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Extracellular Vesicles: A New Frontier for Research in Acute Respiratory Distress Syndrome

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Abstract

Recent research on extracellular vesicles (EVs) has provided new insights into pathogenesis and potential therapeutic options for acute respiratory distress syndrome (ARDS). EVs are membrane-bound anuclear structures that carry important intercellular communication mechanisms, allowing targeted transfer of diverse biologic cargo, including protein, mRNA, and microRNA, among several different cell types. In this review, we discuss the important role EVs play in both inducing and attenuating inflammatory lung injury in ARDS as well as in sepsis, the most important clinical cause of ARDS. We discuss the translational challenges that need to be overcome before EVs can also be used as prognostic biomarkers in patients with ARDS and sepsis. We also consider how EVs may provide a platform for novel therapeutics in ARDS.

Keywords: acute lung injury; extracellular vesicles; acute respiratory distress syndrome; sepsis

Acute respiratory distress syndrome (ARDS) is an inflammatory disorder of the lungs that can develop after various clinical insults, the commonest etiologies being pulmonary and nonpulmonary sepsis. Patients develop acute hypoxemic respiratory failure after inflammatory injury to the alveolar epithelium and the lung and systemic endothelium (1). Our understanding of ARDS pathogenesis has increased in the 50 years since this syndrome was first described; however, there are many issues that require more research (1). Which biomarkers will make it possible to identify critically ill patients with sepsis who are at higher risk of developing ARDS? Which components of the inflammatory cascade can be targeted to attenuate lung and organ injury? Advances in ventilation strategies and fluid management have reduced mortality and morbidity; however, mortality still remains unacceptably high at 35–45% (2). Despite numerous clinical trials, there is still no effective pharmacotherapy available for patients with ARDS. However, the rapidly developing field of study of extracellular vesicles (EVs) may provide a major new opportunity for an improved understanding of ARDS pathogenesis, valuable biomarkers of injury, and targets or vehicles for new therapies.

EVs are structures released naturally by cells, which are surrounded by a phospholipid bilayer membrane and lack a functional nucleus (3). Cells release EVs during health, injury/activation, and apoptosis. EVs contain diverse biologic cargo, including proteins, lipids, mRNA, micro-RNA (miR), and mitochondria (4). Surface markers on EVs can identify the parent cell from which they were derived, and may determine which cells can incorporate specific EVs (5). However, EVs may also be taken up by nonspecific endocytosis (6), membrane fusion (7, 8), and micropinocytosis (9). There is a growing appreciation of the role EVs play in intercellular communication in both health and disease states, facilitating transfer of genetic material, proteins, and organelles between cell types (10). Distinctions between the two main subtypes of EVs were previously based on subcellular origin (11): Exosomes are smaller (<150 nm diameter), and may have an endosomal origin. Microvesicles are larger (up to 1 μm diameter) and derived from the cell membrane. However, the most recent position statement from the International Society for EVs (3) recommends moving away from the terms "exosome" and "microvesicle," as it is rarely possible to determine the specific biogenesis pathway for a given EV. Recommendations are made to instead classify EVs by physical

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characteristics (e.g., size and density), biochemical composition, or cell of origin (3). EVs have been found to contribute to the pathogenesis of other inflammatory diseases, including rheumatoid arthritis, in which platelet-derived EVs deliver IL-1 cargo to fibroblast-like synoviocytes, thereby driving joint inflammation (12). EVs have been shown to contribute to cancer pathogenesis, including tumor growth and development of metastases; EVs are being considered as biomarkers and/or therapeutic targets for different cancers (13).

In this review, we highlight selected studies that have deepened our understanding of the contributions of EVs in both mediating and attenuating inflammatory lung injury in ARDS as well as sepsis. We also discuss the translational challenges that need to be overcome before EVs can be used as diagnostic and prognostic biomarkers and targets for novel therapeutics in ARDS.

Contribution of EVs to the Pathogenesis of ARDS

Endothelial EVs

Preclinical models of ARDS have shown that EVs released after lung injury can be proinflammatory and have an injurious effect (Table 1). Endothelial injury is often the earliest pathological event leading to the development of both sepsis and ARDS (14). Endothelial cells release EVs after diverse modes of injury, including with LPS, plasminogen activated inhibitor-1, and mechanical stretch (15–17). In these studies, endothelial EVs were isolated by ultracentrifugation and characterized by flow cytometry. These endothelial EVs can impair nitric oxide release and vasodilation of arterioles (16). Administration of endothelial EVs in rodents induced inflammatory lung injury, with alveolar neutrophilic infiltration, pulmonary edema, elevated inflammatory cytokines (myeloperoxidase, IL-1β, and TNF-α), and increased lung endothelial permeability (16–19). In "two-hit" murine models of lung injury, an initial endothelial injury triggered release of endothelial EVs, which then primed the lung for a greater inflammatory response when exposed to a subsequent infectious insult (16, 18). This may explain for one pathway how ARDS develops in the clinical setting when more than one insult precedes the development of overt lung injury. This idea of multiple insults may apply to infectious and sterile causes of acute lung injury, including transfusion-associated lung injury (20).

Elevated circulating concentrations of endothelial EVs containing nitrated sphingosine-1-phosphate receptor-3 are associated with higher mortality in patients with sepsis, with or without ARDS (21). These endothelial EVs are also released after LPS treatment of endothelial cells, indicating an association with infectious injury (21). Endothelial EVs could therefore represent a potential biomarker, and/or offer a novel therapeutic target for ARDS. Simvastatin treatment given concurrently with intravenous LPS in mice reduced endothelial EV release and lung endothelial permeability (22). This is a particularly interesting finding, because a secondary analysis of an ARDS trial showed that simvastatin reduced mortality in patients with a hyperinflammatory endotype, suggesting that statin therapy might have worked in part by inhibiting EV release that enhanced the severity of lung injury (23).

EV Release in an *Ex Vivo* Perfused Human Lung Model

Recently, the results of studies in the *ex vivo* perfused human lung model have provided compelling new evidence for the potential role of EVs in mediating lung injury in ARDS. In an *ex vivo* perfused human lung model of gram-negative *Escherichia coli* pneumonia, injury with intrabronchial *E. coli* led to the release of EVs by lung tissue into the perfusate (24). These predominantly endothelial- and platelet-derived EVs were isolated by ultracentrifugation, then characterized by flow cytometry, EM, and nanoparticle tracking analysis. Administration of the *E. coli*–induced EVs either into the perfusate or intrabronchially in naive, uninjured human lungs induced injury similar to that observed with *E. coli* pneumonia with pulmonary edema, impaired alveolar fluid clearance, neutrophilic infiltration, and elevated BAL TNF-α (24). *E. coli*–induced EVs contained high levels of TNF-α and IL-6 mRNA, which in part explained their proinflammatory effects. Monocyte uptake of *E. coli*–induced EVs resulted in increased secretion of TNF-α and IL-6 (24). High-molecular-weight hyaluronic acid bound CD44 on the surface of *E. coli*–induced EVs, thus reducing their uptake by monocytes. Intravenous administration of high-molecular-weight hyaluronic acid to *ex vivo* human lungs injured with *E. coli* or *E. coli*–induced EVs attenuated lung injury, pulmonary edema, BAL TNF-α levels, and histologic evidence of lung injury (24). Thus, this study showed that, after acute bacterial pneumonia, human lung tissue releases pathogenic EVs that can mediate more severe inflammatory lung injury. The results suggest that strategies to sequester EVs could prevent their uptake and biologic cargo delivery to target cells, thereby reducing subsequent inflammatory injury; thus, EV sequestration could offer a therapeutic strategy in ARDS.

Platelet EVs

As described previously here, large numbers of platelet EVs were released in a human *ex vivo* lung model of ARDS (24). Activated platelets can play an important role in ARDS pathogenesis (25), and platelet EVs may similarly contribute to alveolar inflammation. Depletion of platelets in a mouse model of transfusion-associated lung injury prevented most of the lung injury (26). Further studies will be required to determine the role of platelet EVs in ARDS.

Epithelial EVs

A significant proportion of alveolar EVs in patients with ARDS are likely derived from alveolar epithelial cells; these EVs contain higher concentrations of tissue factor and exert a procoagulant effect (27). Alveolar epithelial cell–derived EVs may therefore also contribute to the increased procoagulant activity in ARDS, and thereby represent a therapeutic target. These EVs were isolated using ultracentrifugation and characterized using EM. A limitation of this study was that only EVs expressing phosphatidylserine were quantified. Epithelial cells injured with LPS can release EVs containing proteases capable of generating neutrophil chemoattractants (28). This finding indicates that an infectious insult to the pulmonary epithelium may stimulate EV release, leading to increased neutrophil infiltration and inflammatory responses as seen in ARDS. After lung injury, proinflammatory and proviral miRs can be transported between cells via EVs. BAL EVs from patients with influenza A–induced ARDS are enriched for miR-17-5p; transfection of alveolar epithelial cells with miR-17-5p

Table 1. Pathological Effects of Extracellular Vesicles in Experimental and Clinical Studies of Acute Lung Injury and Acute Respiratory Distress Syndrome

Cell Origin	Authors (Ref. No.)	EV Source	EV Effect
Endothelial	Densmore *et al.* (16), Letsiou *et al.* (17), and Buesing *et al.* (18)	Injured human endothelial cells	Endothelial EV treatment of murine or human arterioles impairs NO release and vasodilation.
	Sun *et al.* (21)	Plasma from patients with ARDS	Endothelial EVs containing S1PR3 may act as a biomarker for mortality in patients with ARDS.
	Liu *et al.* (24)	Perfusate from human *ex vivo* lungs injured with *E. coli*	Administration in naive human *ex vivo* lungs induces neutrophilic infiltration, pulmonary edema, and inflammatory cytokine release.
	Li *et al.* (15, 19)	Plasma of intratracheal LPS mice	Administration in mice induces lung injury similar to that observed in human *ex vivo* lungs.
Epithelial	Bastarche *et al.* (27) and Scheller *et al.* (29)	BAL from patients with ARDS	AEC EVs contain tissue factor and have a procoagulant effect. Patients with influenza-induced ARDS have elevated AEC EVs containing miR-17-5p, which promotes viral replication.
	Szul *et al.* (28)	Human bronchial epithelial cells	LPS activation of TLR-4 on BECs induces release of EVs containing prolyl endopeptidase.
	Lee *et al.* (32)	BAL from sterile murine lung injury	Epithelial EVs upregulate TLR-2 expression in macrophages.
Monocyte	Mitra *et al.* (31)	LPS-stimulated human monocytes; plasma from patients with ARDS	Monocyte EVs contain gasdermin D and activated caspase 1, which induce pulmonary endothelial cell death. These EVs were enriched in plasma from patients with sepsis-related ARDS.
Alveolar macrophage	Soni *et al.* (30)	BAL of intratracheal LPS mice	AM EVs deliver TNF-α to AECs, increasing KC and ICAM-1 expression. Administration in mice induces lung injury: increased BAL KC, protein permeability, and neutrophil infiltration.

Definition of abbreviations: AEC = alveolar epithelial cell; AM = alveolar macrophage; ARDS = acute respiratory distress syndrome; BECs = bronchial epithelial cells; *E. coli* = *Escherichia coli*; EV = extracellular vesicle; ICAM-1 = intercellular adhesion molecule-1; KC = keratinocyte-derived chemokine; miR = microRNA; NO = nitric oxide; Ref. = reference; S1PR3 = sphingosine-1-phosphate receptor-3; TLR = Toll-like receptor.

results in increased viral replication (29). These EVs were isolated by precipitation, and RNA was extracted before sequencing.

Alveolar Macrophage EVs

Different alveolar cell types release EVs in sequential order after infectious lung injury (30). Alveolar macrophage–derived EVs are rapidly released first, followed by endothelial EVs, and then neutrophil EVs. These EVs were isolated using ultracentrifugation and characterized by flow cytometry alone. The temporal difference in BAL EV release by different alveolar cell types may give insight into the pathological mechanisms underpinning ARDS. Alveolar macrophage–derived EV release may subsequently trigger proinflammatory EV release by epithelial cells and neutrophils. The alveolar macrophage EVs can deliver high concentrations of TNF-α cargo to alveolar epithelial cells, resulting in increased production of keratinocyte-derived chemokine (a neutrophil chemoattractant) and expression of intercellular adhesion molecule-1. Alveolar macrophage EVs containing TNF-α may play a significant role in initiating the inflammatory cascade in early ARDS; therefore, alveolar macrophage EVs could be considered as potential novel biomarkers and/or therapeutic targets.

Monocyte EVs

EVs containing gasdermin D are enriched in the plasma of patients with sepsis-related ARDS compared with healthy control subjects (31). LPS-stimulated monocytes release EVs containing gasdermin D and activated caspase 1; coculture of these EVs with human pulmonary vascular endothelial cells induces cell death (31). These findings indicate that an initial infectious injury may induce release of pathogenic EVs from monocytes, which then could damage the pulmonary epithelium and endothelium in ARDS.

Differential EV Release Dependent on Mode of Lung Injury

Different modalities of clinically relevant lung injury can induce release of EVs from different cell types, which have divergent functional effects. In a model of sterile lung injury (induced by hyperoxia or hydrochloric acid), most BAL EVs were

derived from type 1 alveolar epithelial cells (32). However, following a model of infectious lung injury, most BAL EVs were derived from alveolar macrophages. BAL EVs generated from both sterile and infectious lung injury models promoted the recruitment of macrophages to the alveolar space. BAL EVs were isolated by ultracentrifugation, then characterized by flow cytometry, EM, and nanoparticle tracking analysis. Differential effects on cytokine release were also observed with BAL EVs: sterile injury EVs upregulated alveolar macrophage release of IL-6 and TNF-α, whereas infectious injury EVs upregulated IL-1β and IL-10 release by alveolar macrophages (32). BAL EVs generated after different modalities of lung injury promote inflammation via different pathways.

EVs Mediate Lung Inflammation after Distant Injury

Remarkably, mouse models have indicated that release of EVs after distant injury (e.g., traumatic brain injury [33] or trauma and hemorrhagic shock [34]) can mediate lung injury. After trauma and hemorrhagic shock, gut-derived EVs are released into the mesenteric lymphatic system. EVs were isolated by ultracentrifugation, then characterized by EM and nanoparticle tracking analysis. Administration of these EVs to naive mice causes lung injury via macrophage Toll-like receptor 4 activation, increased alveolar vascular permeability, and inflammatory cell infiltration (34).

Table 2. Protective Effects of Extracellular Vesicles in Experimental and Clinical Studies of Acute Lung Injury and Acute Respiratory Distress Syndrome

Cell Origin	Authors (Ref. No.)	EV Source	EV Effect
Leukocyte	Guervilly *et al.* (36)	BAL and plasma from patients with ARDS	Elevated BAL and plasma leukocyte EVs were associated with increased survival and VFDs.
Neutrophil	Neudecker *et al.* (41)	BAL and stimulated human *ex vivo* neutrophils from patients with ARDS	Neutrophil EVs transfer miR-223 to AECs, reducing protein permeability and inflammatory cytokine release in AEC monolayers and murine models of staphylococcal lung injury.
	Eken *et al.* (39) and Gasser *et al.* (40)	Human *ex vivo* neutrophils	Binding to MerTK on macrophages increased secretion of TGF-β and decreased TNF-α and IL-8.
Bone marrow MSCs	Zhu *et al.* (52) and Tang *et al.* (62)	Cultured adult human bone marrow MSCs	MSC EV treatment in murine intratracheal LPS lung injury reduced alveolar neutrophil influx, protein permeability, inflammatory cytokines, and pulmonary edema. Effects mediated partly by MSC EV transfer of KGF mRNA to AECs and Ang-1 mRNA to endothelial cells.
	Monsel *et al.* (53) and Hao *et al.* (64)	Cultured adult human bone marrow MSCs	MSC EV treatment in murine *E. coli* lung injury reduced alveolar neutrophil influx, bacterial load, protein permeability, and inflammatory cytokines. Effects mediated partly by MSC EV transfer of miR-145 to macrophages.
	Li *et al.* (56)	Cultured murine bone marrow MSCs	Prophylactic MSC EV treatment increased survival and reduced pulmonary edema and inflammation in rat traumatic lung injury. Effects mediated partly by EV transfer of miR-124-3p.
	Khatri *et al.* (54)	Cultured pig bone marrow MSCs	MSC EV treatment in pig influenza-induced lung injury reduced lung injury, alveolar protein permeability, and inflammatory cytokines by reducing viral replication in AECs
	Morrison *et al.* (58)	Cultured adult human bone marrow MSCs	MSC EVs transfer mitochondria to AMs, inducing a modified M2 phenotype; these AMs then attenuated inflammatory lung injury when administered to intratracheal LPS–injured mice.
	Park *et al.* (55)	Cultured adult human bone marrow MSCs	MSC EV treatment in an *E. coli* pneumonia model using *ex vivo* human lungs increased alveolar fluid clearance and reduced alveolar bacterial load and protein permeability.

Definition of abbreviations: Ang-1 = angiopoetin-1; KGF = keratinocyte growth factor; MerTK = Mer tyrosine kinase; MSC = mesenchymal stromal cell; TGF-β = transforming growth factor-β; VFDs = ventilator-free days.

These findings indicate that similar mechanisms may be present in patients who develop ARDS after similar distant (indirect) insults. This pathway could even help to explain, in part, the development of neurogenic pulmonary edema as well as lung injury after shock and ischemia–reperfusion.

By learning the mechanisms and pathways by which EVs mediate inflammation and increase endothelial and epithelial permeability, it will be possible to gain greater insight into the protein and RNA pathways involved in the pathogenesis of endothelial and epithelial injury in ARDS and sepsis. These pathogenic EVs might be used as diagnostic and prognostic biomarkers, or as targets for novel therapeutic strategies.

Protective Effects of EVs in ARDS

In the prior section, we discussed the evidence for how EVs may contribute to ARDS pathogenesis from infectious and noninfectious causes; these harmful EVs are predominantly derived from specific cell types (endothelium, alveolar epithelial cells, and alveolar macrophages). There is also evidence that EVs from other cell types may have a protective, antiinflammatory role in the context of ARDS (Table 2). The data suggest that EVs within a given biofluid cannot be considered as a homogenous entity; the origin and cargo of EVs from different cell types at different stages of ARDS are likely to transmit divergent effects. Heterogeneity in cellular function and transcriptome has been related to patient outcomes in sepsis-related ARDS (35). It is therefore likely that heterogeneity in EV profiles will similarly impact on patient outcome.

Leukocyte EVs

Some clinical studies have reported that total leukocyte EV numbers were associated with a better prognosis in patients with ARDS. An observational study characterized BAL and circulating EVs from 52 ventilated patients with ARDS; control groups included ventilated patients without ARDS and nonventilated patients undergoing outpatient bronchoscopy (36). The majority (90%) of patients with ARDS had direct lung injury; 73% had pneumonia. BAL from patients with ARDS contained elevated leukocyte- and neutrophil-derived EVs compared with control subjects. EVs were isolated by centrifugation and characterized by flow cytometry; a limitation of this study is that EM was not also used to characterize EVs. In early ARDS, elevated BAL and plasma concentrations of leukocyte-derived EVs were associated with increased survival and ventilator-free days, thus suggesting a potential role for BAL and serum leukocyte EVs as favorable prognostic biomarkers in early ARDS. In a separate study, total plasma EV concentrations were measured in 280 critically ill patients at the time of ICU admission; 90 of these patients subsequently developed ARDS (37). Elevated plasma EV concentrations were associated with a lower risk of developing ARDS; the strongest associations were observed in patients with sepsis. However, this study could only detect EVs via phosphatidylserine expression, and could not detect the cell of origin. Another group of investigators (38) found that a subset of circulating leukocyte EVs expressing α2-macroglobulin were associated with better survival in intensive care patients with sepsis secondary to pneumonia, but not in patients with sepsis secondary to fecal peritonitis. α2-macroglobulin EV treatment *in vitro* reduced endothelial cell permeability, and increased bacterial phagocytosis by neutrophils, potentially indicating a mechanism of action.

Neutrophil EVs

Neutrophil EVs may have a protective, antiinflammatory role in lung injury. Binding of neutrophil EVs to MerTK (Mer tyrosine kinase) receptors on macrophages increased secretion of the prorepair TGF-β (transforming growth factor-β), and decreased secretion of proinflammatory cytokines TNF-α and IL-8 (39, 40). Therefore, neutrophil EVs can have an antiinflammatory effect on macrophages. Neutrophil EVs containing miR-223 also have an antiinflammatory effect on alveolar epithelial cells via suppression of poly(ADP-ribose) polymerase-1 (41). In murine staphylococcal or ventilator-induced lung injury, administration of EVs containing miR-223 reduced inflammatory cytokine release, alveolar protein permeability, and lung injury.

Proresolving lipids, including lipoxin A4, can aid resolution of inflammatory lung injury (42). After *in vitro* stimulation, or isolation from an *in vivo* model of peritoneal sepsis, neutrophils release EVs containing precursors for proresolving lipid mediators, including resolvins, lipoxins, and protectins (43, 44). Incubation of these neutrophil EVs with macrophages enhanced efferocytosis (clearance of apoptotic cells) and stimulated macrophage biosynthesis of proresolving lipid mediators, including resolvin E1, resolvin E2, maresin 1, and protectin D1 (43). EVs were isolated by ultracentrifugation and then characterized by flow cytometry.

Infiltration of neutrophils into the alveolar space is a hallmark of ARDS pathogenesis, and their proinflammatory role is well established. However, this evidence suggests that neutrophils may also have a concurrent antiinflammatory role via release of EVs, which modulate alveolar macrophage and alveolar epithelial cell functions (45). Future studies are required to assess the inflammatory activity of neutrophils concurrently with the protective effect of neutrophil EVs, to determine whether there is a homeostatic relationship between these functions. In addition, it will be necessary to determine whether the inflammatory effects of neutrophils, or the protective effects of neutrophil EVs, have the dominant effect on alveolar epithelial and endothelial function in the context of ARDS.

Innate Mechanisms Protect against Pathogenic EVs

Innate mechanisms that inhibit release or promote clearance of proinflammatory EVs may be present in patients with ARDS. For example, alveolar macrophages can phagocytose inflammatory BAL EVs via MerTK binding to prevent EV uptake by alveolar epithelial cells (46). The inflammatory BAL EVs have a more injurious effect on alveolar epithelial cells compared with alveolar macrophages. As explained in the previous section, LPS-stimulated macrophages release EVs containing TNF-α, which can initiate an inflammatory cascade. LPS-stimulated alveolar epithelial cells can release IL-25, which acts on macrophages to suppress release of inflammatory EVs containing TNF-α (47). Strategies to enhance IL-25 signaling or alveolar macrophage phagocytosis of EVs could have therapeutic benefit in ARDS.

There is limited evidence suggesting that EVs derived from the same cell type may have divergent effects on different

target cell types. An *in vitro* model of the blood–brain barrier showed that uptake of neutrophil EVs by cerebral microvascular endothelial cells led to tight junction dysfunction and increased permeability (48). This is in contrast to the protective role of neutrophil EVs identified in models of lung injury, as described previously here. Therefore, the biological effect of EVs derived from a given cell type will likely vary depending on the microenvironment generated by the disease and the target cell type that takes up the EVs. Further studies using different target cell/tissue types are required to investigate these divergent responses to EVs.

Subsets of leukocyte-derived EVs appear to have a protective role in ARDS, which may be related in part to delivery of antiinflammatory miRs. Mechanisms also exist to either inhibit release of inflammatory EVs (47) or prevent their uptake by susceptible cell types (46). Therapeutic strategies to upregulate innate protective EVs or enhance existing protective mechanisms may attenuate inflammation in ARDS.

Benefits of EVs Derived from Mesenchymal Stromal Cells in ARDS Models

EVs derived from nonpulmonary cell types can have a protective effect in some models of ARDS. Mesenchymal stromal cells (MSCs) can attenuate inflammation and lung injury in preclinical models of ARDS, due to their intrinsic antiinflammatory and proresolution properties (49). MSCs mediate their effects via cell–cell contact and via release of paracrine factors; administration of MSC-conditioned media was previously shown to attenuate lung injury in intratracheal LPS–injured mice and in the *ex vivo* perfused human lung (50, 51). MSC-derived EVs attenuated inflammation, alveolar protein permeability, and lung injury in both intratracheal LPS and *E. coli* pneumonia models of murine lung injury (52, 53), and in a pig model of influenza-induced lung injury (54). MSC EV treatment of alveolar epithelial cells reduced viral replication and virus-induced apoptosis (54). In an *E. coli* pneumonia model using *ex vivo* human lungs, administration of MSC EVs reduced bacterial load within the alveolar space, reduced protein permeability, and increased alveolar fluid clearance (55). Prophylactic treatment with MSC EVs increased survival in rats undergoing traumatic lung injury; inflammatory cytokines, infiltrating leukocytes, and the degree of pulmonary edema were all reduced (56). These effects were in part attributed to MSC EV transfer of miR-124-3p (56). In addition, an experimental model in newborn mice of infant respiratory distress syndrome and bronchopulmonary dysplasia showed a therapeutic effect of MSC EVs in both reducing lung injury and restoring lung function, in part mediated by induction of antiinflammatory and proresolving macrophages (57).

MSC EVs can transfer mitochondria to alveolar macrophages, inducing a modified M2 (proresolving) phenotype (58). MSCs stimulated with IL-1β release EVs containing miR-146a, which also induces an M2 macrophage phenotype (59). These MSC EV–modified alveolar macrophages have proresolving characteristics (increased secretion of antiinflammatory cytokine IL-10 and reduced secretion of inflammatory cytokines TNF-α and IL-8), but also increased phagocytic activity against bacteria (50, 58, 60, 61). MSC EVs modulate alveolar macrophages to clear bacteria more effectively, while minimizing surrounding tissue injury.

MSC EVs also contain mRNA for keratinocyte growth factor and angiopoetin-1, which can be transferred to alveolar epithelial cells and endothelial cells (52, 62), thereby increasing the integrity of the alveolar–capillary barrier. These findings explain, in part, the ability of MSC EVs to restore alveolar fluid clearance in *ex vivo* human lungs (63). A recent mouse study indicated that MSC EV transfer of miR-145 to macrophages was responsible for the increased bacterial phagocytosis, in part mediated by increased levels of leukotriene B4 (64).

MSCs mediate their antiinflammatory and prorepair effects in part by release of EVs, which deliver miR, mRNA, and mitochondrial cargo to different alveolar cell types (*see* Table 2). Administration of MSC EVs may therefore offer a novel therapeutic strategy for patients with ARDS.

Potential Clinical Applications for EVs in ARDS

EVs have wide-ranging potential clinical applications in ARDS, including use as prognostic biomarkers. Human endothelial EVs can induce lung injury in mice (16), and a subset of endothelial EVs has been associated with mortality in patients with ARDS (21). Thus, endothelial EVs could be used as prognostic biomarkers in ARDS. EVs containing DNA methyltransferase mRNA may have a prognostic role in sepsis and potentially ARDS (65). Therapeutic targeting of circulating and BAL pathogenic EVs in ARDS could reduce transfer of proinflammatory genetic material and proteins to uninjured cells. Nonspecific EV sequestering with hyaluronic acid (24) would target all EVs expressing CD44 (a widely expressed glycoprotein), but has a potential disadvantage of sequestering both pathogenic and beneficial EVs.

Alveolar macrophages can phagocytose pathogenic EVs via MerTK receptors, thereby preventing EV uptake by alveolar epithelial cells and subsequent inflammatory injury (46). Therapeutic strategies to upregulate MerTK expression on alveolar macrophages may accelerate the uptake of pathogenic EVs and attenuate inflammation in ARDS. However, the signaling pathways initiated by MerTK-mediated EV uptake need to be explored. Strategies to inhibit pathogenic EV release also require consideration: alveolar epithelial cells can secrete IL-25 to inhibit the release of pathogenic alveolar macrophage EVs (47). Simvastatin can inhibit the release of pathogenic endothelial EVs in murine models of lung injury (22), and this may be relevant in the setting of ARDS (23). Neutrophil EVs can have an antiinflammatory role in sepsis and ARDS (40, 41). Therefore, strategies to stimulate EV release by neutrophils *in vivo*, or administration of neutrophil EVs generated *ex vivo*, could be considered.

As described previously here, therapeutic use of MSC EVs have shown efficacy at reducing lung injury in several preclinical models. MSC EVs could also be modified to package custom drugs or protective RNA cargo, which could be delivered to specific cell types as determined by the EV surface markers (66).

Translational Challenges and Future Directions

Before EVs can be used as prognostic biomarkers, or as therapeutic targets for ARDS, there remain several major

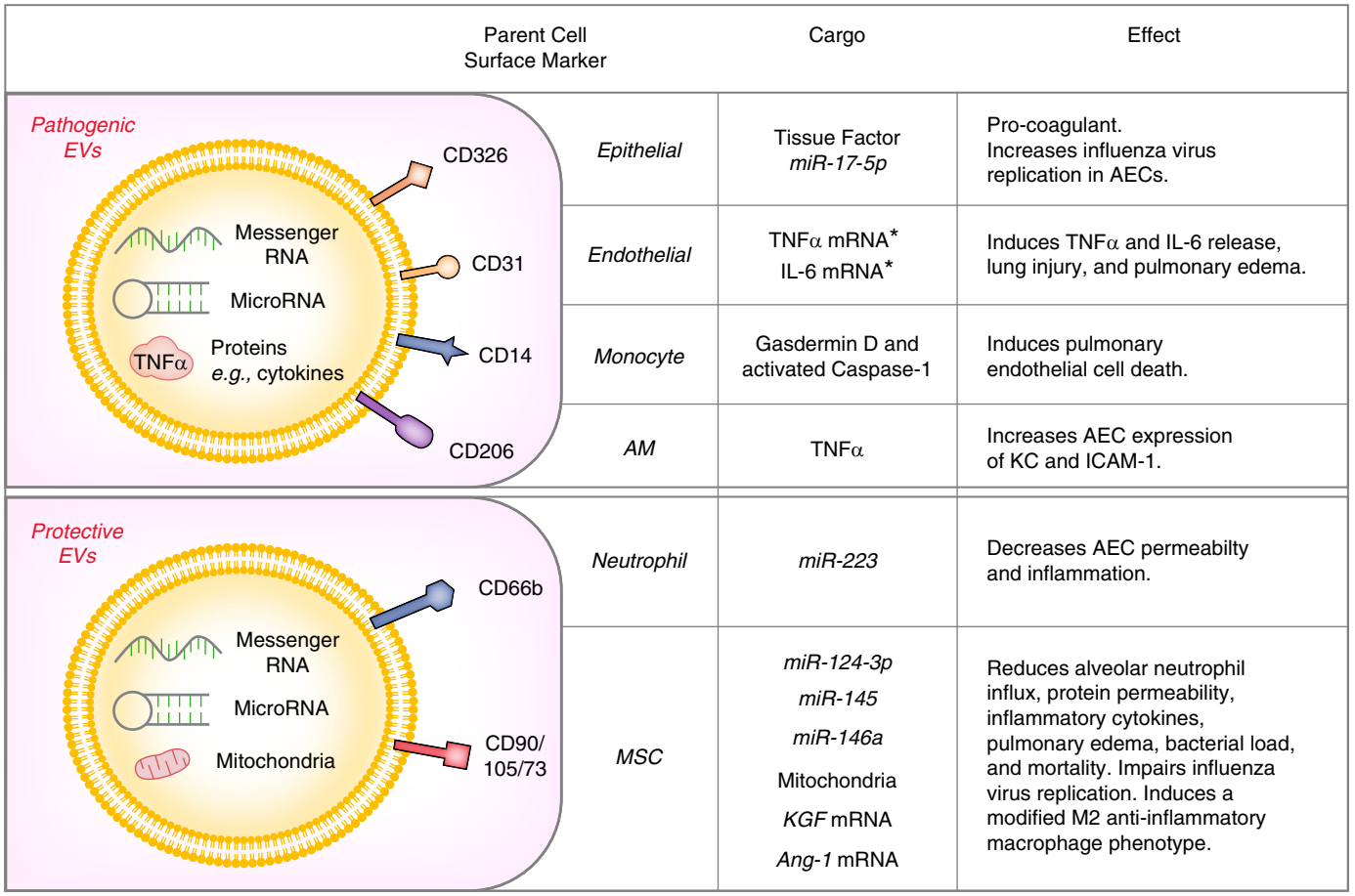

Figure 1. Cell-specific extracellular vesicles (EVs) and their cargo in acute respiratory distress syndrome (ARDS). EV shown with phospholipid bilayer and potential cargo. EV cargo can include microRNA, mRNA, mitochondria, and protein (e.g., cytokines). (Upper panel) Pathogenic EVs and their cargo. (Lower panel) Protective EVs and their cargo. EV parent cells are identified by surface marker expression (e.g., $CD31^+$ indicates endothelial-derived, $CD14^+$ indicates monocyte-derived, $CD206^+$ indicates alveolar macrophage–derived, $CD326^+$ indicates epithelial-derived, $CD66b^+$ indicates neutrophil-derived, and $CD90/105/73^+$ indicates mesenchymal stromal cell–derived EV). Some of the surface markers (e.g., CD31 and CD14) are expressed by multiple cell types and not only the cells shown here. In practice, the presence and absence of a combination of surface markers would be used to identify the cellular origin of EVs. The downstream effects of EV cargo in the context of ARDS are shown in the right panel. *The origin of EVs containing TNF-α and IL-6 mRNA is predominantly endothelial derived but may be from multiple cell types. AEC = alveolar epithelial cell; AM = alveolar macrophage; Ang-1 = angiopoetin-1; ICAM-1 = intercellular adhesion molecule-1; KC = keratinocyte-derived chemokine; KGF = keratinocyte growth factor; miR = microRNA; MSC = mesenchymal stromal cell.

translational challenges that need to be overcome. We give an overview of these challenges, which others have previously described in greater depth (67–69). Most studies investigating the role of EVs in ARDS have been done in animal and *in vitro* models, as well as recently in the *ex vivo* perfused human lung. Future studies must characterize BAL and circulating EVs from patients with ARDS and from those with sepsis relative to the cell of origin, assess their cargo (RNA/protein/organelle content), and determine their biological effect on human cells/tissues or murine models. By understanding how EVs mediate intercellular transfer of genetic material, organelles, and proteins among different cell types in the alveolar space, it will be possible to learn how RNA and protein pathways of injury are involved in ARDS pathogenesis. Our current knowledge of EV cargo in the context of ARDS is summarized in Figure 1.

Standardized methods of biologic fluid collection and RNA isolation from EVs are now possible, so that the data will be comparable and generalizable (70). However, a standardized method of EV isolation from biological fluids is yet to be established. Each isolation technique has its own advantages and disadvantages (71). Differential ultracentrifugation is the most widely used EV isolation technique due to low cost and ability to process large sample volumes; however, high centrifugation speeds may damage some EVs. Ultracentrifugation may be combined with use of a density gradient (e.g., sucrose) to increase purity. Size exclusion chromatography uses porous beads to separate EVs based on size. This technique is fast and preserves EV integrity; however, there are sample volume limitations. Polymeric precipitation kits use hydrophilic polymers to precipitate EVs. This technique is simple and quick; however, contamination with non EV proteins is common. For immunoprecipitation, samples are passed through beads coated with antibodies with specificity to EV transmembrane proteins (e.g., tetraspanins

CD9, CD63, and CD81). This technique gives high purity and selectivity of isolated EVs; however, it is expensive and difficult to detach EVs from the antibodies. A systematic comparison of EV isolation techniques across different human biofluids in healthy and diseased states is required to identify an optimal method that can be standardized. This will allow direct comparison of EV studies undertaken by different groups in patients with ARDS.

Characterization of EV size and concentration can be performed by nanoparticle tracking analysis (based on principles of Brownian motion), nanoscale flow cytometry, or single-particle interferometric reflectance imaging sensing. Use of fluorescently labeled antibodies with the latter two methods also allows identification of EV surface markers and thus cellular origins. Some single-particle interferometric reflectance imaging sensing platforms (e.g., Exoview R100; Nanoview Biosciences) allow characterization of EVs without prior isolation from biofluids, thereby reducing the chance of EV loss during the isolation process (72). However, although microvesicles are derived from cell membrane budding and will therefore express cell-specific surface markers, exosomes are derived from endosomal vesicles, and thus cellular origin is more challenging to identify. A recent position statement from the International Society of EVs (3) recommends identification of transmembrane/lipid-bound or cytosolic proteins to demonstrate the presence and purity of EVs within a biofluid preparation. This statement also recommends that characterization of single EVs should be undertaken using two different, but complementary, techniques, with one being EM based and the other based on light scatter (e.g., nanoparticle tracking analysis and flow cytometry) or resistive pulse sensing (3).

Studies of EV cargo have shown that specific RNAs are enriched in EVs compared with their parent cells; this process is regulated by signaling pathways, indicating that the RNA cargo within EVs from a given cell type may differ in healthy and diseased states (73). However, the relationship between changes in EV cargo composition and differences in EV functional effects is not fully understood. Studies often focus on a single cargo molecule, without addressing the thousands of other cargo molecules contained within the same EV. It may be that changes in classes of cargo molecules (e.g., proinflammatory vs. antiinflammatory) result in different EV functional effects, rather than changes at the level of a single cargo molecule. Changes in target cell surface receptor expression may also alter the functional effects of EVs with no change to the cargo composition. Undertaking RNA sequencing and proteomic studies of EV cargo, in conjunction with identification of EV subtype and cell of origin, will make it possible to further characterize the functional role of EVs within disease processes such as ARDS. This may allow identification of EVs with a specific RNA or protein cargo signature as biomarkers and therapeutic targets in ARDS.

Several animal studies have shown that, after intravenous administration of EVs, the majority of EVs are cleared from the circulation within an hour (74, 75). The duration for which EVs persist within the circulation is partly dependent on size, with larger EVs persisting for longer (76). Macrophages appear to be essential for EV clearance (77); recognition of phosphatidylserine on EVs by macrophage efferocytosis receptors (e.g., MerTK, as described previously here) likely constitutes an important clearance mechanism (46). EVs may also be cleared by excretion via the urinary system (78). The cellular origin of EVs partly determines the target tissues by which EVs are taken up (79). However, the affinity of EVs to different tissues can be artificially modified; one murine study showed that glycosylation of EV surface markers increased EV uptake within the lungs (80). Further studies are required to understand EV biodistribution and clearance before we can effectively target pathogenic EVs or use protective EVs as therapeutic tools.

EV profiles from patients with ARDS should be compared with those from animal models and *ex vivo* human lung models to determine how closely the EVs released in these models correlate with those observed in the clinical setting of ARDS. Clinical studies to test the utility of EVs as diagnostic and prognostic biomarkers in patients with ARDS are also needed. Eventually, clinical trials of MSC EVs may also be warranted to determine therapeutic utility in patients with ARDS as well as infant respiratory distress syndrome and bronchopulmonary dysplasia.

Conclusions

EVs constitute an important intercellular communication mechanism, which allows targeted transfer of diverse cargo among different cell types. New evidence indicates that EVs may be critical to the induction and resolution of injury in ARDS and sepsis. Consequently, there are a wide range of potential clinical applications for EVs, ranging from use as biomarkers to therapeutic targets, and possibly EVs that can be used for therapeutics in both sepsis and ARDS. ■

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References

1. Matthay MA, Zemans RL, Zimmerman GA, Arabi YM, Beitler JR, Mercat A, *et al*. Acute respiratory distress syndrome. *Nat Rev Dis Primers* 2019;5:18.
2. Bellani G, Laffey JG, Pham T, Fan E, Brochard L, Esteban A, *et al*.; LUNG SAFE Investigators; ESICM Trials Group. Epidemiology, patterns of care, and mortality for patients with acute respiratory distress syndrome in intensive care units in 50 countries. *JAMA* 2016;315:788–800.
3. Théry C, Witwer KW, Aikawa E, Alcaraz MJ, Anderson JD, Andriantsitohaina R, *et al*. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. *J Extracell Vesicles* 2018;7: 1535750.
4. Fujita Y, Kadota T, Araya J, Ochiya T, Kuwano K. Extracellular vesicles: new players in lung immunity. *Am J Respir Cell Mol Biol* 2018;58: 560–565.

5. Morelli AE, Larregina AT, Shufesky WJ, Sullivan ML, Stolz DB, Papworth GD, *et al*. Endocytosis, intracellular sorting, and processing of exosomes by dendritic cells. *Blood* 2004;104:3257–3266.
6. Tian T, Wang Y, Wang H, Zhu Z, Xiao Z. Visualizing of the cellular uptake and intracellular trafficking of exosomes by live-cell microscopy. *J Cell Biochem* 2010;111:488–496.
7. Montecalvo A, Larregina AT, Shufesky WJ, Stolz DB, Sullivan ML, Karlsson JM, *et al*. Mechanism of transfer of functional microRNAs between mouse dendritic cells via exosomes. *Blood* 2012;119: 756–766.
8. Parolini I, Federici C, Raggi C, Lugini L, Palleschi S, De Milito A, *et al*. Microenvironmental pH is a key factor for exosome traffic in tumor cells. *J Biol Chem* 2009;284:34211–34222.
9. Fitzner D, Schnaars M, van Rossum D, Krishnamoorthy G, Dibaj P, Bakhti M, *et al*. Selective transfer of exosomes from oligodendrocytes to microglia by macropinocytosis. *J Cell Sci* 2011; 124:447–458.
10. Gupta R, Radicioni G, Abdelwahab S, Dang H, Carpenter J, Chua M, *et al*. Intercellular communication between airway epithelial cells is mediated by exosome-like vesicles. *Am J Respir Cell Mol Biol* 2019; 60:209–220.
11. Shah R, Patel T, Freedman JE. Circulating extracellular vesicles in human disease. *N Engl J Med* 2018;379:958–966.
12. Boilard E, Nigrovic PA, Larabee K, Watts GF, Coblyn JS, Weinblatt ME, *et al*. Platelets amplify inflammation in arthritis via collagen-dependent microparticle production. *Science* 2010;327: 580–583.
13. Becker A, Thakur BK, Weiss JM, Kim HS, Peinado H, Lyden D. Extracellular vesicles in cancer: cell-to-cell mediators of metastasis. *Cancer Cell* 2016;30:836–848.
14. Matthay MA, Zemans RL. The acute respiratory distress syndrome: pathogenesis and treatment. *Annu Rev Pathol* 2011;6: 147–163.
15. Li H, Meng X, Gao Y, Cai S. Isolation and phenotypic characteristics of microparticles in acute respiratory distress syndrome. *Int J Clin Exp Pathol* 2015;8:1640–1648.
16. Densmore JC, Signorino PR, Ou J, Hatoum OA, Rowe JJ, Shi Y, *et al*. Endothelium-derived microparticles induce endothelial dysfunction and acute lung injury. *Shock* 2006;26:464–471.
17. Letsiou E, Sammani S, Zhang W, Zhou T, Quijada H, Moreno-Vinasco L, *et al*. Pathologic mechanical stress and endotoxin exposure increases lung endothelial microparticle shedding. *Am J Respir Cell Mol Biol* 2015;52:193–204.
18. Buesing KL, Densmore JC, Kaul S, Pritchard KA Jr, Jarzembowski JA, Gourlay DM, *et al*. Endothelial microparticles induce inflammation in acute lung injury. *J Surg Res* 2011;166:32–39.
19. Li H, Meng X, Liang X, Gao Y, Cai S. Administration of microparticles from blood of the lipopolysaccharide-treated rats serves to induce pathologic changes of acute respiratory distress syndrome. *Exp Biol Med (Maywood)* 2015;240:1735–1741.
20. Toy P, Gajic O, Bacchetti P, Looney MR, Gropper MA, Hubmayr R, *et al*.; TRALI Study Group. Transfusion-related acute lung injury: incidence and risk factors. *Blood* 2012;119:1757–1767.
21. Sun X, Singleton PA, Letsiou E, Zhao J, Belvitch P, Sammani S, *et al*. Sphingosine-1-phosphate receptor-3 is a novel biomarker in acute lung injury. *Am J Respir Cell Mol Biol* 2012;47:628–636.
22. Yu Y, Jing L, Zhang X, Gao C. Simvastatin attenuates acute lung injury via regulating CDC42-PAK4 and endothelial microparticles. *Shock* 2017;47:378–384.
23. Calfee CS, Delucchi KL, Sinha P, Matthay MA, Hackett J, Shankar-Hari M, *et al*.; Irish Critical Care Trials Group. Acute respiratory distress syndrome subphenotypes and differential response to simvastatin: secondary analysis of a randomised controlled trial. *Lancet Respir Med* 2018;6:691–698.
24. Liu A, Park JH, Zhang X, Sugita S, Naito Y, Lee JH, *et al*. Therapeutic effects of hyaluronic acid in bacterial pneumonia in *ex vivo* perfused human lungs. *Am J Respir Crit Care Med* 2019;200: 1234–1245.
25. Middleton EA, Weyrich AS, Zimmerman GA. Platelets in pulmonary immune responses and inflammatory lung diseases. *Physiol Rev* 2016;96:1211–1259.
26. Looney MR, Nguyen JX, Hu Y, Van Ziffle JA, Lowell CA, Matthay MA. Platelet depletion and aspirin treatment protect mice in a two-event model of transfusion-related acute lung injury. *J Clin Invest* 2009; 119:3450–3461.
27. Bastarache JA, Fremont RD, Kropski JA, Bossert FR, Ware LB. Procoagulant alveolar microparticles in the lungs of patients with acute respiratory distress syndrome. *Am J Physiol Lung Cell Mol Physiol* 2009;297:L1035–L1041.
28. Szul T, Bratcher PE, Fraser KB, Kong M, Tirouvanziam R, Ingersoll S, *et al*. Toll-like receptor 4 engagement mediates prolyl endopeptidase release from airway epithelia via exosomes. *Am J Respir Cell Mol Biol* 2016;54:359–369.
29. Scheller N, Herold S, Kellner R, Bertrams W, Jung AL, Janga H, *et al*. Proviral microRNAs detected in extracellular vesicles from bronchoalveolar lavage fluid of patients with influenza virus–induced acute respiratory distress syndrome. *J Infect Dis* 2019;219:540–543.
30. Soni S, Wilson MR, O'Dea KP, Yoshida M, Katbeh U, Woods SJ, *et al*. Alveolar macrophage–derived microvesicles mediate acute lung injury. *Thorax* 2016;71:1020–1029.
31. Mitra S, Exline M, Habyarimana F, Gavrilin MA, Baker PJ, Masters SL, *et al*. Microparticulate caspase 1 regulates gasdermin D and pulmonary vascular endothelial cell injury. *Am J Respir Cell Mol Biol* 2018;59:56–64.
32. Lee H, Zhang D, Laskin DL, Jin Y. Functional evidence of pulmonary extracellular vesicles in infectious and noninfectious lung inflammation. *J Immunol* 2018;201:1500–1509.
33. Kerr NA, de Rivero Vaccari JP, Abbassi S, Kaur H, Zambrano R, Wu S, *et al*. Traumatic brain injury-induced acute lung injury: evidence for activation and inhibition of a neural–respiratory–inflammasome axis. *J Neurotrauma* 2018;35:2067–2076.
34. Kojima M, Gimenes-Junior JA, Chan TW, Eliceiri BP, Baird A, Costantini TW, *et al*. Exosomes in postshock mesenteric lymph are key mediators of acute lung injury triggering the macrophage activation via Toll-like receptor 4. *FASEB J* 2018;32:97–110.
35. Leligdowicz A, Matthay MA. Heterogeneity in sepsis: new biological evidence with clinical applications. *Crit Care* 2019;23:80.
36. Guervilly C, Lacroix R, Forel JM, Roch A, Camoin-Jau L, Papazian L, *et al*. High levels of circulating leukocyte microparticles are associated with better outcome in acute respiratory distress syndrome. *Crit Care* 2011;15:R31.
37. Shaver CM, Woods J, Clune JK, Grove BS, Wickersham NE, McNeil JB, *et al*. Circulating microparticle levels are reduced in patients with ARDS. *Crit Care* 2017;21:120.
38. Lashin HMS, Nadkarni S, Oggero S, Jones HR, Knight JC, Hinds CJ, *et al*. Microvesicle subsets in sepsis due to community acquired pneumonia compared to faecal peritonitis. *Shock* 2018;49:393–401.
39. Eken C, Martin PJ, Sadallah S, Treves S, Schaller M, Schifferli JA. Ectosomes released by polymorphonuclear neutrophils induce a MerTK-dependent anti-inflammatory pathway in macrophages. *J Biol Chem* 2010;285:39914–39921.
40. Gasser O, Schifferli JA. Activated polymorphonuclear neutrophils disseminate anti-inflammatory microparticles by ectocytosis. *Blood* 2004;104:2543–2548.
41. Neudecker V, Brodsky KS, Clambey ET, Schmidt EP, Packard TA, Davenport B, *et al*. Neutrophil transfer of *miR-223* to lung epithelial cells dampens acute lung injury in mice. *Sci Transl Med* 2017;9: eaah5360.
42. Fang X, Abbott J, Cheng L, Colby JK, Lee JW, Levy BD, *et al*. Human mesenchymal stem (stromal) cells promote the resolution of acute lung injury in part through lipoxin A4. *J Immunol* 2015;195:875–881.
43. Dalli J, Serhan CN. Specific lipid mediator signatures of human phagocytes: microparticles stimulate macrophage efferocytosis and pro-resolving mediators. *Blood* 2012;120:e60–e72.
44. Norling LV, Spite M, Yang R, Flower RJ, Perretti M, Serhan CN. Cutting edge: humanized nano-proresolving medicines mimic inflammation-resolution and enhance wound healing. *J Immunol* 2011;186: 5543–5547.
45. Dengler V, Downey GP, Tuder RM, Eltzschig HK, Schmidt EP. Neutrophil intercellular communication in acute lung injury: emerging roles of microparticles and gap junctions. *Am J Respir Cell Mol Biol* 2013;49:1–5.

46. Mohning MP, Thomas SM, Barthel L, Mould KJ, McCubbrey AL, Frasch SC, *et al*. Phagocytosis of microparticles by alveolar macrophages during acute lung injury requires MerTK. *Am J Physiol Lung Cell Mol Physiol* 2018;314:L69–L82.
47. Li ZG, Scott MJ, Brzóska T, Sundd P, Li YH, Billiar TR, *et al*. Lung epithelial cell–derived IL-25 negatively regulates LPS-induced exosome release from macrophages. *Mil Med Res* 2018;5:24.
48. Ajikumar A, Long MB, Heath PR, Wharton SB, Ince PG, Ridger VC, *et al*. Neutrophil-derived microvesicle induced dysfunction of brain microvascular endothelial cells *in vitro*. *Int J Mol Sci* 2019;20: E5227.
49. Laffey JG, Matthay M A. Fifty years of research in ARDS: cell-based therapy for acute respiratory distress syndrome. Biology and potential therapeutic value. *Am J Respir Crit Care Med* 2017;196:266–273.
50. Ionescu L, Byrne RN, van Haaften T, Vadivel A, Alphonse RS, Rey-Parra GJ, *et al*. Stem cell conditioned medium improves acute lung injury in mice: *in vivo* evidence for stem cell paracrine action. *Am J Physiol Lung Cell Mol Physiol* 2012;303:L967–L977.
51. Lee JW, Fang X, Gupta N, Serikov V, Matthay MA. Allogeneic human mesenchymal stem cells for treatment of *E. coli* endotoxin–induced acute lung injury in the *ex vivo* perfused human lung. *Proc Natl Acad Sci USA* 2009;106:16357–16362.
52. Zhu YG, Feng XM, Abbott J, Fang XH, Hao Q, Monsel A, *et al*. Human mesenchymal stem cell microvesicles for treatment of *Escherichia coli* endotoxin–induced acute lung injury in mice. *Stem Cells* 2014; 32:116–125.
53. Monsel A, Zhu YG, Gennai S, Hao Q, Hu S, Rouby JJ, *et al*. Therapeutic effects of human mesenchymal stem cell–derived microvesicles in severe pneumonia in mice. *Am J Respir Crit Care Med* 2015;192: 324–336.
54. Khatri M, Richardson LA, Meulia T. Mesenchymal stem cell–derived extracellular vesicles attenuate influenza virus–induced acute lung injury in a pig model. *Stem Cell Res Ther* 2018;9:17.
55. Park J, Kim S, Lim H, Liu A, Hu S, Lee J, *et al*. Therapeutic effects of human mesenchymal stem cell microvesicles in an *ex vivo* perfused human lung injured with severe *E. coli* pneumonia. *Thorax* 2019;74: 43–50.
56. Li QC, Liang Y, Su ZB. Prophylactic treatment with MSC-derived exosomes attenuates traumatic acute lung injury in rats. *Am J Physiol Lung Cell Mol Physiol* 2019;316:L1107–L1117.
57. Willis GR, Fernandez-Gonzalez A, Anastas J, Vitali SH, Liu X, Ericsson M, *et al*. Mesenchymal stromal cell exosomes ameliorate experimental bronchopulmonary dysplasia and restore lung function through macrophage immunomodulation. *Am J Respir Crit Care Med* 2018;197:104–116.
58. Morrison TJ, Jackson MV, Cunningham EK, Kissenpfennig A, McAuley DF, O'Kane CM, *et al*. Mesenchymal stromal cells modulate macrophages in clinically relevant lung injury models by extracellular vesicle mitochondrial transfer. *Am J Respir Crit Care Med* 2017;196: 1275–1286.
59. Song Y, Dou H, Li X, Zhao X, Li Y, Liu D, *et al*. Exosomal miR-146a contributes to the enhanced therapeutic efficacy of interleukin-1β–primed mesenchymal stem cells against sepsis. *Stem Cells* 2017;35:1208–1221.
60. Kim J, Hematti P. Mesenchymal stem cell–educated macrophages: a novel type of alternatively activated macrophages. *Exp Hematol* 2009;37:1445–1453.
61. Krasnodembskaya A, Samarani G, Song Y, Zhuo H, Su X, Lee JW, *et al*. Human mesenchymal stem cells reduce mortality and bacteremia in gram-negative sepsis in mice in part by enhancing the phagocytic activity of blood monocytes. *Am J Physiol Lung Cell Mol Physiol* 2012;302:L1003–L1013.
62. Tang XD, Shi L, Monsel A, Li XY, Zhu HL, Zhu YG, *et al*. Mesenchymal stem cell microvesicles attenuate acute lung injury in mice partly mediated by ang-1 mRNA. *Stem Cells* 2017;35:1849–1859.
63. Gennai S, Monsel A, Hao Q, Park J, Matthay MA, Lee JW. Microvesicles derived from human mesenchymal stem cells restore alveolar fluid clearance in human lungs rejected for transplantation. *Am J Transplant* 2015;15:2404–2412.
64. Hao Q, Gudapati V, Monsel A, Park JH, Hu S, Kato H, *et al*. Mesenchymal stem cell–derived extracellular vesicles decrease lung injury in mice. *J Immunol* 2019;203:1961–1972.
65. Dakhlallah DA, Wisler J, Gencheva M, Brown CM, Leatherman ER, Singh K, *et al*. Circulating extracellular vesicle content reveals *de novo* DNA methyltransferase expression as a molecular method to predict septic shock. *J Extracell Vesicles* 2019;8:1669881.
66. Ju Z, Ma J, Wang C, Yu J, Qiao Y, Hei F. Exosomes from iPSCs delivering siRNA attenuate intracellular adhesion molecule-1 expression and neutrophils adhesion in pulmonary microvascular endothelial cells. *Inflammation* 2017;40:486–496.
67. Lener T, Gimona M, Aigner L, Börger V, Buzas E, Camussi G, *et al*. Applying extracellular vesicles based therapeutics in clinical trials—an ISEV position paper. *J Extracell Vesicles* 2015;4:30087.
68. Rohde E, Pachler K, Gimona M. Manufacturing and characterization of extracellular vesicles from umbilical cord–derived mesenchymal stromal cells for clinical testing. *Cytotherapy* 2019;21: 581–592.
69. Willis GR, Kourembanas S, Mitsialis SA. Toward exosome-based therapeutics: isolation, heterogeneity, and fit-for-purpose potency. *Front Cardiovasc Med* 2017;4:63.
70. Srinivasan S, Yeri A, Cheah PS, Chung A, Danielson K, De Hoff P, *et al*. Small RNA sequencing across diverse biofluids identifies optimal methods for exRNA isolation. *Cell* 2019;177:446–462, e16.
71. Carnino JM, Lee H, Jin Y. Isolation and characterization of extracellular vesicles from Broncho-alveolar lavage fluid: a review and comparison of different methods. *Respir Res* 2019;20:240.
72. Daaboul GG, Gagni P, Benussi L, Bettotti P, Ciani M, Cretich M, *et al*. Digital detection of exosomes by interferometric imaging. *Sci Rep* 2016;6:37246.
73. Das S, Ansel KM, Bitzer M, Breakefield XO, Charest A, Galas DJ, *et al*.; Extracellular RNA Communication Consortium. The extracellular RNA communication consortium: establishing foundational knowledge and technologies for extracellular RNA research. *Cell* 2019;177:231–242.
74. Lai CP, Mardini O, Ericsson M, Prabhakar S, Maguire C, Chen JW, *et al*. Dynamic biodistribution of extracellular vesicles *in vivo* using a multimodal imaging reporter. *ACS Nano* 2014;8:483–494.
75. Takahashi Y, Nishikawa M, Shinotsuka H, Matsui Y, Ohara S, Imai T, *et al*. Visualization and *in vivo* tracking of the exosomes of murine melanoma B16-BL6 cells in mice after intravenous injection. *J Biotechnol* 2013;165:77–84.
76. Lundy DJ, Chen KH, Toh EK, Hsieh PC. Distribution of systemically administered nanoparticles reveals a size-dependent effect immediately following cardiac ischaemia-reperfusion injury. *Sci Rep* 2016;6:25613.
77. Imai T, Takahashi Y, Nishikawa M, Kato K, Morishita M, Yamashita T, *et al*. Macrophage-dependent clearance of systemically administered B16BL6-derived exosomes from the blood circulation in mice. *J Extracell Vesicles* 2015;4:26238.
78. Everaert C, Helsmoortel H, Decock A, Hulstaert E, Van Paemel R, Verniers K, *et al*. Performance assessment of total RNA sequencing of human biofluids and extracellular vesicles. *Sci Rep* 2019;9:17574.
79. Wiklander OP, Nordin JZ, O'Loughlin A, Gustafsson Y, Corso G, Mäger I, *et al*. Extracellular vesicle *in vivo* biodistribution is determined by cell source, route of administration and targeting. *J Extracell Vesicles* 2015;4:26316.
80. Royo F, Cossío U, Ruiz de Angulo A, Llop J, Falcon-Perez JM. Modification of the glycosylation of extracellular vesicles alters their biodistribution in mice. *Nanoscale* 2019;11:1531–1537.