

# Stem-cell extracellular vesicles and lung repair

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**Abstract:** Four out of the ten leading causes of morbidity and mortality worldwide are lung diseases. Despite advances in comprehending the pathophysiological mechanisms involved in these disorders, for several respiratory diseases, there is still no effective treatment able to stop their natural history or reverse the morphological and functional damage they cause. In this context, recent research has supported a potential role of cell therapy for lung diseases and critical illness. The anti-inflammatory, antifibrotic, and microbicidal effects of stem cells are mainly attributed to their secretome, which contains proteins, lipids, microRNAs, and mRNAs. These are secreted in the conditioned medium and are also present in extracellular vesicles (EVs). This review will provide a detailed discussion of the role of EVs produced by mesenchymal stromal cells in preclinical experimental models of pulmonary disorders and critical illness, as well as in ongoing clinical trials.

**Keywords:** Extracellular vesicles (EVs); microvesicles; exosomes; lung diseases; regenerative medicine

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## Stem cells and lung repair

Cell-based therapies have been widely used in experimental and clinical studies as a new therapeutic approach for lung diseases, since they modulate inflammation and affect the remodeling process (1).

The main source of adult stem cells for cell therapy is the bone marrow. Hematopoietic stem cells have been studied due to their capacity to differentiate into immune cells and modulate immune-cell proliferation and activity (1,2). Mesenchymal stem (or, more properly, stromal) cells (MSCs) are also present in bone marrow, and play a primary role in stimulating the maintenance, growth, and survival of other cells. MSCs are multipotent and have been found in many sources, such as adipose tissue, amniotic fluid, cord blood,

and the lungs (3). Beyond their stromal properties, MSCs are known to have plastic capacity and immunomodulatory, antifibrotic, and microbicidal properties (4), which has motivated many groups to study their therapeutic potential in experimental lung diseases and test them in phase I and II trials for acute respiratory distress syndrome (ARDS) (5-7), chronic obstructive pulmonary disease (COPD) (8-10), silicosis (11), and idiopathic pulmonary fibrosis (IPF) (12).

The mechanisms by which MSCs might mitigate inflammation and injury are not completely understood, and likely involve multiple pathways mediated by the release of soluble mediators, extracellular vesicles (EVs), and/or organelle transfer, as well as through cell-to-cell contact (1,4). Secretory mediators were first proposed as a

mechanism of action for stem cells because very few stem cells engraft after injection into recipient animals (13). Recent data from a variety of preclinical lung disease models, including ARDS, asthma, emphysema, and pulmonary arterial hypertension (PAH), have demonstrated that systemic administration of conditioned media (CM) obtained from MSCs alone can lead to protective effects similar to those of MSCs (14-21). Recent data suggest that EVs, also known as exosomes, microvesicles, or microparticles, which are released by the MSCs and present in CM, may yield beneficial effects (21-26). However, the specific mediators responsible, such as soluble proteins, EV components, or other components of the CM, have not yet been identified, and are likely to be different depending on the lung injury model (22). Information has emerged regarding the roles of specific miRNAs and other EV components as mediators of the protective effects of MSC administration in preclinical lung disease models, but much remains unknown (21-26).

## EVs

Recently, in order to standardize the nomenclature, the International Society for Extracellular Vesicles established a definition of EVs and the minimal experimental requirements for research about EVs (27). EVs are defined as small membrane vesicles, which includes exosomes, microvesicles and apoptotic bodies. They are distinguished by specific membrane markers, origin, and size (exosomes, 40–150 nm; microvesicles, 0.1–2 µm; apoptotic bodies, 1–4 µm). Because of the overlapping sizes and lack of specific markers for each EV component, the International Society for Extracellular Vesicles recommends use of the term EVs to describe all types of such vesicles. EVs are considered mediators of intercellular communication, as they contain several proteins, microRNAs, mRNAs, long noncoding RNAs, lipid mediators, and even organelles with biological relevance (28).

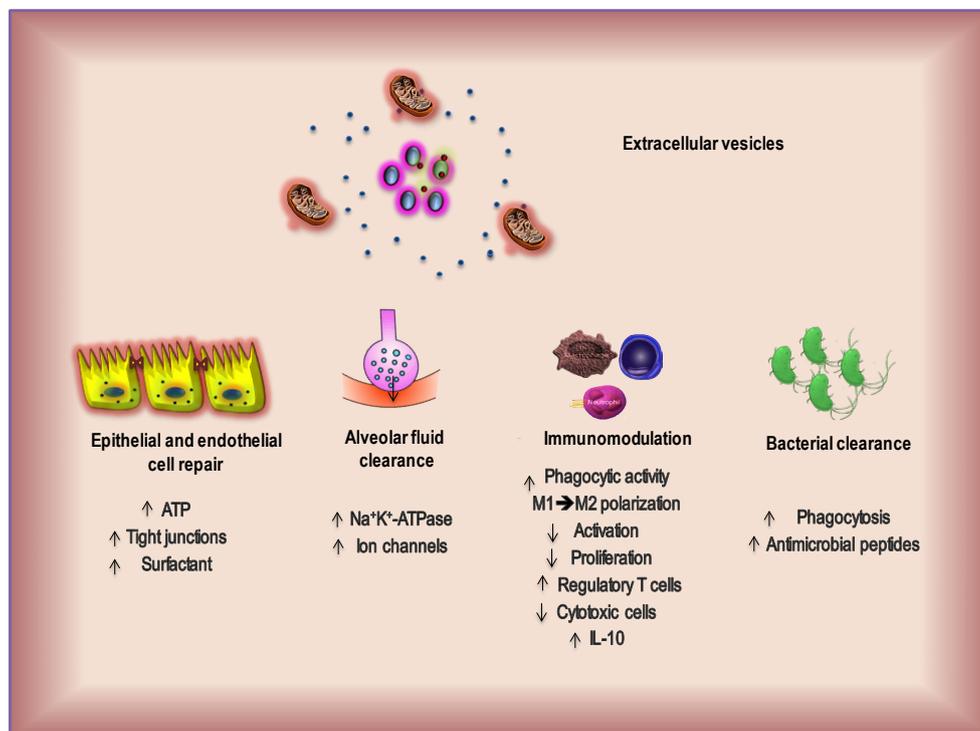
A wide variety of cell types have been shown to release EVs, including immune cells, epithelial cells, endothelial cells, and tumor cells. EVs have been isolated and characterized from different body fluids, such as plasma, urine, and bronchoalveolar lavage fluid (BALF). Of interest to respiratory medicine, EVs are reportedly released from both immune and structural cells in the lungs, and have recently been reported to play a role in pathophysiology of asthma (29), COPD (30), and pulmonary artery hypertension (29,31). Potential applications of EVs as

biomarkers for lung diseases and novel therapeutic targets have emerged (32-35). In this line, MSC-derived EVs can be an important tool for obtaining the clinical benefits of MSC treatment (22). Recently, a Good Manufacturing Practices-grade standard protocol for obtaining exclusively human MSC-derived EVs was proposed (36). The characterization and establishment of MSC-derived EVs will help to identify active components in therapeutic EVs for future clinical applications.

## Effects of EVs *in vitro*

A significant body of literature obtained in animal models of inflammation has shown that stem cell-derived vesicles are also immunosuppressive, probably through the transfer of both RNA and proteins carried by EVs (37-41). EVs derived from MSCs exposed to normoxic or hypoxic conditions are efficiently internalized by bone marrow macrophages, eliciting their switch from M1 to M2 phenotype, downregulating interleukin (IL)-6 and nitric oxide synthase, and upregulating arginase 1 and chitinase-like 3 protein—typical markers of alternative macrophage activation (37). Mechanistically, it has been shown that the MSC exosomal miR-146a, a well-known anti-inflammatory microRNA, when transferred to macrophages, results in M2 polarization and increases survival in septic mice (42). In lipopolysaccharide-primed human monocytes, EV transfer of microRNAs and mitochondria have restored intracellular ATP, reduced levels of pro-inflammatory mediators, and greatly increased their phagocytic properties (43,44). Moreover, MSC-EV treatment induces tolerogenic signaling through promotion of T regulatory cells, apoptosis of effector T cells, and an increase in immunosuppressive cytokine IL-10 concentration when co-cultured with T lymphocytes (39,45).

EVs are taken up by other cell types. Injured human monocytes, as well as alveolar epithelial cells, uptake hMSC-derived EVs through the CD44 receptor (44). MSC exosomes obtained from human umbilical cord MSCs act directly on hypoxic vascular endothelial cells, inhibiting STAT3 signaling (23). Moreover, EVs have strong antiapoptotic and pro-proliferative effects *in vitro* (46-48). Finally, MSC-derived EVs have been implicated in the tissue-restoring effects of MSCs, including wound healing (49), antioxidant and antitumor effects (50), and microbicidal activity (51) (Figure 1). In short, EVs released from MSCs, which can be rapidly isolated by ultracentrifugation and filtration, exhibit anti-inflammatory



**Figure 1** Effects of extracellular vesicles in the lungs. Microvesicles, exosomes, and vesicles containing mitochondria induce endothelial and epithelial repair, promote alveolar fluid clearance, and exert immunomodulatory and microbicidal effects.

properties, decrease oxidative stress, increase ATP, reduce alveolar edema, and can promote bacterial clearance. These properties suggest they could be safely and easily used for therapy of lung diseases.

### *Effects of EVs in vivo*

Stem cell-derived EVs have been tested in experimental lung injury, including models of asthma, ARDS, COPD, IPF, pneumonia, pulmonary artery hypertension, and silicosis (Table 1).

### **Asthma**

Asthma is a common respiratory disease (affecting 1–18% of the population in different countries) usually characterized by chronic airway inflammation. It is defined by a history of respiratory symptoms such as wheezing, shortness of breath, chest tightness, and cough, which vary over time and in intensity, as well as variable expiratory airflow limitation (60). Among patients with asthma, 5–10% have severe disease, a result of mixed Th2/Th17-mediated

neutrophilic airway inflammation. These patients experience poor clinical control and are resistant to corticosteroids and most other available treatments. Hence, new therapeutic options are still needed (61).

In this context, an increasing number of studies on cell therapy have demonstrated beneficial effects of systemic or local administration of syngeneic, allogeneic, or xenogeneic MSCs derived from bone marrow, adipose tissue, placenta, and other sources in a wide spectrum of preclinical asthma models. Therapy with MSCs during either antigen sensitization or challenge mitigates both airway hyperresponsiveness and lung inflammation in a variety of asthma models (21,52,62–74).

There is a growing experience demonstrating the benefit of MSC-derived EV therapy in experimental asthma (21,52). When administered systemically, both CM and, in particular, EVs isolated from human and murine bone marrow-derived MSCs at the onset of antigen challenge in previously sensitized mice were as potent as MSCs themselves in mitigating Th2/Th17-mediated allergic airway inflammation in a mouse model of severe refractory clinical asthma. Human MSCs (hMSCs), CM, and EVs

**Table 1** Experimental studies with stem cell-derived extracellular vesicles in lung diseases

Study	Experimental model	Cell type	Effects
Cruz <i>et al.</i> (21)	<i>Aspergillus</i> extract hyphae-induced asthma	Murine and human BM-MSCs	EVs improved lung mechanics, reduced peribronchial inflammation and Th2/Th17 responses
de Castro <i>et al.</i> (52)	OVA-induced asthma	Human AD-MSCs	EVs reduced lung elastance, collagen deposition and local inflammation
Zhu <i>et al.</i> (25)	LPS-induced ARDS	Human BM-MSCs	EVs reduced lung edema, lung protein permeability, inflammation and effects were partially due to KGF
Song <i>et al.</i> (42)	CLP induced ARDS	Human UC-MSCs	Exosomes resulted in M2 polarization, increased survival
Morrison <i>et al.</i> (44)	LPS-induced ARDS	Human MSCs	EVs transferred microRNAs and mitochondria, promoting an anti-inflammatory and highly phagocytic macrophage phenotype
Monsel <i>et al.</i> (53)	<i>E. coli</i> pneumonia	Human MSCs	Microvesicles increased macrophage phagocytosis, increased ATP levels of epithelial cells and reduced inflammation
Kim <i>et al.</i> (54)	Elastase-induced COPD	Human AD-MSCs	Nanovesicles inhibited morphofunctional changes mainly through FGF
Choi <i>et al.</i> (55)	Silica-induced lung fibrosis	Human BM-MSCs	EVs reduced collagen and inflammation in the lungs
Phinney <i>et al.</i> (56)	Silica-induced lung fibrosis	BM-MSCs	EVs reduced lung inflammation and fibrosis, through mitochondria and microRNAs transfer, which inhibit macrophage activation and increase their bioenergetics
Shentu <i>et al.</i> (57)	Bleomycin-induced lung fibrosis	Human MSCs	EVs downregulated pro-fibrotic mediators and collagen deposition
Lee <i>et al.</i> (23)	Hypoxia-induced PAH	Human HC-MSCs	Exosomes inhibited PAH through suppression of hyperproliferative pathways, including STAT-3 mediated signaling
Aliotta <i>et al.</i> (58)	Monocrotaline-induced PAH	MB-MSCs	Exosomes reduced PAH, based on their increased levels of anti-inflammatory, anti-proliferative miRNAs including miRs-34a, -122, -124, and -127
Chen <i>et al.</i> (59)	Monocrotaline-induced PAH	Rat BM-MSCs	EVs reduced pulmonary artery pressure and cor pulmonale

Therapy with extracellular vesicles (EVs) in lung diseases. AD, adipose-derived; ARDS, acute respiratory distress syndrome; BM, bone marrow; COPD, chronic obstructive pulmonary disease; IPF, idiopathic pulmonary fibrosis; LPS, lipopolysaccharide; CLP, cecal ligation and puncture; MB, menstrual blood; miRs, microRNAs; MSC, mesenchymal stromal cells; OVA, ovalbumin; PAH, pulmonary arterial hypertension; UC, umbilical cord.

were effective in this immunocompetent mouse model, ameliorating *Aspergillus* hyphae extract-provoked increases in airway hyperreactivity, lung inflammation, and the antigen-specific cluster of differentiation (CD)-4 T-cell T helper (Th)-2 and Th17 phenotype. Notably, both CM and EVs from hMSCs were generally more potent than those from mouse MSCs (mMSCs) in most of the outcome measures (21). When both soluble mediators and EV secretion were blocked by the cross-linking agent 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, the observed effects of hMSCs were fully abolished, whereas

with the administration of mMSCs, they were partly ameliorated. These results demonstrated potent xenogeneic effects of CM and EVs in an immunocompetent mouse model of allergic airway inflammation (21).

A recent study assessed the effects of systemically administered adipose tissue-derived MSCs and their EVs. Both presented beneficial effects in ovalbumin-induced allergic asthma, acting on the inflammatory process and reversing tissue remodeling (52). While the effects of each were largely similar, differences were observed in outcome assessment of lung mechanics and inflammation: MSCs and

EVs provoked different effects on eosinophil cell counts, levels of eotaxin, IL-4, and IL-13 in lung parenchyma, CD3<sup>+</sup>CD4<sup>+</sup> T cells in BALF, and lung mechanics (52). This highlights the importance of in-depth studies of the differential mechanisms by which MSCs versus EVs might act in respiratory diseases.

## ARDS

ARDS is a devastating condition that affects around 200,000 people each year in the United States alone, with mortality rates around 34.9–46.1% (75). Over the last decades, many novel therapeutic approaches have been tested for the treatment of ARDS, but none has proven efficient at targeting disease-specific pathways or reducing mortality; thus, supportive care measured, including antibiotics, protective mechanical ventilation strategies, and fluid restriction, remain the mainstays of therapy (76). With recent progress in the field of stem cells revealing their immunomodulatory, antibacterial, and regenerative properties (4), cell therapy has emerged as a potential candidate for ARDS treatment (77–79). Currently, eight clinical trials assessing the safety of cell therapy in patients with ARDS are registered in ClinicalTrials.gov (4–7); of these, several have demonstrated that administration of MSCs is not associated with adverse events (5–7), and one reported beneficial results when administered to two patients in a compassionate use setting (6).

Therapy with MSC-derived CM has already been tested and shown to lead to improvement of acute lung injury in mice (16,17). Additionally, EVs derived from human MSCs, when administered following *E. coli* endotoxin-induced acute lung injury in mice, reduced extravascular lung water by 43% and total protein levels in BALF by 35%, with a reduction in pulmonary edema and lung protein permeability (25). EVs also reduced neutrophil infiltration and macrophage inflammatory protein-2 levels in BALF by 73% and 49%, respectively, indicating a reduction in inflammation. Silencing KGF via siRNA pretreatment of MSCs partially abolished the therapeutic effects of the secreted EVs, suggesting that KGF played an important role in the underlying mechanism (25). In recent ARDS research, MSCs were shown to promote an anti-inflammatory and highly phagocytic macrophage phenotype through EV-mediated mitochondrial transfer. MSC-induced changes in macrophage phenotype depend critically on enhancement of macrophage oxidative phosphorylation. Furthermore, adoptive transfer of alveolar macrophages

previously treated with MSC-derived EVs has been shown to reduce lung damage (43,44).

## Pneumonia

Despite rapid advances in our armamentarium of antimicrobials, bacterial pneumonia is still associated with respiratory failure, and the case-fatality rate for this widely prevalent disease remains high in critically ill patients. In some settings, common treatment options may actually contribute to poor outcomes, as rapid lysis of pathogenic bacteria on the backdrop of an activated immune system may lead to inflammatory damage in the lung (80,81).

Therapy with MSCs has shown to be interesting due to its antimicrobial properties, as they are involved especially in dynamic coordination of the pro- and anti-inflammatory elements of the immune system or in increasing phagocyte activity, and directly by secretion of antimicrobial peptides and proteins (AMPs) (51). AMPs are evolutionarily conserved, gene-encoded small effector molecules that interact with different molecular targets either on the cell surface or within cells. Importantly, in some specific cases, AMPs can be active against pathogens that are resistant to conventional antibiotics (e.g., multidrug-resistant bacteria). In this context, MSCs from different sources or origins have shown ability to reduce the burden of pathogens in different preclinical models of pneumonia, regardless of the route, dose, or timing of administration (81,82).

Administration of human MSC-derived EVs decreased the influx of inflammatory cells, cytokines, protein, and bacterial load, resulting in higher survival rates of mice with bacterial pneumonia, in a mechanism partially dependent on keratinocyte growth factor secretion. The antimicrobial effect of BMSC-derived EVs was partly attributed to enhancement of monocyte phagocytosis of bacteria while decreasing inflammatory cytokine secretion, as well as to increased intracellular ATP levels in injured alveolar epithelial type 2 cells. The therapeutic effects of released EVs could be further enhanced by pre-stimulation of BMSCs with a TLR-3 agonist before isolation (53).

## COPD

COPD, characterized by small-airway disease and parenchymal destruction, affects 5% of the global population and is the third leading cause of death worldwide, representing a substantial economic and social burden. COPD is inexorably progressive despite available pharmacologic treatments,

which are mostly geared toward symptom relief (60). A growing number of investigations on MSC-based cell therapies for COPD are being conducted in experimental and clinical scenarios (8-10,54).

Recently, exosomes obtained from adipose-derived MSCs and artificial nanovesicles generated from the same cells were used in an elastase-induced emphysema model. Nanovesicles were generated by using sequential penetration through polycarbonate membranes, displayed a size (100 nm) and spherical shape resembling natural exosomes, and expressed both exosomal and stem-cell markers (54). Despite their beneficial effects, the disadvantage of exosomes for clinical applications is that they are only released naturally in very small amounts compared to nanovesicles (83). The proliferation rate of lung epithelial cells was increased in cells treated with MSC-derived artificial nanovesicles compared with cells treated with MSC-derived natural exosomes; a lower dose of MSC-derived artificial nanovesicles had similar regenerative capacity compared with a higher dose of MSCs and MSC-derived natural exosomes. Taken together, these data indicate that lower doses of ASC-derived artificial nanovesicles may have beneficial effects similar to those of higher doses of ASCs or ASC-derived natural exosomes in experimental emphysema, suggesting that artificial nanovesicles may have economic advantages that would warrant future clinical studies (54).

In parallel, the contribution of EVs derived from macrophages, epithelial cells, and endothelial cells to COPD pathophysiology highlights their potential as novel therapeutic targets. Elimination of these EVs, which contain nucleic acids or proteins as mediators of intracellular communication involved in disease pathogenesis, may be achieved through several different therapeutic approaches, including capture of circulating EVs, disruption of EV uptake by recipient cells, and inhibition of EV production or secretion (30,84).

### Silicosis

Silicosis is the most common pneumoconiosis, with higher prevalence and incidence in developing countries. To date, there is no effective treatment to halt or reverse progression of the disease caused by silica-induced lung injury (2,11,85). Cell therapy has been tested in several studies and showed prominent effects, reducing lung fibrosis and promoting improvement in lung mechanics (2,85-88).

In this context, EVs derived from MSCs could reduce neutrophil and lymphocyte accumulation in BALF and

reduce collagen deposition in lung parenchyma in silicotic mice (55,56). The same group showed that MSCs manage intracellular oxidative stress by targeting depolarized mitochondria to the plasma membrane via arrestin domain-containing protein 1-mediated microvesicles (56). The resulting vesicles are then engulfed and reutilized by macrophages, enhancing their bioenergetics. Furthermore, they have shown that MSCs simultaneously shed exosomes enriched with micro-RNAs that inhibit macrophage activation by suppressing Toll-like receptor signaling, thereby desensitizing macrophages to the ingested mitochondria. Collectively, these studies mechanistically link mitophagy and MSC survival with macrophage function, thus providing a physiologically relevant context for the innate immunomodulatory activity of MSCs both *in vitro* and in an *in vivo* model of lung injury (56).

### IPF

IPF is a chronic, progressive, and inevitably fatal scarring lung disease, with a median survival as short as 3 years from the time of diagnosis, despite pharmacological therapies already approved by the U.S. Food and Drug Administration and in Europe (89). As such, the administration of MSCs is being investigated as a new therapeutic strategy for pulmonary fibrosis (90) in preclinical and clinical studies. MSCs can migrate to injured sites and secrete multiple paracrine factors, followed by regulation of endothelial and epithelial permeability, decrease of inflammation, enhancement of tissue repair, and inhibition of bacterial growth (90).

The recent discovery of therapeutic applications of EVs released from hMSCs has generated interest in their mechanisms of targeting and action. An *in vivo* efficacy study demonstrated that intravenous delivery of hMSC-EVs 14 days after induction of pulmonary fibrosis with intratracheal bleomycin significantly downregulated  $\alpha$ -smooth muscle actin expression and decreased histopathological fibrosis, indicating therapeutic effects of these vesicles for established lung fibrosis through modification of the myofibroblastic phenotype (57).

### PAH

PAH is a disease that mainly affects the pulmonary vascular bed. It is characterized by a proliferative disorder and resistance to apoptosis of the smooth muscle cells present in the pulmonary artery. This culminates in pulmonary artery remodeling and constriction, promoting an increase

in pulmonary vascular resistance; this, in turn, is associated with compensatory hypertrophy of the right ventricle, which can rapidly progress to heart failure. The mean survival of untreated patients is 2.8 years after diagnosis, versus 3.6 years in treated patients (91).

Intravenous delivery of EVs derived from mouse MSC-CM suppressed influx of macrophages and the induction of proinflammatory and pro-proliferative mediators, including monocyte chemoattractant protein-1 and hypoxia-inducible mitogenic factor, in a murine model of hypoxic pulmonary hypertension. EVs also inhibited vascular remodeling and consequent pulmonary hypertension through suppression of the hypoxic activation of signal transducer and activator of transcription 3 (*STAT3*) and upregulation of the miR-17 superfamily of microRNA clusters, whereas it increased lung levels of miR-204, a key microRNA, expression of which is decreased in human pulmonary hypertension (58).

Recently, a group sought to determine which EV subpopulation plays a regulatory role in the reversal of PAH in mice. They found that the exosome fraction of EVs isolated from murine MSCs (MSC-EXOs) prevents and reverses PH in a monocrotaline-induced model of PAH. Furthermore, MSC-EXOs contain increased levels of miRNAs that blunt angiogenesis, inhibit proliferation of neoplastic cells, and induce senescence of vascular smooth muscle cells and endothelial progenitor cells. EXOs isolated from human MSCs were just as effective as those from murine MSCs in reversing pulmonary hypertension in mice (59). Together, these findings suggest a prominent role of EXOs in mediating the pulmonary vascular remodeling seen in PAH, and point to a promising therapeutic approach for its treatment (58,59).

## Conclusions

Most studies analyzing the therapeutic effect of EVs have been performed in small-animal models and required only a small amount of EVs; therefore, large-scale manufacturing systems are needed to translate EV technology to the clinical trial setting. Furthermore, the same issue involved in isolating targeted EVs described below is still a limitation to clinical studies. In recent years, clear evidence of the involvement of EVs, especially exosomes and ectosomes, in the pathogenesis of lung diseases has emerged. The number and type of circulating EVs changes according as the natural history of lung diseases; the contents of EVs, such as microRNAs, are also changed by the disease condition. Therefore, EVs are

promising candidates as novel biomarkers for lung diseases. EVs also act as a shuttle for transport of small molecules to distant cells, and modulate the function of the recipient cells. Due to this unique capability, EVs are also expected to have potential as a drug delivery system and as novel therapeutic targets. Research into EVs can provide new insights into the pathogenesis of various lung diseases and elucidate novel therapeutic approaches for respiratory medicine.

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## Footnote

*Conflicts of Interest:* The authors have no conflicts of interest to declare.

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