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Review

The mercurial nature of neutrophils: still an enigma in ARDS?

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Williams AE, Chambers RC. The mercurial nature of neutrophils: still an enigma in ARDS? *Am J Physiol Lung Cell Mol Physiol* 306: L217–L230, 2014. First published December 6, 2013; doi:10.1152/ajplung.00311.2013.—The acute respiratory distress syndrome (ARDS) is a life-threatening lung condition resulting from direct and indirect insults to the lung. It is characterized by disruption of the endothelial-epithelial barrier, alveolar damage, pulmonary edema, and respiratory failure. A key feature of ARDS is the accumulation of neutrophils in the lung microvasculature, interstitium, and alveolar space. Despite a clear association between neutrophil influx into the lung and disease severity, there is some debate as to whether neutrophils directly contribute to disease pathogenesis. The primary function of neutrophils is to provide immediate host defense against pathogenic microorganisms. Neutrophils release numerous antimicrobial factors such as reactive oxygen species, proteinases, and neutrophil extracellular traps. However, these factors are also toxic to host cells and can result in bystander tissue damage. The excessive accumulation of neutrophils in ARDS may therefore contribute to disease progression. Central to neutrophil recruitment is the release of chemokines, including the archetypal neutrophil chemoattractant IL-8, from resident pulmonary cells. However, the chemokine network in the inflamed lung is complex and may involve several other chemokines, including CXCL10, CCL2, and CCL7. This review will therefore focus on the experimental and clinical evidence supporting neutrophils as key players in ARDS and the chemokines involved in recruiting them into the lung.

ARDS; inflammation; neutrophil; chemokine; lung injury

ACUTE LUNG INJURY (ALI) and its more severe form acute respiratory distress syndrome (ARDS) are common life-threatening conditions leading to respiratory failure. The current definition of ARDS now includes ALI as mild ARDS and is based on clinical criteria regarding diagnostic timing, bilateral opacities following chest imaging, pulmonary edema, hypoxemia, and a minimal requirement for positive end-expiratory pressure applied by mechanical ventilation to maintain oxygenation (47). The severity of ARDS is classified as mild, moderate, or severe based on partial pressure of arterial oxygen to fraction of inspired oxygen ratios (Pa_{O_2}/Fi_{O_2}), whereby mild has <200 $Pa_{O_2}/Fi_{O_2} \leq 300$, moderate <100 $Pa_{O_2}/Fi_{O_2} \leq 200$, and severe $Pa_{O_2}/Fi_{O_2} \leq 100$ (47). ARDS arises from a variety of local and systemic insults, of which sepsis, pneumonia, and trauma are the most common. The incidence of ARDS is 79/100,000 per year (151) with a mortality rate of 30–65% (130). ARDS is characterized by increased alveolar-capillary barrier permeability, lung edema, impaired oxygenation, and hypoxemia. Disease pathogenesis is also associated with an increased release of several inflammatory mediators, including TNF, which can directly trigger epithelial cell death (7). Furthermore, the accumulation of neutrophils in the lung interstitium and alveolar space is a key characteristic of ARDS. The rapid development of interstitial and intra-alveolar fibrosis in ARDS can further contribute to prolonged respiratory failure and increased mortality (113). Despite some improvements with use of lung-protective ventilator strategies, both morbidity and mortality remain high.

Neutrophils are the first leukocytes to be recruited to sites of inflammation in response to chemotactic factors released by activated macrophages and pulmonary epithelial and endothelial cells (104, 180, 181). At sites of inflammation neutrophils release several antimicrobial factors such as reactive oxygen species (ROS), antimicrobial peptides, and multiple proteinases, the latter of which also help degrade the extracellular matrix during migration (57, 138), together with the formation of neutrophil extracellular traps (NETs). Although neutrophils provide a first line of defense against microbes, excessive recruitment and activation can lead to bystander tissue damage and further loss of lung function (67). Therefore neutrophils and their associated chemotactic factors are thought to significantly contribute to the progression of ARDS. This review will focus of the role that neutrophils play during ARDS and the chemokines thought to be critical for neutrophil recruitment into inflamed lung.

Contribution of Neutrophils to ARDS

The evidence that neutrophils have a direct influence on the development of ARDS comes from both human clinical data and animal studies. It has been reported that the concentration of neutrophils in the bronchoalveolar lavage fluid (BALF) of patients with ARDS correlates with the severity of disease and with poor outcome (1, 3, 115). In addition to having elevated neutrophil numbers, BALF obtained from patients with ARDS

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is highly chemotactic for human neutrophils compared with BALF from normal subjects (141). Leukocyte counts from the blood were reduced in patients that developed ARDS compared with at risk patients (169), further indicating that neutrophil sequestration into the lungs from the circulation is indicative of ARDS (77). The kinetics of neutrophil recruitment may also be important in disease pathogenesis, as neutrophil counts remain higher in those patients who eventually die as a result of sepsis-induced ARDS compared with survivors (162). Neutrophils isolated from sepsis patients with a confirmed clinical diagnosis of ARDS were also shown to compromise the integrity of endothelial cell monolayers in vitro (51). However, despite an obvious association between ARDS and neutrophil recruitment, it has been difficult to establish a direct causal relationship between neutrophilia and endothelial-epithelial barrier disruption or clinical outcomes in ARDS. For example, neutropenic patients can still develop ARDS, as measured by arterial hypoxemia and diffuse bilateral pulmonary infiltrates, in the absence of invading neutrophils (137). This may reflect the heterogeneity of ARDS, since both neutrophil-dependent and neutrophil-independent processes are thought to influence the progression of disease.

Although clinical data does suggest a central role for neutrophils in ARDS, the majority of studies have been performed in animal models. Neutrophil-dependent models of ARDS include LPS-induced lung injury (2, 30), acid-induced lung injury (49), ventilator-induced lung injury (89), and transfusion-related lung injury (111). Neutrophil depletion in these models ameliorates certain features of ARDS, including endothelial-epithelial cell damage and capillary-alveolar permeability, thereby supporting the notion that neutrophils are central mediators of disease pathogenesis. In a rat model of LPS-induced lung injury, treatment with steroids also reduced neutrophil infiltration and cytokine levels, resulting in a consequential attenuation of lung damage, although these findings have been poorly translated into ARDS patients (48, 136, 178). The association between neutrophil influx and barrier permeability is likely to be complex. For example, in the mouse model of LPS-induced lung injury only the initial phase of neutrophil recruitment (3 h after LPS administration) was associated with a reduction in BALF protein levels, as an indication of microvasculature leak, whereas depleting neutrophils at 24 h had no effect on BALF protein (30). This could be explained by the recruitment of different neutrophil subpopulations into the lung, since marginated neutrophils within the pulmonary microvasculature are known to have a more proinflammatory phenotype compared with newly released neutrophils from the bone marrow (50), the former accounting for the initial infiltrate and the latter the secondary infiltrate.

In contrast, oleic acid-induced and hyperoxia-induced models of ARDS are independent of neutrophil recruitment (67), since depletion of neutrophils does not prevent capillary-alveolar leak (75, 143). Because of the conflicting findings between various animal models it is still unclear whether the actual process of neutrophil recruitment into the lungs is sufficient to cause endothelial-epithelial barrier disruption on its own (41). Indeed, the recruitment of neutrophils into normal human lung, in response to the leukotriene LTB4, did not cause significant alveolar epithelial permeability (114). It therefore seems likely that neutrophil activation is required to cause bystander tissue damage (156) and that several factors combine to induce lung injury (Fig. 1), through the release of proinflammatory cytokines, the generation of ROS or free radicals, mechanical stress to the endothelium or epithelium, and the release of neutrophil proteinases or other host defense proteins (13, 85). Similar events are also likely to occur in human ARDS, to varying degrees, depending on individual responses, the cause of initial injury, and the involvement of various microorganisms, including *Streptococcus pneumoniae* (116).

Whereas ROS are generated in neutrophils by NADPH oxidase and nitric oxide synthase pathways, many soluble factors are prestored in neutrophil granules, the contents of which are released following transmigration and activation of neutrophils within the lung. Inhibiting the release of neutrophil granule contents has been shown to reduce lung injury and vascular permeability following challenge with the *Streptococ-*

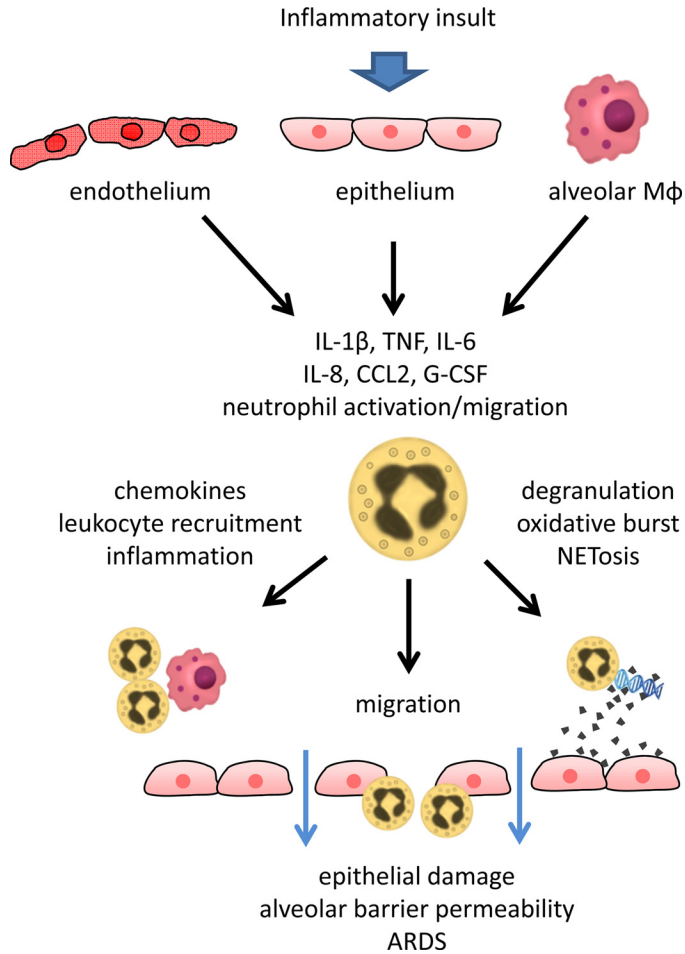

Fig. 1. Role of neutrophils in acute respiratory distress syndrome (ARDS). An initial inflammatory insult to the lung results in the increased expression and release of proinflammatory cytokines, such as IL-1β, TNF, and IL-6, and chemokines, such as IL-8 (CXCL8) and CCL2 (MCP-1). This results in the activation and recruitment of neutrophils into areas of inflamed lung. Activated neutrophils are capable of releasing chemokines that enhance leukocyte recruitment and exaggerate the inflammatory response. The release of reactive oxygen species, granule contents, and neutrophil extracellular traps cause bystander damage to host cells. The migration of neutrophils across the endothelium, and in particular the epithelium, augments tissue damage. Disruption of the endothelial-epithelial barrier allows protein-rich fluid to enter the alveolar space, eventually resulting in alveolar flooding and respiratory failure. Alveolar Mϕ, alveolar macrophage; NETosis, the process of cell death involving neutrophil extracellular trap (NET) formation.

cus pyogenes M1 protein (159), further illustrating the contribution that neutrophils can make in promoting lung injury. An important proteinase released from neutrophil granules is neutrophil elastase, which is also elevated in human ARDS samples (43). The inhibition of neutrophil elastase has been demonstrated to reduce epithelial injury in several animal models, including a rat model of cystic fibrosis, a mouse model of pulmonary fibrosis, LPS-induced lung injury, mechanical ventilation-induced lung injury, and in colonic epithelial cells in vitro (56, 60, 74, 88, 170), although mice deficient in neutrophil elastase also have impaired host defense against gram-negative bacteria (14). The mechanisms by which neutrophil elastase causes lung injury are contentious, because it is unclear whether this proteinase directly damages endothelial or epithelial cells or whether tissue damage is the result of degradation of the alveolar basement membrane (26, 60). The use of neutrophil elastase inhibitors for the treatment of ARDS has therefore received much attention. For instance, sivelestat is routinely used in ARDS patients in Japan. However, a recent review of the available clinical data suggests that neutrophil elastase inhibition has no effect on mortality (81).

Other neutrophil-derived proteinases may also contribute to lung injury, namely proteinase-3, cathepsin-G, and several matrix metalloproteinases (MMPs). However, the nonspecific nature of their proteolytic activity means that multiple downstream effects can occur, other than extracellular matrix degradation. Proteinases have been shown to be capable of both activating and inactivating proinflammatory cytokines and chemokines, which is of particular relevance to ARDS. For example, MMP-9 increases the activity of IL-8 (CXCL8) through amino terminal processing but degrades CXCL1 (Gro-α) (175). The activation of chemokines enhances neutrophil migration, which may augment lung injury (160, 165), whereas the inactivation of proinflammatory cytokines and chemokines may be beneficial for the resolution of ongoing neutrophilia and lung inflammation (64, 119). MMP-9 endopeptidase activity also results in the generation of the extracellular matrix breakdown product of collagen, *N*-acetyl Pro-Gly-Pro (Ac-PGP), which is highly chemotactic for neutrophils and has been associated with inflammatory lung disease (57, 179). Ac-PGP can signal through the chemokine receptors CXCR1 and CXCR2 expressed by neutrophils, which in turn release more MMP-9 and thereby initiate a self-perpetuating cycle of neutrophilic inflammation (184). As a consequence of these multiple biological effects, it has been difficult to experimentally examine the relative importance of each proteinase during disease progression. Targeting neutrophil proteinases in the ARDS disease setting may also be impractical because of their numerous enzymatic targets.

Another important component of innate host defense is the release of NETs, through a process of cell death known as NETosis (19). NET formation involves the disintegration of the nuclear membrane, chromatin condensation, and the release of DNA and granule proteins into the extracellular space (20). NETosis can be initiated by various viral, bacterial, or fungal components and by host factors such as granulocyte-macrophage colony-stimulating factor, HMGB1, and activated platelets (29, 167). NET formation is also dependent on oxidant production (139), a deficiency that has been shown to reduce pathogen clearance from the lungs of infected mice in a chlorine gas model of lung injury (59). Although NETs have potent antimicrobial properties, they contain histones, enzymes, and peptides that are directly toxic to host cells. For example, inhibition of histones reduced mortality in mouse models of LPS-, TNF-, and cecal ligation-induced sepsis, whereas extracellular histones directly caused alveolar damage and pulmonary hemorrhage (183). Following infection with influenza virus H1N1 NETs were found to be associated with damaged alveoli in the lungs of challenged mice, whereas neutrophils released NETs onto the surface of influenza-infected epithelial cells in vitro and caused cell damage (133). The release of NETs has also been associated with lung injury following LPS, bacterial and fungal challenge (44, 174). Indeed, Toll-like receptor engagement on neutrophils is a potent activator of NET formation. NETs have also been observed in sterile transfusion-related acute lung injury (TRALI) in both mouse models and in human patients (168). Inhibiting extracellular histones or using DNaseI to disrupt NETs protected against TRALI (27). Furthermore, the lack of NET clearance from inflamed areas of the lung may further potentiate disease pathogenesis (86). Inhibiting NET formation, or key components of NETs, may therefore provide potential targets for the treatment of ARDS.

Neutrophil Heterogeneity

Rather than being short-lived, end-stage cells, increasing evidence suggests that different neutrophil subsets may be able perform distinct functions during inflammation and have the capacity to interact with various cells of the innate and adaptive immune systems (91). For example, it has been known for some time that the expression of CD16 (FcγRIII) varies on healthy human neutrophils (93). Those neutrophils that express abundant levels of CD16 ($\sim$80–85% of blood neutrophils) are highly chemotactic to *N*-formyl-peptides, whereas those that weakly express CD16 ($\sim$15–20%) do not respond. Three neutrophil subpopulations have been separated based on the expression of CD16 and CD62L (L-selectin): conventional (mature) neutrophils ($CD16^{bright}/CD62L^{bright}$), banded (immature) neutrophils ($CD16^{dim}/CD62L^{high}$), and a population of regulatory neutrophils ($CD16^{bright}/CD62L^{dim}$) (84), the latter of which have reduced endothelial adhesion and reduced chemotactic capacity. These regulatory neutrophils are also able to modulate T cell proliferative responses via an interaction with the integrin $\alpha M\beta 2$ (145). Furthermore, irrespective of underlying inflammation, neutrophils assume an activated phenotype ($CD16^{bright}/CD62L^{dim}/CD11b^{bright}$) after they migrate into the lung (50), suggesting that neutrophil migration across pulmonary endothelium and/or epithelium involves distinct molecular interactions that may be exclusive to the lung but independent of inflammation.

Neutrophil functional heterogeneity was further illustrated in an infection model, whereby neutrophils isolated from mice that were resistant to methicillin-resistant *Staphylococcus aureus* had a proinflammatory phenotype, whereas neutrophils from susceptible mice had an anti-inflammatory phenotype (173). Whereas proinflammatory neutrophils expressed IL-12 and CCL3 (MIP-1α), anti-inflammatory neutrophils expressed IL-10 and CCL2 (MCP-1). These phenotypically divergent neutrophils could also induce alternative macrophage populations (M1 vs. M2) (173), thereby modulating inflammatory vs. anti-inflammatory responses, respectively. Furthermore, in a

human model of LPS-induced endotoxemia the circulating neutrophil compartment exhibited considerable functional heterogeneity (146). Following systemic inflammation, activated neutrophils ($CD16^{bright}$) exhibited less chemotactic activity and increased ROS production compared with immature neutrophils ($CD16^{dim}$) recently released from the bone marrow. It has also been suggested that the immunosuppression observed in the latter stages of systemic inflammatory response syndrome may be attributed to an increase in immature $CD16^{dim}$ neutrophils that have an unreactive phenotype, whereas reactive $CD16^{bright}$ neutrophils contribute to the initial phase of tissue damage (16, 145). This may suggest that inflammation results in a population of mature neutrophils that are capable of causing tissue damage, whereas immunosuppression is associated with an immature population that is more refractory to inflammatory signals (146). Neutrophils isolated from sepsis patients also have reduced CXCR2 expression and a corresponding loss of chemotactic activity (40), produce less ROS (5), and secrete the immunomodulatory cytokine IL-10 (87). These factors may contribute to the immunosuppressive state in progressive sepsis and account for the increased susceptibility to bacterial pneumonia. However, it is still uncertain whether divergent subsets of neutrophils represent distinct cell lineages (33) or whether they just reflect functional heterogeneity (16) because of their inherent phenotypic plasticity (82).

It has recently been demonstrated that neutrophils are capable of undergoing retrograde transendothelial migration to reenter the circulation (21). In so doing, they undergo a phenotypic change characteristic of inflammatory neutrophils ($ICAM\text{-}1^{high}/CXCR1^{low}$) that are primed for ROS production. These neutrophils may also contribute to the dissemination of systemic inflammation, as $ICAM\text{-}1^{high}$ neutrophils that were generated following ischemia-reperfusion injury were sequestered to the pulmonary microvasculature and caused lung inflammation and increased endothelial-epithelial barrier permeability (182). Neutrophils that have undergone retrograde transendothelial migration may account for the activated subsets ($CD16^{bright}/CD62L^{dim}$) found in the circulation following inflammation (146) and may therefore contribute to the pathogenic state of inflammatory lung diseases. Targeting specific neutrophil subpopulations may be a useful approach for modulating the inflammation associated with ARDS.

Mechanisms of Neutrophil Recruitment into the Lung

Numerous factors have been associated with neutrophil recruitment into the lung and alveolar spaces in animal models of ARDS, including cell adhesion molecules, proteinases, oxidants, cytokines, and chemokines. The substantial pulmonary vasculature, and in particular the capillary bed located within the distal lung, contribute to making this organ a unique site for neutrophil egress. In most organs, neutrophils gain access to tissue compartments by migrating across high endothelial venules (HEVs), which are associated with high flow rates and sheer forces. However, in the distal lung, neutrophils migrate across narrow pulmonary capillaries, which have low tidal forces and diameters smaller than the neutrophils themselves [≥ 2 μm compared with 6–8 μm (22)]. These unique migratory parameters lengthen the time available for transmigration and allow neutrophils to extravasate without the need for the conventional rolling and adhesion processes that occur in HEVs. For example, neither L-selectin nor β_2-integrins are essential for neutrophil migration across alveolar capillaries (97, 128). Furthermore, it has been estimated that neutrophils sequestered within the pulmonary microvasculature account for a large pool of neutrophils that likely exceeds the size of the circulating pool by as much as fivefold (42). Such a large marginated pool of neutrophils within the lung microvasculature makes them ideally located to respond immediately to an inflammatory insult. Indeed, neutrophils can rapidly cross the endothelial cell wall in as little as 2 min, preferentially at the corner junctions where three endothelial cells connect (23). Migration at endothelial tricell corners is thought to avoid disrupting intercellular tight junctions, thereby maintaining normal capillary barrier function. Considering that the lungs are in continuous contact with environmental insults, the transmigration across the endothelium and the activation of neutrophils within this unique organ are tightly regulated.

In the context of the lung microenvironment, neutrophils must undergo extensive shape change to migrate across both the capillary endothelium and alveolar epithelium. Neutrophil extravasation is also likely to involve the release of proteinases and elastases (90) that allow movement through the basement membrane by degrading the extracellular matrix, although the precise role of specific proteinases, such as elastase and gelatinase, remains controversial (22, 76). Migration of neutrophils out of the endothelium and across the pulmonary interstitium is thought to be mediated by fibroblasts, which establish defined conduits that assist neutrophils to gain access to the basal surface of the epithelium (177), while at the same time maintaining capillary-alveolar integrity (157). These conduits are likely to be anatomically different to the interalveolar connections, known as the pores of Kohn, which connect adjoining alveolar sacs (37). These interalveolar connections are the size of an alveolar epithelial cell and act to maintain gaseous exchange throughout the respiratory cycle. Although alveolar macrophages are known to migrate between adjacent alveoli through pores of Kohn (142), there is no evidence that neutrophils use these structures during transepithelial migration.

The migration of neutrophils across the alveolar epithelium is also thought to involve several key steps (192) including adhesion, migration, and postmigration events. Adhesion of primed neutrophils to the basolateral surface of the epithelium likely involves the binding of β_2-integrins (CD11b/CD18) on the surface of neutrophils to ICAM-1 on the epithelium (10). Furthermore, ICAM-1 is upregulated on alveolar epithelial cell surfaces in response to several inflammatory stimuli (171, 172). Migration of neutrophils across the epithelium is thought to occur between adjacent epithelial cells through the paracellular space, although it is uncertain whether the mere mechanics of this process is sufficient to increase alveolar permeability. In studies using intestinal epithelial cells, for example, neutrophil migration causes the loss of epithelial tight junctions without any apparent disruption in epithelial permeability (125, 134). Neutrophils are also thought to utilize specific epithelial invasion sites, at least when migrating across the intestinal epithelium (134, 140), which may lessen barrier disruption. Alveolar epithelial cell injury was shown to be dependent on the activation of neutrophils and the release of proteinases and elastase, although independent of ROS generation or direct cytolysis (7, 156). Zemans et al. (191) demonstrated that

neutrophil migration across human epithelial type II cells causes disruption of the monolayer, quickly followed by reepithelialization, a process that was dependent on β-catenin signaling (193). Therefore, this would suggest that excessive neutrophil migration across the alveolar epithelium, and the associated release of degrading enzymes, overwhelms normal physiological migration pathways and directly contributes to epithelial damage and permeability within the inflamed lung. The ability of the pulmonary epithelium to repair itself after injury may be a critical factor in determining the severity of disease (39).

Postmigration events involve the adhesion of neutrophils to the apical surface of epithelial cells within the alveolar lumen. However, it is not clear what molecular interactions are involved in this process. One possibility is an interaction between neutrophil selectins and epithelial ICAM-1 (10, 131, 132), since this interaction clearly influences neutrophil migration into the air space. However, the role that this interaction plays in alveolar neutrophil margination remains to be confirmed. Another possibility is that neutrophil Fc receptors bind to soluble antibody attached to the apical surface of epithelial cells, which has been demonstrated on the intestinal epithelium in certain gut pathologies (148). Furthermore, ARDS has been associated with a deficiency in the clearance of neutrophils from the alveolar space, which may be directly related to a defect in neutrophil apoptosis and a corresponding prolonged lifespan (117). Indeed, several cytokines and growth factors such as granulocyte colony-stimulating factor (G-CSF) and IL-8, which are elevated in ARDS, have prosurvival effects on neutrophils (3). A decrease in neutrophil apoptosis, combined with a deficiency in cell clearance, may also contribute to disease pathogenesis (106).

Role of Chemokines in Neutrophil Recruitment to the Lung

The chemokine IL-8, otherwise known as CXCL8, and the rodent homologues CXCL1 (KC) and CXCL2 (MIP-2) are thought to be central to neutrophil recruitment into the lung during ARDS. Important correlations have been made from clinical ARDS samples, including pulmonary edema aspirates and BALF, between increased IL-8 concentrations, disease severity (65, 126), and neutrophil migration into air spaces (127). These studies provide important information regarding the role that neutrophils and IL-8 play in the pathogenesis of ARDS. Although the related CXC chemokines CXCL5 (ENA-78) and CXCL1 (Gro-α) are also elevated in BALF from ARDS patients (63), only IL-8 consistently correlates with the number of neutrophils and disease severity (61, 176). Furthermore, IL-8 has been demonstrated to be the most potent neutrophil chemoattractant in BALF from ARDS patients and was the predominant neutrophilic chemokine released from LPS-stimulated human alveolar macrophages (62, 101, 127). Therefore IL-8 is considered the main neutrophil chemoattractant in ARDS. The levels of IL-8 in ARDS BALF also correlate with survival, as IL-8 levels are lower in survivors compared with nonsurvivors (12). Neutrophil activation also directly correlates with disease severity and with the levels of IL-8, TNF, and IL-6 (31). Another study revealed that IL-8, as well as G-CSF, correlates with neutrophil influx and the severity of ARDS (3). Wiedermann et al. (180) further demonstrated that IL-8 correlates with neutrophil numbers in BALF of ARDS patients, and G-CSF with circulating neutrophil numbers, whereas CXCL5 (ENA-78) did not show any correlation (Table 1).

Table 1. *Chemokines in ARDS — clinical studies*

Chemokine	Disease	Effect	Reference
CXCL1 (Gro-α)	ARDS	↑ in BALF. Levels consistent with neutrophil numbers. Poor neutrophil chemokine in ARDS BALF.	51, 71, 176
	In vitro	Induced neutrophil chemotaxis, shape change and activation	51
CXCL2 (Gro-β)	Sepsis	Polymorphism associated with higher rate of mortality.	109
	In vitro	Induced neutrophil chemotaxis, shape change and activation	51
CXCL4 (PF-4)	ARDS	↑ in BALF in association with CCL5-CXCL4 heteromers. Possible associated with neutrophil influx.	66
CXCL5 (ENA-78)	ARDS	↑ in BALF. Levels inconsistent with neutrophil numbers. Weak neutrophil chemokine in ARDS BALF.	51, 63, 180
CXCL8 (IL-8)	ARDS	↑ in BALF and pulmonary edema fluid. Levels correlate with neutrophil numbers. Levels correlate with disease severity. Levels correlate with outcome. Predominant neutrophil chemokine in ARDS BALF.	3, 12, 31, 32, 61, 65, 107, 126, 176
	In vitro	Removal of IL-8 or use of anti-IL-8 antibody blocks neutrophil activation and chemotaxis. Chemotaxis not completely removed however.	52, 71, 127
CCL2 (MCP-1)	ARDS	↑ in BALF. Levels correlate with lung injury.	63
CCL3 (MIP-1α)	ARDS	↑ in BALF. No correlation with neutrophil number.	63
CCL4 (MIP-1β)	LPS ex vivo lung	↑ in BALF. Increased levels coincided with increased neutrophils. (IL-8, CXCL5, CCL3, and CCL2 also upregulated).	135
CCL5 (RANTES)	ARDS	↑ in BALF in association with CCL5-CXCL4 heteromers. Possible association with neutrophil influx.	66
	COPD	↑ in bronchial biopsies. Associated with increased activation and upregulation of CC-chemokine receptors on neutrophils.	38, 70

ARDS, acute respiratory distress syndrome; Gro-α/β, growth stimulating activity-α/β; BALF, bronchoalveolar lavage fluid; PF-4, platelet factor-4; ENA-78, epithelial cell-derived neutrophil-activating peptide-78; IL-8, interleukin-8; MCP-1, monocyte chemoattractant protein-1; MIP-1α/β, macrophage inflammatory protein-1α/β; RANTES, regulated on activation, normal T cell expressed and secreted; LPS, lipopolysaccharide; COPD, chronic obstructive pulmonary disease.

IL-8 is able to bind to either of two receptors, CXCR1 or CXCR2, on the surface of human neutrophils, although the relative contribution of each receptor to disease pathogenesis is still uncertain. Until recently, CXCR2 was thought to be the only functional homologue in rodents, whereas extensive neutralization and antagonism studies clearly demonstrated its importance for neutrophil migration into the inflamed lung (92). A functional CXCR1 homologue has now been discovered in mice, although its importance in neutrophil migration is not clear (129). In humans, both receptors are internalized following ligation with IL-8 but only CXCR1 is rapidly reexpressed on the cell surface (34). It has also been demonstrated that IL-8 stimulation of CXCR2 acts to prime neutrophils for CXCR1 receptor activation, as measured by Ca^{2+} mobilization (71). CXCR1 is therefore thought to be the major receptor for IL-8 on human neutrophils. Continued ligation of CXCR1 with IL-8 or CXCL1 is also able to prevent neutrophil apoptosis and may therefore be involved in the abnormal clearance of neutrophils in ARDS (45).

Numerous animal studies, notably using the LPS model of lung inflammation or the acid aspiration-induced lung injury model, clearly demonstrate the central role of IL-8 homologues in driving neutrophil influx into the inflamed lung (67). For example, administration of an anti-IL-8 antibody prevented neutrophilic lung inflammation and extravascular leak in an acid-aspiration rabbit model (49) and an LPS-induced rabbit model of lung injury (189). The importance of IL-8 in recruiting neutrophils to sites of inflammation has also been demonstrated in several nonpulmonary disease models, as anti-IL-8 antibody treatment was able to prevent neutrophil-mediated tissue damage in rabbit models of LPS-induced dermatitis, LPS/IL-1-induced arthritis, lung reperfusion injury, and acute immune complex-type glomerulonephritis (69). IL-8 is therefore considered to be the archetypal neutrophil chemoattractant.

Mouse CXCL1 (KC), the functional homologue of human IL-8, also binds to the receptors CXCR1 and CXCR2. However, the chemokine network is considered to possess a high degree of redundancy and pleiotropy, as the related ELR+ chemokines CXCL2 (MIP-2α), CXCL3 (MIP-2β), CXCL5 (ENA-78), and CXCL15 (lungkine) can also bind CXCR1 and CXCR2. However, these chemokines have varying levels of neutrophil chemotactic potency in humans, with CXCL8 being the most potent followed by CXCL1, CXCL5, CXCL2, and CXCL3, in that order (52). As well as inducing varying levels of chemotaxis, these chemokines also have differential effects on neutrophil activation, exocytosis, and respiratory burst (58), indicating that related CXC-chemokines have different functional activities on neutrophils despite sharing the same receptors (Table 2). In addition, these chemokines may be able to functionally compensate for the absence of CXCL1 (or IL-8), suggesting that therapeutically targeting a single ELR+ chemokine may not be an appropriate approach in ARDS. Neutralizing CXCL1 in the bronchoalveolar lavage fluid of LPS-treated rats also reduced neutrophil migration but only by ~50% (53), suggesting that other neutrophil chemotactic factors may be present in the lavage fluid from the inflamed lung. For example, CXCL2 has also been shown to regulate neutrophil migration and function in a mouse model of LPS-induced (6) and sepsis-induced lung injury (108). CXCL1 and CXCL2 both signal through CXCR2 and, although they have broadly similar effects on neutrophil recruitment, they alter neutrophil function in slightly different ways (110). The local distribution of CXCL1 and CXCL2 within the lung may be important in determining the extent of neutrophil migration, activation, and subsequent tissue injury. Antagonism of CXCR2 is therefore felt to provide a more suitable approach than targeting individual chemokines, as this would inhibit the signaling and chemotactic potential of several chemokines, in particular CXCL1 and CXCL2, and of the PGP tripeptide (158). Indeed, mice deficient in CXCR2 ($CXCR2^{-/-}$) have been shown to have diminished neutrophil recruitment into the lung in response to LPS (150). Neutrophil migration out of the bone marrow (46) and across the pulmonary endothelium following lung injury has also been shown to be regulated by CXCR2 (92, 164).

Extrapolating information from studies performed in animal models of lung injury call for caution, however, because of the differences between mouse and human chemokine systems. For example, CXCL5 does not consistently correlate with human disease severity, despite mouse models of ARDS clearly demonstrating an important role for this chemokine in neutrophil recruitment (180). Furthermore, CXCR1 acts only as a weak receptor for neutrophil chemoattraction in mice, since CXCR2 seems to be more important, whereas in humans CXCR1 appears to be the dominant neutrophil receptor. Neutrophils isolated from the blood of sepsis patients also express lower levels of CXCR2 compared with neutrophils from healthy individuals (40), whereas IL-8 and CXCL1 both downregulate CXCR2 upon stimulation (45). Another caveat is that CXCR2 neutralization may reduce the effectiveness of innate immune defenses and consequently result in uncontrolled bacterial or fungal infection (73, 120). It will therefore be important to consider the appropriateness of targeting CXCR2, or indeed the neutrophilic innate immune response, in certain patient groups with an underlying infection. Despite these species differences, and the potential for microbial outgrowth, a number of promising CXCR2 antagonists are being developed for use in humans to treat neutrophilic lung disease (28, 78, 92, 102).

IL-8 Immune Complexes in ARDS

A significant fraction of the IL-8 recovered from the BALF of ARDS patients is complexed to endogenous IgG, mainly of the IgG3 and IgG4 subclasses (98, 166). Furthermore, elevated levels of IL-8 immune complexes have been associated with poor clinical outcome in ARDS patients and in patients who are at risk of developing ARDS (98–100). IL-8 immune complexes may therefore provide a useful biomarker in ARDS (55). It has been shown that these anti-IL-8:IL-8 complexes retain both their chemotactic properties for neutrophils and their ability to activate them, resulting in the release of oxidants and MPO, a function that is dependent on the Fc receptor, FcγRIIa (96). Furthermore, anti-IL-8:IL-8 complexes inhibit neutrophil apoptosis, which is associated with an increase in the expression of Bcl-xL and a decrease in Bak and Bax (54). Indeed, it is well established that apoptosis of neutrophils is delayed in patients with ARDS (103). In addition, these IL-8 immune complexes activate endothelial cells, which upregulate ICAM-1 and increase ERK, JNK, and Akt phosphorylation (95), which may suggest that IL-8 immune complexes can

Table 2. *Chemokines in ARDS — animal studies*

Chemokine	Model of Lung Injury	Animal	Effect	Reference
CXCL1 (KC/CINC)	LPS-induced	Mouse	↑ in BALF. Neutralization decreased neutrophil recruitment.	6, 190
	Endotoxemia/LPS-induced	Rat	↑ in BALF. Neutralization decreased neutrophil recruitment. Recombinant CXCL1 increased neutrophil influx.	25, 53, 187
	Acute pancreatitis-induced lung injury	Rat	Neutralization decreased lung permeability and MPO activity.	17
	Ventilator-induced lung injury	Mouse	↑ in BALF. CXCR2$^{-/-}$ mice had reduced neutrophil sequestration and lung injury.	15
	Klebsiella infection	Mouse	CXCL1 mediated upregulation of CXCL2/CXCL5. Host defense dependent on CXCL1. Required for ROS generation in neutrophils.	11, 24
CXCL2 (MIP-2)	LPS-induced	Mouse	↑ in BALF. Endogenous DARC sequestration or syndecan-1 shedding decreased neutrophil recruitment.	6, 72, 149
	LPS-induced	Rat	↑ in BALF. Neutralization diminished neutrophil accumulation. Released by alveolar macrophages and epithelial cells.	68, 112, 152, 155
	Endotoxemia	Mouse	Neutralization decreased neutrophil recruitment into lung. Recued lung permeability.	2, 161
	Acid-induced	Rat	↑ in BALF. Neutralization decreased neutrophil recruitment, leak and improved oxygenation.	154
	Ventilator-induced lung injury	Mouse	↑ in BALF. Increased levels correlated with neutrophil accumulation and lung injury.	15
	Ventilator-induced lung injury	Rat	↑ in BALF. Neutralization decreased neutrophil recruitment and leak.	147
CXCL4 (PF-4)	LPS-, sepsis-, and acid-induced	Mouse	Neutralization abolished neutrophil recruitment, permeability and lung damage. CCL5-CXCL4 formed heterodimers.	66
CXCL5 (ENA-78)	LPS-induced	Mouse	CXCL5 dominated neutrophil recruitment into the lung over CXCL1 and CXCL2.	121
	Escherichia coli infection	Mouse	CXCL5$^{-/-}$ mice had impaired neutrophil influx into the lung.	121, 122
CXCL8 (IL-8)	LPS-induced	Rabbit	↑ in BALF. Neutralization decreases neutrophil recruitment, permeability and gas exchange.	69, 186, 189
	LPS/oleic acid double hit	Rabbit	↑ in BALF. Anti-IL-8 neutralization blocks neutrophil infiltration.	9
	Lung reperfusion injury	Rabbit	↑ in BALF. Neutralizations prevented neutrophil infiltration and tissue injury.	153
	Acid-aspirate injury	Rabbit	↑ in BALF. Neutralization decreased neutrophil accumulation, leak, and restored lung function and oxygenation. Neutralization improved fluid clearance.	49
CXCL10 (IP-10)	Oxidative stress	Mouse	↑ in BALF. Neutralization decreases neutrophil accumulation.	124
CXCL12 (SDF-1)	LPS-induced	Mouse	Chemoattractant and anti-apoptotic mediator for lung neutrophils ex vivo.	185
CCL2 (MCP-1)	LPS-induced	Mouse	↑ in lung. Neutralization decreased neutrophil recruitment.	83, 123
	Endotoxemia	Mouse	↑ in plasma and lung. Anti-CCL2 resulted in increased mortality and TNF levels.	194
	E. coli infection	Mouse	CCL2$^{-/-}$ mice had reduced bacterial clearance. Recombinant CCL2 induced neutrophil influx. Neutrophils responded in CCL2 In vitro.	8
	IgG immune complex	Rat	Neutralization had no effect on neutrophil recruitment.	18
CCL3 (MIP-1α)	Trauma-hemorrhage	Mouse	↑ in serum. CCL3$^{-/-}$ mice had decreased edema and lung MPO activity.	79
	Lung reperfusion injury	Rat	↑ in lungs. Neutralization decreased neutrophil infiltration.	94
CCL4 (MIP-1β)	IgG immune complex	Rat	Neutralization decreased neutrophil recruitment and vascular leak.	18
CCL5 (RANTES)	LPS-, sepsis-, and acid-induced	Mouse	↑ in BALF. Neutralization abolished neutrophil recruitment, permeability and lung damage. CCL5-CXCL4 heterodimers may be involved.	66
	IgG immune complex	Rat	No change in expression. Inhibition had no effect on neutrophil recruitment.	18
CCL7 (MCP-3)	Oxidative stress	Mouse	Neutralization decreased neutrophil recruitment.	124
	LPS-induced	Mouse	↑ in lung. Neutralization decreased neutrophils recruitment into air space.	123

KC, keratinocyte-derived chemoattractant; CINC, cytokine-induced neutrophil chemoattractant; ROS, reactive oxygen species; DARC, Duffy antigen receptor for chemokines; IL-8, interleukin-8; IP-10, interferon γ-induced protein 10; SDF-1, stromal cell-derived factor-1; MCP-1/3, monocyte chemoattractant protein-1/3; IgG, immunoglobulin-G.

induce endothelial cells to release proinflammatory mediators. Indeed, FcγRIIa is expressed on several cell types in the ARDS lung, including endothelial cells, neutrophils, and cells of the myeloid lineage, such as macrophages, monocytes, and dendritic cells (4).

The significance of these IL-8 immune complexes remains uncertain, however. The original paper by Kurdowska et al. (98) suggests that each complex comprises just one IL-8 molecule and one IgG molecule. Even though these immune complexes colocalize with FcγRIIa in ARDS lungs (4), it is unclear how these anti-IL-8:IL-8 complexes signal via FcγRIIa. For example, cross-linking is normally required for Fc receptor activation, unless these antibodies are tethered to a cell surface. This is in contrast to the proinflammatory and prochemotactic activity of IL-8 antibody complexes that are dependent on FcγRIIa (96). The other explanation is that these complexes represent a means of regulating the IL-8 response by sequestering the chemokine and preventing functional activity (98), although later studies suggest that these complexes are inflammatory, not regulatory (96). The situation may be further complicated by the expression of FcγRIIb within the lung, which is a known inhibitory receptor that may have opposing downstream effects to FcγRIIa (35). Another question arises: are these autoantibodies produced by everybody or are they limited to patients with ARDS? Are these autoantibodies produced by such individuals prior to ARDS or are they generated in response to ongoing inflammation? Furthermore, IgG antibody isotypes are normally located in the circulation rather than in the lumen of mucosal tissues. Considering these IL-8 immune complexes are readily recovered from BALF, it is therefore unclear whether they are generated locally or whether they enter the alveolar space as a consequence of microvascular leak. Finally, does ARDS therefore have an autoimmune component? This is unlikely, as ARDS is neither a spontaneous disease of endogenous origin nor a chronic inflammatory disease.

Other Chemokines Involved in Neutrophil Migration

It has been demonstrated that human neutrophils alter their cell surface expression of several chemokine receptors in the lung during episodes of inflammation, which may have important implications for ARDS pathogenesis. For example, Hartl et al. (70) demonstrated that human neutrophils isolated from the BALF of chronic obstructive pulmonary disease (COPD) patients expressed higher levels of CCR1, CCR2, CCR3, CCR5, CXCR3, and CXCR4 compared with circulating neutrophils (they found similar upregulation on neutrophils from the synovium of arthritis patients). The same study showed that neutrophils isolated from COPD BALF were able to chemotax toward CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1β), CCL11 (eotaxin), CXCL11 (I-TAC), and CXCL12 (SDF-1). Although these samples were obtained from COPD and not ARDS patients, this does suggest that neutrophils are potentially capable of responding to a number of CC and CXC chemokines other than IL-8. Some of these chemokines, CXCL11 and CCL5 for example, are also elevated in the BALF of COPD patients (38). CCL2 and CCL3 levels were also demonstrated to be consistently elevated in the BALF of ARDS patients, although they did not correlate with either neutrophil or macrophage numbers (63). In addition, it has been reported that human neutrophils downregulate CXCR1 and CXCR2 after recruitment to the airways (144), which may have important consequences for the therapeutic inhibition of these receptors in lung disease. Indeed, human neutrophils have been shown to undergo rapid gene expression changes following transepithelial migration, resulting in delayed apoptosis and reduced capacity for IL-8-mediated chemotaxis (36).

The platelet-derived chemokines CCL5 and CXCL4 are also elevated in human ARDS samples and mouse models of lung injury. These chemokines have been shown to form heteromers and their expression correlates with neutrophil efflux into mouse lung tissue (66). Disruption of these heteromers attenuated experimental sepsis and LPS-induced lung injury. LPS-treated mice also have elevated levels of the related chemokine CXCL12 and increased migration of CXCR4+ neutrophils (185). Moreover, CXCL12 was shown to reduce neutrophil apoptosis in inflamed lungs. Struyf et al. (163) further demonstrated that CXCL6 can synergize with CCL7 (MCP-3) to promote neutrophil migration, albeit in a mouse model of peritoneal inflammation. CCL7, together with CXCL10, was also shown to orchestrate neutrophil migration into the lung in response to oxidative stress (124), further suggesting that neutrophil responses are influenced by a variety of mediators in certain inflammatory conditions. CXCL10 has also been implicated in acid-induced lung injury and ARDS resulting from influenza infection of mice (80). Neutrophils that expressed CXCL10 were able to signal in an autocrine manner via CXCR3, which enhanced neutrophil oxidative burst, chemotaxis, and lung inflammation. Furthermore, CXCR3 was specifically expressed on neutrophils that infiltrated the lung, suggesting that this subset of neutrophils may provide a suitable target for the treatment of ARDS (80).

In a mouse model of *Escherichia coli*-induced lung injury, MCP-1$^{-/-}$ mice showed reduced CCR2+ neutrophil recruitment into the lungs (8). CCR1$^{-/-}$ and CCR2$^{-/-}$ mice also demonstrated reduced neutrophilic inflammation following LPS challenge, although no differences were observed for CCR3$^{-/-}$ mice (188). In contrast, inhibition of CCR3 expressed on pulmonary epithelial cells reduced IL-8 secretion and neutrophil recruitment in response to LPS (105). CCR1 and CCR2 are also upregulated on neutrophils isolated from adjuvant-challenged rats, allowing them to respond CCL2 (MCP-1) in in vitro chemotaxis assays (82). However, neutrophil migration into the lung has been shown to be independent of CCR2 (and CCL2) but cooperatively dependent on CCR2+ monocyte recruitment (118). Likewise in an IgG immune complex-induced rat model of lung injury, neutralization of CCL2 had no effect on neutrophilic inflammation, although neutralization of CCL4 (MIP-1β) did reduce vascular permeability, neutrophil recruitment, and TNF production in BALF (18).

More recently, we have demonstrated that neutralizing CCL2, or the related chemokine CCL7, significantly diminishes neutrophil accumulation in the air spaces of LPS-challenged mice (123). Furthermore, neutrophils isolated from inflamed lungs displayed decreased expression of cell surface CXCR2 and increased expression of CCR1 and CCR2 (but not CCR3). It is therefore becoming increasingly clear that the neutrophilic chemokine network within the lung is more complex than previously thought and may be dependent on the

contextual influence of several inflammatory and chemotactic factors. It is also likely that the migration of neutrophils into various tissue microcompartments, for example endothelial vs. epithelial, is regulated by distinct sets of chemotactic cues differentially expressed within certain lung microenvironments (123), suggesting there may exist a significant amount of nonredundancy within the lung chemokine network (Tables 1 and 2). The functional capacity of individual chemokines may need to be considered within the context of the surrounding inflammatory milieu and the tissue compartment in which they operate.

SUMMARY

Neutrophils play an important role in innate immune defense by phagocytosing pathogenic bacteria and preventing further invasion through the release of ROS, proteinases, antimicrobial peptides, and NETs. However, the excessive accumulation of activated neutrophils can cause unwanted bystander tissue damage, which can negatively affect the normal physiology of the lung by modulating endothelial-epithelial barrier integrity, resulting in pulmonary edema and hypoxemia (Fig. 1). The migration of large numbers of neutrophils across the alveolar epithelium may significantly contribute to epithelial permeability. In addition, proteinases such as neutrophil elastase and matrix metalloproteinases directly degrade the extracellular matrix and components of the paracellular space. The excessive release of reactive mediators, such as oxidants, cationic antimicrobial peptides, and lipid mediators, by neutrophils attached to the apical surface of the alveolar epithelium, may also cause damage and contribute to endothelial-epithelial barrier permeability. Capillary-alveolar permeability is a major hallmark of ARDS, resulting in alveolar leak, lung edema, deficient oxygenation, and hypoxemia. Therefore, targeting excessive neutrophil accumulation within this disease setting may represent a viable approach for the treatment of ARDS.

Studies performed with clinical ARDS samples and animal models of ARDS have revealed a central role for IL-8 (CXCL8) in regulating neutrophil recruitment into the lungs, and consequent tissue damage, capillary-alveolar permeability, and oxygen deficiency. However, the chemokine network regulating neutrophil migration in the lung may involve multiple chemokine ligand families, including several CC-chemokines such as CCL2, CCL4, and CCL7, thereby expanding the number of potential therapeutic targets in this disease setting. The mechanism of neutrophil recruitment into the lung is a multistep process involving extravasation across the pulmonary endothelium, trafficking across the lung interstitium, and migration across the alveolar epithelium into air spaces. This involves multiple chemotactic molecules acting at each of the spatial-temporal checkpoints. Further research needs to be undertaken to better understand these complex chemokine networks and the contribution of individual chemokines during the development of both pulmonary and nonpulmonary diseases associated with neutrophilic inflammation.

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No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

A.E.W. prepared figures; A.E.W. drafted manuscript; A.E.W. and R.C.C. edited and revised manuscript; A.E.W. and R.C.C. approved final version of manuscript.

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