Unique Structural Features That Influence Neutrophil Emigration Into the Lung

ALAN R. BURNS, C. WAYNE SMITH, AND DAVID C. WALKER

I. Introduction

The migration of neutrophils from blood into tissue at sites of inflammation has been modeled as a series of sequential adhesive steps proceeding from tethering on endothelium under shear conditions in postcapillary venules (244). This model has been investigated in a variety of vascular beds (147) and in vitro with monolayers of endothelial cells in parallel plate flow chambers (244). The tethering event in this model depends on adhesion molecules in the selectin family, E-selectin and P-selectin on the endothelium and L-selectin on the neutrophil, as well as ligands for the selectins expressed on both cell types. These adhesion molecules are necessary to efficiently initiate the cascade of adhesive steps ultimately leading to firm attachment of the neutrophils to the endothelium. Many of the molecular and functional aspects of this model have been recently reviewed and are not repeated here (144, 244).

In the lung, a principal site of leukocyte emigration in...
response to inflammation is the alveolar capillary bed. A distinctive characteristic of this bed is the complex interconnecting network of short capillary segments where the path from arteriole to venule crosses several alveolar walls (often >8) and contains often >50 capillary segments (9, 53, 102, 151, 248). Compared with blood in the large vessels of most vascular beds, the blood in this complex network contains ~50-fold more neutrophils and manyfold more lymphocytes and monocytes (56). Videomicroscopic study (82, 201) of these vessels revealed that the transit of neutrophils through this network required a median time of 26 s and mean time of 6.1 s. The increased transit time was due primarily to the time neutrophils spent stopped within this vascular network. In contrast, the transit times of red blood cells (RBC) ranged from 1.4 to 4.2 s. The longer time required for the neutrophils to pass through this bed apparently accounts for their increased concentration.

Recruitment of neutrophils into the lungs through the alveolar capillary network contrasts with the recruitment of neutrophils through postcapillary venules at sites of inflammation in a number of important ways. The tethering mechanisms required to capture neutrophils from flowing blood in larger vessels are apparently not necessary in the alveolar capillary bed. The diameters of spherical neutrophils (6–8 μm) are larger than the diameters of many capillary segments (2–15 μm), and ~50% of the capillary segments would thereby require neutrophils to change their shape to pass through (55, 82, 104, 166). Given the large number of capillary segments through which a neutrophil must pass (often >50), most neutrophils must change shape during transit from arteriole to venule. Morphometric analysis of neutrophils in the alveolar capillary beds revealed significant deviation from spherical shape (55, 82). Computational models of the capillary bed describing flow, hematocrit, pressure gradients, and the effects of deformation on the capillary transit times of neutrophils support the concept that the structure of the capillary bed and the deformation of neutrophils are critical under normal conditions (110).

During inflammation, much of the sequestration and infiltration occurs through vessels so narrow that physical trapping is sufficient to stop the flowing neutrophil (51, 53, 56, 61, 82, 99, 150, 231). A number of investigations have provided evidence that activation of neutrophils further contributes to the failure of transit through the alveolar capillary bed (63, 91, 92, 106, 115, 187, 194, 199, 278, 290), and changes in mechanical properties after activation may be of major importance. Binding of mediators such as chemotactic factors (e.g., the complement fragment C5a) to neutrophil receptors induces a transient resistance of the cells to deformation (27, 35, 60, 62, 69, 115, 289). Because neutrophils must deform to pass through the capillary bed, leukocyte activation by inflammatory mediators could effect further concentration of neutrophils at the alveolar walls (51, 52, 102, 115, 180, 289). The role of mechanical factors in the initial sequestration of neutrophils in the alveolar capillaries is supported by evidence that neither L-selectin nor β2-integrins are required (51, 64, 132). In contrast, both selectins and β2-integrins are required for the localization of neutrophils in postcapillary venules at sites of inflammation.

The events following the initial sequestration of neutrophils within alveolar capillary beds are apparently influenced by adhesion molecules. For example, simple systemic activation of neutrophils by intravenous injection of chemotactic factors [e.g., interleukin (IL)-8 or C5a] results in rapid (<1 min) neutropenia with massive sequestration of neutrophils within alveolar capillaries. This event is not dependent on L-selectin or β2-integrins, but the retention times within this capillary bed are influenced by these adhesion molecules (51, 132), and this adhesion is likely an interaction of leukocyte adhesion molecules and endothelial adhesion molecules. Blockade of the adhesive mechanism (e.g., using blocking monoclonal antibodies) results in release of neutrophils from the lungs (51, 58, 59, 64, 158). Mediator-induced decreases in deformability are temporally correlated with upregulation of adhesivity of β2-integrins (e.g., both occurring within ~1 min of exposure to IL-8), allowing both physical trapping and sticking to the vascular wall within the alveolar capillary bed. A similar phenomenon occurs in the liver where sequestration apparently does not depend on adhesion, but retention and liver injury are heavily dependent on β2-integrins (120).

Pulmonary infection or injury often results in pronounced emigration of neutrophils from these capillary beds into the alveolar space. Doerschuk et al. (54) demonstrated a number of years ago that this process can occur through at least two distinct pathways. One pathway requires β2-integrins, whereas the other does not, and the distinction depends on the stimulus (54, 96, 126, 137, 180, 185, 203, 205, 234). There is also evidence from studies in the peritoneal cavity that macrophages are essential for production of a stimulus that elicits β2-integrin-independent neutrophil emigration (173). Furthermore, even within the pathway requiring β2-integrins, 20–30% of the migration appears to be independent of β2-integrins. Studies of intercellular adhesion molecule (ICAM)-1, a major ligand for β2-integrins, reveal some interesting differences with regard to the stimuli inducing the pulmonary inflammation (32). In response to intratracheal Escherichia coli lipopolysaccharide (LPS), a stimulus that induces β2-integrin-dependent neutrophil emigration, ICAM-1 expression on capillary endothelial cells and alveolar type II cells was increased. In response to Streptococcus pneumoniae, a stimulus that induces β2-integrin-independent migration, ICAM-1 was only increased on alveolar type II cells but not on capillary endothelial cells.

The mechanisms accounting for these apparently dis-
Distinct pathways for neutrophil emigration from alveolar capillaries are poorly understood. In vitro studies of neutrophil locomotion indicate that different chemotactic factors may select different pathways. For example, neutrophil emigration through human umbilical vein endothelial cell monolayers is not blocked by antibodies to $\beta_2$-integrins if the stimulus is IL-8 or leukotriene (LT) B$_4$, but transendothelial migration in response to the chemotactic tripeptide $\{N$-formyl-methionyl-leucyl-phenylalanine (fMLF) $\}$ is $\beta_2$-integrin dependent (179). Clear evidence for such chemotactic factor selection in vivo has not been published.

The pathways recruited by different stimuli may also depend on the route of exposure to the alveolar capillary bed. For example, unilateral intrabronchial instillation of hydrochloric acid in rat lungs induces $\beta_2$-integrin-independent sequestration and emigration in alveolar capillaries. Neutrophil sequestration also occurs in the contralateral lung, and the retention of neutrophils there is $\beta_2$-integrin dependent (180). Intratracheal E. coli LPS results in $\beta_2$-integrin-dependent emigration, while intravenous administration results in sequestration that is $\beta_2$-integrin independent (54, 69).

II. ALVEOLAR WALL

A. Cellular Anatomy and Organization of the Alveolar Wall

Understanding how neutrophils move from alveolar capillary lumen to alveolar airspace requires that we know something about the anatomy and organization of the lung. For our purposes, the lung can be thought of as having two parts: the central airways (trachea, bronchi, and terminal bronchioles) and the peripheral parenchyma (respiratory bronchioles, alveolar ducts, and alveoli). In adult human lung, there are 300 million alveoli, and each alveolus is $\sim 0.25$ mm in diameter. The walls between alveoli have a single capillary network that is in contact with alveolar air on both sides. Each alveolus contains $\sim 1,000$ capillary segments, and the entire capillary network is connected to 300 million arterial end branches and an equal number of veins (103, 284). By transmission electron microscopy, a cross-sectional view of the alveolar wall (Fig. 1) reveals one side of the wall is relatively thin ($\leq 0.2 \mu$m in humans and even less in small mammals ($0.1 \mu$m]), consisting of an endothelium joined to an overlying epithelium through a shared basement membrane. The other side of the wall is much thicker and more variable in its dimensions depending on the nature of the interstitial components (extracellular matrix, fibroblasts, and less frequently mast cells and pericytes) that lie between the capillary endothelium and alveolar epithelium (284).

In humans, estimates of the alveolar surface area are impressive at 100 m$^2$ (nearly the size of a tennis court) with the capillary endothelial surface being smaller, by $\sim 10$–$20\%$, than the alveolar surface (284). Two types of epithelial cells cover the alveolar surface. Type I pneumocytes are squamous epithelial cells that send out extensive cytoplasmic sheets that penetrate pores of Kohn (holes within the alveolar wall connecting one alveolus to another) and cover portions of neighboring alveoli. Type II pneumocytes are cuboidal epithelial cells that synthesize and secrete the pulmonary surfactant lining the alveolus.
oliar surface (135). Type II cells also possess the capacity to proliferate and make more type II cells, or differentiate and replace injured type I pneumocytes (1). The cell volume of a squamous type I cell is approximately twice that of a type II cell, while its alveolar surface is 50 times that of a type II cell. Expressed as a percentage of the total number of cells within the alveolar wall, type I pneumocytes account for 8%, type II pneumocytes 16%, capillary endothelial cells 30%, interstitial cells 36%, and alveolar macrophages 10%. Hence, epithelium and endothelium make up about one-fourth each of the alveolar wall. Interestingly, the surface covered by one type I cell is 4,000–5,000 μm² compared with ~1,000 μm² for an endothelial cell, which means endothelial cells are ~4 times more numerous than epithelial cells (284).

**B. Extracellular Matrix of the Alveolar Wall**

The extracellular matrix (ECM) of the alveolar septum has two principle components: a basement membrane which underlies alveolar epithelial and capillary endothelial cells and an interstitial matrix which is maintained by fibroblasts. When viewed by electron microscopy (Fig. 1), the alveolar epithelial basement membrane (ABM) is organized as follows: immediately beneath the epithelium lies an electron translucent layer referred to as the lamina rara externa (LRE). Adjacent to this layer lies the basal lamina proper, which is referred to as the lamina densa (LD). The LD contains proteoglycans (perlecan, heparan sulfate, and chondroitin sulfate), fibronectin, entactin, laminin, and type IV collagen; the globular protein core of perlecan is embedded in the LD, while its heparan sulfate side chains are positioned within the LRE. Beneath the LD lies the lamina fibroreticularis (LF) in which type VII collagen-anchoring fibrils link the LD to the fibrillar (e.g., type III collagen) ECM. In general, the capillary endothelial basement membrane (CBM) is less compact than the ABM, and the layer lying between the capillary lamina densa and the endothelium is referred to as the lamina rara interna (LRI) (212, 223, 225, 264, 265).

Microdomains exist within the basement membrane as defined by marked differences in chemical composition. For example, at the ultrastructural level, there are distinct differences between the ABM and CBM in the distribution of electron-dense ruthenium red anionic sites. Based on selective enzyme degradation, ruthenium red appears to react with heparan sulfate proteoglycan, and the LRI of the adult rat lung has one-fifth the number of ruthenium red anionic binding sites compared with the LRE. Interestingly, this difference is not observed in neonatal rats; hence, the diminished reactivity of the LRI appears to be an age-acquired phenotype (26, 264, 265). In the ABM of adult rats and rabbits, differences in microdomains between type I and II cells have been reported. Specifically, there are significantly fewer sulfated molecules beneath type II cells (223). In vitro experiments suggest sulfation of basement membrane components is a critical determinant of type II cell responses to growth factors that regulate proliferation and differentiation (225).

It is generally accepted that alveolar epithelial and endothelial cells synthesize and maintain the basement membrane. However, in vitro studies suggest fibroblasts contribute to the process by reorganizing the collagen matrix or by supplying soluble factors (79). In vitro studies of neonatal lung mesenchymal cells suggest these cells may be the primary producers of entactin during lung development (228) and that extracellular entactin is fairly stable, a finding consistent with the absence of intracellular staining for entactin in adult rodent tissues (23).

The interstitial matrix of the lung is comprised of a fiber system (collagen and elastin) embedded within a proteoglycan gel (Fig. 1). Collagen fibers resist tensile stresses while elastin provides tissue elasticity and recoil. Collagen and elastin are embedded in a hydrated porous proteoglycan gel that resists compressive forces. While hoops of elastic fibers are prominent around alveolar openings and alveolar ducts (191, 212), they also form a reticulum surrounding and enclosing each alveolus (176). Type III fibrillar collagen is found within alveolar walls and appears aggregated at the entrance rings to alveolar ducts while the distribution of type I collagen is more irregular and less prominent (204).

**III. NEUTROPHIL MIGRATION ACROSS VASCULAR ENDOTHELIUM**

Much of what we know about neutrophil transendothelial migration comes from in vivo studies of postcapillary venules in the systemic circulation and from in vitro models of neutrophil trafficking using endothelial cells derived from large veins (e.g., umbilical veins). Hence, generalizations in the literature regarding neutrophil transendothelial migration may not be appropriate to the lung. The primary reason for this is because, as discussed in section 1, the principal site for neutrophil emigration in the lung is the alveolar capillary bed. What follows is an overview of issues surrounding our current understanding of neutrophil transendothelial migration. Whenever possible, data gathered from studies in the lung are presented and compared with data obtained in nonpulmonary tissues.

It is generally accepted that neutrophils migrate across the endothelium by penetrating junctions that lie within the intercellular cleft. This paradigm is based on studies of nonpulmonary tissues and originated with Julius Arnold (1875), who used the injection of cinnabar to demonstrate the borders of endothelial cells. He sug-
gested leukocytes move across blood vessel walls (diapedesis) by passing between endothelial cells at either points of dense staining, “stigmata,” or circles of stain, “stomata” (13). Nearly 100 years later, with increased resolution, transmission electron micrographs taken by Marchesi and Florey seemed to confirm Arnold’s observation that interendothelial junctions were the main sites through which neutrophils (and other leukocytes) pass from blood to tissue (162, 163). Since firmly adherent neutrophils can migrate quickly (<2 min) across the endothelium (29, 250), it has been suggested that endothelial junctions must open and close rapidly during the migration process (39).

Cytokine-activated human umbilical vein endothelial cell (HUVEC) monolayers are a commonly used in vitro model for studying neutrophil trafficking (29, 31, 50, 108, 245). In a manner similar to Arnold’s use of cinnabar (13), we have used silver staining to reveal the presence of endothelial borders as an aid in determining the site of neutrophil diapedesis (Fig. 2). We find that >70% of migrating neutrophils cross the endothelium at tricellular corners where the margins of three endothelial cells converge (29). Under physiological flow conditions (2 dyn/cm²), rolling neutrophils arrest (become firmly adherent) on cytokine-activated endothelium, and the distance an arrested neutrophil moves before it migrates across the endothelium is only 5.5 ± 0.70 μm (under static conditions in the absence of flow, the distance is similar 6.8 ± 0.9 μm) (89). This distance is less than one neutrophil cell diameter, suggesting the surface properties of endothelial borders and tricellular corners favor neutrophil arrest.

Using immunofluorescence microscopy, we (29) and others (18, 107) have noticed gaps (0.25–2 μm across) in endothelial border staining patterns for tight junction proteins (occludin, ZO-1, and ZO-2) at tricellular corners (Fig. 2). Assuming an upper gap value of 2 μm for each corner and 5 corners/endothelial cell, tricellular corners comprise <10% of the available border area. Because neutrophil transmigration on IL-1β-treated HUVEC monolayers occurs exclusively at cell borders, mathematical probability predicts that <10% of the neutrophils should migrate at tricellular corners if the process is random (29). This value is significantly less than observed values (~70%), suggesting neutrophil migration is not a random process. Moreover, a comparison across species (human, dog, and mouse) reveals the preference for tricellular corners is widespread (77, 64, and 47%, respectively). Although the percentage of neutrophils migrating at corners is significantly different between species (human > dog > mouse), in each case the number migrating at corners greatly exceeds that predicted by random chance (i.e., <10%) (33).

With respect to leukocyte emigration, ICAM-2 and P-selectin (CD62P) are the only adhesion molecules to show preferential expression on endothelial borders.

ICAM-2 (CD102) is constitutively expressed on vascular endothelial cells (45) and is a ligand for leukocyte β₂-integrins CD11a/CD18 and CD11b/CD18 (145, 249, 291). In vitro, ICAM-2 appears to play a role in neutrophil (116) and eosinophil (83) transendothelial migration. However, prolonged (24 h) exposure of HUVEC monolayers to IL-1β or tumor necrosis factor (TNF)-α decreases ICAM-2 surface expression by 50%, and its association with cell borders is lost (170). In our laboratory, loss of ICAM-2 from HUVEC borders is evident as early as 4 h after stimulation with IL-1β (Burns, unpublished observations). Hence, if ICAM-2 participates in neutrophil arrest on cell borders, its actions may be limited to the initial phase of an inflammatory response.

Does ICAM-2 play a role in neutrophil emigration in the lung? Based on light microscopic immunohistochemistry of mouse lung, Gerwin et al. (83) reported ICAM-2

![Fig. 2. Neutrophils migrate preferentially at tricellular corners where the borders of three endothelial cells converge.](image-url)
staining in alveolar capillaries and large pulmonary vessels. Using an ovalbumin model of allergic inflammation, they found that mice deficient in ICAM-2 showed prolonged eosinophil accumulation in the lung interstitium. Although this finding reveals an essential role for ICAM-2 in eosinophil emigration during allergic inflammation, similar studies with neutrophils are lacking and will require further investigation.

In vitro, using HUVEC monolayers, it is very clear that P-selectin can play an important role in recruiting neutrophils to endothelial borders (30). Although not expressed constitutively on the endothelial surface, P-selectin is stored in endothelial cytoplasmic granules known as Weibel-Palade bodies. Histamine and thrombin trigger rapid (minutes) fusion of Weibel-Palade bodies with the endothelial surface. This results in preferential P-selectin upregulation along endothelial borders where, under physiological flow conditions, it mediates neutrophil rolling on endothelial borders. Of significance to the lung, scanning electron microscopic observations of large pulmonary vessels show that after histamine infusion, leukocytes are captured along endothelial borders (30). This is consistent with intravital observations showing selectin-mediated leukocyte rolling in pulmonary arterioles and venules (134). However, neutrophils do not roll in capillaries (134), the principal site of neutrophil emigration, and current evidence does not favor a role for P-selectin-mediated capture in pulmonary capillaries. Using confocal microscopy, Yamaguchi et al. (292) report that P-selectin is absent from rat pulmonary microvessels. However, this result should be interpreted with caution since the level of P-selectin expression may be so low as to exceed the limit of immunohistochemical detection.

Weibel-Palade bodies have been observed in alveolar capillaries of rats (125) and humans (279), but they appear to be far more numerous in larger pulmonary vessels (236). Whether capillary Weibel-Palade bodies contain enough P-selectin that can be mobilized to the endothelial surface to influence neutrophil localization at borders remains to be determined.

With the exception of one report, precise information concerning the site of neutrophil migration across the alveolar capillary endothelium is lacking. Moreover, the conventional model of neutrophil tethering, rolling, and arrest on inflamed endothelium is not applicable to lung capillaries. Approximately 50% of the capillary segments are narrower than the mean diameter of a spherically shaped neutrophil. This size discrepancy forces neutrophils to deform and assume a “sausage” shape as they enter and squeeze through narrow capillary segments (55, 102) (Fig. 3). For this reason, in the pulmonary circulation, neutrophil tethering and rolling on inflamed endothelium is restricted to larger diameter pulmonary arterioles and venules (133, 134, 136). We have used scanning electron microscopy (SEM) to examine some of these larger vessels in a rabbit model of streptococcal pneumonia (Fig. 2). Of 28 neutrophils observed in the process of transmigration, 14 (50%) were observed crossing at tricellular corners, 7 (25%) at bicellular borders, and 7 (25%) were crossing transcytotically (i.e., through the endothelial cytoplasm) (33). Unfortunately, the small size and segmented nature of the capillary bed makes it difficult to determine the site of neutrophil migration using SEM. However, using transmission electron microscopy and serial section reconstruction of pulmonary capillaries, we reconstructed the migration paths of five neutrophils (275). Three were migrating at capillary tricellular corners, one was migrating between two endothelial cells, and another was migrating transcytotically through the endothelial cytoplasm.

Again, as found in vitro using HUVEC monolayers, the marked preference for tricellular corners (~50% in large vessels and ~60% in capillaries) in the pulmonary circulation is far greater than that predicted by chance (<10%). Importantly, freeze-fracture electron microscopy shows that alveolar capillary endothelial tight junctions are discontinuous at tricellular corners (276). We made similar observations in our HUVEC monolayers (31), which leads us to hypothesize that preferential migration at tricellular corners allows neutrophils to pass around rather than through endothelial tight junctions. While early studies suggested neutrophil transendothelial migration is associated with widespread proteolytic degradation of endothelial junctions (5, 50), subsequent studies established that widespread degradation is a technical artifact and unrelated to the migratory process (31, 177) (see sect. iv).

In the absence of proteolysis, lateral displacement of endothelial junctional complexes (tight and adherens
junctons) would seem necessary for neutrophil diapedesis to occur at tricellular corners. Freeze-fracture observations on pulmonary capillaries show the size of the tricellular pore created by the tight junction discontinuity is only 27 nm in diameter (276). Transmission electron microscopic observations suggest that neutrophils migrate through pores that are 1–2 µm wide (31, 163). From in vitro and in vivo studies (33), it is also clear that a significant fraction (25–30%) of neutrophils migrate between pairs of adjacent endothelial cells (i.e., biccullary). Even though tight junction fibrils between pairs of adjacent endothelial cells can be discontinuous, gaps between these fibrils are typically too small (3–6 nm) to allow neutrophils to pass (237, 238). Hence, we hypothesize that lateral displacement of junctional complexes is necessary to create a pore wide enough (1–2 µm) to accommodate a transmigrating neutrophil.

There is good evidence that tight junctions can slide within the membrane. In a study of mechanically stretched trachea, freeze-fracture electron microscopy shows the height of the epithelial tight junction complex shortens by as much as 25%, and small gaps appear in the fibrils (33). Moreover, during an acute inflammatory response to cigarette smoke exposure, plasma exudate expands tracheal epithelial tricellular corners, seemingly driving the tight junctions apart (33). The idea that junctional complexes “slide” apart during neutrophil diapedesis gains support from studies on VE-cadherin, a transmembrane protein involved in the formation of endothelial adherens junctions. Shaw et al. (232), using HUVEC monolayers transfected with green fluorescent protein-tagged VE-cadherin and real time imaging, obtained compelling evidence that adherens junctions open and close (much like a curtain) during leukocyte diapedesis. VE-cadherin and its role in leukocyte transendothelial migration are described in detail below (see sect. n.C).

Displacement of junctional complexes may require the neutrophil to signal the endothelium. Neutrophil adhesion is known to induce a coordinate increase in endothelial cytosolic free calcium, phosphorylation of serine-19 and threonine-18 on endothelial myosin regulatory light chains, and isometric tension generation by endothelial monolayers (80, 101, 109, 222). Neutrophil-mediated outside-in endothelial signaling likely involves the same molecules that mediate neutrophil adhesion to the endothelial surface. On HUVEC monolayers (pretreated with LPS or histamine to upregulate surface adhesion molecule expression), monoclonal antibodies directed against E-selectin, P-selectin, and vascular cell adhesion molecule (VCAM)-1 induce transient increases in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) and alterations in F-actin distribution. Interestingly, antibody ligation of platelet/endothelial cell adhesion molecule (PECAM)-1 or ICAM-1 appeared to have no effect, not even after application of a secondary antibody to induce cross-linking (156).

The inability of ICAM-1 or PECAM-1 to induce an increase in [Ca\(^{2+}\)]\(_i\) may relate to the type of antibody used or the species/tissue being examined. In a separate study, endothelial calcium increases were detected after antibody ligation of PECAM-1, even with Fab fragments, and the authors noted the effect was specific for certain antibodies (93). In vitro experiments with rat brain microvesel endothelial cells show cross-linking ICAM-1 results in Rho activation, phosphorylation of cytoskeletal proteins and transcription factors, and ICAM-1 association with the cytoskeleton (11, 70). Similarly, in cultured human pulmonary microvascular endothelial cells treated with TNF-α for 24 h, ICAM-1-dependent neutrophil adhesion or ICAM-1 cross-linking induces cytoskeletal remodeling and phosphorylation and activation of p38 mitogen-activated protein kinase (MAPK). Interestingly, in this model, the majority (60%) of adherent neutrophils localize to endothelial cell borders, and this response is attenuated by pretreating the endothelium with SB203580, an inhibitor of p38 MAPK (280–282). Although this finding suggests ICAM-1 plays a role in regulating neutrophil migration toward endothelial junctions, one must keep in mind that these are cultured cells treated with TNF-α for 24 h to increase ICAM-1 surface expression. We know from ultrastructural immunogold studies in mice that pulmonary capillary endothelial cells express very little constitutive ICAM-1 (32, 121). In humans, ICAM-1 is not detectable on alveolar capillaries by immunohistochemical staining; it is detectable on larger veins and arteries (75). Moreover, in mice, 4 h after airway instillation of S. pneumoniae, large numbers of neutrophils are present in the alveolar air-space, yet capillary endothelial ICAM-1 surface expression is not increased (32). In rabbits, 2 h after instillation of S. pneumoniae, 75% of the migrating neutrophils adhere and crawl through endothelial borders (33). Although the level of ICAM-1 expression on rabbit pulmonary capillaries was not determined, there is no reason to think it will be different from that in mice and humans. The fact that neutrophils can migrate into alveolar space using ICAM-1-independent mechanisms should not be taken to imply that ICAM-1 signaling is not important; rather, ICAM-1 is unlikely to be the only mechanism for recruiting neutrophils to endothelial borders.

Endothelial cytoskeletal rearrangements appear to be necessary for leukocyte transmigration. In a study of human microvascular endothelial monolayers, disruption of endothelial microfilaments by cytochalasin B or latrunculin A inhibits monocyte transmigration (128). On HUVEC monolayers, inhibition of myosin light-chain kinase (MLCK) diminishes neutrophil transmigration and inhibits endothelial F-actin formation, myosin filament formation, and myosin light chain (MLC) phosphorylation (101, 222). As mentioned in the preceding paragraph, neutrophil adhesion to human pulmonary microvascular endothelial cells (HPMVEC) treated with TNF-α also...
leads to endothelial cell stiffening and F-actin reorganization. Interestingly, unlike HUVEC monolayers, endothelial stiffening does not appear to rely on MLCK or MLC phosphorylation (280). This may reflect phenotypic differences between HUVEC and HPMVEC. Alternatively, activation of MLCK and MLC phosphorylation is triggered by neutrophil transendothelial migration but not adhesion to the endothelial surface. In any event, it is clear that adhesive interactions between leukocytes and endothelial cells trigger endothelial cytoskeletal changes. Because tight junction transmembrane proteins (occludin and claudin) and adherens junction transmembrane proteins (VE-cadherin) are anchored to the cytoskeleton through accessory cytoplasmic linker proteins (e.g., ZO-1, ZO-2, catenins) (78, 117, 118, 252, 253), current evidence suggests outside-in signaling and cytoskeletal remodeling following endothelial adhesion molecule ligation is a mechanism for displacing endothelial junctions.

A puzzling observation from the streptococcal pneumonia studies (33, 275) is that a significant fraction (~20% in capillaries and ~25% in larger pulmonary vessels) of neutrophils migrate directly through endothelial cytoplasm (i.e., transcytotically). Studies in skin (73, 105) and spinal tissue (157) also document a transcytotic route for neutrophils in venules which in one case (73) appears to be the sole route across the endothelium. The regulatory mechanism(s) behind transcytotic migration in the lung (and elsewhere) is unknown. However, neutrophil-derived vascular endothelial growth factor (VEGF) may play a critical role in determining whether neutrophils emigrate through interendothelial clefts or transendothelial pores.

While VEGF functions as an endothelial growth and survival factor, it also increases endothelial permeability (189). Topical administration or subcutaneous injection of VEGF in animals induces endothelial gaps (transcellular pores) and increased permeability within 1–10 min (72, 213). Pores large enough to accommodate red blood cells and platelets have been described (72). The mechanism behind VEGF-induced gap formation may be related to caveolar fusion (74). Endothelial caveolae are micropinocytic vesicles ~60 nm in diameter. Dvorak and colleagues (66, 267) believe fusion between adjacent caveolae gives rise to larger structures known as vesiculo-vacuolar organelles (VVOs). The high-affinity VEGF receptor-2 (FLK-1, KDR) localizes to caveolae (74) and VVOs (71). In response to VEGF, transcytotic increases in macromolecular permeability are mediated through caveolae (74), and transendothelial membrane-lined pores develop through VVO fusion with the apical and basal endothelial surfaces (66). These observations are important and relevant to neutrophil migration in the lung for two reasons. First, pulmonary capillary endothelial cells contain large numbers of caveolae (95, 220) (Fig. 4). Second, >70% of human neutrophil VEGF localizes to specific granules and, after stimulation, VEGF secretion is detected within 15–30 min (81, 255). The localized release of VEGF at sites of firm neutrophil adhesion would provide specificity to the process by restricting caveolar fusion and transendothelial pore formation in the vicinity of the neutrophil. While the mechanism remains speculative, the circumstantial evidence is compelling and warrants further investigation.

IV. ROLE OF ENDOTHELIAL ADHESION MOLECULES IN NEUTROPHIL TRANSENDOTHELIAL MIGRATION

Although much is known about the role adhesion molecules play in sequestering neutrophils in pulmonary capillaries, much less is known about their role in regulating neutrophil migration across the endothelium. Most studies focus on the importance of interendothelial clefts as primary routes for neutrophil migration. Support for this notion stems from findings that PECAM-1 (CD31), junctional adhesion molecules (JAMs), VE-cadherin, and CD99 localize to interendothelial clefts and antibodies raised against these molecules can inhibit (anti-PECAM-1, anti-JAM-1, anti-CD99) or enhance (anti-VE-cadherin) leukocyte transendothelial migration (4, 17, 49, 90, 140, 141, 154, 167, 181, 183, 184, 195, 226). Although these studies suggest these molecules function as “gate keepers” regulating neutrophil transendothelial migration, specific studies addressing their role in neutrophil emigration in the lung are scarce.

FIG. 4. Transmission electron micrograph of a canine alveolar capillary (Cap) showing numerous caveolae (arrows) within the endothelial cytoplasm. As, airspace. Bar is 500 nm.
A. PECAM-1

PECAM-1, a 130-kDa transmembrane protein and member of the immunoglobulin (Ig) superfamily, is expressed on endothelial cells, leukocytes, and platelets. In human placental blood vessels, ultrastructural immunogold labeling showed PECAM-1 localizes to basolateral membranes of interendothelial clefts (17). The extracellular region of PECAM-1 is organized into six globular domains that engage in homophilic interactions (3) as well as heterophilic interactions with glycosaminoglycans (GAGs) (283). A signaling function for PECAM-1 is suggested by recent studies showing it becomes phosphorylated on tyrosine residues Y663 and Y686 and associates with at least four cytoplasmic signaling molecules (SHP-1, SHP-2, SHIP, and phospholipase C-γ1) (98, 119, 202). PECAM-1 may also influence the endothelial cytoskeleton as recent data suggest it functions as a scaffolding molecule for β- and γ-catenins, accessory cytoplasmic molecules that link transmembrane proteins to the cytoskeleton (114).

The evidence that PECAM-1 plays a critical role in leukocyte emigration originated with the observation that anti-PECAM-1 antibody or soluble recombinant PECAM-1 blocks monocyte and neutrophil transendothelial migration (184). Subsequent studies established that globular domains 1 and 2 are necessary for leukocyte migration across inflamed endothelium while domain 6 regulates migration across the subendothelial basement membrane (148, 149). It has been suggested that homophilic interactions between leukocyte PECAM-1 and endothelial PECAM-1 may occur during transendothelial migration. This “zipper” hypothesis proposes that endothelial permeability is preserved during leukocyte migration because as the leading edge (pseudopod) of the neutrophil unzips interendothelial junctions, junctional contacts are replaced by endothelial PECAM-1 interactions with neutrophil PECAM-1 (250). Although this is an attractive hypothesis, it assumes a sufficient level of PECAM-1 on the neutrophil surface exists to engage in homophilic interactions with the endothelium. Resting neutrophils express relatively low levels of PECAM-1 compared with the high levels (>10^6 copies/cell) found on endothelial cells (48). In a peritoneal model of inflammation in mouse, PECAM-1 is downregulated on emigrated neutrophils. In vitro, mouse neutrophil migration across TNF-α-treated (4 h) HUVEC monolayers is associated with a loss of PECAM-1 from the neutrophil surface, and PECAM-1 downregulation appears to require more than just chemotactic stimulation, since neither TNF-α nor fMLF alone altered PECAM-1 expression (36). The latter observation contrasts with a separate study on human neutrophils showing treatment with IL-8, fMLF, or phorbol 12-myristate 13-acetate (PMA) alone downregulates PECAM-1 surface expression. PECAM-1 downregulation was also seen after PECAM-1 antibody ligation, a situation that potentially mimics neutrophil PECAM-1 engagement with endothelial PECAM-1 (68). Hence, it is not clear if sufficient PECAM-1 remains on the neutrophil surface to engage with endothelial PECAM-1 and form an effective zipper during neutrophil transendothelial migration.

There is only one published study demonstrating a role for PECAM-1 in neutrophil emigration in the lung. In a rat model of IgG immune complex deposition, neutrophil emigration into the alveolar airspace was inhibited 75% using a polyclonal rabbit antibody to human PECAM-1 which recognizes rat PECAM-1 (266). Although this study did not determine the site (endothelium or basement membrane) at which migration was inhibited, in vitro studies show this antibody blocks human neutrophil transmigration at the level of the endothelium (184). However, this does not mean we can conclude PECAM-1 is important for neutrophil migration across the alveolar capillary endothelium. In a separate study in rat mesentery activated by IL-1β (4 h), the same rabbit polyclonal antibody had no effect on neutrophil transendothelial migration, but migration across the venular basement membrane was inhibited by 50% (269). Hence, while antibody inhibition of PECAM-1 can interfere with neutrophil emigration in the lung, the role of PECAM-1 plays in neutrophil migration across the alveolar capillary endothelium and basement membrane remains unclear and more studies are needed.

Given the ability of antibodies and soluble recombinant PECAM-1 to inhibit leukocyte emigration, it was surprising to find that mice with a targeted deletion in PECAM-1 show only a mild and transient defect in leukocyte emigration. Specifically, neutrophil and monocyte migration across inflamed endothelium appears normal, whereas neutrophils, but not monocytes, experience a delay in crossing the basement membrane (65, 260). The delay appears to be temporary as neutrophil emigration into the peritoneal cavity is reduced at 4 h after IL-1 stimulation, but not at 24 h (65). The delay also appears to be cytokine specific, since it occurs in response to IL-1β but not TNF-α (260). In an attempt to explain discrepancies between PECAM-1 inhibition studies and PECAM-1 knock-out studies, it has been argued that PECAM-1-deficient mice exhibit compensatory changes in adhesion molecule usage. Normally, PECAM-1 blockade inhibits leukocyte transendothelial migration by 80–90%. Hence, molecules and mechanisms accounting for residual migration (10–20%) might be amplified in the knock-out animal. Alternatively, some unknown molecule(s) mediates leukocyte transendothelial migration in the knock-out (182). If a compensatory change exists, it must account for the temporary nature of the delay at the basement membrane, explain why neutrophils and not monocytes experience this enhanced delay, and provide insight into why this delay is cytokine specific. Until discrepancies...
between PECAM-1 inhibition and deletion are resolved, the certainty with which we attribute a role for PECAM-1 in leukocyte transendothelial migration must be questioned.

B. JAMs

JAMs belong to a novel subset of Ig molecules that belong to the larger cortical thymocyte Xenopus (CTX) family (16). The JAM family has three members (JAM-1, JAM-2, and JAM-3), and full-length amino acid sequences for these molecules suggest they exist as transmembrane proteins with two globular extracellular domains, a single membrane-spanning segment, and a short intracellular tail. Simultaneous discovery of these molecules by different laboratories has resulted in confusing JAM nomenclature when comparing human and mouse systems. Presently, mouse JAM-1 is equivalent to human JAM-1, mouse JAM-2 is the homolog of human JAM-3, and mouse JAM-3 is equivalent to human JAM-2, which is also known as VE-JAM-2 (16). After transfection into Madin-Darby canine kidney (MDCK) II cells, JAM proteins are enriched at cell-cell borders of transfected cells and not at borders of transfected and nontransfected cells, suggesting JAMs engage in homophilic adhesive interactions (16, 186). At least for JAM-1, homophilic adhesion appears to involve JAM-1 cis-dimerization and trans-interaction of the NH2-terminal domains with JAM-1 dimers on the adjacent cell (19, 46, 130). What follows is a description of each JAM member and a determination as to whether it might play a role in neutrophil emigration in the lung.

1. JAM-1

In mice, JAM-1 localizes to epithelial cells, endothelial cells, and megakaryocytes. It received its name based on its predominant expression in epithelial and endothelial intercellular clefts. In humans, JAM-1 localizes to epithelial and endothelial cells as well as hematopoietic cells (neutrophils, monocytes, lymphocytes, and erythrocytes) (161, 192, 288). Comparisons between human JAM-1 and mouse JAM-1 show 68% identity and 79% similarity over the entire protein amino acid conservation in the putative transmembrane, and cytoplasmic tail region is 84% identical and 92% similar (288). A functional role in maintaining epithelial and endothelial barrier function was suggested by the ultrastructural immunogold finding that JAM-1 is positioned apically within the epithelial intercellular cleft of mouse duodenum, colocalizing with tight junctions (167). Comparable immunogold studies of endothelial cells are lacking.

While dual label confocal microscopy and optical sectioning suggest JAM-1 colocalizes with endothelial tight junction proteins (AF-6 and cingulin) (67, 167), these findings must be interpreted with caution. The practical resolution limit for the confocal microscope in the z-axis is 0.5 μm, and endothelial cell borders can be as thin as 0.2 μm (31). Hence, while merged fluorescence images of thin samples (e.g., endothelia) give the appearance of colocalization, in reality the molecules may be separated along the z-axis by as much as 0.5 μm. Accurate spatial localization of JAM-1 within the interendothelial cleft will require ultrastructural immunogold techniques. The anticipated result is JAM-1 will localize to endothelial tight junctions, since biochemical findings from immunoprecipitation and glutathione S-transferase (GST) pull-down experiments (using epithelial cells and endothelial cells) show the cytoplasmic PDZ-binding domain of JAM-1 interacts with junctional proteins ZO-1 and AF-6 (20, 67).

By Northern blot analysis, JAM-1 expression is high in murine lung tissue. At the light microscope level, frozen sections of human lung show JAM-1 staining in both bronchi and alveolar epithelial cells (154). Although staining in pulmonary capillaries was not reported, reliable discrimination of epithelial and endothelial JAM-1 staining is difficult at the level of the light microscope, owing to the thinness (≤0.2 μm) of the alveolar wall and juxtapositioning of epithelial and endothelial cells. Clearly, this is another example where immunogold electron microscopy studies are needed to determine whether alveolar capillary endothelial cells express JAM-1 and if it localizes to the intercellular cleft.

Studies showing human JAM-1 localization to endothelial cells and leukocytes raise the possibility that homophilic interactions between endothelial and leukocyte JAM-1 may facilitate neutrophil transendothelial migration. The observation that JAM-1 remains on the neutrophil surface after activation with interferon (IFN)-γ, LPS, or formyl peptide (288) is consistent with the idea that it might engage endothelial JAM-1 during diapedesis. While mouse leukocytes do not express JAM-1, anti-murine JAM-1 monoclonal antibody, BV11, inhibits spontaneous and chemokine-induced [monocyte chemoattractant protein (MCP)-1 or MCP-3] human monocyte migration across cultured mouse endothelial monolayers. BV11 also inhibits human monocyte migration across LPS-treated cultured mouse endothelium (167). In vivo, BV11 partially inhibits monocyte emigration in a mouse model of skin inflammation (167) and monocyte and neutrophil emigration in a mouse model of cytokine-induced meningitis (49). Interestingly, BV11 did not inhibit leukocyte influx in a mouse model of infectious meningitis, implying fundamental differences in the mechanisms underlying leukocyte migration in the two models (i.e., cytokine-induced vs. organism-induced inflammation) (142). Hence, a role for JAM-1 in neutrophil emigration may vary depending on the nature of the inflammatory insult. In certain inflammatory settings, neutrophil diapedesis may be JAM-1 independent.

A new level of complexity was added to the JAM-1...
story when Ostermann et al. (192) reported that human JAM-1 is a heterophilic ligand for the leukocyte β2-integrin CD11a/CD18 (LFA-1). Deletional analysis suggested the membrane proximal domain 2 of JAM-1 was responsible for adhesive interactions with LFA-1. Using blocking antibodies, the authors found IL-8-stimulated neutrophil migration across resting HUVEC monolayers was largely mediated by LFA-1 (80%) and ICAM-1 (45%). Pretreatment of HUVEC monolayers with anti-JAM-1 alone or in combination with anti-ICAM-1 reduced neutrophil migration by ~60%. Because LFA-1 binds to the membrane proximal domain 2 region of JAM-1, there is a possibility for homophilic interactions between endothelial JAM-1 domain 1 and neutrophil JAM-1 domain 1. Hence, complex interactions between homophilic binding of endothelial JAM-1 to endothelial JAM-1, heterophilic binding of neutrophil LFA-1 to endothelial JAM-1 and homophilic binding of neutrophil JAM-1 and endothelial JAM-1 may provide another molecular zipper for transendothelial migration (192).

Finally, both JAM-1 and PECAM-1 have been suggested to also play negative roles in leukocyte transendothelial migration. Endothelial cultures treated for 8–24 h with a combination of TNF-α and IFN-γ show substantial loss of these molecules from the intercellular cleft; treatment with either cytokine alone has little or no effect. While the bulk of the evidence suggests the loss is due to redistribution from cleft to cell surface (193, 215), internalization and decreased synthesis may also be involved (211). Conceivably, loss of JAM-1 and PECAM-1 cleft molecules may shut down the inflammatory response by preventing leukocyte migration. Indeed, using static assays, where neutrophils are allowed to settle and adhere to endothelial cells in the absence of flow, a marked reduction in neutrophil (and monocyte) migration across TNF-α- and IFN-γ-treated HUVEC monolayers has been noted (211, 233). However, this reduction was not seen when the assay was performed under flow conditions (1 dyn/cm²) (233). Surprisingly, under flow conditions, antibody against PECAM-1 inhibits monocyte transmigration by 66% when the monolayer is costimulated with TNF-α and IFN-γ (no data given for neutrophils) (233). This observation suggests PECAM-1 participates in transmigration even when not associated with interendothelial clefts, indicating a potential signaling function (190). The issue of flow and its ability to affect neutrophil transmigration is an interesting consideration for the lung. Because leukocyte emigration occurs preferentially in alveolar capillaries, flow may be temporarily reduced or absent in select capillary segments as margined neutrophils deform to pass through the microvasculature (102). Moreover, in a rabbit model of streptococcal pneumonia, this effect is enhanced as capillary blood flow to the pneumonic regions decreases 20–50% over the first 8 h (57). Although the mechanism of reduced blood flow is likely related to hypoxic pulmonary vasoconstriction (88, 100, 152), it remains to be determined whether, under conditions of reduced or no blood flow, redistribution of JAM-1 or PECAM-1 away from interendothelial clefts is a mechanism for downregulating neutrophil emigration in the lung.

2. JAM-2 and JAM-3

In mice, Aurrand-Lions et al. (15) identified a novel Ig superfamily molecule whose expression was restricted to endothelial cells, particularly endothelial cells in high endothelial venules, lymphatic vessels in lymphoid organs, and vessels within the kidney. The molecule was termed JAM-2 because it shares 31% sequence identity with JAM-1 at the protein level. Moreover, it localizes to cell-cell contacts and decreases paracellular permeability when transfected into Chinese hamster ovary cells. When transfected into epithelial cells (MDCK) expressing tight junctions, immunofluorescence staining for JAM-2 codistributed with ZO-1 and occludin (15). Simultaneously, in another laboratory, Arrate et al. (14) cloned human JAM-3, a novel Ig superfamily molecule with 32% identity to human JAM-1 at the protein level. By Northern analysis, human JAM-3 is present in lung tissue, but the highest levels occur in kidney, brain, and placenta (14). Arrate et al. (14) suggest mouse JAM-2 isolated by Aurrand-Lions et al. (15) is the homolog of human JAM-3. If this is the case, then differences in tissue distribution exist between species because mouse JAM-2 is poorly expressed in lung and absent in brain. Hence, based on tissue distribution, although there may be a role for human JAM-3 in neutrophil emigration in the lung, a role for murine JAM-2 seems unlikely.

During this time, human JAM-2 was isolated and its protein sequence found to be 35% identical to human JAM-1 and mouse JAM-1 (41). Northern blot analysis revealed human JAM-2 is highly expressed in heart and poorly expressed in lung. Human JAM-2 shares 36% protein sequence homology with human JAM-3, and its sequence is identical to another human molecule, VE-JAM, which localizes exclusively to endothelial borders and is expressed prominently in high endothelial venules (HEV) (195). Subsequently, mouse JAM-3 was identified and the coding sequence suggested it was the mouse equivalent of human JAM-2 (16). Mouse JAM-3, like human JAM-2, is poorly expressed in lungs and shows stronger expression in heart (16). Hence, given the poor levels of expression for human JAM-2 and mouse JAM-3 in lung, these molecules seem unlikely to play important roles in neutrophil emigration from the pulmonary microvasculature. In humans, a more likely role for JAM-2 is in lymphocyte trafficking across HEV, since human JAM-2 is a counter-receptor for human JAM-3 and activated lymphocytes express human JAM-3 (14). A similar situation may exist...
in the mouse, since HEVs express all three JAM family members (16) and mouse JAM-3 is a counterreceptor for mouse JAM-2, which is expressed on lymphocytes (and monocytes) (B. Imhof, personal communication).

C. VE-cadherin (Cadherin-5)

VE-cadherin belongs to the cadherin family, and its expression is restricted to endothelial intercellular clefts. Conceptually, in the presence of calcium, cadherin monomers become rigid and competent to undergo cis-dimerization. Trans-dimerization follows when cadherin dimers on adjacent cells engage in homophilic interactions (129, 200, 251). In general, the cadherin cytoplasmic tail binds β- or γ-catenin, which in turn links to α-catenin. Catenins link cadherins to the cytoskeleton, and these interactions are critical to the formation of adherens junctions, belt-like structures that lie within the intercellular cleft below the level of the tight junction (251).

VE-cadherin plays a critical role in maintaining the paracellular barrier properties of the endothelium. Measuring Evans blue dye extravasation in heart and lungs, Corada et al. (38) found that a rat monoclonal antibody against mouse VE-cadherin (BV13) induced a concentration- and time-dependent increase in vascular permeability. In vitro experiments suggest the antibody affects VE-cadherin specifically as no change was observed in the endothelial border staining patterns for a variety of other molecules (PECAM-1, JAM-1, and two tight junction markers, ZO-1 and cingulin). In the lung, 7–9 h after antibody administration, electron microscopy showed neutrophils adhering to denuded capillary basement membranes and migrating across the alveolar wall (38). In a mouse model of thioglycollate-induced peritonitis, VE-cadherin antibody administration accelerated neutrophil emigration into the peritoneal cavity (90). These studies establish a clear role for VE-cadherin (adherens junctions) in maintaining the integrity of the endothelium, but are adherens junctions really barriers to leukocyte transendothelial migration?

As discussed above, neutrophil migration in the lung (at least in response to S. pneumoniae) occurs preferentially (50%) at tricellular corners where the margins of three endothelial cells converge and tight junctions are inherently discontinuous (33). Immunofluorescence images of HUVEC monolayers suggest adherens junctions are also discontinuous at tricellular corners (29). Hence, tricellular corners may provide the neutrophil with a portal for moving around (rather than through) both adherens junctions and tight junctions. Still, during streptococcal pneumonia, ~25% of the migrating neutrophils move between pairs of adjacent endothelial cells (bicellular migration) where adherens junctions appear to have fewer constitutive gaps (33). Whether bicellular migration involves adherens junction proteolysis has been the subject of much debate.

Initially, Dejana et al. (46), using an in vitro model of leukocyte trafficking, reported that neutrophil adhesion and migration induced widespread proteolytic cleavage of the adherens junction complex. Subsequently, this was shown to be an artifact arising from postfixation proteolysis (177). Allport et al. (6) renewed the debate with a migration study of monocytes and differentiated U937 leukocyte cells interacting with HUVEC monolayers under flow conditions. Using immunofluorescence microscopy, they observed focal disruptions in VE-cadherin border staining at the site of leukocyte migration and suggested this was not due to artifactual postfixation proteolysis, since monocytes have significantly lower protease levels than neutrophils and differentiated U937 cells lack fixation-resistant elastase (6). However, these cells still contain other proteolytic enzymes and since a conventional fixation procedure was employed, rather than a modified one that minimizes postfixation proteolysis (31), the results are inconclusive. In a subsequent study, Shaw et al. (232) overcame fixation issues by using HUVEC monolayers transfected with VE-cadherin coupled to green fluorescent protein (GFP) and real-time fluorescence microscopy (232). Under flow conditions (1.5 dyn/cm²), leukocytes (monocytes and neutrophils) could be seen migrating through preexisting gaps (holes) in VE-cadherin-GFP border fluorescence, and some gaps were located at tricellular corners. At other times, a de novo gap appeared at the site of leukocyte arrest on the endothelial surface. Migrating leukocytes appeared to push VE-cadherin aside as they moved across the monolayer and then, within 5 min, the displaced material diffused back to refill the junction. The opening and resealing of adherens junctions is likely mediated through catenin interactions with the cytoskeleton (251).

In summary, despite the obvious contribution VE-cadherin makes to the maintenance of the pulmonary microvascular integrity, in vitro studies suggest VE-cadherin is not a significant barrier to neutrophil transendothelial migration. Widespread proteolytic cleavage of the adherens junction complex is not required for neutrophil emigration. Instead, neutrophils trigger a reversible lateral displacement of the VE-cadherin complex enabling the endothelium to reseal after the migration event. This “curtain” effect also seems to operate at tricellular corners where small constitutive discontinuities in the adherens junction complex are expanded to accommodate migrating neutrophils.

D. CD99

As this review was being written, a new interendothelial cleft molecule was identified that has the potential
to regulate leukocyte trafficking in the lung. The molecule is CD99, a heavily O-glycosylated 32-kDa type I transmembrane protein whose previous expression had been noted only on hematopoietic cells. Schenkel et al. (226) report that CD99 is expressed at endothelial cell borders, and a monoclonal antibody (hec2) against CD99 blocks monocYTE migration across resting or cytokine-treated (IL-1β or TNF-α) HUVEC monolayers by >90%. Anti-CD99 does not affect monocyte adhesion, and blocking CD99 on the monocyte is just as effective as blocking CD99 on the endothelium. Additional studies with CD99 transfectants suggest monocyte transendothelial migration likely involves homophilic interactions between endothelial CD99 and monocyte CD99. Interestingly, anti-CD99 causes migrating monocytes to arrest within the intercellular cleft, at a point distal to the blockade effect seen with domain 1 or 2 PECAM-1 antibodies which prevent monocytes (and neutrophils) from entering the interendothelial cleft (149, 184). Whether CD99 regulates neutrophil transendothelial migration is unknown, but Schenkel et al. report (data not shown) that neutrophils express CD99. The distribution of CD99 within the vasculature is largely unknown, but hec2 antibody reacts with human arterial and venous endothelium (umbilical cords) as well as microvessels (dermis). Although it remains to be seen whether CD99 is expressed in the pulmonary vasculature, further investigation is warranted given the potential of this molecule to regulate leukocyte trafficking.

V. NEUTROPHIL MIGRATION ACROSS BASEMENT MEMBRANE

A. Role for Proteases?

Following diapedesis across the alveolar capillary endothelium, neutrophils must cross the subendothelial basement membrane to gain access to the interstitial space. Whether this process involves proteolytic degradation of the basement membrane is controversial. Observations of leukocyte migration have not provided evidence of significant disruption of endothelial basement membranes (112, 113, 160, 164, 231). Neutrophil elastase and gelatinase B each has a high capacity for extracellular matrix degradation. Delclaux et al. (47) report that in response to formyl peptide, neutrophil migration across Matrigel (a matrix derived from mouse sarcoma) is inhibited by 50% using gelatinase B or elastase inhibitors. However, this observation is difficult to interpret, since Matrigel by itself is capable of stimulating elastase and gelatinase release (178). The stimulatory effect of Matrigel contrasts with a report showing that fibronectin associated with subendothelial basement membrane acts as a protective substrate and inhibits neutrophil activation (168). Similarly, it has been reported that neutrophil contact with type IV collagen (a major constituent of the alveolar capillary basement membrane) (10, 127, 221, 293) inhibits subsequent neutrophil activation by a variety of mediators (25). This effect appears to be mediated through the α5β1 chain of type IV collagen, and this may decrease the potential for damage as neutrophils traverse the capillary wall (178).

The endothelium may be another determinant that regulates the neutrophil’s proteolytic arsenal. Mackarel et al. (159) found that neutrophil migration across cultured human pulmonary endothelium, and its associated subendothelial basement membrane was not affected by inhibitors of elastase or gelatinase. Similarly, Huber and Weiss (111) reported that neutrophil migration across human umbilical vein endothelial cells and their subtending basement membrane was not dependent on neutrophil elastase or cathepsin G and was resistant to inhibitors directed against neutrophil collagenase, gelatinase, and heparanase. Moreover, scanning electron microscopic examination of the transmigrated basement membrane showed no evidence of holes, even though basement membrane barrier function (as measured by the passage of monastral blue) was compromised. The presence of the endothelium was critical as denuded basement membranes did not support neutrophil migration. Because endothelial cells synthesize serine and metalloproteinases and are able to degrade basement membrane components, it was suggested that the endothelium prepared the basement membrane for neutrophil invasion (111). In any event, these studies provide compelling evidence that neutrophil proteases are not required for passage across the basement membrane. Recent in vivo studies uphold this concept by showing neutrophil emigration at inflammatory foci is normal in mice genetically deficient in elastase (22) or gelatinase B (24).

B. Preexisting Holes in the Basement Membrane

So how do neutrophils penetrate the basement membrane if they don’t use proteases? Is there a mechanical explanation? Walker and colleagues (21, 274) believe that in vivo the basement membrane is perforated with preexisting holes and neutrophils gain access to the interstitial space by migrating through these holes (Fig. 5). The next few paragraphs examine the evidence that holes in the basement membrane are portals for neutrophil emigration.

In rabbits and mice, the majority (75–80%) of capillary endothelial tight junctions are located at the intersection of thick and thin walls of the alveolar septum (274). Since the majority of neutrophil migration in the alveolar capillaries appears to occur at tricellular corners (275), it follows that thick walls are the principal site into which neutrophils migrate. Pericyte profiles are clustered at or
near the intersection of the thin and thick wall. Endothelial tight junctions are associated with pericyte profiles in a nonrandom fashion, and ~40% of the pericyte profiles around alveolar capillaries are within 0.5 μm of endothelial tight junctions (242, 274). In other tissues, pericytes surround capillaries and make numerous contacts with the endothelium (209, 239, 241, 261). Others (77, 270–273) have shown that numerous cytoplasmic interdigitations occur between endothelial cells and pericytes in immature capillaries of human granulation tissue. These observations suggest there are holes in the basement membranes of endothelial cells through which contacts are made with pericytes.

Using transmission electron microscopy and serial section reconstruction, Walker and colleagues determined that fibroblasts are also clustered at the intersection of thick and thin alveolar walls. Fibroblast extensions were observed penetrating the endothelium through slit-like holes (0.43–1.0 μm in length and 0.16–0.48 μm in width), and all holes were located at intersections of thick and thin walls. During streptococcal pneumonia in rabbits, migrating neutrophils appeared to pass through small holes in the basement membrane, as evidenced by their hourglass-shaped appearance. Importantly, neutrophils penetrated the basement membrane at the same loci where holes occupied by fibroblasts were observed in control rabbits (274). Collectively, these observations support the concept that basement membrane holes occupied by fibroblasts are avenues through which neutrophils migrate into the extracellular compartment of the alveolar wall.

Before a neutrophil could pass through a hole in the basement membrane, the fibroblast extension would have to be withdrawn or displaced. Fibroblast retraction can be triggered by mechanical stress as shown by experiments in dermal explants subjected to sinusoidal stretching. Stretching causes stellate fibroblasts with long cytoplasmic processes to become spherical and lose contact with the ECM. The ECM relaxes, and interstitial pressure becomes more negative, which results in a rapid (5–10
min) rise in tissue edema (165). Whether mechanical stress plays a role in fibroblast retraction during neutrophil emigration in the lung is unknown, but tissue edema frequently accompanies lung injury (87, 258). In the event that fibroblasts retract, many of the exposed holes would still be too small to allow a neutrophil to pass. Estimates from electron microscopy suggest the hole must be at least 1 μm in diameter to permit neutrophil emigration (274). In the absence of proteolysis, the neutrophil would have to mechanically enlarge the hole, and this seems feasible since the basement membrane is thought to be somewhat elastic (76). An example of this elasticity can be found in earlier observations by Majno and Palade (160) showing red blood cells assuming deformed shapes as they pass through the basement membrane in vivo.

VI. FIBROBLASTS: A SOURCE OF CONTACT GUIDANCE AND STIMULATION FOR NEUTROPHIL MIGRATION

As neutrophils pass through the endothelial basement membrane, they emerge into the thick wall interstitium. Within the interstitium two types of fibroblasts exist. The first type of fibroblast is intimately associated with fibrous connective tissue elements and oriented parallel to the epithelium, whereas a second type is stellate and oriented perpendicular to the epithelial cells of the alveolar walls (Figs. 1 and 5). The latter has been characterized as a myofibroblast (122, 123) and is thought by some to be capable of contraction (124, 143). It is the myofibroblast that Walker et al. (274) describe as having cytoplasmic extensions that penetrate the endothelial basement membrane.

Sims (240) demonstrated that fibroblasts within the alveolar wall are connected by adherens-like junctions on cytoplasmic extensions of the fibroblasts. Adherens-like junctions also occur between fibroblasts and the basement membranes of type I and II epithelial cells (123, 240, 274). Walker and co-workers (21) determined that individual myofibroblasts provide a physical bridge from pre-existing holes in the endothelial basement membrane, through the interstitium, to similar holes in the basement membrane of epithelial type II pneumocytes, simultaneously keeping all three cells in physical contact. Taken together, these observations define a reticulum of fibroblasts within the alveolar wall that simultaneously link the endothelium to the epithelium at morphologically distinct points through apertures (holes) in the respective basement membranes (Fig. 5).

Several other studies (2, 26, 265) demonstrated discontinuities in the basement membranes of rat type II pneumocytes through which extensions of cytoplasm contact interstitial fibroblasts. Through morphometric analysis they estimated these contacts occur with a frequency of 0.5 and 0.7 per pneumocyte, respectively. In rabbit lung, Walker et al. (274) reported similar round holes that range in diameter from 0.14 to 0.7 μm in the basement membranes of type II pneumocytes at points of contact with fibroblasts. Using serial sections from three type II pneumocytes, Walker et al. (274) showed not only does the basement membrane of each pneumocyte have multiple holes (Fig. 5), but also each pneumocyte is connected to more than one fibroblast.

During streptococcal pneumonia in rabbits, migrating neutrophils are always in close contact with alveolar wall fibroblasts (21). Morphometric estimates suggest that on average, 30% of the neutrophil surface is close enough (≤15 nm) to a fibroblast surface to engage in adhesive interactions. The idea that this close association is an adhesive contact is supported by the presence of a layer of dense fibrillar cytoplasm along areas of neutrophil contact with fibroblasts. In spite of the mild edema that occurs in the alveolar walls of S. pneumoniae-infected rabbit lung, ∼70% of the surface area of migrating neutrophils is close enough to either extracellular matrix elements or fibroblast plasma membranes to be adherent. Although in vitro experiments suggest chemokines (e.g., IL-8) bind to ECM proteoglycans like heparin and heparin sulfate (50a, 138a), and promote neutrophil haptotaxis (directed migration in response to an immobilized attractant) (216a), evidence showing that such a mechanism operates in vivo is lacking. Moreover, ECM elements (collagen, elastin, etc.) are not physically arranged in a manner that provides a continuous and unique structural “bridge” between holes in the endothelial and epithelial basement membranes. Hence, the ECM may play more of a “hand-hold” role than a directional role. Conversely, because fibroblasts connect points of neutrophil entry into the interstitium to points at which neutrophils leave the interstitium (21), we believe they play a unique directional (guidance) role in neutrophil migration.

Although the mechanisms of neutrophil adhesion are not completely understood, several in vitro studies found that PMA-activated human neutrophils showed enhanced CD18-dependent adhesion to skin and lung fibroblasts (86, 235). Armstrong and Lackie (12) found activated rabbit neutrophils can both adhere to and travel along the surface of cultured chicken fibroblasts. Burns et al. (28) determined that canine neutrophils adhere to cultured canine lung fibroblasts in the presence of platelet-activating factor (PAF), but not IL-8. The combination of PAF and IL-8 allowed neutrophils to adhere and crawl on the fibroblast surface. Fibroblasts treated with TNF-α secrete IL-8 protein into the medium, and secreted IL-8 was chemotactic for neutrophils (28). Collectively, these findings suggest lung fibroblasts can function not only as an adhesive substrate for neutrophils, but also as a source of stimulation for neutrophil migration. Burns et al. (28) found neutrophil crawling was completely inhibited by...
anti-CD18 monoclonal antibody. In contrast, neutrophil adhesion was only partially (70%) CD18 dependent (28). While the identity of the CD18-independent component of adhesion is unknown, the $\beta_4\alpha_{x\gamma}$ integrin family of adhesion molecules is a likely candidate. In a slightly different model that examines neutrophil migration through a human lung fibroblast barrier, Shang and Issekutz (23) identified a role for CD18, as well as $\alpha_x\gamma$, $\alpha_y\gamma$, and $\alpha_5\beta_1$-integrins. With the use of a cocktail of blocking antibodies to these molecules, neutrophil migration was inhibited by 60%. Further inhibition (75%) in migration was seen when an antibody to the neutrophil $\alpha_5\beta_1$-integrin was added to the cocktail (229).

Neutrophil adhesion to cultured lung fibroblasts is also partially (50%) dependent on fibroblast ICAM-1, a ligand for CD18 (28). The identity of other fibroblast ligands has not been determined, but apparently, it reportedly does not involve extracellular matrix proteins or blood proteins since PMA-mediated neutrophil adhesion to lung fibroblasts does not require serum, albumin, type I or type IV collagen, or fibronectin (235). More recently, it has become apparent that cytokines and mast cell products will induce VCAM-1 expression on human lung fibroblasts (172, 230, 246). While $\beta_2$-integrins $\alpha_4$ and $\alpha_9$ are known to bind VCAM-1 (230, 257), it remains to be determined whether VCAM-1 expression on lung fibroblasts mediates $\alpha_x\gamma$- and $\alpha_9$-dependent neutrophil adhesion and motility.

VII. ROLE OF INTEGRINS IN NEUTROPHIL MIGRATION THROUGH EXTRACELLULAR MATRIX

The adhesive properties of various ECM elements affect both the direction and rate of leukocyte locomotion (139). For example, fibronectin and proteoglycans (e.g., heparin) are widely distributed throughout the interstitium, either in association with collagen fibers or free in the matrix (84, 224, 227, 256). In vitro, the addition of heparin, fibronectin, or laminin to type I collagen gels decreases neutrophil motility. It seems fibronectin and laminin coat the collagen and create a substrate that favors adhesion over motility. Heparin causes collagen fibers to aggregate, and this increases the mesh size of the gel to the point that neutrophil motility is compromised (138). While elastin is probably not a good substrate for cell attachment because of its highly hydrophobic nature (44), in vivo studies suggest microfibrillar proteins associated with elastin (40) support adhesion of migrating cells to elastic fibers (198).

Clearly, adherence to ECM is important for neutrophil localization to inflammatory sites. Integrins are the major class of cell adhesion receptor that mediate cell-matrix interactions. ECM ligands for integrins include fibronectin, fibrinogen, laminin, collagens, entactin, tenascin, thrombospondin, and vitronectin (8, 97, 219). The ligand for many integrins is an RGD (Arg-Gly-Asp) sequence contained in many extracellular matrix components including fibronectin, laminin, and the collagens (214, 218, 219). While leukocyte $\beta_2$-integrins CD11b/CD18 and CD11c/CD18 are known to bind fibrinogen following leukocyte activation (7, 155), recent studies suggest $\beta_\gamma$-integrins are not essential for interactions with other ECM molecules. In vitro, activated CD18 null neutrophils adhered to fibronectin and vitronectin through $\alpha_x\beta_3$ and $\alpha_5\beta_3$, respectively, while adhesion to endothelial laminin 10 is through $\alpha_5\beta_1$ (243). In rat mesentery, after chemotactic stimulation, $\beta_3$-integrins were found to be more critical than $\beta_2$-integrins in supporting neutrophil motility in the interstitium. Specifically, with the use of antibodies or peptides recognizing various integrins, $\beta_3$-integrin blockade inhibited neutrophil migration velocity by 70% $\beta_2$-integrin blockade inhibited migration velocity by only 20%. Additional experiments determined that neutrophil locomotion was critically dependent on $\alpha_2\beta_1$ (70%), but not $\alpha_4\beta_1$ or $\alpha_5\beta_1$ (286, 287).

While $\beta_1$-integrins have limited expression on circulating neutrophils, expression levels increase markedly after transendothelial migration (131, 217, 286, 287). Antibody cross-linking experiments suggest ligation of $\beta_\gamma$-integrin provides a signal for $\beta_1$-integrin upregulation (285). In a similar context, PECAM-1 ligation has been shown to generate an inside-out signal to activate $\beta_1$, $\beta_2$, and $\beta_3$-integrins (reviewed in Ref. 182). Taken together, the process of neutrophil adhesion and transmigration appears to be associated with $\beta_1$-integrin activation.

With respect to the lung, $\beta_1$-integrins clearly play a role in neutrophil emigration. In rats, neutrophil emigration into the alveolar airspace is partially (20%) CD18 dependent. Antibody blocking studies suggest the CD18-independent component to this migration is largely (~80%) mediated by the $\beta_1$-integrins $\alpha_4$ and $\alpha_5$ (34). In mice, while neutrophil emigration following LPS treatment is largely (80%) CD18 dependent, migration into the airspace still requires $\beta_1$-integrins, particularly $\alpha_5$ and $\alpha_6$. In a situation where neutrophil emigration is entirely CD18 independent (e.g., instillation of KC, the murine ortholog of human IL-8), antibody inhibition of $\beta_1$-integrin or specific $\alpha$-subunits ($\alpha_2$, $\alpha_4$, $\alpha_5$, and $\alpha_6$) blocks neutrophil emigration into the alveolar airspace. Ultrastructural examination of lung tissue 4 h after KC stimulation and neutrophil localization to inflammatory sites. Integrins are the major class of cell adhesion receptor that mediate cell-matrix interactions. ECM ligands for integrins include fibronectin, fibrinogen, laminin, collagens, entactin, tenascin, thrombospondin, and vitronectin (8, 97, 219). The ligand for many integrins is an RGD (Arg-Gly-Asp) sequence contained in many extracellular matrix components including fibronectin, laminin, and the collagens (214, 218, 219). While leukocyte $\beta_2$-integrins CD11b/CD18 and CD11c/CD18 are known to bind fibrinogen following leukocyte activation (7, 155), recent studies suggest $\beta_\gamma$-integrins are not essential for interactions with other ECM molecules. In vitro, activated CD18 null neutrophils adhered to fibronectin and vitronectin through $\alpha_x\beta_3$ and $\alpha_5\beta_3$, respectively, while adhesion to endothelial laminin 10 is through $\alpha_5\beta_1$ (243). In rat mesentery, after chemotactic stimulation, $\beta_3$-integrins were found to be more critical than $\beta_2$-integrins in supporting neutrophil motility in the interstitium. Specifically, with the use of antibodies or peptides recognizing various integrins, $\beta_3$-integrin blockade inhibited neutrophil migration velocity by 70% $\beta_2$-integrin blockade inhibited migration velocity by only 20%. Additional experiments determined that neutrophil locomotion was critically dependent on $\alpha_2\beta_1$ (70%), but not $\alpha_4\beta_1$ or $\alpha_5\beta_1$ (286, 287).

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sider the effects this treatment might have on other cell types.

For example, fibroblast adhesive contacts with collagen are critically dependent on β1-integrins. Fibroblasts are able to exert tension on the ECM, and antibodies against β1-integrins interfere with this adhesive interaction. β1-Integrin blockade decreases connective tissue fiber tension, and this increases negative interstitial pressure, which results in rapid edema formation (206, 207). Hence, although anti-β1-integrin antibody clearly inhibits neutrophil emigration, the effect may be indirect. β1-Integrin blockade may adversely affect the organization of the interstitial matrix and render it unsuitable for neutrophil motility. Interstitial chemotactic gradients may be diluted and fibroblast networks disrupted (retracted) resulting in diminished contact guidance for neutrophils.

VIII. NEUTROPHIL MIGRATION ACROSS ALVEOLAR EPITHELIUM

Compared with what we know about the regulation of neutrophil migration across the endothelium, we really know very little about migration across the alveolar epithelium. Basic information is lacking regarding the behavior of tight junctions and adherens junctions during neutrophil transepithelial migration in the lung. It will be important to determine if neutrophil transepithelial migration is associated with junctional proteolysis or whether the junctions slide apart like a curtain (232) to accommodate the migrating neutrophil. The migration mechanisms for crossing the epithelium could turn out to be very different from those used to cross the endothelium. It is important to remember that unlike crossing the endothelium where the movement is in the apical to basal direction, neutrophils are now moving in a basal to apical direction as they cross the epithelium. For example, ICAM-1 is expressed in abundance on the apical surface of the alveolar epithelium, and it would not be surprising to find that it serves as an adhesive ligand for CD18-dependent neutrophil adhesion (262, 263), functioning as an adhesive tether to retain neutrophils at specific locations (196). However, because ICAM-1 is not expressed on the basolateral aspect of alveolar epithelial cells (32), it is unlikely to play a role in neutrophil transepithelial migration in the physiologically relevant basal to apical direction.

Much of what we do know about neutrophil transepithelial migration comes from in vitro studies using intestinal epithelial monolayers. Studies of neutrophil migration across intestinal epithelial monolayers suggest neutrophils take a paracellular route as they migrate in the basal to apical direction, and this involves focal disruption of epithelial tight junctions. The notion that neutrophil transepithelial migration is associated with tight junction disruption is largely based on studies showing reversible loss of epithelial barrier function as measured by tracer and flux studies (reviewed in Ref. 196). However, this claim is not supported by morphological evidence, since freeze-fracture images of epithelial tight junctions appear normal during neutrophil transmigration (188), just as they do in endothelial cells (31, 33). Migration across the epithelium does not appear to depend on neutrophil oxidant production or protease release (196). Nash et al. (188) conclude that if junctional abnormalities exist, they must be highly focal.

During neutrophil migration across intestinal epithelial monolayers, neutrophils tend to accumulate in clusters beneath the epithelium at so-called invasion sites (39, 188, 197). The presence of nonrandom invasion sites and general preservation of tight junction morphology during neutrophil transepithelial migration can be explained if neutrophil migration occurs preferentially at epithelial tricellular corners. Intestinal epithelial tight junctions are known to be discontinuous at tricellular corners (247), and intestinal epithelial cells used for neutrophil migration studies (e.g., T84 cells) have on average six corners per cell (85). Hence, there are six nonrandom sites associated with each cell where tight junctions are discontinuous, and these sites could allow neutrophils to migrate around rather than through tight junctions without compromising tight junction barrier function. Despite numerous reviews and articles on neutrophil transepithelial migration, the issue of tricellular corner migration has been largely ignored.

Earlier in this review, we postulated that neutrophils find their way to type II pneumocytes by crawling along fibroblasts in the interstitial space. The neutrophil crawls into the basal lateral space of the type II pneumocyte by passing through a hole in the basement membrane that was once occupied by a type II pneumocyte contact with an underlying fibroblast (Fig. 5). From here, neutrophils appear to preferentially enter the alveolar lumen adjacent to type II pneumocytes (33, 42, 274). In fact, in a study of dog lung, Damiano et al. (43) report that all observed cases of neutrophil egress from the interstitium were associated with type I/type II junctions. When viewed from the alveolar surface (33, 42), the site at which the neutrophil emerges is at an epithelial tricellular corner where the border of two type I pneumocytes meet the border of a type II pneumocyte (Fig. 6). Just as in endothelial cells, freeze-fracture images of tricellular corners between type I and II pneumocytes show tight junction discontinuities (Walker, unpublished observation). Tricellular corner discontinuities are a common feature for a variety of epithelia including tracheal (276, 277), intestinal (247), nasal (171), bladder (268), and caput epididymal (254). In the lung, the type I/II corner discontinuity pro-
vides a natural path through which the neutrophil can migrate around rather than through the tight junction. This may be particularly important for transepithelial migration, since epithelial tight junctions are more substantial (deeper), particularly at tricellular corners (Fig. 6), than those found on capillary endothelial cells. The increased tight junction complexity may partially account for the observation that the alveolar epithelium is 10 times less leaky than the alveolar capillary endothelium (259).

Studies in the intestinal epithelium suggest CD11b/CD18 and CD47 play critical roles in neutrophil transmigration (reviewed in Ref. 196). Neutrophils from patients with leukocyte adhesion deficiency (LAD) failed to migrate across an intestinal epithelial cell monolayer. The dependency on CD18 was mimicked by antibody against CD11b/CD18 but not against CD11a/CD18 or CD11c/CD18. In the lung, CD18 is not always required for neutrophil emigration into the alveolar airspace. For example, in response to *S. pneumoniae*, neutrophil emigration in the lung is CD18 independent (54). An autopsy performed on an LAD patient suffering from a complete deficiency of CD11/CD18 showed emigrated neutrophils in the lung (94). Hence, unlike the intestinal epithelium, CD18 is not absolutely required for migration across the alveolar epithelium. CD47 is expressed on all hematopoietic cells and most other cell types (169, 208). In vitro, antibody blockade of epithelial or leukocyte CD47 inhibits neutrophil migration across intestinal epithelia, collagen-coated filters, and endothelia (37, 153). In the neutrophil, CD47 is stored in secondary specific granules, and its translocation to the cell surface appears to facilitate neutrophil migration. Transfection of CD47 into CD47-deficient epithelial cells (Caco-2) also results in enhanced neutrophil transepithelial migration. Exactly how CD47 regulates neutrophil migration is not clear, but it has been proposed that in its activated state, CD47 induces downstream tyrosine kinase-linked signaling events which result in enhanced neutrophil migration (153). Although specific experiments demonstrating a role for CD47 in neutrophil migration across the alveolar epithelium are lacking, a recent study suggests CD47 is important for monocyte emigration in the lung. Using an in vitro model of monocyte migration across cultured alveolar epithelial cells, Rosseau et al. (216) found transepithelial migration largely depends on CD11b/CD18 and CD47. The additional engagement of β1-integrins (α4, α5, and α6) was required for optimal migration (216).

In the lung, JAM-1 is present on alveolar epithelial cells (154). Whether JAM-1 plays a role in human neutrophil migration across the alveolar epithelium is unknown. On the basis of findings with a human T84 colon cancer epithelial cell line, a role for JAM-1 in regulating interepithelial tight junction assembly has been suggested by Liu and colleagues. They identified a panel of monoclonal antibodies against the extracellular portion of human

![Image](https://example.com/image.png)
JAM-1 that inhibit epithelial barrier repair after disruption by calcium depletion. Immunofluorescence microscopy suggests these anti-JAM-1 antibodies inhibit both occludin and JAM-1 in reassembling tight junctions. Although this study is important because it provides the first direct evidence for JAM-1 having an important role in interepithelial junction assembly, it is important to note that these antibodies do not inhibit human neutrophil migration across T84 epithelial monolayers, human microvascular endothelial cell monolayers, or HUVEC monolayers (154, 233). Because domains 1 and 2 of JAM-1 recognize JAM-1 domain 1 and leukocyte CD11a/CD18, respectively (192), and Liu and colleagues did not map the epitope sites (i.e., domain 1 or 2) recognized by their anti-JAM-1 antibodies, it is difficult to interpret the lack of inhibition seen in neutrophil transmigration. Simultaneous antibody blockade of both domains 1 and 2 may be necessary to block neutrophil transepithelial migration.

Finally, in closing, it is necessary to point out that neutrophil emigration in the lung is not always an ordered process regulated by adhesion molecules. In his monograph on the lung, William Snow Miller (176) suggests fibrinogen exudates and their conversion to fibrin strands are common features of lobar pneumonias. As far back as the 1920s, using a light microscope, Miller reported that early inflammatory stages of acute lobar pneumonia begin with engorgement of blood capillaries followed by a serous exude into the alveoli in which epithelial cells, leukocytes, and red blood cells are present (174, 175). Nearly 80 years later, using a scanning electron microscope, we have confirmed Miller’s observation and are able to show fibrin strands erupting into the alveolar space through macroscopic discontinuities in the alveolar wall. The strands organize into netlike structures (Miller described these as broad sheets of fibrin) providing an adhesive substrate for emigrated neutrophils (Fig. 7). Because fibrin is also an adhesive substrate for bacteria, neutrophil adhesion and migration into fibrin gels is critical for bacterial killing. In vitro, complement fragment C5a and LTB4 appear to enhance neutrophil migration into fibrin gels and bacterial killing (146). While the origin of the alveolar wall discontinuities is unknown, Miller suggests the serous exude accumulates in the restricted space beneath the epithelium (i.e., interstitium) and the cells are pushed off by the increased pressure. Support for this “pressure” mechanism can be found in a transmission electron micrograph showing large numbers of neutrophils simultaneously exploding into the alveolar lumen through a macroscopic discontinuity in the alveolar wall (Fig. 8). Preexisting holes in the basement membrane might be involved in this process, since the holes represent the path of least resistance for movement of serous fluids and cells from blood to airspace. Clearly, we have overlooked (ignored?) Miller’s observations in our rush to explain how adhesion molecules regulate neutrophil emigration. Under conditions where serous exudates and leukocytes are spewed into the alveolar lumen, neutrophil migration from blood to airspace may bypass the adhesion mechanisms discussed in this review.

IX. CONCLUSIONS AND FUTURE DIRECTIONS

With respect to inflammation, the pulmonary circulation is very different from the systemic circulation. Capillaries, not venules, are the primary site for neutrophil emigration. The molecular paradigm explaining how adhesion molecules regulate neutrophil tethering, rolling, arrest, and transmigration in the systemic circulation does not apply to the pulmonary capillary bed.

During inflammation in the lung, neutrophils sequester in capillaries through a process involving mechanical and adhesive changes not only in the neutrophil, but also in the endothelium. Mechanical changes in the cytoskeleton of activated neutrophils cause them to stiffen and

FIG. 7. Fibrin exudates erupt into the alveolar space during streptococcal pneumonia in rabbit lung. Four hours after tracheal instillation of Streptococcus pneumoniae, scanning electron microscopy reveals fibrin strands emerging into the alveolar space through macroscopic discontinuities (poles) in the alveolar wall (A). B shows how fibrin strands organize into a reticulum that functions as an adhesive substrate for neutrophils as they emerge into the alveolar lumen. Bar is 5 μm.
Streptococcus pneumoniae. Four hours after tracheal instillation of *S. pneumoniae*, transmission electron microscopy reveals a discontinuity (pore) in the alveolar capillary wall through which multiple neutrophils emerge simultaneously into the alveolar space (As). The margins of the pore are identified by arrows. Capillary lumens (*) appear empty because the tissue was fixed by vascular perfusion. Bar is 10 μm.

FIG. 8. Macroscopic discontinuities (pores) in the alveolar wall provide another mechanism for neutrophil emigration into the alveolar space. Four hours after tracheal instillation of *S. pneumoniae*, transmission electron microscopy reveals a discontinuity (pore) in the alveolar capillary wall through which multiple neutrophils emerge simultaneously into the alveolar space (As). The margins of the pore are identified by arrows. Capillary lumens (*) appear empty because the tissue was fixed by vascular perfusion. Bar is 10 μm.

Compared with what we know about neutrophil interactions with endothelial cells, we know even less about neutrophil migration through the interstitium and across the alveolar epithelium. Studies of neutrophil migration through extracellular matrices suggest there is a role for adhesion molecules, particularly β1-integrins. Conversely, neutrophil-mediated proteolysis of the matrix does not seem to be required for migration. In the lung, a neutrophil travels a remarkably short distance (typically <1 μm) in going from blood to airspace. It shows a preference for emigration into the alveolus by migrating at tricellular corners where the margins of two type I pneumocytes converge on a type II pneumocyte. Intervertebral fibroblasts may play a key role in directing neutrophils to these preferred epithelial migration sites because single fibroblasts form cellular bridges between capillary endothelia and type II pneumocytes. Fibroblasts make intimate contacts with these cells through preexisting holes in the basal lamina. There is morphological and in vitro evidence that after migrating through holes in the endothelial basal lamina, neutrophils adhere to and crawl on interstitial fibroblasts. By way of its unique position within the alveolar wall interstitium, the fibroblast guides the neutrophil to the space between the type II pneumocyte and its underlying basement membrane. Preferential migration across the epithelium occurs at tricellular corners where type I and II pneumocytes meet because epithelial junctions are inherently discontinuous at these sites. This schema for neutrophil migration across the alveolar wall is summarized in Figure 9.

From the preceding statements, it can now be appreciated that neutrophil emigration in the lung is significantly regulated by its unique microanatomy. Future insights into molecular mechanisms regulating neutrophil emigration in the lung will benefit from careful consideration of the anatomical issues. If we hope to learn more from in vitro models, we must move in a direction that incorporates the unique positioning of fibroblasts within the matrix in ways that allow for the formation of intimate contacts with endothelial and epithelial cells through holes in the basement membrane. We must also pay attention to the pathways (paracellular and transcellular) taken by neutrophils as they cross the endothelium and epithelium. Although it is likely the pathway taken will be stimulus dependent, it is also likely the pathway will change during the course of the inflammatory episode. For example, mild inflammation may begin in a regulated fashion with paracellular neutrophil emigration, but as the inflammatory process intensifies and chemokine concentrations shift, transcellular migration may be favored. Only by understanding these types of issues can we hope to design effective therapeutics for treating acute and chronic inflammatory diseases like adult respiratory distress syndrome, emphysema, multiorgan failure, ischemia/reperfusion injury, graft versus host disease, and autoimmune disease.
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Address for reprint requests and other correspondence: A. R. Burns, Dept. of Medicine and Pediatrics, Sect. of Cardiovascular Science, Baylor College of Medicine, Rm. 515B, One Baylor Plaza, Houston, TX 77030 (E-mail: aburns@bcm.tmc.edu).

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