Regulatory mechanisms of neutrophil migration from the circulation to the airspace

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Abstract

The neutrophil, a short-lived effector leukocyte of the innate immune system best known for its proteases and other degradative cargo, has unique, reciprocal physiological interactions with the lung. During health, large numbers of 'marginated' neutrophils reside within the pulmonary vasculature, where they patrol the endothelial surface for pathogens and complete their life cycle. Upon respiratory infection, rapid and sustained recruitment of neutrophils through the endothelial barrier, across the extravascular pulmonary interstitium, and again through the respiratory epithelium into the airspace lumen, is required for pathogen killing. Overexuberant neutrophil trafficking to the lung, however, causes bystander tissue injury and underlies several acute and chronic lung diseases. Due in part to the unique architecture of the lung's capillary network, the neutrophil follows a microanatomic passage into the distal airspace unlike that observed in other end-organs that it infiltrates. Several of the regulatory mechanisms underlying the stepwise recruitment of circulating neutrophils to the infected lung have been defined over the past few decades; however, fundamental questions remain. In this article, we provide an updated review and perspective on emerging roles for the neutrophil in lung biology, on the molecular mechanisms that control the trafficking of neutrophils to the lung, and on past and ongoing efforts to design therapeutics to intervene upon pulmonary neutrophilia in lung disease.

Abbreviations

AT	Alveolar epithelial type
DARC	Duffy antigen receptor for chemokines
ELR	Glu-Leu-Arg
ICAM	Intercellular adhesion molecule
IL	Interleukin
LPS	Lipopolysaccharide
NF	Nuclear factor
PMN	Neutrophil
VCAM	Vascular cell adhesion molecule
VLA	Very late activation antigen

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Introduction

The lung, by virtue of its unique tissue architecture, continual exposure to the environment, and mandate to balance robust antimicrobial host defense with the maintenance of vital gas exchange, has a relationship to the circulating neutrophil (PMN) unlike that of all other organs. Rapid recruitment of PMNs from the bloodstream to the airspace is required for host defense. However, bystander tissue injury from excess or recurrent PMN recruitment contributes to nearly all categories of lung disease. Although the molecular mechanisms of PMN recruitment to the airspace have been studied for decades, foundational discoveries continue to be made. PMN trafficking to the lung differs fundamentally from that in other organs. In part due to the technical challenges of imaging and intervening upon PMN trafficking in the lung in vivo, in several cases, mechanisms of PMN trafficking worked out in other tissues (e.g., cremaster muscle, intestine) have been extrapolated to the lung. In this review, after first discussing intriguing reciprocal homeostatic interactions between the lung and PMN and the unique journey

that the intravascular PMN follows into the alveolar space during pneumonia, we highlight established and emerging mechanisms for chemokines, the endothelium, and the respiratory epithelium in the regulation of PMN trafficking into the airspace. Last, we close with a perspective on PMNs in lung disease and potential opportunities for therapy.

Homeostatic margination and function of PMNs in healthy lung

Mechanisms of PMN margination in the lung

Decades ago, studies of rabbits, sheep, and other animals revealed that non-inflamed lungs during health contain a pool of PMNs that is ~ 2–3 times the number of PMNs in the free circulation [1, 2]. These 'marginated' intravascular PMNs largely localize within alveolar capillaries. A series of landmark studies clarified that PMNs traveling from pulmonary arterioles to venules transit ~ 8–17 alveolar walls involving ~ 40–100 capillary segments, and that, due to size mismatch, at least half of these capillary segments require PMNs to deform and sometimes to even stop once or more during passage (reviewed in [2]). Likely due to their slower deformability, PMNs have a much longer transit time through the lungs than do erythrocytes [2], accounting, at least in part, for their marked relative concentration within the lung microvasculature. By contrast, studies using intravital labeling have generally reported relatively few extravascular (i.e., interstitial, subepithelial) PMNs in the healthy murine lung, ranging from minimal [3–5] to ~ 14% of lung PMNs [6]. One group, however, did observe substantial numbers of extravascular PMNs by two-photon microscopy [7]. Whether this discrepancy reflects technical or environmental (microbiome, caging/bedding) differences is unclear. Virtually no PMNs are found in the alveolar space during health.

Arguing against an important role for molecular adhesion in steady-state PMN margination in the lung, E-, L-, and P-selectins have been shown not to play a role in margination [2, 8–10]. By contrast, PMN CXCR4 may contribute to margination via ligation to pulmonary capillary endothelial CXCL12, although reports differ, with one showing that the CXCR4 antagonist AMD3100 demarginates PMNs from the lungs of mice and non-human primates [11], while another found that it increases pulmonary PMN content in mice [12].

Emerging roles for the lung in the PMN life cycle

Recent reports that there is a complex community of marginated immune cells in the lung in addition to PMNs (i.e., natural killer [NK] cells, CD4+ T cells, CD8+ T cells, invariant NK T cells) suggest that the pulmonary microvasculature may provide a niche for transcellular immune communications [4]. The PMN content of the lung microvasculature, in particular, exceeds that of large vessels by ~ 50-fold [2], suggesting that, quantitatively, alveolar capillaries are an important stop in the PMN life cycle. Of interest, it was recently reported that aged CD11b^{hi}L-selectin^{lo}CXCR4$^+$ PMNs constitute a large proportion of PMNs in the lung vasculature, that PMNs physically interact with B cells in the lung via CD18, and that this interaction induces PMN apoptosis, committing PMNs to phagocytic removal ('efferocytosis') [13]. This report, taken together with others that aged PMNs may also be preferentially recruited to the lung during inflammation [14], suggests that the lung niche, rather than serving as a simple mechanical sieve for PMNs, may be selective, acting as a major final resting ground for, and homeostatic regulator of PMNs. Of interest, epinephrine induces rapid release of PMNs from the lungs [11]. Conversely, intravenous injection of complement fragments or activated plasma induces rapid and marked retention of PMNs in alveolar capillaries and associated neutropenia [15, 16]. Collectively, this suggests that the lung serves as an emergency sink and source for PMNs during times of acute systemic stress.

Emerging roles for marginated PMNs in the lung

In the liver and spleen, intravascular macrophages play an important role in the clearance of pathogens from the bloodstream. By contrast, the lungs in mice and humans are not thought to contain intravascular macrophages. Of interest, a recent report using intravital microscopy indicates that marginated PMNs may serve this surveillance role in the lungs [5]. Yipp and colleagues reported that pulmonary intravascular PMNs crawl short distances along the endothelium in a CD11b-dependent fashion in the steady state, but that these distances increase within minutes of exposure to LPS or bacteria. Moreover, intravascular bacteria were noted to be captured by the pulmonary endothelium through adherence, where they were rapidly targeted by migratory PMNs for phagocytic clearance. Suggesting that PMNs may have a unique affinity for the lung niche, it has been noted that PMNs adhere to pulmonary endothelium in the steady state, unlike dermal endothelium [5]. Beyond CXCL12 [11], the identity of the pulmonary endothelial molecules that specifically address PMNs to the lung in the steady state remain to be identified, as does whether biomechanical signals induced in compressed intracapillary PMNs or in the endothelial cells contacting them poise marginated PMNs and pulmonary microvascular endothelial cells for their cooperative surveillance roles.

Overview of the microanatomic passage of PMNs into lung during inflammation/infection

In the lungs, the conducting airways branch > 20 times before the terminal alveolar spaces ('alveoli') are reached [17]. In humans, the alveolar surface area has been approximated at ~ 100 m^2 [18]. Alveoli are lined by two types of epithelial cells—squamous alveolar epithelial type 1 (AT1) cells (involved primarily in gas and solute exchange), and cuboidal type 2 (AT2) cells (involved in surfactant synthesis and recycling). Additional cells in the alveolar septal wall during health include endothelial cells, fibroblasts, pericytes, macrophages, and mast cells [18]. Transmission electron microscopy (EM) studies of the alveolar wall have suggested a 'thin' (≤ 0.2 μm) side with near apposition of epithelial and endothelial cells overlying a shared basement membrane, and a 'thick' side with interstitial components (e.g., extracellular matrix [ECM], fibroblasts) separating epithelium from endothelium [18].

Reports to date have generally supported a model wherein PMNs emigrating during lung inflammation do not leave the vasculature at the level of high endothelial venules as in other tissues, but primarily at the level of alveolar capillaries [19]. Work by Walker and colleagues using transmission EM and serial section reconstruction suggests that PMNs exit the vasculature paracellularly (i.e., between endothelial cells) at the junction of the thin and thick portions of the alveolar septal wall at a location where interstitial fibroblasts penetrate pre-existing (anatomic) holes in the basement membrane and reach the endothelium [18, 20, 21]. Emigrating PMNs are thought to follow the track of these fibroblasts through the interstitium to pre-existing holes in the subepithelial basement membrane, extensively contacting the fibroblast along the way, and then to enter the alveolus paracellularly at tricellular junctions of two AT1 cells with an AT2 cell [18, 21–23]. During this passage, it is presumed that PMNs use β$_2$ (CD18)- and β$_1$-integrins to interact directly with ECM components (i.e., fibronectin, vitronectin, laminin, collagen). That said, the identity and relative importance of specific integrin ligands during different pulmonary exposures, the existence and nature of haptotactic gradients (ECM-bound chemokine gradients) in the lung interstitium, as well as the specific role of interstitial fibroblasts (as well as whether and how they retract to make space for emigrating PMNs) all remain poorly defined.

While paracellular egress of PMNs at the level of the alveolar capillary is generally held as the canonical path for PMNs in the inflamed lung, it is important to note that this has not been firmly established for all pulmonary exposures. Some studies have suggested that a substantial percentage of PMNs may emigrate transcytotically during pneumonia (i.e., directly through the endothelial cytoplasm) [18]. Moreover, PMNs may in some settings emigrate from larger pulmonary vessels rather than capillaries [18, 24]. Although the course that PMNs follow when they infiltrate the walls of conducting airways during inflammatory airways diseases (e.g., bronchiolitis, bronchitis, bronchiectasis) is less well defined than it is for alveolar diseases (i.e., pneumonia, acute lung injury), it is thought that PMNs in this case egress from the bronchial arterial rather than pulmonary arterial circulation. As the bronchial arteries derive from the systemic circulation (i.e., proximal descending aorta), it is presumed that PMN emigration into the airways may abide by mechanisms similar to those that have been worked out in extrapulmonary tissues such as the cremaster muscle rather than those worked out in the alveolus (discussed below). Moreover, the multicellular architecture of the airway wall, which varies in thickness and complexity along the length of the respiratory tract, almost certainly imposes different constraints on PMNs as they traverse into the airway lumen. The degree to which the specific PMN route into the lung is dependent on the type, deposition site, and dose of the exposure remains largely undefined.

Although most studies of pulmonary neutrophilia have simply quantified PMNs in bronchoalveolar lavage, advanced methods, typically involving pre-mortem intravenous injection of labelling antibodies, have been described that can distinguish intravascular and extravascular (interstitial) PMNs in the lung [3, 4, 6]. As up to 50% of intravascular PMNs may remain in the lung even after prolonged post-mortem vascular perfusion [3], techniques such as these are invaluable for characterizing the stepwise passage of PMNs from bloodstream to alveolus. Using these methods, Reutershan and colleagues have shown that, in the first hour following LPS inhalation in mice, increased PMNs are sequestered within the pulmonary vasculature, that PMNs then diapedese into the interstitium starting at ~ 1 h and increasing to a plateau at 12–24 h, and that transepithelial migration (TEpM) into the alveolar lumen is delayed, starting after 2 h and reaching a peak at ~ 24 h [6]. PMNs alter their surface levels of multiple adhesion molecules as they pass through different intrapulmonary tissue compartments (endovascular, interstitial, intra-alveolar), implying that specific adhesive interactions regulate compartmental checkpoints along the journey [25].

Cytokine and chemokine induction initiates PMN attraction to the infected lung

Importance of the IL-17—ELR+ CXC chemokine axis

Cytokine/chemokine induction by sentinel airspace-resident cells (alveolar macrophages, epithelial cells) upon detection of microbes and other pro-inflammatory stimuli is the key initiating event for PMN attraction to the lung (the sequence of events likely differs for PMN recruitment to the lung in response to systemic insults such as endotoxemia but has not been as clearly defined). The transcription factor nuclear factor (NF)-κB acts as a master regulator for cytokine induction in the lung [26–29]. Important roles have been identified for several pro-inflammatory cytokines (i.e., TNFα, IL-1β, G-CSF) in PMN recruitment to the lung through upregulation of endothelial, epithelial, and PMN adhesion molecules, prolongation of PMN survival, and other mechanisms [30, 31]. The potential for complex cytokine interactions is suggested by IL-6-null mice, which have elevated airspace TNFα and increased alveolar neutrophilia following LPS inhalation [30, 31].

A central role for chemokines, in particular, Glu-Leu-Arg (ELR)+ CXC chemokines (so named for their conserved amino acid sequence) has been identified [31]. These chemokines, which include CXCL1, -2, and -5 in mice and homologues for these plus IL-8 in humans, directly chemoattract PMNs via ligation of the receptor CXCR2 (and CXCR1 in humans). Indeed, LPS-induced alveolar neutrophilia is virtually abolished in CXCR2-null mice, suggesting a critical requirement for this axis [32]. Whereas pro-inflammatory cytokines and most chemokines are thought to be generated predominantly by alveolar macrophages, CXCL5 and CXCL15, another ELR+ CXC chemokine with an as-yet poorly defined receptor, interestingly are produced by respiratory epithelial cells (AT1, AT2, and Club cells for CXCL5 [33–35]; airway epithelial cells for CXCL15 [36]) and both play important roles in PMN recruitment to the lung [37–39]. Indeed, it has recently been reported that circadian fluctuations in Club cell CXCL5 in response to clock gene-sensitive regulation of the gene by endogenous glucocorticoids underlies circadian variations in the magnitude of alveolar neutrophilia triggered by inhaled LPS in mice [35].

Although isolated AT2 cells release cytokines and chemokines in vitro upon LPS challenge [34], critical upstream roles for hematopoietic cell-derived IL-23 and IL-17 have been identified in vivo in the potentiation of pro-neutrophilic cytokines and chemokines. IL-17 is required for intrapulmonary induction of CXCL2 and G-CSF and for early PMN recruitment to the lung following infection with *K. pneumoniae* [40], a response that is likely driven, at least in part, by epithelial responsiveness to the cytokine via IL-17A and IL-17C receptors [41]. Co-administration of IL-17 and TNFα to the airspace of mice is sufficient to induce ELR+ CXC chemokines and G-CSF in the airspace as well as CXCL5-dependent alveolar neutrophilia [42]. IL-17 can itself be acutely produced by several cell types in the infected lung, including Th17 cells, γδ T cells, invariant NKT cells, and lymphoid tissue inducer-like cells [43–45]. Yet further upstream, IL-23 has been shown to be a potent activator of IL-17-producing innate cells [46, 47]. IL-23 is produced in the infected lung by dendritic cells and macrophages [48, 49], and is required for downstream induction of IL-17A and IL-17F, as well as IL-17-induced targets (G-CSF, CXCL1-, -2, -5), pathogen clearance, and survival during Gram-negative bacterial pneumonia [48]. Taken together, these reports identify a transcellular hierarchy of sentinel and messenger cytokines that ultimately induce effector chemokines and cytokines that act directly upon PMNs.

Additional chemoattractants that regulate PMN trafficking into the lung

The tripeptides *N*-acetyl proline-glycine-proline (PGP) and PGP are generated in the LPS-exposed airspace in a somewhat delayed fashion compared to ELR+ CXC chemokines and sustain PMN trafficking to the airspace through direct chemotactic activity upon CXCR1/2 [50, 51]. These so-called 'matrikines' are generated by the sequential proteolytic activity of MMP8/9 and prolyl endopeptidase upon collagen in the lung [51], thus relaying local ECM damage in the inflamed lung as a feedforward neutrophilic signal. PGP, which is elevated in the airspace of acute respiratory distress syndrome (ARDS) patients [52] and correlates with disease severity in chronic obstructive pulmonary disease (COPD) [53], is degraded by leukotriene A4 hydrolase (LTA4H) [54, 55], an enzyme better known for its generation of the pro-inflammatory PMN chemoattractant LTB4.

Eicosanoids, bioactive lipids that are generated from arachidonic acid (AA) released from membrane phospholipid by phospholipase A2, have been shown to play complex, collaborative roles in PMN trafficking to the lungs. PMN-derived LTB4 and epithelial-derived hepoxilin A3 (HXA3) have, in particular, been firmly implicated in this regard. LTB4, a lipid that is synthesized by the sequential activities of 5-lipoxygenase (5-LO) and LTA4H upon AA, has long been known as a potent PMN chemoattractant that is upregulated in pro-inflammatory conditions [56] and more recently shown to chemoattract PMNs via the high-affinity BLT1 receptor [57]. PMN-derived LTB4 plays an important role in PMN recruitment to the *K. pneumoniae*-infected mouse lung [58]. Cytosolic phospholipase A2-*α*-dependent LTB4 generation in PMNs amplifies TEpM in response to

bacterial infection [59]. Elevated LTB4 has been measured in the lungs of patients with COPD, asthma, and ARDS [60]. Although not all experimental interventions on the LTB4-BLT1 pathway have succeeded in attenuating pulmonary neutrophilia in animal models, LTA4H inhibition and select BLT1 antagonists have been shown to have efficacy [60, 61]. HXA3, a 12-LO-derived lipid, is produced by intestinal (T84) and respiratory (A549, H292) epithelial cell monolayers upon Gram-negative bacterial infection, generating an apical-to-basolateral gradient that drives PMN TEpM [62–64]. Respiratory infection of mice with *P. aeruginosa* also induces airspace HXA3 [64]. Interesting differences between LTB4 and HXA3 have been documented. LTB4 but not HXA3 induces PMN degranulation [62] and eosinophil TEpM [65]. It is thought that initial PMN TEpM triggered by epithelial HXA3 may subsequently be amplified by PMN-derived LTB4 [66].

Role of extracellular matrix and endothelium in chemokine gradient formation

Chemokines released by alveolar macrophages and epithelial cells are thought to directionally chemoattract PMNs into the airspace through the formation of concentration gradients through lung tissue and into the vascular lumen. Nearly all chemokines bind to heparin sulfate, chondroitin sulfate, and other tissue glycosaminoglycans via a positively charged C-terminal domain, thereby establishing tissue-bound gradients [67, 68]. Of interest, it has been shown that matrilysin (i.e., matrix metalloproteinase [MMP]-7) is required to generate a transepithelial gradient of CXCL1 in the lung by ectodomain shedding of syndecan-1, the predominant heparin sulfate proteoglycan on epithelia [69]. The relative potency of chemokines to induce PMN accumulation in the airspace may relate to their association-dissociation kinetics from tissue glycosaminoglycans. Thus, it was reported that, when instilled into the murine airspace, CXCL8 mutants deficient in heparin-binding appeared in plasma at higher concentrations than wild type protein and recruited more PMNs [70]. Similarly, CXCL1 was found to have a higher heparin association-dissociation constant than CXCL2, to appear in plasma more rapidly than CXCL2, and to attract more PMNs to the airspace [70]. Parallel findings were reported in rats [71]. Collectively, these findings imply distinct roles for CXCR2-active chemokines in spatiotemporal control of PMN trafficking to the lung, perhaps explaining why pulmonary chemokines are not fully redundant in vivo [31].

Of interest, CXCL5 was recently reported to antagonize CXCL1- and CXCL2-dependent PMN attraction to the mouse lung during *E. coli* pneumonia by competitive displacement of the latter two chemokines from the scavenger protein duffy antigen receptor for chemokines (DARC) on circulating erythrocytes, thereby increasing plasma levels of CXCL1 and CXCL2, disrupting their gradients, and desensitizing PMN CXCR2 [39]. Further suggesting non-redundancy of chemoattractants, evidence supports a 'hierarchy' of responsiveness at the level of the PMN [72, 73], with bacterial-derived signals having increased priority over host-derived signals, perhaps to ensure accurate terminal targeting of PMNs to sites of infection. Thus, the bacterial-mimetic chemoattractant formylated Met-Leu-Phe (fMLP) outcompetes IL-8 for PMN attraction, even at 1/1000th the concentration, and appears to do so through competitive phosphatase and tensin homolog (PTEN)-mediated suppression of Akt signaling [74, 75].

Ensuring delivery of chemokine gradients to the intravascular PMN, endothelial cells transfer interstitial chemokines from their basolateral to the apical surface. Regulated transcytosis rather than paracellular diffusion is thought to predominate [76]. Prior to overt chemoattraction, endothelium-bound chemokines activate integrins on luminal PMNs, causing firm adhesion and arrest, to be further discussed below. Heparan sulfation on endothelial cells plays an important role in these events, as mice with endothelial heparan sulfate deficiency have reductions in chemokine transcytosis, endothelial display of chemokines, and PMN firm adhesion and migration [77]. Endothelial DARC also mediates basolateral-to-apical transcellular transport of chemokines, supporting PMN emigration [78]. Endothelial DARC was found to transfer CXCL1, enhancing CXCL1-induced PMN transendothelial migration; thus, reduced PMNs were attracted to the airspaces of DARC-null mice after intratracheal instillation of CXCL8 [79]. Studies using bone marrow chimeras to discriminate endothelial and erythrocyte DARC have suggested that both are required for intact PMN migration into the airspace in response to inhaled LPS, likely due to distinct roles in forming chemokine gradients [80]. By contrast, another group has reported that DARC-null mice have elevated airspace chemokines and PMNs after LPS inhalation and that this phenotype tracks with erythrocyte DARC deficiency [81].

Endothelial cells are themselves also an important source of chemokines for PMN recruitment to tissues, expressing CXCL1 in secretory granules basally [82] and upregulating it in response to cytokines [83, 84]. The finding that endothelial cells null for the common IL-6 family receptor subunit gp130 display increased surface CXCL1, causing inappropriate integrin-dependent PMN arrest and impaired PMN transmigration, indicates that endothelial cell-intrinsic chemokines play key roles in regulated, signal-dependent PMN traffic into tissues [85]. Of interest, PMN-derived chemokines have also been shown to provide feed-forward signals, augmenting further PMN recruitment to the lung [86]. In mouse models of acute lung inflammation, PMN CXCL10 signals in an autocrine fashion through its receptor

CXCR3 to augment further PMN chemotaxis as well as the PMN oxidative burst [87]. These findings may account for reports of PMN swarming-like behavior in the lung and other organs during inflammation [88]. Finally, a role for endothelial CXCR2 in PMN migration into the LPS-exposed lung, perhaps through induction of endothelial actin stress fibers, has also been suggested by bone marrow chimeric mice [32].

PMN-endothelial interactions and transendothelial migration into the lung

Sequence of events preceding diapedesis: unique features in the lung

Work over the past several decades, much of it anchored by intravital microscopy of postcapillary venules in the cremaster muscle and mesentery, has suggested a canonical sequence of events by which PMNs engage and then transit the endothelium as follows: selectin-dependent tethering and rolling, PMN activation, integrin-dependent firm adhesion, intraluminal crawling, and, finally, transmigration [89, 90]. The situation is different, however, in the lung, where PMNs leave the vasculature largely at the level of the alveolar capillary. Here, it has been shown that PMN tethering and rolling likely do not occur, instead of being replaced by mechanical sequestration as cytokine-induced PMN cytoskeletal stiffening due to F-actin polymerization provokes dramatic slowing of PMNs within the narrow-caliber capillaries [19, 91–95]. Selectins have been shown to play no role in PMN recruitment to the lung under certain conditions, such as *S. pneumoniae* infection [10] despite being required for PMN emigration during *Pneumococcal* peritonitis [96, 97]. Although selectin-mediated tethering per se likely does not occur in alveolar capillaries, antibody blockade studies have nonetheless identified roles for E- and L-selectin, as well as the PMN common selectin counterreceptor, P-selectin glycoprotein ligand-1, in LPS-induced recruitment of PMNs to the lung [98]. L-selectin has also been shown to support PMN recruitment to *the K. pneumoniae*-infected lung, at least in part, through regulation of integrin signaling and integrin-dependent PMN migration [99]. Challenging the paradigm of non-adhesive, mechanical entrapment of PMNs within pulmonary capillaries during inflammation, a recent report identified a role for pulmonary capillary endothelial dipeptidase-1 in adhesive sequestration of PMNs during endotoxemia [100]. Moreover, another report found that heparanase promotes PMN sequestration in the lung during endotoxemia, likely by thinning the endothelial glycocalyx and exposing underlying adhesion molecules [101].

CD18-dependent and -independent PMN recruitment

Following the slowing of intravascular PMNs, firm adhesion to the endothelium next initiates the steps leading to diapedesis. The heterodimeric β_2-integrins, CD11a/CD18 ($\alpha_L\beta_2$; LFA-1) and CD11b/CD18 ($\alpha_M\beta_2$; Mac-1) have been shown to play important roles in PMN trafficking to the lung and other tissues, in particular, mediating firm adhesion of PMNs to endothelium and facilitating PMN chemotaxis across ECM [102]. Of the two β_2-integrins, antibody neutralization studies have supported a more important role for Mac-1 in PMN recruitment to the lung [103]. Landmark reports by Doerschuk and colleagues revealed that pulmonary exposures can be grossly categorized as eliciting alveolar neutrophilia that is CD18 (i.e., β_2-integrin)-dependent vs. CD18-independent, with most exposures favoring but not completely relying upon a single pathway (e.g., LPS-induced neutrophilia is $\sim$80% CD18-dependent) [104–108] (Table 1).

Studies revealing that CD18-dependent stimuli like LPS upregulate the CD18 ligand ICAM-1 on pulmonary capillary endothelium, whereas CD18-independent stimuli such as *Pneumococcus* do not [109] have suggested that PMN integrin interactions with endothelium are critical. That said, the

Table 1 Inhaled exposures eliciting alveolar neutrophil accumulation through CD18-dependent vs. CD18-independent mechanisms

CD18-dependent stimuli		CD18-independent stimuli	
E. coli LPS	[104, 105, 111]	*S. pneumoniae*	[104, 105]
P. aeruginosa	[105, 107]	CXCL1	[111]
E. coli	[279]	HCl (ipsilateral lung)	[104, 280]
HCl (contralateral lung)	[280]	*S. aureus***	[279]
IgG immune complexes	[281]	Group B *Streptococcus*	[282]
		Hyperoxia	[283]
		IgG immune complexes	[281]

The cited reports measured alveolar neutrophilia in mice, rats, rabbits, or guinea pigs with either CD18 deletion (mice) or antibody neutralization of CD11 or CD18 β_2-integrin components. Partial effects on neutrophilia using the latter method may not decisively distinguish between technical (i.e., dosing) and biological causes

**Partially CD18-dependent

presence of integrin ligands in the pulmonary interstitium and on respiratory epithelial cells (discussed below) suggests that integrins also regulate important post-endothelial checkpoints during the journey of PMNs to the alveolus. Indeed, the recent finding that mice with the abrogation of high-affinity β_2-integrin activation have increased pulmonary interstitial PMNs during *P. aeruginosa* pneumonia suggests that activated integrins may slow PMN migration into or through the extravascular space in the lung [110]. Moreover, alveolar neutrophilia elicited by CXCL1, as well as the CD18-independent component of LPS-induced neutrophilia, are both mediated by the β_1-integrin CD29, which appears to play a predominant role in the movement of PMNs from interstitium into alveoli (i.e., TEpM) [111]. Of interest, CD29/CD49e (i.e., very late activation antigen [VLA]-5) and CD29/CD49f (VLA-6) heterodimers regulate CD18-independent PMN trafficking to both LPS and CXCL1, whereas heterodimers of CD29 with CD49b (VLA-2) and CD49d (VLA-4) are utilized only in trafficking to CXCL1 [111]. VLA-4 also mediates a minor portion of the CD18-independent PMN trafficking to *Pneumococcal* pneumonia [112]. By contrast, PMN integrin β_3 is reportedly not involved in PMN recruitment to the lungs in *Pneumococcal* pneumonia [113]. Finally, non-integrin PMN adhesion proteins such as CD47 have also been shown to regulate PMN trafficking to the lung during *E. coli* pneumonia [114], as further discussed below.

PMN diapedesis

Although LFA-1 and Mac-1 both bind to ICAM-1 (and other ligands), recent studies have shown that they serve distinct functions in PMN transendothelial migration. In CXCL2-treated postcapillary venules imaged by time-lapse intravital microscopy, it was found that LFA-1 mediates initial PMN adhesion and that this is followed by Mac-1 (PMN)-, Vav-1 (PMN)-, and ICAM-1 (endothelial)-dependent intraluminal crawling, often perpendicular to or against the flow of blood, to optimal emigration sites [115, 116]. Wild type PMNs crawled to intercellular junctions and emigrated paracellularly most (~ 86%) of the time, whereas only ~ 39% of Mac-1-null PMNs emigrated paracellularly, with the remainder emigrating transcellularly, and over a longer period of time [115]. Although PMNs generally appear to favor the paracellular route [23, 117], prolonged stimulation of endothelium with TNFα may increase transcellular migration in a manner dependent upon upregulation of and signaling by ICAM-1 [118]. The existence of transcellular migration in vivo has been supported by studies using serial section transmission EM and scanning EM [119–122]; however, to what extent and under what contexts this occurs in the lung is poorly understood.

Whether transmigrating via the paracellular or transcellular route, PMNs are engaged by concentrated patches of ICAM-1 presented upon microvilli-like projections from the endothelial surface; these patches subsequently further recruit VCAM-1, tetraspanins, and actin, ultimately forming enveloping structures that have been described as 'docking structures' or 'transmigratory cups' [123, 124]. These dome-like, enveloping structures are thought to preserve endothelial barrier integrity during PMN transit [124]. Of interest, intravital two-photon imaging has also revealed that focal collections of intravascular monocytes support PMN transendothelial migration into the lung [7]. Platelets interacting directly with PMNs via P-selectin have also been shown to facilitate PMN recruitment to the LPS-exposed lung [98, 125], although the mechanism remains somewhat obscure.

PMNs transiting the paracellular route engage in multiple, sequential molecular interactions with junctional endothelial proteins, including platelet/endothelial cell adhesion molecule (PECAM)-1 (also known as CD31), ICAM-2, junctional adhesion molecules (JAM)-A and -C, and CD99. Molecules that regulate PMN transendothelial migration are catalogued in Table 2. Homophilic interaction between PMN and endothelial PECAM-1 is an early event in junctional engagement by PMNs; PECAM-1 antagonism blocks PMN passage through endothelial junctions, although PECAM-1-dependence of PMN transmigration appears to vary by genetic background in mice [126, 127]. Moreover, it is reported that PECAM-1 does not play a role in PMN emigration from pulmonary capillaries in response to either *E. coli* (a CD18-dependent exposure) or *S. pneumoniae* (a CD18-independent exposure) [128]. Endothelial JAM-A serves as a ligand for PMN β_2-integrins, supporting LFA-1-dependent transmigration [129]. JAM-A deletion or blockade reduces LPS-induced airspace neutrophilia, but increases PMNs in lung digests, suggesting impaired trafficking from vasculature to alveolus [130]. JAM-C also mediates PMN transmigration in a Mac-1-dependent manner [131] and has been shown to support alveolar neutrophilia in the LPS inhalation model [132].

PECAM-1, JAM-A, and CD99 not only localize to the junctional surface of endothelial cells, but also to a contiguous, invaginated subjunctional intracellular structure of interconnected tubules and vesicles, the so-called 'lateral border recycling compartment' (LBRC) [133]. Microtubule-dependent surface recycling of LBRC molecules is required for PECAM-1 homophilic interactions with PMNs and for transendothelial PMN migration [133, 134], and relies on a transient increase in cytosolic free calcium ion concentration in endothelial cells [135, 136]. Endothelial transient receptor potential canonical family member six (TRPC6), a cation channel, appears to underlie this event [137]. Homophilic interactions between PMN and endothelial CD99 are

Table 2 Adhesion molecules that regulate neutrophil transendothelial migration

	Transendothelial migration				
	PMN	endothelium	PMN-endothelium interaction site	Model	Ref
CD11a/CD18 (LFA-1)	+	a	Apical, lateral	Primary cell	[284, 285]
CD11b/CD18 (Mac-1)	+	a	Apical, lateral	Primary cell	[284–286]
CD11c/CD18 (p150/95)	+	a	ND	Primary cell	[285]
CD18-independent integrins	+	a	ND	Primary cell	[287, 288]
PECAM-1 (CD31)	–	+/–*	Lateral	Primary cell, peritonitis, dermatitis, pneumonia	[127, 128, 289]
CD44	+	+/–	ND	Cremaster muscle, peritonitis, dermatitis	[155, 156]
CD47	+	+	Lateral	Primary cell, pneumonia	[114, 290, 291]
ICAM-1 (CD54)	?	+	Apical, lateral, basal	Primary cell	[118, 123, 284, 286, 292, 293]
CD55	+	+	ND	Cell-free system, primary cell	[193, 294–298]
E-selectin (CD62E)	a	+/–	Apical	Primary cell, peritonitis, lung inflammation, lung injury, pneumonia	[10, 90, 98, 285]
P-selectin (CD62P)	a	+/–	Apical	Peritonitis, lung inflammation, lung injury, pneumonia	[10, 16, 90, 98]
L-selectin (CD62L)	+	a	Apical	Primary cell, cremaster muscle, peritonitis, lung inflammation, lung injury, pneumonia	[16, 90, 98, 99, 299]
CD99	+/–	+	Lateral	Primary cell, cremaster muscle, peritonitis	[138, 139, 300]
ICAM-2 (CD102)	?	+	Apical, lateral	Cremaster muscle, peritonitis	[301–303]
VCAM-1 (CD106)	a	+	Apical	Primary cell	[123, 304]
PSGL-1 (CD162)	+	?	Apical	Intestine, cremaster muscle, peritonitis, lung inflammation	[98, 305–307]
JAM-A (CD321)	+	+/–	Lateral or interaction-independent	Primary cell, peritonitis, cremaster muscle, cardiac ischemia reperfusion	[129, 308–310]
JAM-C (CD323)	a	+	Lateral	Primary cell, peritonitis, cremaster muscle	[131, 311]
ESAM	a	+	Lateral	Cremaster muscle, peritonitis	[312]
RAGE	?	+	ND	Cremaster muscle, peritonitis	[183, 313, 314]
Dipeptidase-1	a	+	Apical	Systemic inflammation	[100]
Carbohydrate	+	+	Apical, lateral	Primary cell, cremaster muscle, peritonitis, dermatitis, hepatitis	[315–317]
Heparanase	?	+	Interaction-independent	Lung injury	[101, 318]
MIF	?	+	Interaction-independent	Lung injury	[142, 319]

+ molecule expressed, regulates transmigration, – molecule expressed, does not regulate transmigration, *?* molecule expressed, role undefined, *ND* not determined

*Not involved in transendothelial migration within lung [128]

aDenote no or limited available evidence for expression in the indicated cell type

required for the completion of paracellular transmigration [138, 139]. Finally, PMNs that have passed through the paracellular barrier are thought to migrate short distances along the basal lamina to sites of a low density of collagen IV and laminin that correspond to gaps in pericyte coverage where they exit into the interstitium in a manner involving

interactions between leukocyte LFA-1 and pericyte ICAM-1 [140, 141]. It was recently shown that endothelial cell-derived macrophage migration inhibitory factor (MIF) signals in a paracrine fashion to its receptor CD74 on pericytes, thereby reducing pericyte contractility and augmenting PMN passage into the lung interstitium and alveolar space in response to LPS inhalation [142].

Vascular endothelial (VE)-cadherin is a type I transmembrane protein exclusively localized in endothelial cells, where it is concentrated at adherens junctions and contributes to endothelial barrier integrity through homophilic cell–cell interactions [143, 144]. VE-cadherin clearance from the site of PMN transmigration by internalization and/or lateral displacement, involving site-specific phosphorylation, is thought to be required for efficient PMN transmigration [117, 145–147]. VE-cadherin phosphorylation is regulated by its complexation with VE-protein tyrosine phosphatase (PTP), and it has been shown that LPS inhalation triggers dissociation of VE-PTP from VE-cadherin in the lung, thereby promoting PMN recruitment [148]. Moreover, mice engineered to stabilize VE-cadherin through a fusion construct with α-catenin were found to have a significant reduction in LPS-induced PMN recruitment into the airspace, confirming a role for VE-cadherin junctional disassembly in PMN diapedesis from pulmonary vessels [149].
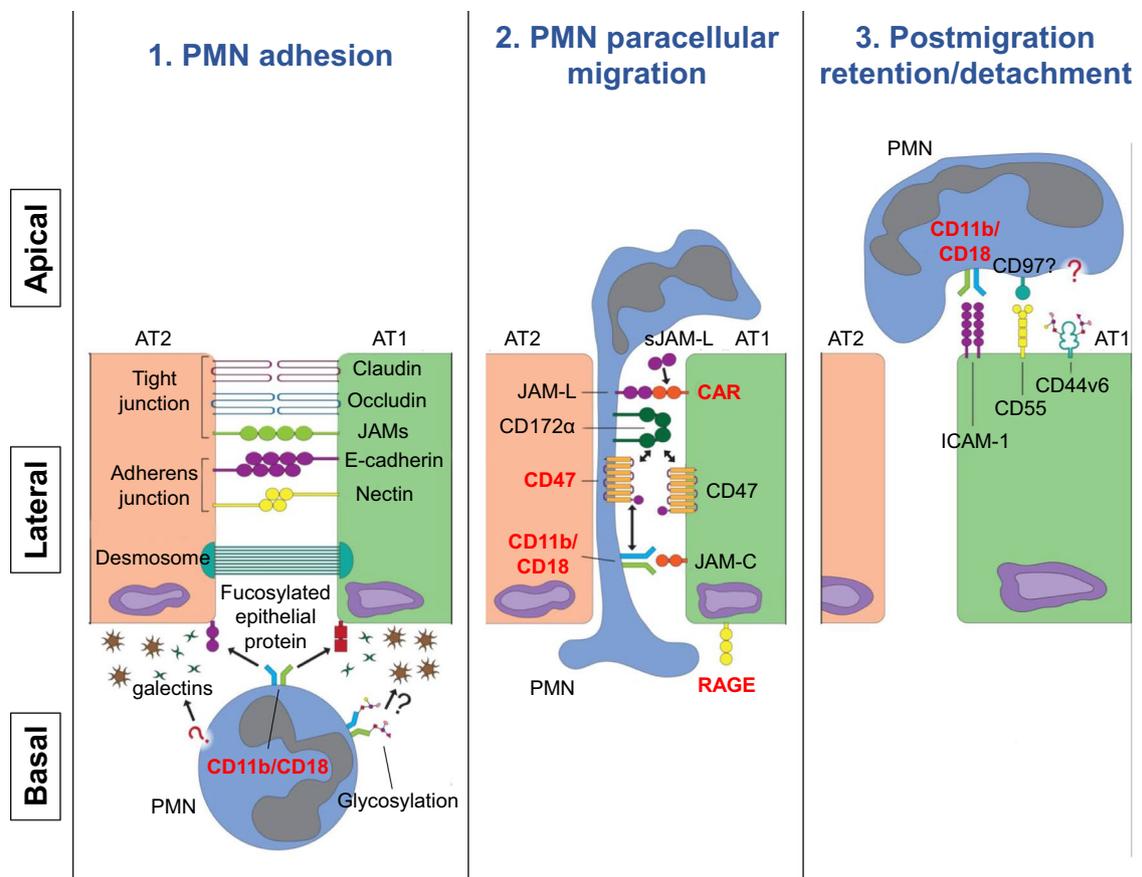
Fig. 1 Transpithelial migration of neutrophils into the airspace. The transepithelial passage of neutrophils (PMNs) from the pulmonary interstitium into the airspace involves the sequential steps of adhesion, paracellular migration, and post-migration adhesion/detachment, as illustrated. Initial adhesive events of PMNs with the basal aspect of epithelial cells may be regulated by PMN CD11b/CD18 interactions with fucosylated proteins and other poorly characterized molecules. Paracellular migration involves serial interactions of PMNs with epithelial junctional proteins, as shown. Finally, apical adhesion is thought to regulate PMN antimicrobial and migratory functions in the airspace and, potentially, to provide positive feedback signals that augment further PMN entry into the paracellular space. The degree to which specific epithelial adhesion proteins are presented by alveolar epithelial type 1 (AT1) vs. AT2 cells is unclear. As discussed in the text, the contribution of some of the adhesion molecules depicted to PMN transepithelial migration in the lung has been largely extrapolated from studies conducted in intestinal systems. Molecules shown in the red font have been confirmed to regulate PMN transepithelial migration in the lungs

Table 3 Adhesion molecules that regulate neutrophil transepithelial migration

	Transepithelial migration									
	PMN	Lung epithelium	PMN-epithelium interaction site	Model	Ref	PMN	Intestinal epithelium	PMN-epithelium interaction site	Model	Ref
CD11a/CD18 (LFA-1)	+/–	a	ND	Cell line, primary cell	[151–153, 320]	–	a	ND	Cell line	[321, 322]
CD11b/CD18 (Mac-1)	+	a	Basal, lateral	Cell line, primary cell	[150–152, 320, 322]	+	a	Basal, lateral, apical	Cell line, intestinal loop	[179, 321, 322]
CD11c/CD18 (p150/95)	+/–	a	ND	Cell line, primary cell	[151, 152, 320]	–	a	ND	Cell line	[321]
CD18-independent integrins	+*	a	ND	Cell line, lung inflammation	[111, 323]	+	a	Basal, lateral	Cell line	[323, 324]
CD44	+	?	ND	Cell-free system, pneumonia, lung injury	[157–159, 325, 326]	+	–	ND	Cell-free system, cell line	[327]
CD44v6	a	?	Expressed basolaterally	Pneumonia	[325, 326, 328]	a	+	Apical	Cell line, intestinal loop	[191, 192]
CD47	+	?	ND	Pneumonia	[114, 329]	+	+/–	Lateral	Cell line, intestinal loop	[160–162]
ICAM-1 (CD54)	?	+	ND	Cell line, primary cell	[25, 152, 292, 320, 330, 331]	?	+	Apical	Cell line, intestinal loop	[190, 292]
CD55	+	+	ND	Cell-free system, cell line	[294–296, 332]	+	+	Apical	Cell-free system, cell line	[193, 294–296, 333]
CD172α (SIRPα)	?	a	ND		[25]	+	a	Lateral	Cell line	[161]
JAM-A (CD321)	?	+	ND	Lung injury	[130, 308]	–	+/–	Interaction-independent	Cell line, intestinal loop, peritonitis	[179, 180, 308]
JAM-C (CD323)	a	?	ND		[175]	+	+	Lateral	Cell line	[181]
TREM-1 (CD354)	+	?	ND	Primary cell, pneumonia	[163, 334, 335]	?	a	ND	Cell line	[163, 336]
JAM-L	?	a	ND		[337]	+	a	Lateral	Cell line, intestinal loop	[166, 182, 338]
CAR	a	+	Lateral	Primary cell, lung inflammation	[176]	a	+	Lateral	Cell line	[182]
RAGE	?	?	Expressed basally		[184–186, 313]	?	+	Lateral	Cell line	[184, 313]
EMP2	a	+	ND	Cell line, lung inflammation, pneumonia	[194]	a	a	ND		
Carbohydrate	?	?	ND		[171, 172, 339]	+	+	Basal, lateral, apical	Cell line, intestinal loop	[169, 192, 340–343]

+ molecule expressed, regulates transmigration, – molecule expressed, does not regulate transmigration, ? molecule expressed, role undefined, *ND* not determined

*CD29/CD49 [111]

a Denote no or limited available evidence for expression in the indicated cell type

Transepithelial migration (TEpM)

Neutrophil adhesion molecules and surface receptors that modulate TEpM

After passage through the interstitium, PMNs arrive at the subepithelial zone, where TEpM commences. This process, comprised of the three sequential steps of epithelial adhesion, paracellular migration, and postmigration retention/detachment, involves multiple engagements between PMN integrins and their counterpart adhesion molecules on epithelial cells (Fig. 1; Table 3). CD11b/CD18 (Mac-1) appears to be the predominant β2 integrin regulating PMN transit in most cases of TEpM. Antibody blockade of CD11b or CD18 efficiently inhibits PMN adhesion or transmigration across airway epithelium [150–152]. In a recent study in which PMNs were fractionated from different compartments of lung during inflammation, peak surface expression of CD11b was found on interstitial PMNs, with a decline upon PMN entry into the airway, suggesting a role for CD11b in support of PMN TEpM [25]. In contrast to Mac-1, CD11a/CD18 (LFA-1) has been shown in a few reports to play a role in PMN adherence to cytokine-stimulated or virus-infected bronchial or nasal airway epithelial cells [152, 153]. However, this LFA-1-mediated PMN attachment may impact epithelial cell injury more than PMN TEpM [153]. Additionally, as discussed earlier, lung-specific PMN recruitment can also be regulated through CD18-independent mechanisms [104, 108, 154], and β1 integrin CD29 in conjunction with various forms of CD49 in this regard has been recognized as mediating PMN trafficking from the interstitium into alveolus in response to select exposures [111].

Non-integrin adhesion molecules expressed on PMNs such as CD44, CD47, CD172α (signal regulatory protein, SIRPα), Junctional Adhesion Molecule-Like protein (JAM-L), as well as surface receptors such as triggering receptor expressed on myeloid cells-1 (TREM-1) have also been identified as key regulators of TEpM. These proteins are discussed individually below and also in Table 3.

CD44 is a transmembrane glycoprotein that serves as a receptor for multiple ligands including hyaluronan, osteopontin, and MMPs, mediating cellular adhesion and mobility. Interestingly, CD44 was shown to interact with E-selectin [155] and is required for PMNs to adhere or migrate through endothelium [156]. On the other hand, in models of pneumonia and lung injury, the presence of CD44 was shown to impair PMN transit from parenchyma to the bronchoalveolar space, resulting in diminished BAL neutrophilia [157–159]. This suggests that CD44 may be a negative regulator of PMN migration across lung epithelium.

CD47 is a transmembrane immunoglobulin superfamily member expressed on diverse cell types, including PMNs and epithelia. Although perhaps best-known as a self-recognition marker that protects host cells from phagocytic clearance, CD47 has also been shown to participate in PMN trafficking to the lung. Experimental evidence to support this notion showed that CD47 deletion on PMNs significantly reduced their recruitment into pneumonic lung tissues [114]. However, the molecular mechanisms underlying CD47 regulation of TEpM have thus far largely been investigated in the setting of the intestinal epithelium. Preincubation of PMNs with anti-CD47 blocking antibodies was demonstrated to reduce the rate of PMN crossing of colonic epithelium, in part through changes in protein tyrosine phosphorylation, an event downstream of CD47 ligation [160]. In addition, a *cis* interaction between CD47 and its ligand CD172α on the plasma membrane of PMNs was found to be critical to CD47-mediated PMN migration across epithelial monolayers [161]. In addition to CD172α, a recent report showed that PMN CD47 could also associate directly with Mac-1 to facilitate TEpM through activation of integrin adhesive function [162].

Despite its status as a cognate ligand for CD47, studies suggest that CD172α has a role in PMN TEpM somewhat distinct from that of CD47. Unlike the ubiquitous expression of CD47, CD172α is primarily restricted to myeloid cells and is detectable on nearly all lung-recruited PMNs with a further increase in expression on interstitial and alveolar PMNs [25]. Although it has long been believed that CD172α on PMNs executes its role in TEpM through simple *trans* interactions with epithelial CD47, a recent study has challenged this. It was shown in an in vitro model that functional blockade of CD172α attenuates PMN TEpM, whereas antagonism of epithelial CD47 delays TEpM, suggesting distinct mechanisms [160, 161]. Moreover, a recent study clearly demonstrated that epithelial CD47 is not required for PMN TEpM in vivo; recruitment of PMNs was normal in mice with CD47 exclusively deleted in the intestinal epithelium [162]. Evidence from both studies indicates that CD172α may have other as yet unclarified functions to control PMN TEpM through mechanism(s) independent of engagement of epithelial CD47.

TREM-1 is another immunoglobulin superfamily member displayed on the surface of PMNs, monocytes, and macrophages. In addition to its originally identified function as an amplifier of pro-inflammatory signaling by pattern recognition receptors, TREM-1 has been shown to be required for PMN migration through respiratory epithelium [163]. Mechanistically, TREM-1 supports NOX2-dependent superoxide production through regulation of the PI3K/AKT signaling pathway, and PMN-derived reactive oxygen species (ROS) support PMN mobility [164]. ROS-supported PMN TEpM has also been demonstrated in both in vitro transmigration assays using human A549 lung epithelial cells and in vivo murine models, where inhibition of ROS significantly

reduced leukotriene B4-mediated PMN trafficking into the airway [165]. However, direct evidence to link TREM-1 to ROS-mediated PMN TEpM is still lacking.

Although additional PMN adhesion molecules have been shown to regulate PMN TEpM, most of these others have been verified in only intestinal and not pulmonary model systems to date. An example, mentioned above, is JAM-L. Interestingly, during TEpM, in addition to PMN membrane-bound JAM-L, cleavage of JAM-L from the PMN surface is reported to create a soluble form (sJAM-L) that enables binding to coxsackie adenovirus receptor (CAR) on the tight junction of epithelial cells. This interaction opens up CAR-dependent epithelial cell-to-cell contact that is believed to facilitate PMN influx across the epithelial barrier to the intestinal lumen [166].

Epithelial adhesion molecules and their regulators that modulate TEpM

The journey of PMNs from the basolateral side of epithelial cells, ultimately into the airspace lumen, relies upon multiple, sequential specific adhesive interactions of PMN adhesion molecules with cognate ligands that are localized to restricted regions of the epithelial plasma membrane, as displayed in Fig. 1.

PMN adhesion

PMN-expressed CD11b/CD18 (Mac-1) is thought to regulate all three steps of TEpM (adhesion, paracellular migration, and postmigration retention/detachment) through engagement with a series of ligands localized to restricted regions of the epithelial membrane. However, despite the documentation of > 30 Mac-1 ligands to date [167, 168], little is known about the identity of the specific Mac-1 ligands expressed on the basolateral aspect of respiratory epithelial cells that impact PMN-epithelial interactions during TEpM. It has been shown that the extracellular domain of CD11b has unique lectin-like structures that allow for its association with epithelial cell surface glycan, in addition to engaging epithelial ligands through protein–protein interactions. In studies of the intestine, Zen et al. identified fucoidin as one of a few carbohydrates that can directly bind to Mac-1 and inhibit T84 intestinal epithelial cell adherence to immobilized Mac-1 isolated from human PMNs. Treatment of T84 cells with fucosidase or a proteoglycan biosynthesis inhibitor resulted in a significant reduction of epithelial adhesion, suggesting that fucosylated proteoglycans serve as epithelial ligands that interact with Mac-1 in mediating PMN adhesion [169]. Additionally, epithelial galectins, a class of animal lectin proteins with an affinity for β-galactose-containing saccharides, have been proposed to play a role in carbohydrate-dependent PMN adhesion [170]. Along these lines,

expression of galectins-1, 3, or 9 is induced in the respiratory epithelium during infection/inflammation, correlating with increased PMN infiltration into the lung [171, 172]. Moreover, human PMN Mac-1 is highly decorated with carbohydrate moieties such as sialyl Lewis X [173], which contains a terminal β-galactose glycan that can be recognized by galectins.

PMN paracellular migration

Following initial adherence to the epithelial surface, PMNs continue their journey by entering the space between epithelial cells, where they interact with various lateral membrane adhesion molecules to facilitate their apical movement. One such family of laterally expressed adhesion proteins are the JAMs. JAMs belong to the cortical thymocyte marker for Xenopus family, which comprises classical members JAM-A, JAM-B, and JAM-C as well as subgroup members such as JAM-4, JAM-L, CAR, CAR-like membrane protein (CLMP), and endothelial cell-selective adhesion molecule (ESAM). Of these, the lung epithelium primarily expresses JAM-A, JAM-C, CAR, and CLMP [174–178]. The tight junction-associated protein JAM-A has recently been implicated in the regulation of PMN TEpM. Using a murine in vivo vascularized proximal colonic segment model, Flemming et al. showed that intestinal epithelium-targeted deletion of JAM-A enhanced intestinal permeability and simultaneously impaired PMN trafficking across colonic epithelium [179]. Intriguingly, the mechanism by which JAM-A mediates PMN TEpM may be β2 integrin-independent, as JAM-A is a ligand for LFA-1 [129] but not Mac-1, and there is no clear evidence, to the authors' knowledge, that LFA-1 plays a role in PMN TEpM. More recently, Luissint et al. provided evidence that the reduction of PMN transmigration into the colonic lumen in JAM-A-deficient mice might be due to increased gut permeability that impairs macrophage-dependent PMN recruitment [180]. In an acute lung injury model, JAM-A deficient mice also had diminished PMN recruitment into the airspace with increased interstitial PMN accumulation, suggesting a similar role for JAM-A in mediating PMN transmigration across the epithelium in the lungs as that observed in the intestines [130]. In contrast to JAM-A, JAM-C serves as a component of desmosomes at the lateral side of intestinal epithelial cells, where it regulates PMN TEpM through direct binding to PMN Mac-1 [181]. JAM-C has also been shown to regulate monocyte migration across influenza A virus-infected murine primary alveolar epithelial cells (AECs) [175]. Although not yet formally demonstrated to our knowledge, a similar role for JAM-C in PMN TEpM appears likely. As discussed above, CAR, another tight junction epithelial protein, enables PMN TEpM via interaction with JAM-L expressed on PMNs or sJAM-L.

CAR-dependent PMN TEpM has been documented in both the intestinal and respiratory systems [176, 182].

The receptor for advanced glycation end products (RAGE), a member of the immunoglobulin superfamily that can bind to Mac-1 [183] among other ligands, may also have a role in PMN TEpM in the lungs. In addition to its role as a pattern recognition receptor in the innate immune response, RAGE has been reported to be a ligand for β2 integrins, suggesting potential for direct roles in leukocyte recruitment. Under inflammatory conditions, RAGE is upregulated and localizes to the lateral membrane of intestinal epithelial cells near apical adherence junctions. Functional blockade of RAGE inhibits T84 cell adhesion to Mac-1 and prevents PMNs from crossing T84 epithelial monolayers, suggesting RAGE may serve as an epithelial adhesive ligand for PMN Mac-1 during TEpM [184]. Given the cellular location of RAGE at the lateral aspect of epithelial cells, but close to apical junctions, its function is likely more similar to JAM-C and CAR (in regulating PMN paracellular migration) than to that of proteins that support initial PMN adhesion. However, in the lungs, RAGE expression is prominent in alveolar epithelial type 1 (AT1) cells and has been reported to predominate on basal membranes of AT1 cells [185, 186]. Anti-RAGE antibody treatment or deletion of RAGE attenuates PMN recruitment and accumulation in the lung parenchyma and airspace [187–189]. Further studies are needed to clarify to what extent this arises from defective PMN TEpM, as RAGE possesses additional immunoregulatory functions.

Postmigration retention/detachment

After migrating paracellularly, PMNs emerge at the apical epithelial surface. Their subsequent retention or detachment is determined by multiple adhesive interactions. ICAM-1, a member of the immunoglobulin superfamily, is one of the best-characterized ligands for β2 integrins. Proinflammatory stimuli upregulate ICAM-1 on the surface of tracheal, bronchial, and alveolar epithelial cells, where the expression appears to be restricted to the apical surface. Of interest, apical ICAM-1 has been shown not only to promote PMN adhesion and locomotion on the apical surface of epithelium, thereby supporting PMN antimicrobial function, but also to augment PMN TEpM [190]. The latter occurs through myosin light-chain kinase-dependent changes in epithelial permeability to PMN traffic, thus suggesting a feedforward mechanism for PMN TEpM.

An additional epithelial adhesion molecule that regulates PMN detachment from the apical side of epithelium is CD44v6, an alternative splice isoform of CD44 [191]. Although the details of how CD44v6 is regulated to engage with PMNs are unclear, it has been reported that post-translational glycosylation of CD44v6 with sialyl Lewis A is required for its interaction with PMNs [192]. In addition,

the decay-accelerating factor, CD55, is another anti-adhesive protein on the apical membrane of epithelium [193]. However, its counter-ligand on PMNs remains unidentified, to our knowledge.

A new epithelial regulator of PMN TEpM in the lung

In addition to the relative paucity of studies to date that have demonstrated roles for TEpM-regulatory epithelial adhesion molecules specifically in the lung, the question has remained of whether the expression of different adhesion molecules is coordinated in respiratory epithelium. Recently, our laboratory identified epithelial membrane protein 2 (EMP2), a tetraspan membrane protein highly expressed by AT1 cells, as a master regulator of PMN TEpM into the airspace [194]. Prior to our report, EMP2 had been identified as a lipid raft-resident protein that supports the recruitment of select integrins (α6β1, αvβ3), adhesion molecules (ICAM-1), and signaling proteins to lipid rafts, and that downregulates caveolins [195–197]. Despite its high expression in the lung, where it is expressed at the protein level in AT1 cells, but not AT2 cells nor alveolar macrophages [198], no function had been established for EMP2 in lung biology. We found that EMP2-null mice have reduced recruitment of PMNs into the airspace in response to inhalation of LPS and reduced neutrophilic lung injury during bacterial pneumonia. In the case of LPS, an excess of interstitial PMNs was found in EMP2-null mice, suggesting defective TEpM. AT1 cells from *Emp2*−/− mice had dysregulated surface display of multiple adhesion molecules (CD47, ICAM-1, β3 integrin). EMP2-silenced epithelial cell cultures recapitulated this altered display and were defective in supporting transmigration of PMNs. The defective adhesion protein display and TEpM of EMP2-deficient epithelia were both rescued by silencing of caveolin-2. Taken together, this suggests that EMP2 coordinates the proper display of multiple adhesion molecules that support PMN TEpM through repression of caveolin proteins in *trans*. Given the high expression of EMP2 in the lung and its potential tractability to inhaled therapeutics, these findings suggest exciting potential for EMP2 as a target in neutrophilic lung disease.

Considerations for intervening upon PMNs in acute and chronic lung disease

The canonical, and, presumably, the evolutionary function of PMN recruitment to the lung is pathogen killing during an acute respiratory infection. In this context, PMN-derived proteases, oxidants, antimicrobial peptides, and extracellular traps contribute to the clearance of bacteria from the alveolus. Of interest, although cell depletion studies have confirmed a critical role for PMNs in host survival during

bacterial pneumonia [199], increased PMN recruitment to the lungs during pneumonia can in some instances be harmful. Thus, mice null for the transcription factor p53, the lipid transporter ABCG1, the cytokine IL-10, or the phosphatase PTEN all have increased pulmonary neutrophilia during Gram-negative bacterial pneumonia; despite enhanced bacterial clearance from the lung, these animals all exhibit increased mortality likely due to exacerbated lung injury [200–203]. Conversely, mice with myeloid deficiency of PTEN have reduced PMN recruitment to the lung during pneumococcal pneumonia with unaltered pathogen burden but improved survival [204]. Recently, our group reported that mice null for EMP2 have improved survival during bacterial pneumonia in the setting of a reduced transepithelial influx of PMNs into the airspace lumen; interestingly, at high bacterial inocula, this airspace PMN deficit was associated with improved pathogen clearance [194]. Others have also shown that PMNs may compromise pulmonary host defense in other contexts, via enhancement of *Pseudomonas* biofilm generation [205] and promotion of bacterial colonization in the lung [206].

Collectively, these studies indicate that PMN recruitment to the lungs during pneumonia may be dissociable from both pathogen clearance and host survival. This suggests that there may be an optimal magnitude or kinetics of pulmonary neutrophilia during infection and/or that qualitative features of pulmonary neutrophils, such as their intrapulmonary localization, local programming, function, or interactions with other cell types may be as important as PMN number. Further suggesting context-dependent roles for the PMN in lung infection, both beneficial and deleterious functions have been reported for PMNs during influenza pneumonia [207]. The degree and timing of neutrophilia, type of influenza virus, and age of the host may be key determinants [86, 208]. Emerging reports that PMN extracellular traps may drive pathogenesis in COVID-19 pneumonia also suggest mixed roles for the PMN in pulmonary defense against SARS-CoV-2 [209].

Although numerous publications have shown that PMNs can injure the lung in both acute and chronic settings, further complicating our expectations for this cell type are reports that PMNs also mediate lung repair. Thus, PMNs activate β-catenin-dependent epithelial repair in the LPS-challenged lung during their transit across the epithelium [210] and also promote re-epithelialization after acid-induced lung injury [211]. PMN-derived MMP9 plays an important role in repair following ventilator-induced lung injury, likely through the processing of extracellular matrix [212]. PMNs also participate in transcellular biosynthetic circuits that generate specialized pro-resolving lipid mediators [213] and suppress lung inflammation via transfer of miR-223 to pulmonary epithelial cells [214]. Apoptotic PMNs sequester chemokines during resolution of inflammation [215] and also program

efferocytic macrophages to resolve lung inflammation [216]. Indeed, PMN depletion reportedly exacerbates lung inflammation and injury induced by influenza virus [217]. Taken together, these reports suggest that PMNs are not monotonic with respect to either pulmonary host defense or lung injury, and that selective or even context-dependent strategies may thus be necessary to intervene upon them in lung disease. Layered on top of this consideration are reports that multiple PMN functions, including chemotaxis, respiratory burst, degranulation, and phagocytosis are dysregulated in chronic lung diseases such as COPD and bronchiectasis [218, 219].

Therapeutic avenues for controlling PMN traffic to the lungs: past and ongoing trials

Multiple therapeutic strategies for modulation of PMN traffic to the lungs have been tested to date, including inhibitors of adhesion molecules, chemokines, and intracellular signaling molecules, as discussed in the sections that follow. It is important to note that few of these strategies have been lung-selective or even PMN-selective and that inhibition of PMN trafficking to the lungs, as with other tissues, may carry untoward effects on host defense. In addition, in many cases, the full collateral impact of these strategies on the antimicrobial functions of PMNs remains to be defined.

Targeting adhesion molecules needed for PMN entry into lungs

Given the crucial role of adhesion molecules in the regulation of PMN migration to inflamed tissues, inhibition of these molecules holds promise as a strategy for curtailing the exacerbated inflammation in neutrophilic acute/chronic lung diseases. As discussed above, adhesion molecules that facilitate PMN recruitment to the lungs/airways include those on (1) PMNs—L-selectin, β2 integrin, α4 integrin; (2) endothelium—E-selectin, P-selectin, ICAM-1, VCAM-1, PECAM-1; (3) epithelium—CD47, ICAM-1, β3 integrin; and (4) platelets—P-selectin. Deficiency of these molecules in humans or mice has been reported to attenuate PMN migration, supporting the potential of these molecules as drug targets. Select adhesion molecules have been tested as therapeutic targets to date, with mixed results.

The therapeutic potential of an inhaled pan-selectin antagonist, TBC-1269 (Bimosiamose), was investigated in patients with COPD and in an ozone-induced human airway inflammation model. The individuals receiving the treatment showed slightly reduced airway inflammation (sputum IL-8, MMP9). However, this marginal effect was mainly attributed to a reduction in recruited macrophages, not PMNs [220, 221]. Additional selectin antagonists, including the pan-selectin inhibitor GMI-1710 (Rivipansel) as well as a

humanized monoclonal antibody against P-selectin SelG1 (Crizanlizumab) and E-selectin-specific inhibitors GMI-1271 (Uproleselan) and GMI-1687 have been/are being examined in trials for diseases such as sickle cell disease or hematological malignancies (multiple myeloma, acute myeloid leukemia). Whether they can be applied to respiratory diseases needs further investigation.

Although integrin and ICAM-1 antagonists have mostly been investigated in non-respiratory diseases, these agents may have the potential for repurposing for lung inflammation. Efalizumab (Raptiva) and Natalizumab (Tysabri) are humanized monoclonal antibodies against β2 and α4 integrins on PMNs. Unfortunately, clinical trials of these two drugs in psoriasis, multiple sclerosis, or Crohn's disease were terminated early due to reports of progressive multifocal leukoencephalopathy [222]. In contrast to the anti-integrin agents, ICAM-1-targeting biologics including the monoclonal antibody BI-505 (Bersanlimab) and antisense oligodeoxynucleotide ISIS 2302 (Alicaforsen) appear to have an improved safety profile. Nonetheless, Bersanlimab was found to be ineffective in multiple myeloma [223], and alicaforsen did not deliver a positive result in Crohn's disease [224, 225]. A modified version of alicaforsen formulated for topical application that was thought promising for ulcerative colitis also failed to meet the primary endpoint in recent trials [226–228]. A new line of a human antibody, MSH-TP15, was recently developed to target ICAM-1 [229]; further confirmation of its safety and efficacy in clinical practice is awaited.

While, to date, most anti-adhesion molecule strategies have been designed with the PMN and/or endothelium in mind, strategies targeting the pulmonary epithelium (i.e., TEpM) carry promise for a lung-selective effect. Along these lines, as discussed above, our laboratory recently reported that EMP2, a protein highly expressed by the alveolar epithelium, regulates epithelial surface display of multiple adhesion proteins. Downregulation of EMP2 reduced PMN TEpM into the inflamed airspace without an untoward effect on antibacterial host defense [194]. Our preliminary study also showed that airway delivery of an anti-EMP2 biologic reduced airspace neutrophilia induced by LPS in wild-type mice. This finding suggests the exciting potential for lung-directed (i.e., inhaled) anti-EMP2 therapeutics in neutrophilic lung disease.

Targeting chemoattractant receptors needed for PMN entry into lungs

An oral CXCR2 antagonist, AZD-8309, developed for the treatment of COPD was tested in healthy individuals challenged with inhaled LPS. Despite an initial positive result showing a ~ 80% reduction in sputum PMNs compared to placebo as well as decreased PMN elastase activity [230],

the development of this drug was reportedly discontinued in 2007.

AZD-5069 is a selective CXCR2 antagonist that was shown to be well tolerated and without overt increased risk for infection. Its efficacy was investigated in trials for moderate-to-severe COPD, bronchiectasis, and severe asthma. A significant decrease in PMN counts in sputum or lung tissues was observed; however, the diminished neutrophilia did not translate into improved disease outcomes [231–234].

Another selective and reversible CXCR2 antagonist, GSK1325756 (Danirixin), has also been tested in patients with COPD. However, no clear benefit was observed with treatment and an increase in pneumonia occurrence was observed in participants receiving the highest dose [235]. GSK1325756 was also tested for safety and efficacy in influenza when co-administered with oseltamivir, a standard antiviral therapy. No severe adverse events were reported, but the efficacy data was inconclusive due to the limited number of participants in the study [236, 237].

SB-656933 (Elubrixin) is another compound developed to target CXCR2 that has shown inhibition of airway neutrophilia induced by ozone challenge in healthy volunteers or in subjects with cystic fibrosis (CF) [238, 239]. However, whether this agent will improve lung function and clinical symptoms remains to be demonstrated.

Among all CXCR2 antagonists examined in clinical trials, SCH527123 (MK-7123; Navarixin) is one of a few which also targets CXCR1 and has been shown to impact disease outcomes. Treatment with SCH527123 at a dose of 50 mg in COPD patients for 6 months decreased airway neutrophilia and resulted in a significant improvement in lung function (forced expiratory volume in 1 s [FEV1]) compared to placebo. Of note, the beneficial effect was only seen in active smokers [240]. The same dose of SCH527123 administered to patients with severe asthma also reduced airway PMNs and reduced asthma exacerbations [241]. However, severe neutropenia was observed in both trials, and the development of this drug for lung diseases was evidently terminated.

Formyl peptide receptors (FPRs), in particular, FPR1 and FPR2, are another family of G protein-coupled receptors expressed on PMNs that induce PMN chemotaxis, among other cellular functions. FPRs recognize *N*-formylated peptides derived from bacterial or mitochondrial proteins during acute infection or tissue injury. An elevated level of mitochondrial-derived formyl peptides with FPR activity has been detected in bronchoalveolar lavage fluid and serum of ARDS patients [242]. Moreover, recent work has suggested FPR1-mediated PMN homing to injured lung may also be involved in the development of pulmonary fibrosis [243]. These data suggest that targeting FPRs might be an attractive approach to manage neutrophilic lung diseases. Several natural and synthetic compounds with FPR antagonism have been reported [244, 245]. However, thus far, these

antagonists have been tested only in preclinical models; further investigation is necessary to explore their potential for clinical use.

After satisfactory results in phase Ib trials, the LTB4 receptor antagonist BIIL 284 BS was tested in a randomized, double blind, multicenter, placebo-controlled phase II trial of pediatric and adult patients with CF [246]. Unfortunately, the trial was terminated early when a planned interim analysis revealed a significant increase in pulmonary-related serious adverse events in adults receiving the drug, in particular, increased respiratory signs and/or symptoms associated with pulmonary exacerbation. A study of the agent in a mouse model of *P. aeruginosa* lung infection confirmed reduced pulmonary PMNs but higher bacterial overgrowth and bacteremia [247].

Targeting PMN migration through interventions on PMN-intrinsic signal transduction

Phosphodiesterase 4 (PDE4) is a cytoplasmic enzyme that catalyzes the hydrolysis of cAMP and thereby regulates PMN migration and other functions (chemokine/cytokine synthesis, ROS release, degranulation). Inhibiting PDE4 with the small molecule roflumilast was recently shown to reduce PMN chemotaxis to CXCL1 and LTB4 through activation of EPAC1 and Rap1 in a cAMP-dependent manner [248, 249]. These findings mechanistically support the use of oral roflumilast, an agent which was approved by the FDA in 2011 for the treatment of COPD, particularly for patients with symptoms of bronchitis and frequent exacerbations. It was shown in these patients that roflumilast effectively suppresses sputum neutrophilia, improves FEV1, and reduces exacerbation rate. A series of additional PDE4 inhibitors designed for inhaled delivery have since been developed, and some have advanced into clinical trials [250]. Among these, CHF6001 has shown promising anti-inflammatory effects in COPD [251] and in atopic asthmatics, where it resulted in attenuation of the late asthmatic response in patients after allergen challenge [252]. Importantly, given the local delivery of CHF6001 to lungs, the observed drug effects may be more restricted [253], thus minimizing side effects caused by systemic agents.

Phosphoinositide 3-kinases (PI3Ks) are lipid kinases that regulate several core cellular functions, including proliferation, differentiation, survival, metabolism, and motility. As discussed above, PI3Ks have been shown to play an important role in PMN chemotaxis. Several studies have shown potential for preclinical PI3K pan-inhibitors and PI3Kδ inhibitors to reduce airspace neutrophilia and other inflammatory measures in mouse models of allergic asthma [254]. A recent study showed that an inhaled prodrug PI3K inhibitor, CL27c, reduced airway inflammation, including airspace neutrophilia in a mouse model of asthma and also reduced

neutrophilic inflammation and lung fibrosis induced by bleomycin [255]. Of interest, impaired PMN directional migration in healthy older adults, a potential cause of increased bystander tissue damage, has been linked to increased PI3K signaling and shown to be correctable by inhibition of PI3Kγ or PI3Kδ [256]; a similar correctable deficit in directional chemotaxis was also noted in PMNs from COPD patients [218]. Although these studies suggest the potential for PI3K inhibition as a strategy for modulating or optimizing PMN traffic to the lung in humans, it remains unclear whether the pleiotropic actions of PI3K inhibitors will complicate their development as lung disease therapeutics.

Targeting PMN migration with statins

HMG-CoA reductase inhibitors (i.e., 'statins') are widely used for cardiovascular disease prevention and treatment. In addition to their well-known cholesterol-reducing effects, statins also possess broad-spanning effects on inflammation and have been shown to inhibit PMN migration to the lung in mice and humans [257–261]. Despite their therapeutic potential in neutrophilic lung disease, negative results were obtained in two large-scale randomized controlled trials in ARDS: HARP-2 and SAILS [262, 263]. More recent post-hoc analyses of these trials found that statin therapy provided a survival benefit in the subgroup of ARDS patients with hyperinflammatory features [264]. Statins have also shown mixed results in studies of COPD [265], but may benefit a subset of COPD patients with cardiovascular disease or exhibiting a high level of C-reactive protein, a marker for systemic inflammation [263, 266, 267]. It has been shown, somewhat paradoxically, that simvastatin may correct the deficient directional PMN migration observed in patients with COPD [265] and also in healthy, older human subjects, especially during infections such as pneumonia [268, 269]. Statins have also been shown to potentially augment the release of antibacterial PMN extracellular traps [270]. Collectively, these studies indicate a potential for statins as PMN-targeting agents in human lung disease, but suggest that patient subphenotyping, potentially involving biomarkers and/or studies of PMN function, may be required to optimize the selection of patients.

Targeting PMN retention in inflamed lungs through the CXCR4/CXCL12 axis

CXCR4/CXCL12 is a well-defined signaling axis that regulates PMN retention in the bone marrow. Administration of granulocyte colony-stimulating factor (G-CSF), which reduces CXCR4 expression on PMNs, or the CXCR4 antagonist, AMD3100 (plerixafor), to disrupt the engagement between CXCR4 and CXCL12 on bone marrow stromal cells, results in rapid release of PMNs from the bone

marrow to the circulation. Interestingly, growing evidence suggests that CXCR4/CXCL12 may also regulate PMN activities within the lungs. In the setting of lung infection/injury, extravascular PMNs reportedly regain CXCR4 surface expression, allowing them to accumulate in inflamed areas where there is increased expression of CXCL12 [271]. Blockade of this pathway suppresses PMN accumulation, perhaps partially due to enhancement of PMN 'reverse transmigration' [271, 272]. High CXCR4 presentation has been reported on lung-infiltrated PMNs from several unresolved human chronic lung inflammatory diseases [273]. Application of AMD3100 locally in diseased lungs to intervene upon CXCR4/CXCL12 may thus have the potential for alleviating PMN retention during resolution of inflammatory lung diseases.

Other strategies

Less selective anti-inflammatory strategies that impact PMN migration to the lung, including macrolides and anticytokine biologics have also been tested [274]. In addition to strategies for modulating PMN trafficking to the lung, several PMN effector molecules have also been targeted over the years in an effort to mitigate neutrophil-mediated inflammation in the lungs and other organs. Examples include PMN elastase inhibitors, myeloperoxidase inhibitors, and MMP inhibitors [274, 275]. Due to their robust infiltration of the inflamed lung, PMNs have themselves also been tested in preclinical models as vehicles for nanoparticle drug delivery to the lung [276].

A final complication for therapeutic development that is worth noting is that PMNs can undergo marked functional and phenotypic changes after recruitment to the airspace. This is particularly well documented in the CF airway, where PMNs exhibit enhanced degranulation and oxidant generation (potentially exacerbating lung injury) but impaired bacterial killing [277]. This PMN phenotype, recently coined 'GRIM' (granule releasing, immunomodulatory, metabolically active), can be mimicked in vitro by inducing PMN TEpM with CF airway secretions [278]. Tractable model systems such as this carry promise for identifying the molecular component(s) of CF sputum that reprogram airspace PMNs as well as for facilitating therapeutic screens.

Conclusions

The lung has unique anatomic features that reflect the mandates of gas exchange, in particular, a highly complex, redundant, narrow-caliber capillary network that is separated by just one cell layer (the alveolar epithelium) from the environment. Perhaps as a consequence, the lung shares a unique relationship with the circulating PMN. In health, PMNs, in

numbers that far exceed that in the peripheral circulation, reside within the lung vasculature, patrolling the extensive endovascular surface for bacteria and then completing their life cycle. During infection, rapid and robust recruitment of PMNs through the pulmonary endothelium and then epithelium into the airspace needs to be balanced with the risk of collateral PMN-mediated tissue damage. Although several molecular checkpoints at the level of the endothelium and epithelium have been identified in the PMNs journey to the airspace, fundamental questions remain for the field (see "Box: outstanding questions"). Layered on top of this, emerging studies that have challenged the traditional monolithic view of the PMN as a simple vector of degradative cargo suggest that a more nuanced understanding of the PMN in pulmonary homeostasis will be required. Taken together, these studies suggest, in principle, the exciting opportunity to manipulate both the intrapulmonary localization and local programming of the PMN to therapeutic benefit. In upcoming years, it is expected that research questions centered on the PMN and on the lung will reciprocally and fundamentally enrich our understanding of the biology of the other.

Box: outstanding questions

What are the local niche signals in the lung that sequentially program the tissue-compartmental localization and function of PMNs during pneumonia and its resolution?

Do pulmonary interstitial fibroblasts directly and/or indirectly regulate the transendothelial and transepithelial passage of PMNs in the lung?

Are there distinct subsets of PMNs that subserve destructive, antimicrobial, and repair roles in the lung?

How do intrapulmonary PMNs and the lung microbiota reciprocally interact during health and chronic lung disease?

Are there therapeutic strategies that can be leveraged to selectively intervene upon PMN traffic to the lung in isolation from other organs?

Are there molecular strategies that can be leveraged to selectively manipulate PMN number within the pulmonary intravascular, interstitial, and intra-alveolar compartments?

What is the relative importance of interstitial vs. intra-alveolar PMNs to host defense during pneumonia?

How does transepithelial traffic of PMNs impact intrapulmonary compartmentalization and clearance of pathogens during pneumonia?

What role does efferocytic clearance of apoptotic PMNs play in pathogen killing in the infected lung?

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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