Neutrophil Intercellular Communication in Acute Lung Injury

Emerging Roles of Microparticles and Gap Junctions

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A hallmark of acute inflammation involves the recruitment of polymorphonuclear leukocytes (neutrophils) to infected or injured tissues. The processes underlying this recruitment are complex, and include multiple mechanisms of intercellular communication between neutrophils and the inflamed tissue. In recent studies of the systemic and pulmonary vasculature, interest has increased in novel forms of intercellular communication, such as microparticle exchange and gap junctional intercellular communication. To understand the roles of these novel forms of communication in the onset, progression, and resolution of inflammatory lung injury (such as acute respiratory distress syndrome), we review the literature concerning the contributions of microparticle exchange and gap junctional intercellular communication to neutrophil–alveolar crosstalk during pulmonary inflammation. By focusing on these cell–cell communications, we aim to demonstrate significant gaps of knowledge and identify areas of considerable need for further investigations of the processes of acute lung inflammation.

Keywords: neutrophil; connexin; gap junction; microparticles; acute lung injury

Although investigations of acute respiratory distress syndrome (ARDS) have yet to yield an efficacious pathophysiology-targeted therapy, they have provided important insights into the intercellular communications regulating neutrophil activation and alveolar transmigration. These communications include juxtacrine and paracrine (e.g., chemokines, cytokines, and proteinases), cross-talk between neutrophils and lung parenchymal cells, and the signaling conducted through cell-surface receptors such as leukocyte integrins and cognate adhesion molecules (e.g., intercellular adhesion molecules) expressed by lung parenchymal cells (1). The past decade has seen an increasing recognition of alternative forms of intercellular communications during the onset and resolution of inflammatory lung disease. These communications include microparticle exchange as well as gap junctional intercellular communication

CLINICAL RELEVANCE

The acute respiratory distress syndrome (ARDS) is a common yet highly morbid critical illness. Despite more than 40 years of investigation, no effective disease process-targeted treatment for ARDS exists. By highlighting promising new mechanisms of cell–cell communications in ARDS, we hope to identify new avenues of research that could yield novel therapeutic approaches.

(GJIC). This Perspective will focus on these alternative forms of communication between neutrophils and lung parenchymal cells during the genesis of ARDS, highlighting opportunities for further investigations.

MICROPARTICLE EXCHANGE

Microparticles are spherical, lipid bilayer-encapsulated extracellular bodies ranging from 50–1,000 nm in diameter. Microparticles can be secreted by almost every cell type, including lung parenchymal cells and inflammatory cells. Multiple forms of microparticles exist, reflecting different modes of production. “Exosomes” and “shedding vesicles” are derived from living cells, whereas “apoptotic bodies” are secreted by apoptotic and/or necrotic cells (2). Exosomes have an endosomal origin, and are stored as intraluminal vesicles within multivesicular bodies (3). Upon stimulation, exosomes are secreted by fusion with the cell membrane, forming a relatively homogenous group of microparticle sizes (50–150 nm). In contrast, shedding vesicles and apoptotic bodies derive from the budding of small cytoplasmic protrusions from the plasma membrane (4). These represent a more heterogeneous group of membrane vesicles (50–1,000 nm).

Although the microparticle release rate in resting cells is very low, the secretion of shedding vesicles can be induced by various inflammatory stimuli associated with altered intracellular calcium concentrations (5, 6). In contrast, exosome secretion can occur independently of any calcium influx, and may happen spontaneously (7, 8). Released microparticles may be ultimately internalized by local or distant recipient cells via mechanisms ranging from specific receptor–ligand interactions (indicating a targeted, cell-specific delivery) to nonspecific internalization via endocytosis or simple fusion with the cell membrane (9–12). Microparticles may therefore serve as a circulating “storage pool” of bioactive effectors (13) that mediate intercellular crosstalk and the horizontal transfer of genetic material, including microRNAs (miRNA) (14). As such, microparticles may participate in biological functions (including

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hemostasis, cell activation, and inflammation) relevant to the pathogenesis of ARDS (15).

GAP JUNCTIONAL INTERCELLULAR COMMUNICATION

In contrast to the distant interactions enabled by microparticle exchange, gap junctions allow for contiguous cell–cell communication. Gap junctions are formed from cell-surface connexins, a 20-member family of transmembrane proteins (16). Connexins associate into hexagonal connexons, which constitute 2–3-nm-diameter pores permeable to small (< 1 kD) molecules. A connexon may exist in an unpaired state (“hemichannel”) or pair with the connexon of a neighboring cell, forming a gap junction capable of direct cytoplasmic exchange. Recent studies have demonstrated the importance of gap junctional intercellular communication (GJIC) to the onset (17) and resolution (18) of pulmonary inflammation.

RELEVANCE OF ALTERNATIVE COMMUNICATION MECHANISMS TO NEUTROPHIL FUNCTION

The physiological significance of microparticle exchange and GJIC has been largely demonstrated in cell types other than neutrophils. However, available evidence supports the relevance of these processes to neutrophil function during inflammation.

Neutrophils rapidly shed microparticles in response to inflammatory stimuli (Figure 1) (19, 20). Shed neutrophil microparticles are heterogeneous, ranging from 50–200 nm in size (21). Neutrophil microparticles typically display surface phosphatidylserine as well as neutrophil markers such as selectins, CD15, CD64, CD66b, and CD66e (20, 22). As will be described, neutrophil-derived microparticle exchange exerts a significant impact on processes germane to organ inflammation, including inflammatory cell adhesion, cytokine production, and chemotaxis.

In contrast to microparticle exchange, the participation of neutrophils in GJIC is less well understood. Activated neutrophils express transmembrane connexins, including connexins 43 (23, 24) and 40 (25, 26). These connexins are capable of forming cell-surface connexons, which, if unpaired, function as channels for the extracellular secretion of small molecules such as ATP (24). Should neutrophil connexons link with the connexons of an adjacent cell, GJIC may occur. Indeed, neutrophil–neutrophil (25) and neutrophil–endothelial (23, 26) GJIC has been documented.

DO THESE COMMUNICATIONS CONTRIBUTE TO ARDS?

Although microparticle exchange and GJIC may be of relevance to neutrophil physiology, their contributions to the pathogenesis

of ARDS remain under investigation. We will discuss studies examining the activated neutrophil at several key stages in the evolution of lung inflammation: (1) the activated, circulating neutrophil, (2) the endothelial-adherent neutrophil, (3) the transmigrating neutrophil within the interstitial space, and (4) the activated neutrophil within the alveolar space.

Circulating Neutrophils during Systemic Inflammation

Systemic inflammatory diseases are characterized by rapid changes in circulating neutrophil morphology and physiology (27), thought to be mediated by the circulating cytokines and chemokines characteristic of the systemic inflammatory response syndrome (SIRS). The role of alternative forms of intercellular communication is less clear.

Neutrophil interactions with non-neutrophil microparticles. Neutrophils are known to interact with circulating microparticles of nonleukocyte origin (28–30). Opportunities for neutrophil–microparticle interaction are increased during SIRS, given a significant increase in circulating endothelial and platelet microparticles (28, 31).

Septic shock is associated with a significant increase in circulating platelet microparticles (31), with evidence of increased microparticle–neutrophil interactions (30). These complexes, although linked to improved patient outcomes, are also associated with augmented leukocyte activation (30). Indeed, platelet microparticles can activate neutrophils (29, 32, 33) and, in doing so, facilitate the progression of intravascular coagulation (34).

Similarly, circulating endothelial microparticles are more abundant in patients with sepsis (31), and complex with circulating leukocytes (28). Monocyte–endothelial microparticle complexes are associated with improved patient outcomes in sepsis (30). Although neutrophil–endothelial microparticle interactions have been similarly observed in patients with SIRS (28), the physiological impact of these interactions is largely unexplored.

Circulating erythrocyte microparticles may additionally activate neutrophils during ARDS (35), a finding of particular interest to the pathogenesis of transfusion-related acute lung injury (TRALI).

Neutrophil production of microparticles. Neutrophils not only receive communications via microparticle exchange, but also produce microparticles, influencing the function of several cell types relevant to lung injury (Table 1). Consistent with these diverse effects, the overall clinical impact of neutrophil-derived microparticles is uncertain. Circulating leukocyte and erythrocyte microparticles isolated from septic rats induced hemodynamic instability in normal rats (43). In contrast, nonplatelet microparticles isolated from humans with sepsis improved the vasoconstrictor response of murine aortic rings (31). Indeed, high circulating concentrations of leukocyte-derived microparticles were associated with improved outcomes in ARDS (44).

Direct cell–cell contact between neutrophils and other circulating cells. Intravascular neutrophils may directly interact with other circulating cells during systemic inflammatory responses. Neutrophils form clusters in response to inflammatory stimuli, with evidence of homotypic neutrophil GJIC (25). Neutrophils may additionally complex with platelets during sepsis (45). Because platelets are capable of forming functional connexons (46, 47), these complexes may allow for neutrophil–platelet GJIC. Interestingly, the presence of circulating platelet–leukocyte conjugates was associated with improved outcomes in septic shock (30). Whether this benefit indicates a protective effect of circulating platelet–leukocyte complexes, or rather reflects a harmful effect of their tissue sequestration, remains uncertain (45, 48). Indeed, platelet–neutrophil complexes release extracellular DNA, forming neutrophil extracellular traps implicated in TRALI (49). The presence and relevance of platelet–neutrophil GJIC in these processes are unexplored.

Figure 1. Microparticle release from activated neutrophils. Scanning electron micrograph of inactive (a) and FMLP-activated (b) neutrophils. Arrowheads (in main image and inset) demonstrate a 150-nm-diameter vesicle budding from the neutrophil surface, consistent with microparticle release. Scale bar, 1.5 μ m (400 nm on high-power inset). Reproduced with permission from Ref. 19.

TABLE 1. EFFECTS OF NEUTROPHIL-DERIVED MICROPARTICLES ON CELL TARGETS IN ARDS

Cell Target	Effect	Mechanism	Reference
Endothelial cells	Proinflammatory	Increased IL-6, MCP-1, tissue factor expression, and JNK-1 activation	(36, 37)
Neutrophils	Anti-inflammatory	Delivery of annexin A1 to neutrophil receptor ALX	(38)
Macrophages	Anti-inflammatory	Increased TGF- β release; decreased IL-8, IL-10, and TNF- α release; down-regulation of TLR2 expression	(11, 39, 40)
Platelets	Prothrombotic	Activated platelet Akt signaling via integrin and selectin binding; delivery of platelet-activating factor	(41, 42)

Definition of abbreviations: ARDS, acute respiratory distress syndrome; JNK-1, c-Jun N-terminal protein kinase-1; MCP-1, monocyte chemoattractant protein-1; TGF- β , transforming growth factor- β ; TLR2, Toll-like receptor-2.

Neutrophils Adherent to the Vascular Endothelial Surface

Adherent neutrophils may elaborate circulating microparticles capable of remotely influencing cells relevant to lung injury (as reviewed in Table 1). In addition, adherent neutrophils may participate in intercellular communications that can directly alter the vascular surface as well as the neutrophil itself.

Neutrophil–endothelial interactions at the pulmonary vascular surface. Endothelial cells express connexins (50, 51) capable of forming functional gap junctions with neighboring endothelial cells (17, 52). Cytoplasmic dye transfer experiments and compatible electron microscopy (Figure 2) have also suggested possible endothelial GJIC with recruited neutrophils (23, 26). Surprisingly, inflammatory insults generally attenuate GJIC (26, 53), despite an increase in the connexin content of cells in the distal lung (18, 54). This discordance may arise from the inflammatory internalization of endothelial connexin 43 (50).

The impact of neutrophil–endothelial GJIC (or the inflammatory loss thereof) upon pulmonary inflammation is unclear. The loss of endothelial connexin 43 was associated with an attenuation of leukocyte adhesion in the cremasteric microcirculation (55). Concordantly, proinflammatory effects of pulmonary connexin 43 were observed, although these findings were thought to reflect endothelial–endothelial GJIC (54). Conversely, an *in vitro* study using human umbilical vein endothelial cells reported that gap junction blockade augmented neutrophil transmigration (26).

Microparticle transfer may complement GJIC in mediating neutrophil–endothelial communication at the vascular surface. GJIC between alveolar mesenchymal stem cells (MSCs) and epithelial cells triggers the calcium-mediated release of MSC microparticles, which transfer MSC mitochondria to the alveolar surface (18). A similar pathway could conceivably occur between adherent neutrophils and the pulmonary endothelium, given observations that adherence is a major stimulant to neutrophil microparticle release (42). The adhesion-stimulated release of neutrophil microparticles may either augment neutrophil

chemotaxis and endothelial transmigration by coating the endothelial surface with L-selectin and P-selectin glycoprotein ligand-1 (56), or conversely, attenuate inflammation via the delivery of annexin-A1 to recruited neutrophils (38).

Neutrophil–platelet interactions at the pulmonary vascular surface. Neutrophils adherent within the pulmonary circulation may directly interact with platelets. After binding to endothelial cells, neutrophils polarize surface activation of the integrin CD11b/CD18 (macrophage-1 antigen, Mac-1), leading to localized platelet–neutrophil adherence that contributes to inflammatory diseases such as TRALI (57). Given that connexins are expressed in both activated neutrophils and platelets (as already discussed), this polarized binding represents a potential opportunity for GJIC between adherent neutrophils and their platelet partners. The presence and/or functional significance of such GJIC remain unexplored.

Neutrophil Transit through the Pulmonary Interstitium

The first step in neutrophil transit into the interstitium involves the release of the leukocyte trailing edge from the endothelial basolateral surface. In cremasteric microvessels, this release is opposed by neutrophil lymphocyte function–associated antigen 1 (LFA-1)–endothelial intercellular adhesion molecule-1 interactions (58). The leukocyte's attempt to pull away from these adhesions induces the release of CD18⁺ microparticles within the subendothelial space (58). The functional significance of this microparticle deposition, observed during both neutrophil and T-cell transmigration, is unknown, but may serve to direct trailing neutrophils to the site of inflammation.

Despite numerous opportunities for cell–cell interactions during the pulmonary interstitial passage of neutrophils, little else is known about neutrophil GJIC or microparticle exchange during transmigration. Given the propensity of transmigrating neutrophils to approximate pulmonary fibroblasts (59), as well as the ability of fibroblasts to participate in GJIC (60), future investigations of neutrophil–fibroblast interactions may be fruitful.

Figure 2. Potential neutrophil–endothelial gap junctional intercellular communication during inflammation. (a) Neutrophils exchange cytoplasm with the pulmonary endothelium during endotoxemia. *Top:* Intravital confocal lung microscopy of an LPS-treated (20 μ g/g body weight, administered via tail-vein injection), endothelial-fluorescent Tie2–green fluorescent protein (GFP) mouse demonstrates alveoli (A) separated by subpleural microvessels. *Bottom:* Cytoplasmic exchange (yellow) occurs between calcein red–labeled polymorphonuclear leukocytes (PMNs) (red, PMNs preincubated with 10 μ M calcein red $\times$ 30 minutes) and endothelial cells (green), 30 minutes

after application of LPS. Scale bar = 10 μ m. (b) Neutrophil–endothelial cytoplasmic exchange may occur via gap junctions, as suggested by a transmission electron micrograph of neutrophil (located at top of image) adhesion to an endothelial surface within a hamster cheek pouch subjected to ischemia–reperfusion injury. Scale bar, 1 μ m. (c) Enlarged view of selected (box in b) area demonstrates close membrane apposition (brackets above cell–cell border in c) consistent with gap junction formation. Scale bar, 100 nm. b and c are reproduced with permission from Ref. 23.

Neutrophils within the Alveolar Space

After transmigration, neutrophils enter into the alveolar space, where they confront invading pathogens and/or cause tissue injury. Despite these important activities, little is known about neutrophil communications occurring within the alveolar space.

Neutrophil–alveolar microparticle exchange. Microparticles detectable within the alveolar space (44, 61) are of varied origin. Epithelial cells have been implicated as a primary source of alveolar microparticles in ARDS (61). Platelet-derived microparticles have been found in porcine bronchoalveolar lavage (BAL) fluid and human tracheal lining fluid (62). Patients with bacterial pneumonia demonstrate increased BAL concentrations of microparticles expressing the neutrophil marker complement receptor–1. These microparticles inhibited the phagocytosis of partly opsonized bacteria *in vitro* (19). The *in vivo* significance of alveolar neutrophil microparticles remains unknown.

Gap junctions. Although epithelial–epithelial gap junctions are of physiologic importance in cystic fibrosis (63), no evidence of neutrophil GJIC with airway epithelia has been observed (64). Alveolar epithelial cells, however, have the potential to form gap junctions with bone marrow–derived cells (MSCs) (18). Whether these processes are relevant to neutrophils remains unknown.

CONCLUSIONS

Although the emerging processes of microparticle exchange and GJIC appear to be of pathophysiologic significance in the inflamed lung, the coordination of these varied intercellular interactions and their overall impact upon lung injury progression are uncertain. However, the pace of scientific discovery regarding microparticle exchange and GJIC is rapid, with multiple recent high-impact publications demonstrating the current enthusiasm for these modes of intercellular communication. Despite these gains, it is particularly striking how little is known about microparticles and/or GJIC after neutrophils exit the vasculature. This gap in knowledge appears to offer fertile ground for future investigations. Improvements in our understanding of neutrophil communication may bring us closer to achieving pathophysiology-targeted therapeutics in ARDS.

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