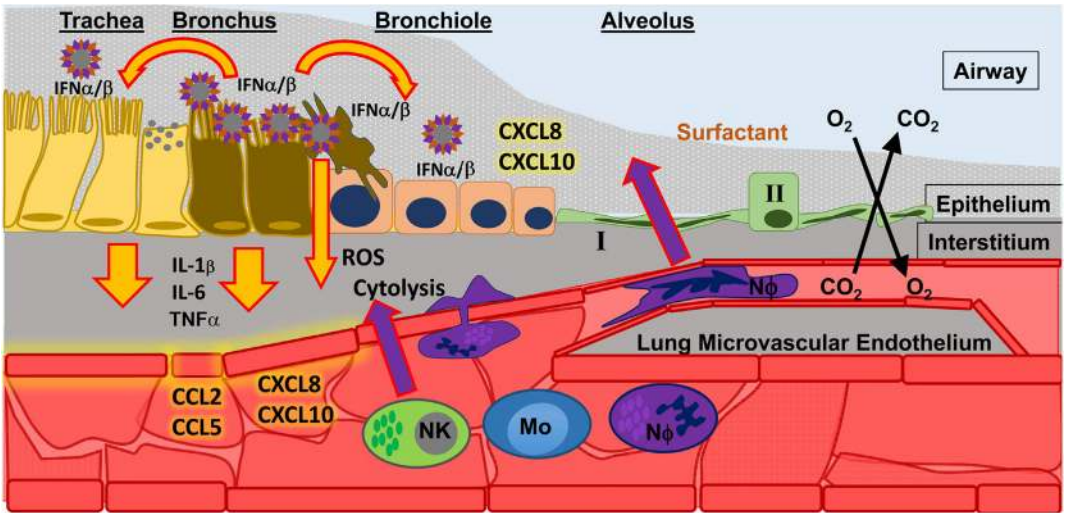

FIGURE 1 | **Influenza A virus (IAV) infection in the upper respiratory tract**. Infection of epithelial cells in the bronchus results in the release of type I interferons (IFN α/β) which signal to nearby cells. The result of IFN α/β signaling is the release of pro-inflammatory cytokines (e.g., IL-1β, IL-6, TNFα) that signal to endothelial cells, which help spread inflammatory signals (chemokines, such as CCL2, CCL5, CXCL8, CXCL10) throughout the blood to recruit innate immune cells to the site of infection. Recruited innate immune cells [such as natural killer cells (NK); monocytes (Mo); and neutrophils (NΦ)] must interact with activated endothelium to leave the blood stream and migrate toward the site of infection. There they can perform effector functions to control infection, such as releasing reactive oxygen species (ROS) and directly killing infected cells (cytolysis).

infection, thereby signaling inappropriate (incorrect or excessive) immune responses (27). Neutrophils, as first-responders to many forms of airway infection, may be a keystone species in determining viral disease outcome; however, neutrophils are poorly studied with respect to viral infection and specifically to respiratory viral disease.

Much research has focused on the role of neutrophils in severe versus mild respiratory disease, as well as their role in bacterial infections. It is, therefore, important to establish a general role of neutrophils in respiratory virus infection to provide a groundwork into more specific questions (e.g., are neutrophils capable of catering to a virus-specific response?). Herein, the evidence for the presence and activities of neutrophils during respiratory viral infection is reviewed. Having established spatio-temporal aspects of the neutrophil response to influenza A virus (IAV), the potential antiviral function(s) of neutrophils during acute virus infection as well as recovery from infection are discussed. The discussion will focus on IAV and neutrophil activities during the course of a "typical" flu infection: activation, migration, and effector functions *in situ* (**Figure 1**). IAV are particularly well-suited for the study of neutrophils in viral respiratory disease, since they are well-studied in humans and animal models, and it is well-established that infection with specific viral variants (i.e., genetic point mutations) alter the course of disease from mild to severe (28). More recently, specific IAV viral variants that affect pathogenicity have been linked to alterations of the neutrophil response (29). Thus, a comparison of the neutrophil response between disease phenotypes of a single virus species (*Influenza A virus*) may elucidate a role for neutrophils in the viral microenvironment. Herein, we review evidence of neutrophil responses during the course of disease in various IAV phenotypes in animal models of infection, as well as comparing these responses to what is known about neutrophil responses during bacterial infection of the airways.

INFLUENZA A VIRAL PHENOTYPES

Influenza A virus poses a concern for global public health due to emergence of strains with increased human transmission and/or increased pathology (30–35). In 2009, a novel virus type, influenza A(H1N1)pdm09 IAV, emerged with an increased transmission rate and greater disease, i.e., moderate to severe pathology relative to seasonal human IAV (36–50). Clinical isolates of influenza A(H1N1)pdm09 have relatively little genetic variability yet cause variable clinical outcomes from moderate to severe pathology, including ARDS (39, 41, 47, 48, 51). Therefore, they are well-suited to understand host and viral contributions to IAV pathogenesis. A detailed discussion of the viral replicative cycle is beyond this review, yet excellent reviews are plentiful [e.g., Ref. (52–54)]. IAV is a well-studied model for virus infection in laboratory animals, such as mice and ferrets, and much is known about the contributions of viral and host determinants to severe disease (55–58). Retrospective and experimental infection studies routinely demonstrate common occurrences in the formation of severe IAV [including severe influenza pneumonia (SIP) and ARDS] in humans, ferrets, and mice; these include increased cytokine secretions in the lung, diffuse alveolar damage ("DAD," bronchointerstitial pneumonia in veterinary pathology), and neutrophilic infiltration (29, 55, 58–66).

In general, IAV infection is an excellent model to investigate the respiratory system's immune response to viral infection, specifically the pathway leading to severe pneumonia and/or ARDS.

Influenza disease is commonly relatively mild and self-limiting, although highly pathogenic forms exist (42, 59, 67–72). The major complication from IAV infection is the formation of SIP which may develop into ARDS (59, 65, 67, 68, 70–73). The reason(s) why infection with IAV may lead to severe viral pneumonia and ARDS is poorly understood, but is thought to involve both host and viral factors. The respective and combined contributions of the host innate immune response and viral factors to the timing and severity of SIP are poorly understood. Neutrophils are present in the respiratory tract during infection with mild seasonal IAV, SIP, and highly pathogenic avian influenza viruses ["HPAI," which includes avian influenza A (H5N1) virus] (50, 51, 65, 67, 68, 74–76). During SIP and HPAI infection, an increase in the number of neutrophils in the lower respiratory tract (LRT) is correlated with disease severity (50, 51, 65, 67, 68, 76).

Although clinical pathology suggests that a spectrum of disease results from IAV infection, there are at least three disease "phenotypes" caused by infection with IAV, listed by increasing case fatality rate: a mild upper respiratory tract (URT) infection, a SIP which can lead to ARDS, and a LRT infection which can lead to hypercytokinemia. The virological basis for disease phenotype is related to adaptations to mammals—most important are receptor specificity and efficiency of replication—and the major mechanisms have been defined through the use of experimental animal models (30, 32, 33, 57, 77–80). An "ideal" viral infection (i.e., one that is successful for the virus and non-lethal for the host) may be considered a balance between virus replication and an immune response necessary to promote viral shedding, typical of mild seasonal ("epidemic") IAV. In general, emergent IAV, directly or indirectly from avian enzootic cycles, have increased pathology in humans, the most fatal form of which is a syndrome of complete immune dysregulation (65, 69, 70, 81–84). IAV is genetically highly variable, and mechanisms for increased disease severity are multifactorial, involving host and viral factors.

Uncomplicated Influenza

The majority of yearly, seasonal IAV infections in the world cause a relatively mild, self-limiting URT disease. Influenza disease is characterized by an abrupt onset fever, myalgia, and malaise, with symptoms similar to other URT infections, such as sneezing, coryza, and rhinorrhea (67, 85). Symptoms can last anywhere from 1–5 days and are clinically indistinguishable from other "flu"-like illnesses, including bacterial and viral infections that cause the common cold [e.g., *Streptococcus pneumoniae*, *Haemophilus influenzae*, human rhinovirus (HRV) infection, *Human respiratory syncytial virus* (hRSV) infection, and coronavirus infection] (85–88). Experimental infection of humans with IAV suggests that the virus is mainly restricted to the URT, although sampling the LRT is difficult (67, 68, 87, 89). While fever typically begins 2 days postinfection, virus is shed from the URT in nasal secretions as quickly as 24 h postinfection, allowing efficient transmission prior to symptom onset and continues until 4–5 days postinfection (86, 87, 89) (**Figure 2**). Rhinorrhea is coincident with neutrophilic rhinitis and shedding of necrotic nasal epithelium (67, 90, 91). Surprisingly, the LRT seems to be involved in uncomplicated IAV infection, although this observation is frequently overlooked or unaddressed in studies (68, 92). In humans, local and systemic concentrations of IL6, CXCL8/IL8, and MCP1/CCL2 correlate with increased disease severity (i.e., symptom severity and increased virus shedding) (87–89, 93) (**Figure 2**).

Severe Influenza Pneumonia

Influenza A(H1N1)pdm09 virus spread quickly throughout the globe, much like previous pandemic viruses, such as the 1918 H1N1 "Spanish flu" IAV. Humans infected with influenza A(H1N1)pdm09 virus also presented with typical flu-like symptoms (e.g., fever, cough); however, there was an increased number of cases presenting with dyspnea, respiratory distress,

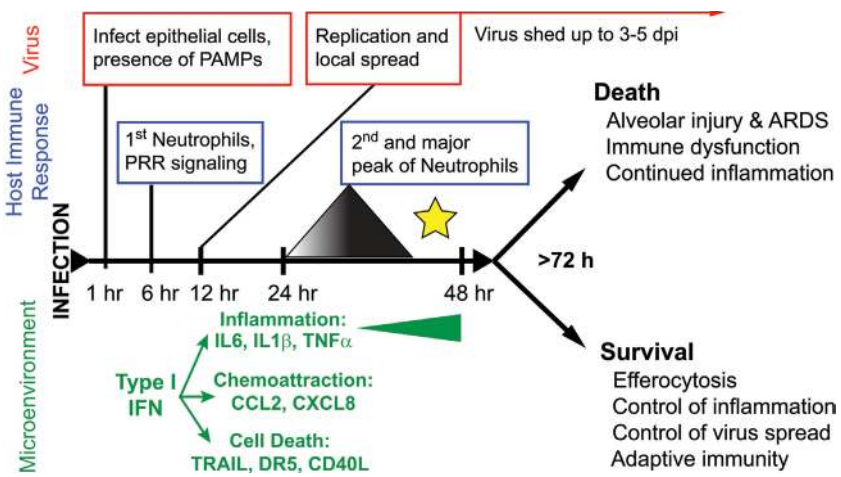

FIGURE 2 | The course of disease following influenza A virus (IAV) infection. A timeline depicting major events in the viral replication cycle (red), the host immune response (blue), and the effects on the host tissue environment (green) during an IAV infection of the airways. A star marks the critical point for the formation of severe disease versus recovery from infection. This review posits that at this timepoint, coincident with a second wave of increasing neutrophilia and inflammation, the outcome of disease is determined.

and pneumonia (36–38, 40–48, 50, 94, 95). Additionally, retrospective assessments show a proportionately greater number of adolescents and adults with severe disease compared to typical seasonal influenza, and patients with comorbidities, such as obesity and asthma, were at higher risk of severe infection (51, 96–98). In general, the virus causes infection of URT, as well as bronchitis and bronchiolitis, and a high proportion of cases presented with severe disease in the form of viral pneumonia (42, 51, 96). Histopathologic changes in autopsies revealed extensive cytonecrosis, desquamation, and inflammatory infiltration of the bronchus and trachea, mild to severe necrotizing bronchiolitis (42, 50, 51, 99). The primary pathologic finding of SIP was sporadic to DAD with hyaline membrane formation, edema, and occasionally hemorrhage (42, 50, 51, 99). As is typical of influenza infections, some patients experienced bacterial coinfection although this was not in a majority of patients, including those dying from ARDS (42, 48, 50, 51, 93, 99–103). This may distinguish the influenza A(H1N1)pdm09 virus from the 1918 H1N1 IAV, for which bacterial superinfection was determined to cause a majority of the deaths (104, 105), although this may more accurately reflect improved hygiene and standard of care. As discussed below, studies of the reconstructed 1918 H1N1 IAV using animal models suggest that this virus was highly pathogenic irrespective of secondary bacterial pneumonia (62, 106–108).

Across many cohorts of clinical patients, serum concentration of IL6, CCL2, and CXCL8 were significantly elevated in severe cases of influenza A(H1N1)pdm09 viral pneumonia compared to patients with other confirmed illnesses including seasonal IAV, milder forms of influenza A(H1N1)pdm09 virus, bacterial pneumonia, or other viral respiratory infection [hRSV, HRV, human adenovirus (hAdv)] (93, 100–102, 109). These cytokines and chemokines remained elevated over time (up to 6 days following hospital admission) in cases of severe influenza A(H1N1)pdm09 viral pneumonia, whereas they decreased as patients recovered from seasonal and mild influenza A(H1N1) pdm09 virus infection (93, 100). In severe cases of influenza A(H1N1)pdm09 virus infection, decreased type I IFN and ISG production was occasionally noticed compared to adult patients with seasonal IAV infection (93). The influenza A(H1N1)pdm09 viruses have received much scrutiny, and a large dataset of the genetics and pathogenic phenotypes of virus isolates exists in human and animal models. The H1N1 subtype IAV are highly important viruses due to their pandemic potential, as supported by the historical record (62, 68, 110). Some influenza A(H1N1) pdm09 viruses can infect the LRT in humans and in the ferret animal model, which makes them excellent laboratory viruses to investigate the involvement of the LRT in pathogenesis, specifically in the development of severe disease (42, 111–115). Their overall genetic similarity makes for excellent comparison studies between natural clinical isolates, and reverse genetics systems exist to study molecular pathogenicity (41, 95, 113, 116–126).

HPAI and Cytokine Storm

Avian IAV has been recorded sporadically entering the human population over the last 20 years, beginning with an H5N1 subtype virus which emerged in China in 1997 (70, 82, 127, 128). It is likely that this avian influenza A (H5N1) virus and other emergent strains result from contact with infected domestic poultry that are infected with HPAI (82). The disease caused by avian influenza A (H5N1) virus is characterized by DAD, alveolar necrosis, and alveolar hemorrhage [human disease, including pathology, is reviewed in Ref. (83)]. There is evidence of viremia and systemic spread; IAV antigen has been detected in the trachea, bronchi, and alveolar pneumocytes (69, 76), as well as infrequently in the brain and gastric epithelium (59, 76, 83). The innate cellular immune response in the lungs was characterized by an increase in inter-alveolar macrophages/histiocytes (59, 65, 69, 71, 81, 129) and only moderate infiltration of lymphocytes and neutrophils in the few patients that were analyzed postmortem (69, 76). Systemically, patient serum had high concentrations of CXCL10, CCL2, IL6, IL8, and IL10 compared to matched-control patients with seasonal H3 and H1 IAV, and these concentrations were correlated with viral load in throat (59, 65, 69). In lethal cases, the result of infection and immune dysregulation led to multiple organ failure (e.g., kidney tubular inflammation, necrotic lesions in brain, impaired liver function) and abnormal clotting. Reactive histiocytes undergoing hemophagocytosis were frequently found in bone marrow and lungs of patients, which is indicative of diseases involving hypercytokinemia (59, 65, 70, 81, 83, 130).

Other events involving avian IAV transmission to humans are known and are often associated with veterinary or other animal workers; for example, a 2004 case of avian influenza A (H7N7) virus infection in a veterinarian in Europe showed severe fatal pneumonia and DAD (131). It was reported that 1 L of serosanguineous fluid was drained from his chest upon autopsy. In 2013, another avian IAV emerged in Southeast Asia; this time an avian influenza A (H7N9) virus (71, 72, 129, 132). The histopathology was similar to avian influenza A (H5N1) virus: severe pneumonia, DAD, and epithelial necrosis were common features of infection with both viruses (71, 72). Therefore, it seems the typical presentation of human patients infected with either virus includes high levels of CXCL10, CCL2, IL-6, and CXCL8 in the plasma, peripheral blood leukopenia, and lung neutrophilia (65, 69, 81, 129, 130, 132), and this is also similar to experimental infection of laboratory animal models (111, 133). There were slightly more bacterial coinfections in cases of H7N9 compared to H5N1 avian IAV (71, 72, 81). In a direct comparison, serum from patients with infected with avian influenza A (H5N1) virus had higher concentrations of IFNα and IFNγ in the blood and lower levels of IL8, whereas the opposite was true for patients with avian influenza A (H7N9) virus (134). Similarly, CXCL9 and CXCL10 were higher in patients with avian influenza A (H5N1) virus, whereas CCL4 concentrations were higher in patients with avian influenza A (H7N9) virus (134). Infection with either virus resulted in higher blood C-reactive protein (CRP) (129). Variability between patients may account for the apparent discrepancies between specific immune responses. Therefore, experimental infection of laboratory animals removes individual variability and provides a clear picture of general disease progression, with important caveats for their comparison to human disease as discussed in more detail in the next section (58, 135).

NEUTROPHILS IN IAV COURSE OF DISEASE

Neutrophils are increased in the lungs and blood after infection with pathogenic IAV in mice, humans, and ferrets (28, 136, 137). Cell depletion studies have demonstrated that neutrophils are necessary for recovery from severe, but not mild, IAV infection (29, 138, 139). Studies in mice show that neutrophils have effects during both early and late stages of disease (140). As discussed in detail below, initial pathogen sensing through various pathogen recognition receptors (PRRs) stimulates inflammatory signals from resident macrophages to initiate neutrophil chemotaxis to the infected airways (**Figure 1**). For example, TLR7 recognition of IAV dsRNA- and Myd88-mediated release of TNFα and CCL3 by mononuclear cells is important for neutrophil recruitment to the site of infection (141, 142). Transgenic mice have been used to study the contribution of specific cytokines and chemokines to inflammation following IAV infection, particularly as this relates to "hypercytokinemia," and are summarized in Tables S1–S4 in Supplementary Material. The signals from the infected lung are propagated systemically by endothelial cells, which recruit and tether neutrophils. The importance of endothelial signaling in the development of severe disease has been shown recently using sphingosine-1 phosphate agonist to prevent severe disease in animal models of both influenza virus and respiratory syncytial virus (143, 144). The complex interactions governing neutrophil extravasation, migration through the interstitium, and crossing the alveolar epithelium are well known in relation to many forms of ALI and the development of ARDS with the exception of conditions surrounding viral infection, although mechanistically they should be quite similar (3–5, 7, 145, 146).

Neutrophil Migration

In the ferret model, the migration of neutrophils to the lungs occurs in two distinct waves: a first wave within hours of challenge, peaking after 24 h then decreasing; and a second wave that increases over time until disease resolution or death (111) (**Figure 2**). In the ferret model, we have shown that the neutrophils become concentrated at specific foci in the lungs coincident with influenza-positive epithelium and the expression of chemoattractant chemokine genes (22). Neutrophil chemotaxis in humans is thought to be mediated by many factors, such as the chemokine CXCL8, cytokines IL-1 and TNFα, and complement C5a (145, 147, 148). During both mild and severe IAV disease, patients show increased blood CRP and activation of C5a (149), as well as increased secretion of CXCL8, TNFα, and IL-1 in nasal washes, which correlate with disease severity (87, 89, 93, 150–152). In the mouse model of influenza virus infection, chemical reduction of C5a during IAV infection reduced lung neutrophilia (153). Similarly, knockout mice deficient in the inflammasome pathway or mice not expressing cytokines, such as IL-1b and IL-6, have decreased neutrophil activation and migration to the lungs during IAV infection (Table S1 in Supplementary Material) (154–156). Mice do not possess CXCL8, but CXCL1 and CXCL2 have equivalent functions. Neutrophils contribute CXCL2 to the IAV-infected mouse lung to further stimulate neutrophil recruitment (157). More recently, it was shown that removing a CXCL1 repressor (*Setdb2*) does not increase recruitment of neutrophils to the lungs of mice infected with IAV PR8, rather it reduces the ability to respond to bacterial superinfection (158). In addition, it was shown that another ISG, CXCL10, operates on a unique subset of CXCR3+ neutrophils present during mouse IAV infection in an autocrine manner, increasing chemotaxis, oxidative burst, and enhancing inflammation (157) (Tables S3 and S4 in Supplementary Material). Finally, aryl hydrocarbon receptor is somehow linked to increases in NO and neutrophilia in the lungs of IAV-infected mice independently of known neutrophil chemoattractants or mechanisms of neutrophil extravasation (159–162).

Neutrophil Extracellular PRRs and Phagocytosis

In cell coculture, human neutrophils were seen to interact specifically with IAV-infected cells (163), although the nature of this interaction in the infected lung is unknown. Neutrophils are phagocytic cells, and their methods for sensing extracellular pathogens rely on TLRs (2, 147). Stimulation of neutrophils through cell surface TLRs has been recorded to promote cytokine secretion (CXCL8 and TNFα *via* NFκB and AP-1), formation of reactive oxygen species (ROS), phagocytosis, granule secretion, neutrophil extracellular trap (NET) formation, and migration (145, 147, 148). Human neutrophils highly express nucleic acid-detecting TLRs, specifically endosomal TLR8 (164, 165), but do not express nor respond to activators of TLR3 or TLR7 (164–166). [Interestingly, $TLR3^{-/-}$ mice have increased neutrophilia and fewer macrophages in the lungs, yet have increased survival after infection with IAV (167, 168).] TLR4 is required for LPS-induced neutrophil migration to the lung (169) and can stimulate immunostimulatory responses *via* TRIF adaptors (170); however, TLR4-stimulation does not lead to the production of type I IFN in neutrophils (166, 171).

Several innate immune effector proteins with opsonizing functions that are present in airway mucosae are known to interact with both IAV and neutrophils. Surfactant protein D, a lung collectin, is an innate immune defense against a variety of viruses, opsonizing the viruses for phagocytosis by neutrophils, which in turn causes the production of ROS (172–178). Human neutrophil defensins are short basic peptides released from neutrophil granules during inflammation (145, 178, 179). They have been shown to interact with IAV, reducing infectivity, and promote neutrophil phagocytosis and clearance of IAV (178–182). Defensins may also buffer the oxidative burst from neutrophils that follows from phagocytosis of viruses that have been opsonized by surfactant protein D (178–180). One study indicated that neutrophils do not interact with immunoglobulin-bound IAV (183); however, another showed that protective anti-IAV antibody therapy only protected mice in the presence of neutrophils (184). The hypothesis that IAV pathogenicity can be partially explained by infection with viral variants that can evade opsonization by innate immune effectors is attractive and deserves further study (176).

Neutrophil Intracellular PRRs

Neutrophils express sialic acid receptors and may become infected with IAV (74, 185, 186). Neutrophils infected with IAV have increased apoptosis, but infection does not result in the production of virus (186). Infection of human neutrophils with IAV treated at 56°C to denature the viral replicase but not HA suggested that infection alone, but not replication, is sufficient to stimulate the release of CXCL8 and CCL4 in human neutrophils (187). Neutrophils infected with IAV have rapid upregulation (<9 hpi) of type I IFN pathways, including cytoplasmic PRRs, IFNβ, and ISGs (186), which is counter to the long-held dogma that neutrophils were incapable of gene expression. This is may be due to RIG-like receptors (RLRs) sensing of viral dsRNA, as neutrophils transfected with poly(I:C) (a viral RNA mimic) have a similar response (166). Additionally, neutrophils express nod-like receptors but it is unclear how these interact with IAV infection (188, 189). In general, inflammasome and pro-IL1 activation following IAV infection is poorly understood (154, 155, 190, 191). However, studies of IAV infection using caspase-1, IL-1β, or IL-1R transgenic mice show modulation of neutrophil infiltration and pathology and suggest that it is an IAV subtype-dependent effect (154, 191–195) (Tables S1 and S2 in Supplementary Material). Furthermore, it has been demonstrated that the HA of some IAV isolates suppresses neutrophil activation, providing further evidence for IAV subtype-dependent effects on neutrophils (185, 186, 196).

Neutrophil Activation and Degranulation

Activation of TLRs and RLRs trigger degranulation and the expression of surface CD11b (166), which pairs with CD18 to form the "Mac-1" integrin dimer that binds collagen (197). This facilitates migration through tissues, and release of gelatinase or collagenase (MMP-2 and MMP-9) from neutrophils assist in clearing connective tissue from the path. At the site of infection, neutrophils release microbial effectors [reviewed in Ref. (145, 198)]. Neutrophils develop granules sequentially (azurophilic, specific, gelatinase, secretory) and secrete granules in the reverse order (145). Secretory and gelatinase granules are released shortly after endothelial transmigration and contain membrane proteins essential for movement [extracellular matrix (ECM)-binding integrins] and pathogen recognition [immunoglobulin (FcR) and complement receptors] (145). Specific and azurophilic granules contain tissue-destroying enzymes and antimicrobial proteins. For example, neutrophil myeloperoxidase (MPO) may contribute to lung injury during IAV infection (199); however, it may have direct antiviral effects on IAV (200). An investigation found no difference between IAV infection of a wild-type and neutrophil elastase knockout mouse, measuring lung function, chemokine secretion, and neutrophil recruitment (201) (Table S5 in Supplementary Material). The contents of the granules can be secreted to destroy ECM (such as MMP-9) or directed toward phagosomes to destroy engulfed microbes. The production of hypochlorous acid (HOCl) is the main oxidant used in phagosomal killing, and its production is dependent on the generation of ROS by the neutrophils. Interestingly, infection by IAV was related to the inhibition of phagosomal killing of bacteria (196).

Neutrophil degranulation primes neutrophils for ROS generation by mobilizing NADPH oxidase components to the plasma membrane (145) and exocytosis of MPO. IAV infection causes the generation of ROS in neutrophils (202). Oxidative burst is thought to have direct microbial effects; however, the direct effect on IAV has not been published (203). IAV infection benefits from the presence of ROS in the environment (204, 205), yet IAV also suppresses NADPH oxidase activity within infected phagocytes (206, 207). Many have investigated the effects of ROS and NO on lung inflammation during IAV infection and found that reduction of oxidative stress in the form of both ROS and NO alieves IAV-dependent lung injury (206, 208–213) (Table S5 in Supplementary Material). For example, oxidized lipids in the lung environment may trigger TLR4, activating immune cells and contributing to increased lung injury (214–216).

Neutrophil Netosis

Neutrophils undergo a form of programmed cell death called netosis, in which NETs are formed (217). NETs are extracellular strands DNA wrapped in histones and enriched in neutrophil effector proteins (e.g., neutrophil elastase and MPO) (218). NETs have the effect of killing many pathogens, including bacteria (146), fungi (219), protozoans (220), and more recently viruses (221). NETs are becoming the focus of study in autoimmune disease atherosclerosis, since they damage endothelium (222–224). Recently, it was shown that hantavirus stimulates NET production during infection, which leads to the generation of autoantibodies and may provide a mechanism for the hemorrhagic fever caused by Old World hantaviruses (225). NETs are typically found with histones, MPO, and neutrophil elastase, and the effect is to isolate the effects of these molecules directly onto the pathogen surface with a "sticky" NET of nucleic acid. NETs contribute to acute lung injury and alveolar capillary damage during IAV infection (139). Yet, very little is known about the relationship between NETs and viral infection in viral disease pathogenesis (226).

In summary, neutrophils are capable of recognizing viruses *via* PRRs as either opsonized virions or *via* endosomal TLR. Although the signaling cascade differs from other phagocytic cells, neutrophils are capable of responding to viral PAMPs with respiratory burst, degranulation of proteases and cytokines, and/or netosis. It is not clear if these responses are effective against influenza virus; in fact, evidence exists that suggest influenza viruses may take advantage of the inflammatory environment. More importantly, it is not clear if there are differences in response to different influenza virus subtypes or strains. As is true for many respiratory etiologies, neutrophil responses must be balanced during influenza virus infection to adequately control of inflammation while promoting pro-immune responses. The timing of neutrophils during disease progression correlates with a key point in divergent disease outcomes (**Figure 2**), and neutrophils may act both globably and locally at foci of infection (22, 111). Thus, neutrophils are focused in the airways at critical timepoints following infection and therefore balancing their potent inflammatory effector functions may determine disease outcome.

COMPARISONS OF NEUTROPHILS IN VIRAL RESPIRATORY DISEASES

As discussed above, there appears to be a correlation between the timing and location of IAV infection and the action of neutrophils, but evidence directly linking these phenomena together remains overall circumstantial. In contrast, during bacterial pneumonia there is direct evidence of the importance of neutrophils in disease: bacterial PAMPs directly upregulate neutrophil activating and chemoattractant chemokines, bacteria have defined anti-neutrophil functions, and some bacteria, e.g., *Mycobacterium tuberculosis*, rely on neutrophils to establish their granulomatous niche (6, 227, 228). Similarly, we suggest that viruses may interact with respiratory cells to create a viral microenvironmental niche. To further substantiate a link between neutrophils and virus infection, evidence for the role of neutrophils in selected viral respiratory diseases is summarized in **Tables 1** and **2**. Discussion below focuses on common patterns of neutrophil responses in severe and mild forms of respiratory viral infection.

Severe Viral Respiratory Disease

For both viral and bacterial etiologies, the most severe clinical complications result from infection of the LRT. Infection of the LRT by viruses, such as human parainfluenza viruses, influenza A(H1N1)pdm09, HPAI, New World hantavirus infections (causing hantavirus pulmonary syndrome), *Severe acute respiratory syndrome-related coronavirus* (SARS-Cov), and Middle East respiratory syndrome coronavirus (MERS-CoV), are all associated with neutrophilic infiltration at sites of infection to various degrees and may develop into ARDS (9–11, 19, 60, 131, 242–245). Clinically defined, ARDS has three phases; and most patents die within the first phase, the acute or "exudative" phase [reviewed in Ref. (12–14)]. This phase is characterized by an increased immune response with high production of pro-inflammatory cytokines and chemokines, increased neutrophil infiltration and accumulation in the alveoli, and disruption of the alveolar epithelial–capillary barrier, which leads to increased vascular permeability and edema (13). The distinctive role for lung neutrophil infiltration in viral infection is summarized as follows: some, but not all, viruses that infect the LRT result in clinically defined ARDS, and lung neutrophil infiltration is associated with viruses that do and do not lead to ARDS (3, 5, 9, 10, 60, 131, 199, 242–244, 246, 247). In ARDS, there are data that directly support the role of neutrophils as both beneficial and detrimental (3, 13, 14). Perhaps, there are general factors (host or virus) that lead to a common antiviral response of neutrophils.

Mild Viral Respiratory Disease

In contrast to emergent highly pathogenic respiratory viruses, notable "mild" human respiratory viruses also involve increased neutrophils at the site of infection (e.g., hRSV). As expected, infection with these viruses is typically associated with the increase of neutrophil chemoattractant chemokines. For example, infection with HRV is a well-studied virus for which there are several studies on the link between neutrophils and disease (229–231, 248) (**Table 2**). HRV virions enter nasal epithelial cells *via* endocytosis, yet, unlike influenza, infection does not cause major

TABLE 1 | (–) Sense RNA respiratory viruses that cause increased neutrophil infiltration during infection.

Virus family	Virus type	Host/model	Primary airway target cell	Pathology	Neutrophil abundance	Reference
Orthomyxoviridae	Influenza A virus	Hu, NHP, Mo, Fe, Sw	Epithelial cells [upper respiratory tract (URT), lower respiratory tract (LRT)]	Mild: necrotic rhinitis and tracheitis; moderate: necrotic bronchiolitis and alveolitis; severe: diffuse alveolar damage and hypercytokinemia	(+++)	(229–231)
Paramyxoviridae	Human respiratory syncytial virus	Hu	Epithelial cells (URT, LRT)	Severe: necrotic bronchiolitis and alveolitis, obstructed bronchioles, and giant cell formation	(+++)	(232, 233)
Paramyxoviridae	Human metapneumovirus	Hu	Epithelium	Mild: airway inflammation and epithelial degeneration	(+)	(234, 235)
Paramyxoviridae	Hendrah/Nipah virus	Hu	Not defined	Severe: interstitial pneumonia, but primarily vasotropic or neurotropic	(++)	(236)
Paramyxoviridae	Measles virus	Hu, Mo	Resident myeloid cells of the lung	Severe: bronchiolitis obliterans	(++)	(237)
Paramyxoviridae	Human parainfluenza virus	Hu	Ciliated epithelium	Bronchiolitis and alveolitis	Not defined	(238)
Bunyaviridae	New World hantavirus	Hu	Lung microvascular endothelium	Hantavirus pulmonary syndrome, endothelial inflammation, and focal antigen-positive sites in lung	(+)	(239)

Hu, human; NHP, non-human primate; Mo, mouse; Fe, ferret; Sw, swine.

TABLE 2 | (+) Sense RNA and DNA respiratory viruses that cause increased neutrophil infiltration during infection.

Virus family	Virus type	Host/ model	Primary cell target	Pathology	Neutrophil abundance	Reference
Picornaviridae (+RNA)	Human rhinovirus	Hu	Epithelium	Mild to moderate: neutrophilic rhinitis; severe: acute LRT, bronchiolitis, and alveolitis	(+++)	(231)
Adenoviridae (dsDNA)	Human adenovirus (HAdv3, HAdv7)	Hu	Epithelium	Bronchitis and alveolitis	(+)	(240)
Coronaviridae (+RNA)	Human coronavirus (NL-63 or OC43)	Hu	Epithelium	Mild	(+/−)	(241)
Coronaviridae (+RNA)	Severe acute respiratory syndrome coronavirus	Hu, Fe	Epithelium	Alveolitis, acute respiratory distress syndrome (ARDS), hypercytokinemia	(++)	(10)
Coronaviridae (+RNA)	Middle east respiratory syndrome coronavirus	Hu, Fe	Epithelium	Alveolitis, ARDS, hypercytokinemia	(++)	(11)

Hu, humans; Fe, ferrets.

damage to the nasal epithelium (249–251). Neutrophilic rhinitis, increased vascular permeability, and mucus hypersecretion are the key pathological features of HRV infection (229–231, 249), and infected epithelium seems to be the source of large amounts of neutrophil chemotactic molecules, particularly CXCL8 and kinins (229, 248, 252, 253). Interestingly, *in vitro* studies have shown that viral recognition of HRV shares features with hRSV, but is somewhat different than with IAV (254–256). It has been established that there are virus-specific and cell-specific differences in sensing RNA viruses *via* primarily TLR- and/or RLR-pathways (and even in a preference for RIG-I versus MDA5), yet these pathways may have similar general endpoints, such as chemokine and cytokine signaling (255, 257–259).

Finally, a key question is whether virus-induced cytopathy drives neutrophilia or whether it is the result of host response to viral infection. Infections with influenza A(H1N1)pdm09 virus, HPAI, SARS-CoV, and MERS-CoV are thought to cause acute lung injury which results in ARDS; characterized by excessive damage to the alveolar epithelium and involving the infiltration of neutrophils (67, 68) (**Tables 1** and **2**). However, less pathogenic strains such as HRV infections do not cause significant damage to the respiratory mucosa, yet neutrophils are present (249, 250). Conversely, viruses that cause moderate, focal cytopathy in the lungs, for example seasonal IAV and hRSV, are known to cause neutrophilic infiltration (250, 252, 253). Therefore, neutrophils are not necessarily associated with direct cytopathic effect nor are they exclusively associated with severe disease.

Bacterial Respiratory Disease

Despite many years of searching, there is no single reliable biomarker to indicate a bacterial versus viral infection (although CRP is a good candidate). This is surprising, given the otherwise significant fundamental differences between the biology of these two types of pathogens; differences which are reflected in general immune responses and begin with pathogen detection. Signaling through TLR on the plasma membrane versus endosomal or cytoplasmic PRRs is controlled by complex intracellular adaptor proteins [reviewed in Ref. (260)]. For example, the complexities of signaling allow the characteristically bacteria-specific TLR4 to signal the upregulation of immunostimulatory type I IFN characteristic of a virus infection (170). As evidenced above, many viral infections associated with neutrophil infiltration have RNA genomes. Host cells detect RNA viruses primarily through RLR as well as TLR, whereas bacteria rely on a different group of PRRs to detect extracellular PAMPs (255, 261–263). Interestingly, the lung microenvironment to hRSV has been shown to be different from IAV, specifically in the presence of IL-4 (264, 265). It is thought that this is driven by the presence of alternatively activated macrophages during RSV infection (266). It is not known if this directs differences in neutrophil chemoattraction, yet IL-4 is known to drive a Th2 ("bacterial" or antibody-biased) immune response (267). In sum, surprisingly little is clinically different between the innate immune responses to viral versus bacterial infection; however, perhaps comparative studies that focus on neutrophils can uncover virus-specific responses.

NEUTROPHILS IN THE VIRAL MICROENVIRONMENT

In general, immune activation pathways that involve the activation of NFκB lead to the secretion of neutrophil chemotactic chemokines [reviewed in Ref. (268)]. This is heavily driven by PAMP recognition and activation pathways, and during a viral infection type I IFNs and ISGs are the unique elements in the virus-inflamed lung environment [reviewed in Ref. (24–26, 269, 270)]. This single difference between viral and bacterial infections could have drastic effects on the actions of neutrophils once in the lung. Moreover, without this information (e.g., pathogenic viruses that suppress type I IFN) neutrophils may respond to inflammation in an inefficient way, potentially with pathologic effects (23, 186, 256, 271–277). Neutrophils are known to respond to IAV and type I IFN by upregulation of ISGs (186). In systemic lupus erythematosus, neutrophils may be a large contributor of type I IFN (278, 279). During increased inflammation, left-shifted or immature neutrophils emerge from the bone marrow—a classic sign for sepsis, but also known to be present during some severe viral respiratory diseases (e.g., HPS) (239). It is known that immature neutrophils do neither express IFNα/β receptor nor many other cytokine receptors (273). It is unknown what affect this would have during increased inflammation during pathogenic influenza infection, although their role in other inflammatory conditions suggests it may affect their functions (271, 273, 280, 281).

Resolving Inflammation

All forms of respiratory infection require resolution of the infection and inflammation. IFNs are essential components

of initiating sterilizing immunity to virus infection *via* the adaptive immune system (i.e., resolution of infection), at which point the resolution of inflammation can effectively proceed (25, 26, 282). Although the mechanisms are poorly understood, through their direct antiviral actions and indirect actions on the lung microenvironment (e.g., efferocytosis of apoptotic neutrophils by macrophages), neutrophils have the ability to influence outcomes toward successful resolution as well as toward the formation of ARDS (3, 5, 12, 13, 21, 74, 166, 184, 198, 199, 283–285). Thus, there is evidence that the role of neutrophils in viral infections of the respiratory system is not limited to inflammation, but likely includes recovery from infection and the initiation of adaptive immunity (74, 138, 284). Apart from initiating adaptive immunity, resolution of inflammation may be partially regulated by secretion of IL-1RA and chemokine-destroying factors by recruited macrophages (286, 287). Additionally, it has been proposed that efferocytosis of apoptotic neutrophils is a key step in resolution of inflammation (288–290), and occurs in the lung during bacterial pneumonia (291). It is unclear if this happens during IAV infection or infection with other respiratory viruses.

Prolonging Inflammation

Factors that prolong the life span of neutrophils in the lungs increase the probability that they may contribute to immunopathology. IL-6 and G-CSF are immune mediators present in the lung during infection and are known to prolong survival of neutrophils in mouse lungs following IAV infection (292). Both neutrophils and macrophages are known to phagocytose apoptotic epithelial cells in mouse lungs during IAV infection (283). The cells may be recruited *via* chemokines or damage receptors. For example, necrotic IAV-infected epithelial cells are a source of CXCL8 (293), which attract neutrophils to dying cells. In addition, neutrophils can detect DAMPs such as S100A9 (294). It has been shown that extracellular S100A9 is abundant during IAV infection in mice (295). Antibody-mediated neutralization of S100A9 decreased lung inflammation in mice and improved disease outcome (295). Apart from potential tissue-destroying effects of neutrophil proteases, the presence of NETs may induce even more inflammation in the lungs (21, 139). Thus, there are limited data supporting directly malevolent actions of neutrophils (Table S5 in Supplementary Material), yet factors that increase their presence and prolong their survival in the lung are correlated with increased disease severity.

CONCLUDING REMARKS

There are substantial data that suggest neutrophils are a part of a viral response to infection. Neutrophils are among the first responders to IAV infection in the lung, and they remain in great numbers throughout the development of ARDS. Although neutrophils are an important component of the general response to infection in the respiratory system, as is discussed herein, neutrophils are capable of recognizing viruses (*via* viral PAMPs), responding to viruses with specific effector functions, and may be instrumental in determining disease outcome. Evidence exists to support the hypothesis that neutrophils respond specifically to the focal nature of viral infection, and they act to influence this microenvironment *via* their virus-specific effector functions. Factors that influence successful recovery from respiratory viral infection (versus lethal outcome) are complex and both host- and virus-specific. However, a better understanding of the role neutrophils, previously underappreciated with respect to viral infections, will reveal important information about disease outcome. Many questions remain before it is determined the part neutrophils play in mild and severe disease.

AUTHOR CONTRIBUTIONS

Both authors contributed to the formulation of this scientific research topic. JC wrote the manuscript as part of his Ph.D. dissertation under the mentorship of CJ (296).

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2017.00550/full#supplementary-material.

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