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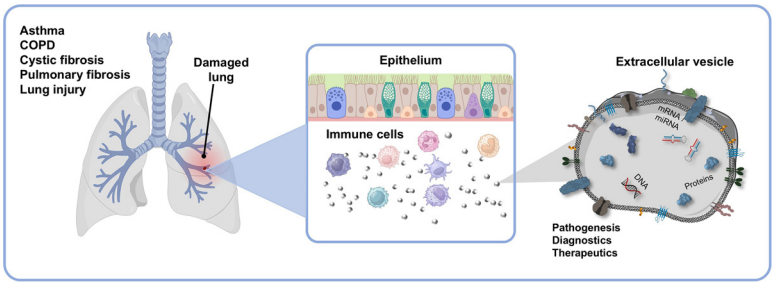
REVIEW ARTICLE

EXTRACELLULAR VESICLES AND THE LUNG: FROM DISEASE PATHOGENESIS TO BIOMARKERS AND TREATMENTS



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KEY WORDS

airways; exosomes; microparticles; microvesicles; pulmonary

CLINICAL HIGHLIGHTS

- Extracellular vesicles (EVs) are released by all cells and can send advanced messages between cells by shuttling functional membrane and cytosolic proteins and nucleic acids between cells. Further, the compositions of EV components change under pathological conditions. EVs are thus involved in both normal tissue homeostasis and pathological processes. EVs can be isolated from biofluids, including bronchoalveolar lavage fluid and blood, and this has led to the discovery of numerous EV-based biomarker candidates for different diseases, including lung diseases.
- EVs can also be used as therapeutics, which is especially relevant in pulmonary disease. For example, mesenchymal stem cell-derived EVs have been used to treat lung injury and chronic obstructive pulmonary disease (COPD), sometimes in well-controlled clinical trials. Further, EVs engineered to express high levels of the anti-inflammatory membrane protein CD24 have also been effective in clinical trials of lung injury.
- Overall, EVs are rapidly emerging as clinical tools in respiratory diseases, including as biomarkers for asthma and COPD subgroups, pulmonary fibrosis, cystic fibrosis, and lung injury. Most importantly, EV-based therapeutics may provide additional anti-inflammatory functions in respiratory disease by targeting inflammatory pathways that are not efficiently treated with glucocorticoids.

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EXTRACELLULAR VESICLES AND THE LUNG: FROM DISEASE PATHOGENESIS TO BIOMARKERS AND TREATMENTS

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Abstract

Nanosized extracellular vesicles (EVs) are released by all cells to convey cell-to-cell communication. EVs, including exosomes and microvesicles, carry an array of bioactive molecules, such as proteins and RNAs, encapsulated by a membrane lipid bilayer. Epithelial cells, endothelial cells, and various immune cells in the lung contribute to the pool of EVs in the lung microenvironment and carry molecules reflecting their cellular origin. EVs can maintain lung health by regulating immune responses, inducing tissue repair, and maintaining lung homeostasis. They can be detected in lung tissues and biofluids such as bronchoalveolar lavage fluid and blood, offering information about disease processes, and can function as disease biomarkers. Here, we discuss the role of EVs in lung homeostasis and pulmonary diseases such as asthma, chronic obstructive pulmonary disease, cystic fibrosis, pulmonary fibrosis, and lung injury. The mechanistic involvement of EVs in pathogenesis and their potential as disease biomarkers are discussed. Finally, the pulmonary field benefits from EVs as clinical therapeutics in severe pulmonary inflammatory disease, as EVs from mesenchymal stem cells attenuate severe respiratory inflammation in multiple clinical trials. Further, EVs can be engineered to carry therapeutic molecules for enhanced and broadened therapeutic opportunities, such as the anti-inflammatory molecule CD24. Finally, we discuss the emerging opportunity of using different types of EVs for treating severe respiratory conditions.

airways; exosomes; microparticles; microvesicles; pulmonary

1. INTRODUCTION

Extracellular vesicles (EVs) are membrane-bound vesicles released by cells into the extracellular environment and play crucial roles in cell-to-cell communication by shuttling multiple types of molecules, including proteins, lipids, and nucleic acids between cells (1, 2). These vesicles include a variety of nanoparticles, such as exosomes and microvesicles, each differing in size, origin, and function (**FIGURE 1**). Exosomes are the smallest vesicles (30–150 nm in diameter) formed within endosomal compartments known as multivesicular bodies (MVBs). Initially, invagination of the plasma membrane forms early endosomes, which mature into late endosomes or MVBs containing intraluminal vesicles (ILVs). These ILVs, formed by inward budding of the MVB membranes, are released into the extracellular space as exosomes upon fusion of the MVB with the plasma membrane (3). In contrast, microvesicles are relatively large (100–1,000 nm in diameter) and shed by directly budding off from the plasma membrane. This process is driven by cytoskeletal reorganization and alterations in membrane lipid composition and protein interactions (4). It is important to remember that the nomenclature in the literature is inconsistent, and the term "exosomes" is often used as a synonym for "extracellular vesicles." In the current article, we will consistently use the term "extracellular vesicles," abbreviated "EVs", regardless of which terminology the authors of the cited articles have used.

Extracellular vesicles can carry a wide range of bioactive molecules, including proteins, lipids, and nucleic acids, that can reflect the physiological and pathological conditions of the EV-producing cells. Once released into the extracellular space, EVs are transported through biological fluids like interstitial fluid, lymph, and blood, where their protective membranes maintain stability.

*K-S. Park and C. Lässer contributed equally to this work.

CLINICAL HIGHLIGHTS

- Extracellular vesicles (EVs) are released by all cells and can send advanced messages between cells by shuttling functional membrane and cytosolic proteins and nucleic acids between cells. Further, the compositions of EV components change under pathological conditions. EVs are thus involved in both normal tissue homeostasis and pathological processes. EVs can be isolated from biofluids, including bronchoalveolar lavage fluid and blood, and this has led to the discovery of numerous EV-based biomarker candidates for different diseases, including lung diseases.
- EVs can also be used as therapeutics, which is especially relevant in pulmonary disease. For example, mesenchymal stem cell-derived EVs have been used to treat lung injury and chronic obstructive pulmonary disease (COPD), sometimes in well-controlled clinical trials. Further, EVs engineered to express high levels of the anti-inflammatory membrane protein CD24 have also been effective in clinical trials of lung injury.
- Overall, EVs are rapidly emerging as clinical tools in respiratory diseases, including as biomarkers for asthma and COPD subgroups, pulmonary fibrosis, cystic fibrosis, and lung injury. Most importantly, EV-based therapeutics may provide additional anti-inflammatory functions in respiratory disease by targeting inflammatory pathways that are not efficiently treated with glucocorticoids.

Target cells take up EVs through various mechanisms, including receptor-ligand binding, endocytosis, macropinocytosis, micropinocytosis, phagocytosis, or direct membrane fusion. The uptake of EVs to specific cells may be guided by surface molecules such as tetraspanins and integrins, facilitating cell-type recognition. Upon internalization in recipient cells, EVs deliver their cargo to the cytoplasm or to certain organelles, thereby modulating signaling pathways and potentially the phenotype of recipient cells (5).

The lung regulates various physiological processes to maintain its role in immune defense against allergens, pathogens, and pollutants, and numerous studies have in recent years shown that EVs are deeply involved in modulating both physiological and pathological events within the respiratory system (6, 7). Indeed, EVs can be identified in bronchial biopsies, as illustrated in **FIGURE 2**. Some of the larger EVs are likely to be stationary in tissues. In contrast, the smaller ones may diffuse through the tissue to be taken up by other cells or to travel through the lymphatic system either to reach cells in regional lymph nodes or to be released into circulation. In lung health, EVs are considered to facilitate homeostasis by intercellular communication between different cell types within the lung, including epithelial cells, endothelial cells, fibroblasts, and immune cells (8, 9). EVs also play crucial roles in modulating immune responses, controlling oxidative stress, promoting tissue repair, and maintaining the integrity of the lung epithelial barrier through the transfer of molecular cargo (10–13).

Because the molecular cargos of EVs change under pathological conditions, EVs are being explored as potential biomarkers for various lung diseases by identifying the disease-specific EV cargo (14, 15). This has been especially studied in cancer, but emerging data suggest that EVs may also function as biomarkers in nonmalignant diseases of the lung. Thus EVs carry molecular signatures indicating the presence of inflammation, fibrosis, apoptosis, oxidation, and responses to therapeutic interventions. EVs are also stable in biological fluids such as bronchoalveolar lavage fluid (BALF), which makes them suitable candidates for robust biomarker identification (16). However, it is crucial to consider that EVs within

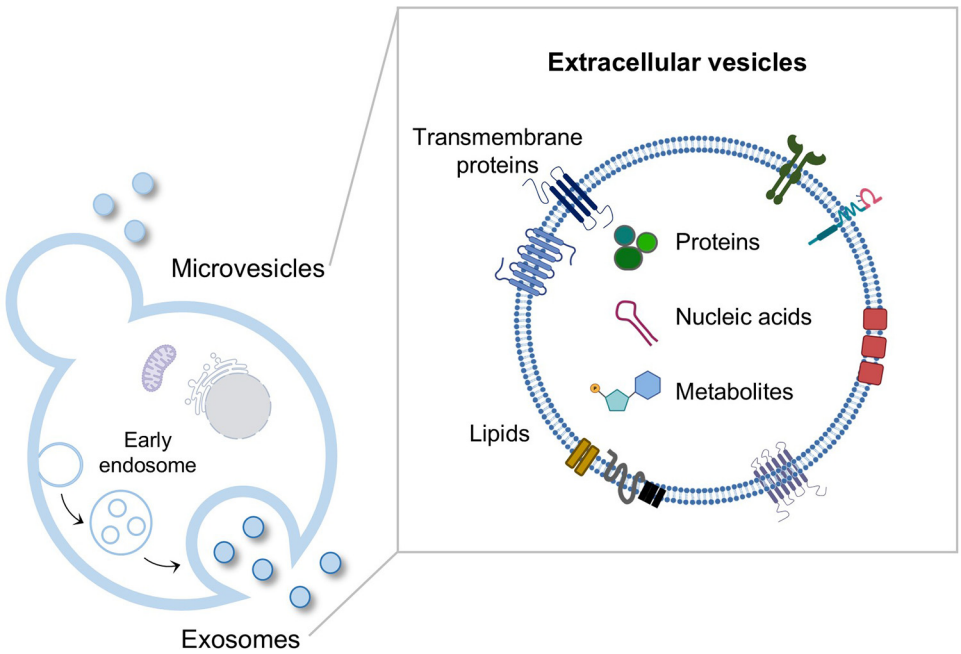


FIGURE 1. Extracellular vesicle (EV) biogenesis and composition. EVs are small, membrane-bound particles released by cells into the extracellular environment, and they play a crucial role in intercellular communication. EVs can generally be released at the level of the plasma membrane by budding (microvesicles) or via the endosomal pathway (exosomes). EVs carry lipids, membrane proteins, intravesicular proteins (cytosolic), metabolites, and nucleic acids, such as RNA and DNA from donor cells. Image was created by BioRender.com with permission.

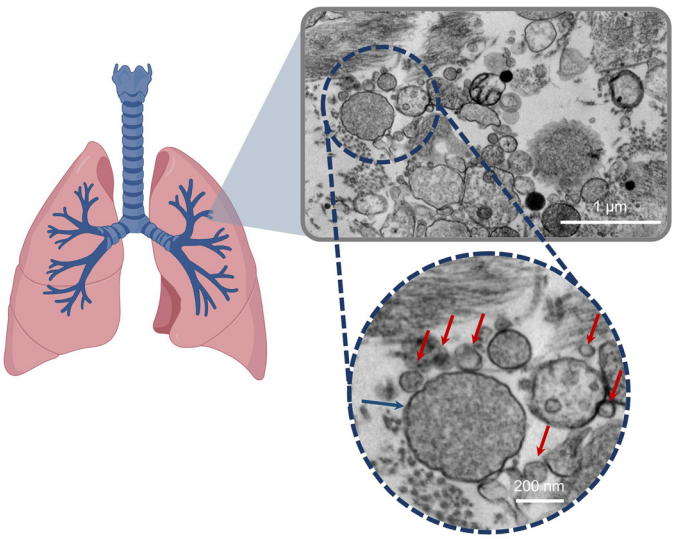


FIGURE 2. Electron microscopy of a bronchial biopsy from a healthy individual. Red arrows indicate the presence of extracellular vesicles in the interstitial tissue of the bronchi. The larger round (blue arrow) structures may illustrate protrusions from cells. The image was created by BioRender.com with permission.

any single biofluid sample derive from a wide variety of cell types, implying that the biomarker of interest may only be present in a fraction of the total EV population in that biofluid. This is particularly relevant for EV biomarker identification in blood, which contains large amounts of EVs from red blood cells, platelets, as well as other potential non-EV contaminants such as various-sized lipoproteins.

EVs have emerged as promising candidates for treating different types of diseases, including cancer and systemic inflammatory processes (17, 18). Mesenchymal stem cell (MSC)-EV-based therapeutics might provide a novel approach to treating severe lung disease by harnessing the immunomodulatory and regenerative properties of the EVs (19, 20). EVs can also be engineered to carry specific cargo, such as therapeutic proteins or different nucleic acid species, potentially increasing the therapeutic potency and efficacy of the EVs. Finally, by engineering the EVs to have relatively specific cell targeting capability and reduced uptake in irrelevant cells, the efficacy of the EV therapeutic may increase in parallel with reduced side effects. Importantly, multiple studies have concluded that MSC-derived EVs are safe in patients with different diseases (21).

In this review, we focus on the role of EVs in major non-cancer pulmonary diseases, including asthma, chronic obstructive pulmonary disease (COPD), cystic fibrosis (CF), pulmonary fibrosis, and acute lung injury. A common trait of all of these diseases is that they are associated with strong immune responses and significant levels of inflammation, processes that are intricately connected to EV biology. We discuss the multifaceted functions of EVs in the lung and their role as potential biomarkers in respiratory diseases.

Finally, we discuss the therapeutic potential of EVs in treating pulmonary disease, some of which are currently undergoing clinical trials for acute lung injury and COPD. We have aimed to cover the entirety of the current scientific literature on EVs in the lung, and we have identified a total of 509 published papers on the topic in PubMed (**FIGURE 3** and Supplemental Table S1), published before the end of 2024. This review is divided into six sections: pathological functions of EVs in respiratory diseases (sect. 2), EV-associated biomarker candidates in respiratory diseases (sect. 3), therapeutic EVs in respiratory disease models (sect. 4), clinical studies with therapeutic EVs (sect. 5), bacterial outer membrane vesicles and lung diseases (sect. 6), and conclusions and future perspectives of EVs in respiratory diseases (sect. 7). These sections also include subsections on asthma, COPD, CF, pulmonary fibrosis, and lung injury. Within this context, we also highlight current challenges for the clinical application of EVs and the potential contribution of bacterial outer membrane vesicles (OMVs) to pathophysiology in the lung.

1.1. Quality Assurance in the Respiratory EV Field

Even though EVs have been known for multiple decades, the volume of publications has grown explosively in the last decade. In 1983, two papers independently showed that the transferrin receptor of reticulocytes is released into the extracellular space in association with ~50-nm vesicles (22, 23). For a decade, one or two additional papers were published each year, focusing on reticulocytes and their clearance of unwanted proteins. However, in the late 1990s, EVs from immune cells were demonstrated to take part in signaling within the immune system and to be able to stimulate the immune system to eradicate tumors in mice (24, 25). This increased the interest in EVs, further boosted by two independent papers published in 2007 and 2008 demonstrating that EVs can shuttle functional RNA between cells (26, 27). Until today, an exponential growth in publications in the field has been observed over the last 10 years (Supplemental Figure S1). This has inspired a large influx of new researchers to the EV field, which has generated many well-executed scientific experiments, reporting important and groundbreaking findings. However, as pointed out later in this review, EVs are usually isolated from complex samples where non-EV material easily contaminates them, which can lead to misinterpretation of the results. To conclude that an observed biological effect is likely to depend on EVs, it is crucial to *1*) choose an EV isolation method that efficiently enriches EVs while reducing non-EV material, *2*) carefully characterize the isolated EVs to determine which EV subtype has been isolated to which purity, and

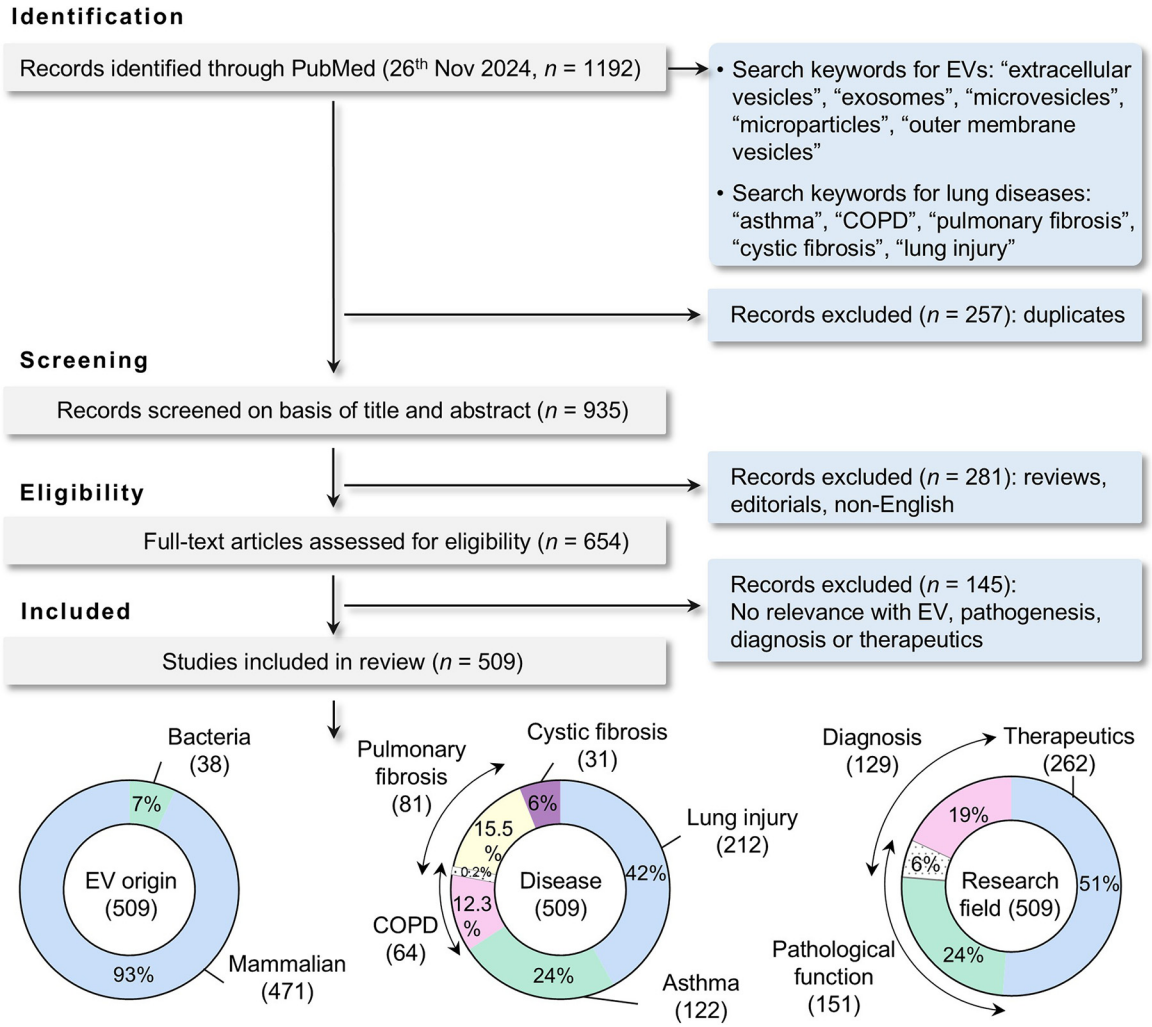


FIGURE 3. Flow diagram of the identification, screening, eligibility, and inclusion of references for this review. This illustrates the search strategy and selection process for the included extracellular vesicle (EV) publications. COPD, chronic obstructive pulmonary disease.

3) that publications describe all details of used methods, for the reader to determine whether the data likely support the conclusions. The International Society for Extracellular Vesicles (ISEV) was established in 2011 and promotes EV research and education by organizing scientific meetings, workshops, massive open online courses, and task forces, as well as establishing EV-focused scientific journals such as the *Journal of Extracellular Vesicles* and the *Journal of Extracellular Biology*.

Importantly, the society has for more than a decade published guidance for EV research (Minimal Information for Studies of Extracellular Vesicles [MISEV]), which are known as "MISEV2014," "MISEV2018," and "MISEV2023" (28–30). These were created through consensus-based discussions within the EV community as a response to the exponential growth of the EV field. In parallel, the Hendrix group in Belgium has developed a tool to determine how diligent research methods have been reported in EV publications. This tool, called EV-TRACK, is a platform to facilitate transparency in EV research by increasing the detailed reporting on EV methodology (31). If isolation and characterization information is well reported in the method section of an article, it will receive a score of 100 in the EV-TRACK metric system. Out of the EV articles covered in this review, only just over 30 have been included in EV-TRACK. It is, therefore, obvious that EV-TRACK is not useful in evaluating the quality/status of the respiratory EV field, as reported in this review. We have, therefore, manually screened all papers included in this review based on isolation methods and characterization of EVs. We used the categories from a recent primer publication (32), specifically: "no isolation/not sufficiently specified," "concentration," "enrichment," and "separation" (**FIGURE 4*A***). Isolation methods that concentrate are methods that increase the concentration of EVs by reducing liquid volume, but in parallel, also concentrate non-EV material. The methods included here are ultrafiltration, tangential flow filtration (TFF), precipitation, and kits. Usually, these methods have high efficiency but low specificity, although there may be

differences in the efficiency among different kits. Isolation methods that enrich for EVs while also removing some of the non-EV materials are considered enrichment methods and included here are differential ultracentrifugation (dUCF), size exclusion chromatography (SEC), asymmetric flow field flow fractionation (AF4), density gradient, density cushion, and affinity capture. Finally, when a combination of two or more of the enrichment methods is used to further purify EVs from non-EV material, we consider this a method that separates EVs from non-EV material but potentially also separates subpopulations of EVs. Such combinations may include "differential ultracentrifugation + density gradient" or "density cushion + SEC." Separation methods have the highest specificity but lower efficiency due to the multiple steps employed.

This analysis suggests that the most commonly performed isolation method is enrichment, followed by concentration methods (**FIGURE 4*B***). Specifically, dUCF or kit-isolation methods were the most common (**FIGURE 4*C*** and Supplemental Figure S2, *A*, *F*, and *K*), and we know that kit isolation is often nonspecific. Further, kit-isolation methods seem to be used more extensively in papers exploring EV-associated biomarkers. It is also essential to be aware that the different isolation methods may isolate different subpopulations of EVs from biofluids or other samples. Most studies using dUCF to isolate EVs use centrifugation protocols biased toward small EVs (**FIGURE 4*D*** and Supplemental Figure S2, *B*, *G*, and *L*). Still, any clinically relevant biomarker or EV function may be associated with other EV subpopulations. Therefore, we suggest that future studies should allow for discoveries of EV functions beyond small EVs, perhaps most importantly for biomarker discovery.

Next, we also evaluated how well the papers characterize EVs. We divided the methods into "biophysical characterization" and "biochemical characterization." Biophysical methods can determine the concentration, size, refractive index, morphology, and/or zeta potential of the EVs, while biochemical characterization methods can determine the presence or absence of biomolecular markers. Some of these methods can also be used to quantify concentration and are therefore included in both biophysical and biochemical characterization depending on what they were used for. Biophysical characterization methods include nanoparticle tracking analysis (NTA), single particle interferometric reflectance imaging sensor (SP-IRIS), nanoFCM, modified flow cytometry, dynamic light scattering (DLS), static light scattering (SLS), multiangle light scattering (MALS), (tunable) resistive pulse sensing [(T)RPS], transmission electron microscopy (TEM), cryo-TEM, and atomic force microscopy (AFM). Biochemical characterization methods include fluorescence NTA, modified flow cytometry, nanoFCM, SP-IRIS, immuno-TEM, superresolution microscopy, Raman spectroscopy, Western blot, enzyme-linked immunosorbent assay (ELISA), proteomics, and bead-based cytometry. Overall, the quality of concentration, enrichment, and characterization methods has improved significantly over the last 15 years, but there are still significant shortcomings in the published EV literature.

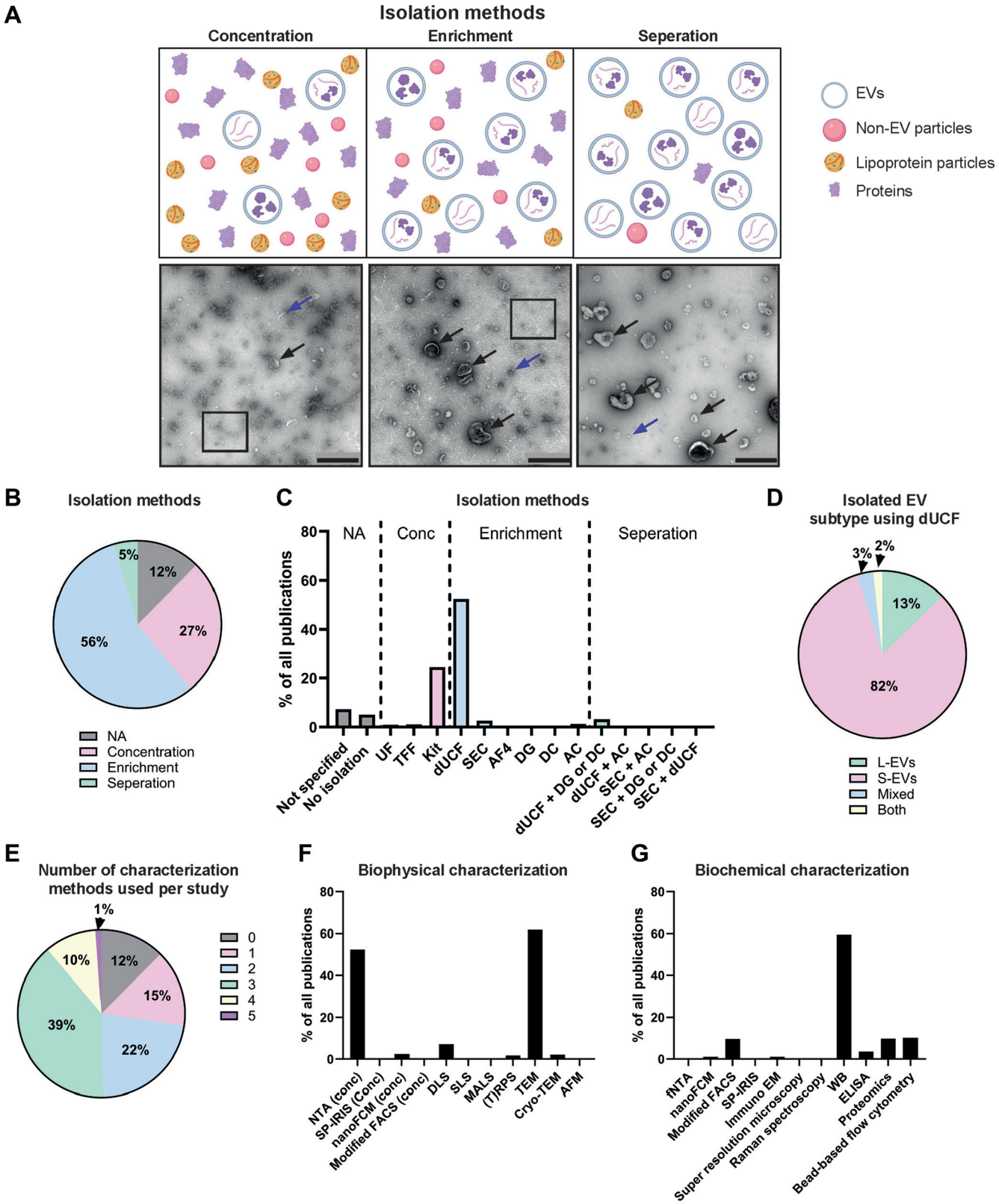
Another historical weakness in EV biomarker studies is that the number of characterization methods for EVs provided is limited or, in some cases, even nonexistent. Most publications provide at least three methods of characterization of the EVs they are studying (**FIGURE 4*E***), which is what is commonly recommended. However, the biomarker discovery field uses less than the pathological functions and therapeutics field (Supplemental Figure S2, *C*, *H*, and *M*). TEM, Western blots, and NTA are the most common characterization methods (**FIGURE 4, *F* AND *G***), with them being used in 60% of the studies of biological functions or developing EV therapeutics but only in 40% of the biomarker discovery papers (Supplemental Figure S2, *D*, *E*, *I*, *J*, *N*, and *O*). For future studies, we recommend using more than three characterization methods in respiratory EV research, specifically as many new high-resolution methods have become available, such as methods to characterize single vesicles. Also, it is essential to know that NTA quantifies particles and is not specific to EVs. Further, NTA provides a size distribution of the sample that is generally not considered accurate, as NTA often cannot identify the smallest vesicles.

Overall, we recommend that the readers of this article who are considering starting a quest to study extracellular vesicles may use this review as an inspiration to consider which methods to use carefully. It is obvious that EV methods improve over time, and conclusions that were reasonable based on results from methods used 10 years ago may not be fully supported by current methods. For example, we suggest that precipitation kits provide less conclusive data regarding the involvement of EVs in an observed scientific finding, whereas a complete modern isolation procedure using enrichment, separation, and modern characterization may provide more conclusive results. Specifically, it is essential to consider using a combination of methods to isolate and purify the EVs, and ideally, these methods should be orthogonal. Thus dUCF combined with density gradient centrifugation or SEC will provide much cleaner EV isolates than only dUCF.

2. PATHOLOGICAL FUNCTIONS OF EVs IN RESPIRATORY DISEASES

The airway epithelium is the first line of defense against inhaled particles, allergens, and pathogens. It forms a physical barrier separating the lungs and the rest of the body from the outer world. However, the airway epithelium is more than just a physical barrier, as it also

regulates numerous physiological processes, including inflammation in respiratory diseases. Many of the respiratory diseases covered in this review are associated with the infiltration of immune cells into various tissues. Together with fibroblasts and other structural cells, epithelial cells and the infiltrating immune cells participate in the pathophysiology of numerous disease conditions. In this section, we focus on the role of EVs in asthma,



COPD, CF, pulmonary fibrosis, and lung injury, and we discuss EVs released by airway epithelial cells, immune cells, fibroblasts, and other cells present in the lung that are considered to be involved in the pathophysiology of these diseases. Furthermore, we discuss results related to biofluid-derived EVs from subjects with respiratory diseases, where the specific cellular origin of the EV might not be known. Finally, this section discusses the potential involvement of EVs in multiple animal respiratory disease models.

2.1. Asthma

Asthma presents with multiple phenotypes and endotypes with somewhat similar physiology, but these days, it is known to be orchestrated by different mechanistic pathways. Typically, T2-high asthma, often associated with an allergic component, involves immunoglobulin E (IgE)-mediated activation of mast cells and the infiltration of eosinophils into the airways. T2-low asthma also consists of multiple subtypes of the disease, where some are associated with eosinophilia and interleukin (IL)-33-driven pathways, and others are not, while some asthma phenotypes have unclear pathogenetic pathways. Another type of asthma may be associated with neutrophilic inflammation and could be mediated by a T17-driven response (33). A weakness of many studies of EVs in asthma is that the patient's phenotype/endotype is not fully considered. Further, animal models are biased toward allergy-associated asthma and thus may not provide accurate information about the roles of EVs in nonallergic asthma.

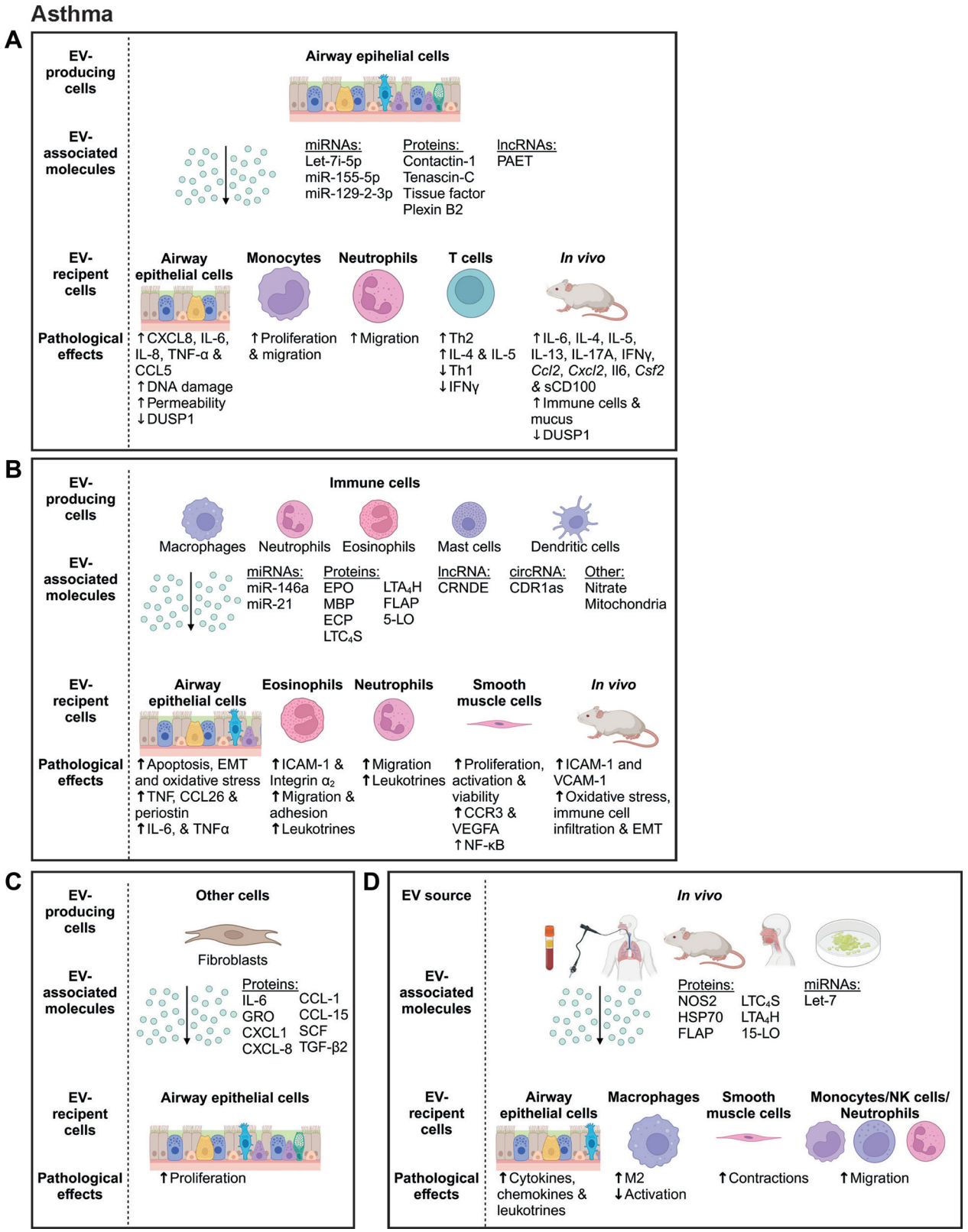
2.1.1. Airway epithelial cell-derived EVs.

This section discusses the details of airway epithelial cell EV release from primary human epithelial cells, primary animal epithelial cells, and cell lines, considering both the cargo and route of release of the EVs (**FIGURE 5*A***). Airway epithelial cells can release EVs from both the apical and the basolateral side of the cells, but to study EVs released at both sides in vitro, air-liquid interphase cultures are required. Air-liquid interphase cultured epithelial cells undergo mucociliary differentiation and comprise a heterogeneous cell population, including ciliated and mucus-secreting cells, representing the airways better than the classical two-dimensional (2-D) cultures. While EVs can be isolated directly from the cell culture medium on the basolateral side, a liquid medium must be added to the apical (air) side for a short time before EVs can be collected from this liquid. In this section, we will cover EV studies on both 2-D and air-liquid interphase cultured epithelial cells.

Most studies using air-liquid interphase-cultured airway epithelial cells have reported EV release on both the basolateral and apical sides (34–36). However, one study detected EV release only on the apical side of the epithelium (37), while two other studies only evaluated the EVs released on the basolateral side (38, 39). Interestingly, Schindler and colleagues (36) demonstrated that air-liquid interphase-cultured bronchial epithelial cells release higher numbers of EVs, but smaller EVs, on the apical side compared to the basolateral side. Furthermore, CD9 and CD81, markers of EVs, were expressed at higher levels on the basolateral-derived EVs than on the apical side. Further, small RNA sequencing suggested that apical EVs contain more microRNAs (miRs) but less long noncoding RNAs (lncRNAs), which are known to be associated with asthma regulation (40), compared to EVs released from the basolateral side. When focusing on miRs, it was shown that 236 miRs were significantly different in EVs from the apical side compared to the basolateral side, with the majority being more abundant in the apical EVs. Many of these miRs from the apical side were members of the miR-30, miR-941, let-7, miR-10, and miR-17 families, while the most represented miR families in the EVs from the basolateral side were the miR-320, miR-181, miR-550, let-7, and miR-154 families. Kyoto Encyclopedia of Genes and Genomes

FIGURE 4. Isolation and characterization methods used in the included extracellular vesicle (EV) publications discussed in this review. *A*: illustration of how pure EV isolates are after different isolation methods demonstrated both graphically and by negative stain electron microscopy. Black arrows indicate EVs, blue arrows indicate non-EV particles, and the black boxes illustrate background proteins. It is evident that there is less non-EV material in samples isolated by separation (right panels). Scale bar = 500 nm. *B*: percent distribution of EV isolation methods among all discussed publications, divided into concentration, enrichment, and separation methods. NA represents the articles that did not specify well enough how the EVs were isolated or did not isolate EVs before analysis. *C*: distribution of the specific isolation methods used in all discussed publications. *D*: distribution of isolated EV subtypes depending on differential ultracentrifugation (dUCF) protocols. Here, we called EVs isolated at 10,000–20,000 *g* large EVs (L-EVs) and EVs isolated above 100,000 *g* small EVs (S-EVs). "Mixed" illustrates the articles that isolated L-EVs and S-EVs together as the 100,000 *g* centrifugation was applied without a prior 10,000–20,000 *g* centrifugation. "Both" illustrates the articles that isolated L-EVs and S-EVs separately but included both EV subtypes in their analysis. *E*: number of characterization methods used in each of all discussed publications. *F* and *G*: distribution of the specific methods used for EV characterization, in all discussed publications, divided into biophysical methods (*F*) and biochemical methods (*G*). AC, affinity capture; AF4, asymmetric flow field flow fractionation; AFM, atomic force microscopy; conc, concentration; DC, density cushion; DG, density gradient; DLS, dynamic light scattering; ELISA, enzyme-linked immunosorbent assay; EM, electron microscopy; FACS, fluorescence-activated cell sorting; fNTA, fluorescence NTA; MALS, multiangle light scattering; NA, no isolation/not sufficiently specified; NTA, nanoparticle tracking analysis; SEC, size exclusion chromatography; SLS, static light scattering; SP-IRIS, single particle interferometric reflectance imaging sensor; TEM, transmission electron microscopy; TFF, tangential flow filtration; (T)RPS, (tunable) resistive pulse sensing; UF, ultrafiltration; WB, Western blot. Image was created by BioRender.com with permission.

(KEGG) pathway analysis of these miRs suggested that the apical EV miRs are associated with the mammalian target of rapamycin (mTOR) and mitogen-activated protein kinase (MAPK) signaling pathways. In contrast, the basolateral EV miRs are associated with T- and B-cell receptor signaling. This suggests that the EVs released apically or basolaterally may belong to different subpopulations of EVs, potentially targeting different recipient cells.



Only one study has compared EVs released from bronchial epithelial cells from healthy versus asthmatic subjects (36). The rest of the studies have instead shown that different asthma-related stimuli can induce the release and also alter the cargo and function of airway epithelial EVs, including proinflammatory cytokines [IL-13, IL-4, IL-17A, and interferon (IFN)-γ], the synthetic viral mimic poly(I:C), allergens [house dust mites (HDMs), ovalbumin (OVA), and respiratory syncytial virus (RSV)], and mechanical compression (34, 37, 41–45).

The study by Schindler et al. (36), who compared EVs released by bronchial epithelial cells from asthma versus healthy subjects, suggested that 32 miRs are altered in EVs on the apical side in asthma patients compared to EVs from healthy subjects, with the majority being upregulated in asthma patients. On the basolateral side, 23 miRs were significantly different, with the majority being downregulated in EVs from asthma patients compared to EVs from cells from healthy subjects. Interestingly, the miR-9 family and miR-21 showed upregulation in EVs released on both sides of the cells in asthma versus healthy controls. This may be important, as increased levels of miR-9 have been explicitly linked to steroid-resistant neutrophilic asthma (46) and miR-21 is altered in both blood and sputum-derived EVs in asthmatics versus healthy controls (47–51). By contrast, miR-34b and miR-34c were found to be downregulated in EVs released from both sides of the cells. Members of the miR34/449 family are known to regulate differentiation in epithelial cells and have previously been shown to be downregulated in airway epithelial brushings from subjects with asthma and to be repressed in cultured human airway epithelial cells upon stimulation with IL-13 (52), suggesting a protective role of these micro-RNAs in allergic disease.

Immune cells infiltrating the lung during the chronic inflammation observed in asthma release multiple cytokines, and several studies have studied the effect of these proinflammatory cytokines on the epithelial cells and their released EVs. In T2-driven asthma, cytokines such as IL-4, IL-5, and IL-13 are considered important, while in T2-low asthma, IL-17A and tumor necrosis factor (TNF)-α may be involved. Kulshreshtha and colleagues (42) have shown that the lung epithelial cell line BEAS-2B releases more EVs after exposure to IL-13, IL-4, and IFN-γ. In contrast, the macrophage cell line MHS releases fewer EVs when stimulated with the same cytokines. The EVs from IL-13-stimulated airway epithelial cells can enhance the proliferation and migration of monocytes, which EVs from nonstimulated airway epithelial cells cannot do, suggesting that IL-13 influences EV cargo and, thus, EV function (42). Furthermore, one study that focused on T17-associated asthma and EVs released on the apical side of air-liquid interphase cultured airway epithelial cells stimulated the cells with either IL-13 and IL-4 to mimic T2 asthma or with IL-17A and TNF-α to mimic T17 asthma. Interestingly, when these cytokines activate, the bronchial epithelial cells release more EVs on their apical side. Subsequent quantitative mass spectrometry showed that the proteins upregulated in the T17-associated EVs were related to neutrophil activation, whereas the EVs from T2-stimulated epithelium were not. This was functionally validated in the study, as only the T17-derived EVs could induce neutrophil migration compared to EVs from T2-stimulated and nonstimulated epithelial cells (37). In addition to IL-13 altering the proteome of bronchial epithelial cell-derived EVs, it also alters the EV miR cargo. For example, Bartel et al. (35) stimulated epithelial cells cultured as air-liquid interphase with IL-13 and showed that this alters the expression level of several miRs in EVs released from both the apical and basolateral sides. Most of the miRs were downregulated, and ingenuity pathway analysis (IPA) suggested that the altered miRs were associated with inflammatory responses (35). Together, this suggests that the epithelium can respond to an ongoing inflammation by changing the protein and RNA cargo of their released EVs, which can then further stimulate or inhibit the ongoing inflammation by affecting immune cell proliferation, polarization, activation, and migration. Dissecting the differences in EVs released during T2 high and the different T2 low inflammatory processes will be critical in future studies to determine their role in their potential underlying mechanisms in asthma endotypes.

Another way of mimicking asthma pathology is to stimulate the bronchial epithelial cells with allergens. Zhang et al. (44) took EVs from the bronchial epithelial cell line BEAS-2B stimulated with HDM and administrated them to mouse airways. The results showed that more immune cells were present in the BALF, parallel

FIGURE 5. Pathological functions of extracellular vesicles (EVs) in asthma. The pathological functions of epithelial cell-derived EVs (*A*), immune cell-derived EVs (*B*), EVs derived from other cells (*C*), and EVs derived from in vivo samples such as body fluids or tissues from either humans or rodent disease models of asthma (*D*). CCL, chemokine (C-C motif) ligand; CCR, C-C chemokine receptor; CDR1as, CDR1 antisense RNA; CRNDE, Colorectal neoplasia differentially expressed; CSF2 colony-stimulating factor 2; CXCL, chemokine (C-X-C motif) ligand; DUSP1, dual specificity protein phosphatase 1; ECP, eosinophil cationic protein; EPO, eosinophil peroxidase; EMT, epithelial-mesenchymal transition; FLAP, 5-lipoxygenase activating protein; GRO, growth regulated protein; HSP, heat shock protein; ICAM, intercellular adhesion molecule; IFN, Interferon; IL, interleukin; LO, lipoxygenase; LTC4S, leukotriene C4 synthase; LTA4H, leukotriene A4 hydrolase; MBP, major basic protein; NF-κB, nuclear factor-kappa B; NGF, nerve growth factor; NK, natural killer; NOS, nitric oxide synthase; PAET, $PM_{2.5}$-associated exosomal transcript; sCD100, soluble CD100; SCF, stem cell factor; TGF, transforming growth factor; Th, T helper; TNF, tumor necrosis factor; VCAM, vascular cell adhesion molecule; VEGFA, vascular endothelial growth factor A. The image was created by BioRender.com with permission.

with increases in lung IL-4, IL-5, IL-13, IL-17A, and IFN-γ concentrations. It was also observed that mucus formation increased when the BEAS-2B cells were treated with HDM before EV isolation compared to phosphate-buffered saline (PBS)-treated controls. This suggests that EVs from HDM-treated airway cells are sufficient to trigger inflammation in the airway. Furthermore, it was shown that the protein contactin 1 (CNTN1), a glycosylphosphatidylinositol (GPI)-anchored membrane protein belonging to the immunoglobulin superfamily, was upregulated in airway epithelium-derived EVs after HDM stimulation and that it could facilitate the recruitment, proliferation, migration, and activation of dendritic cells (DCs) via notch receptor 2 (NOTCH2) signaling (44). Furthermore, Yao et al. (45) showed that EVs derived from HDM-stimulated airway epithelial cells release EVs that induce T-helper 2 (Th2) differentiation in $CD4^+$ T cells; this was further enhanced if the epithelial cells had been stimulated with both HDM and RSV. However, HDM-stimulated cell-derived EVs suppressed Th1 cell differentiation. In line with this, the Th2 cytokines, IL-4, and IL-5 expression were increased, while the Th1 cytokine IFN-γ was decreased. The authors suggest that miR-155-5p in the EVs was responsible for the exaggerated Th2 inflammation observed (45).

Additionally, airway epithelial cells harvested from mice and stimulated with OVA release more EVs, and proteomic analyses show that the protein Plexin B2, a molecule involved in cell migration, is the most upregulated protein in these EVs after OVA stimulation. When these EVs were administered to mouse airways, the transcripts for the proinflammatory cytokines and chemokines *Ccl2*, *Cxcl2*, *Csf2*, and *Il6* were upregulated in the lung. Additionally, sensitization with these EVs increased OVA-challenged-induced airway inflammation. Furthermore, Plexin B2 is a ligand for CD100, and it was shown that CD100-expressing macrophages in the lung could take up the epithelial-derived EVs when administered intranasally to mice. This leads to an upregulation of matrix metalloproteinase 14 (MMP14) and, subsequently, a decrease in cell membrane-bound CD100 but a parallel increase in soluble CD100 in BALF. However, an appropriate control group using EVs from PBS-treated epithelial cells was lacking in some of the experiments in this study, which is a recommended control (43). Together, these studies argue that airway epithelial cells alter the protein cargo of EVs upon allergen stimulation, resulting in the release of proinflammatory EVs that can directly activate immune cells.

Some subjects with asthma get exacerbated by airway virus infections, such as the common cold. Poly(I:C) is commonly used as a synthetic viral mimic as it is structurally similar to double-stranded RNA and can interact with Toll-like receptor 3 (TLR3). Mills et al. (41) showed that EVs from poly(I:C)-stimulated epithelial cells induced CXCL8, IL-6, and CCL5 release from unstimulated epithelial cells and that poly(I:C) increased their release of the EV-associated glycoprotein tenascin-C (TN-C) in parallel with the release of free TN-C, suggesting that stimulated epithelium can activate neighboring cells via EVs. However, because EVs contain a vast number of molecules, it is not yet clear which EV molecules mediate the changed functionality of stimulated epithelial EVs. Additionally, it has been demonstrated that bronchial epithelial cells exposed to mechanical compression also increase their release of the EV-associated TN-C in parallel with the release of free TN-C (34). Together, these studies suggest that viral stimuli, or physical stress mimicking bronchospasms, can induce TN-C release from bronchial epithelial cells in asthma patients and at least some of the TN-C is associated with EVs. This is interesting because TN-C is a protein that shows increased expression in asthmatic patients and is related to atopic status and disease severity (53–59) and inflammation and wound healing in general.

Other studies have also evaluated the effect of compressive stress on airway epithelial cells, which mimics the bronchoconstriction occurring during asthma exacerbation. It was then shown that air-liquid interphase-cultured bronchial epithelial cells in the presence of compressive stress release tissue factor-expressing EVs on the basolateral side of the cells, and if the air-liquid interphase-cultured bronchial epithelial cells are stimulated with IL-13 before the compressive stress, more tissue factor EVs are released (34, 38, 39). Tissue factor is a membrane protein that initiates blood coagulation, and it is elevated in the sputum and BALF of patients with asthma (60–62). This suggests that EVs carrying tissue factors participate in tissue homeostasis and/or airway remodeling in asthma.

Finally, air pollution is a significant environmental problem that can affect respiratory diseases, as small particles can penetrate the lung tissue, activate immune cells, and induce inflammation in the lung, which can exacerbate respiratory diseases such as asthma and COPD and may even cause lung cancer. It has been demonstrated that human epithelial cells exposed to particulate matter smaller than 2.5 μm ($PM_{2.5}$) release EVs with altered RNA cargo. Specifically, Wang et al. (63) showed that miR-129-2-3p is enriched in EVs after $PM_{2.5}$ exposure and that these EVs enhanced permeability in naïve epithelial cells as well as increasing their IL-6, IL-8, and TNF-α secretion. Furthermore, Zheng et al. (64) found that 118 miRs were upregulated and 144 were downregulated after $PM_{2.5}$ exposure. Among the upregulated miRs was let-7i-5p, which is particularly interesting since let-7i-5p is also upregulated in plasma EVs isolated from children with asthma. It was further

suggested that the RNA-binding protein ELAV-like protein 1 is responsible for loading let-7i-5p into EVs and that let-7i-5p can reduce the expression of dual specificity protein phosphatase 1 (DUSP1), downregulating inflammatory processes in recipient epithelial cells. Further, when EVs from $PM_{2.5}$-treated epithelial cells were injected into a murine asthma model (OVA exposure), let-7i-5p was increased in the lung tissue. At the same time, DUSP1 was decreased, resulting in upregulation of IL-6 as a sign of increased inflammation. Additionally, when bronchial epithelial cells are stimulated with $PM_{2.5}$, they release EVs that may induce DNA damage in naïve bronchial epithelial cells in a process proposed to be orchestrated by a lncRNA and associated with the accumulation of reactive oxygen species (65). Together, these studies suggest that air pollution can stimulate the airway epithelial to release proinflammatory EVs that also can disrupt the epithelium.

In summary, epithelial cells respond to multiple triggers, such as viral mimics and allergens, by altering the cargo and, hence, the function of their released EVs. Furthermore, proinflammatory cytokines commonly present during the ongoing inflammation in the airways of subjects with asthma and mechanical stress alter the cargo and function of EVs released by epithelial cells. This implies that epithelial EVs can participate in both the initiation and preservation of the inflammation by affecting naïve epithelial cells and initiating the recruitment, proliferation, migration, and activation of immune cells (**FIGURE 5*A***).

2.1.2. Immune cell-derived EVs.

In asthma, multiple types of immune cells accumulate in the airways, including T-lymphocytes and eosinophils, as well as macrophages, dendritic cells, and neutrophils. The role of immune cell-derived EVs in asthma pathophysiology has been extensively studied (**FIGURE 5*B***). Eosinophils are considered important in several asthma endotypes and are recruited from the bone marrow to enter the asthmatic airways. It has been shown that eosinophils from asthma patients release more EVs than eosinophils from healthy subjects and that the EVs from eosinophils contain typical eosinophilic granule proteins, including eosinophil peroxidase (EPO), major basic protein (MBP), and eosinophil cationic protein (ECP) (66, 67). Furthermore, Cañas et al. (67) showed that EVs isolated from eosinophils from asthma patients have increased secreted nitrate levels, which induce eosinophil migration and enhance adhesion, partly via increased expression of the intercellular adhesion molecule-1 (ICAM-1) and integrin α_2-expression. Furthermore, eosinophil-derived EVs also affect airway epithelial cells and smooth muscle cells. Thus EVs isolated from eosinophils derived from asthma patients delay wound repair and induce apoptosis in small airway epithelial cells in vitro but induce proliferation and activation in bronchial smooth muscle cells. Additionally, the EVs from the asthmatic eosinophils can stimulate TNF, CCL26, and periostin expression in small airway epithelial cells and CCR3 and vascular endothelial growth factor A (VEGFA) expression in bronchial smooth muscle cells (68). Mazzeo and colleagues (66) have demonstrated that IFN-γ stimulation of eosinophils increases their EV release. This suggests that eosinophils may modify eosinophil functions via an EV-mediated autocrine route but can also influence the development of the pathological features of asthma by affecting structural cells.

Although eosinophils are strongly associated with asthma, neutrophilic inflammation and mixed eosinophilic and neutrophilic inflammation can be observed in many asthma patients. It has been shown that lipopolysaccharide (LPS) stimulated neutrophils to release EVs that contain significantly increased levels of the lncRNA colorectal neoplasia differentially expressed (CRNDE), a molecule known to be involved in inflammation. Furthermore, this lncRNA can be transferred to airway smooth muscle cells via EVs, which results in the activation of nuclear factor-kappa B (NF-κB) via phosphorylation of the inhibitor of NF-κB kinase β (IKKβ). This leads to increased cell viability and proliferation in the recipient airway smooth muscle cells and is hypothesized to be involved in smooth muscle remodeling in asthma. This is further supported by an in vivo study, where CRNDE was knocked out in neutrophils in a mouse model of asthma, resulting in reduced thickness of the bronchial smooth muscle layer (69). In horse heaves, an asthma-like disease, two studies have shown that EVs isolated from neutrophils stimulated with LPS can upregulate proteins associated with immune responses in parallel with increased proliferation of airway smooth muscle cells (70, 71). Together, these results thus support the hypothesis that neutrophil-derived EVs can contribute to the airway remodeling observed in asthma.

Mast cells are an essential player in allergic asthma as the binding of allergens to IgE on mast cells is a crucial component of the disease mechanism. This leads to the release of leukotrienes, histamines, and different proteases, contributing to the pathophysiology of asthma, including airflow obstruction. Several studies have, therefore, evaluated EVs from mast cells and shown that other stimuli, including IL-33, IgE, ozone, oxidative stress, and LPS, affect the RNA cargo of mast cell-derived EVs (72–75). Briefly, mast cells stimulated with IL-33 release EVs containing higher levels of the inflammation-regulating molecule miR-146a, and mice injected with IL-33 accumulate more miR-146a in their plasma EVs (73). Wang and Wang (76) have suggested that IgE-stimulated mast cells release EVs containing increased levels of the circular

RNA cerebellar degeneration-related protein 1 antisense RNA (CDR1as). This molecule has been suggested to protect cells from hypoxia/reoxygenation injury (76). When these EVs were injected into mice, they were taken up by vascular endothelial cells, which resulted in the upregulation of ICAM-1 and vascular cell adhesion molecule-1 (VCAM-1). The authors suggested that mast cell-derived EVs thereby might participate in the inflammation-mediated atherosclerosis seen in this animal model (74). Stimulating mast cells and macrophages with LPS and ozone results in EVs with increased miR-21 content, an RNA species implicated in vascular remodeling and vascular damage (72, 77). Furthermore, miR-21 is upregulated in lung tissue in both mouse and rat models of asthma. While these EVs induce oxidative stress, immune cell infiltration, and epithelial-mesenchymal transition in the airways of mice and cultured tracheal epithelia cells, miR-21 inhibition reduces these effects (72, 77). By contrast, miR-21 is downregulated in neutrophil EVs from horses with heaves compared to those from healthy horses (78), which suggests that there are differences in EVs from mast cells versus neutrophils and that non-RNA cargo may also confer some of the biological effects that are observed.

It is well understood that macrophages and monocytes contribute to airway inflammation by promoting the recruitment and activation of inflammatory cells by releasing factors that affect structural cells such as fibroblasts or smooth muscle cells. For example, leukotrienes are proinflammatory lipid mediators involved in host defense and inflammatory diseases. Specifically, they can increase vascular permeability, smooth muscle contraction, and the chemotactic effect on migrating immune cells, and their overproduction contributes to inflammation in asthma. Importantly, enzymes involved in the leukotriene biosynthesis pathway, such as leukotriene C4 synthase (LTC_4S), leukotriene A4 hydrolase (LTA_4H), 5-lipoxygenase activating protein (FLAP), and 5-lipoxygenase (5-LO), are present in EVs from human macrophages and dendritic cells (79). Notably, the enzymes are functional because these EVs can convert LTA_4 to LTC_4 and LTB_4 (79). The EVs can also convert arachidonic acid into 5-LO products, but the concentrations are considerably lower than the formation of leukotrienes from LTA_4 (79). Polymorphonuclear leukocytes also migrate toward the cell-derived EVs (79). These findings suggest that leukotriene metabolism can occur outside of cells within EVs, but it is unclear how relevant such production is to that occurring in cells.

Similarly to small particles affecting the EVs released from airway epithelial cells (discussed in sect. 2.1.1), their effect on EVs released by immune cells has also been investigated. EVs isolated from particle-stimulated (0.3–2.5 μm) monocytes and macrophages stimulate airway epithelial cells to release IL-6 and TNF-α, while EVs from nonstimulated cells induced IL-8 release. Together, this suggests that immune cell-derived EVs may be involved in the mechanism of the inflammatory response associated with particle exposure and that these EVs potentially have different biological functions depending on cell exposures (80).

Finally, EVs isolated from airway myeloid-derived regulatory cells (MDRCs) contain mitochondria that can be transferred to T cells, and the EV-transferred mitochondria can integrate with the recipient cell's mitochondrial network (81). However, there are no observed differences in mitochondrial transfer via EVs from MDRCs from asthma patients versus EVs from healthy subjects.

Together, this suggests that EVs from immune cells can induce apoptosis in small airway epithelial cells and proliferation and activation in smooth bronchial muscle cells. Additionally, they can increase migration and leukotriene production in eosinophils and neutrophils. These are all features of immune cell EVs that may contribute to the airway remodeling observed in asthma.

2.1.3. EVs derived from other cells.

Although epithelial and immune cell-derived EVs are mainly studied in asthma, one study has evaluated fibroblast-derived EVs. Haj-Salem et al. (82) observed that primary human bronchial fibroblasts from patients with severe asthma release EVs with increased quantities of several cytokines, including IL-6, growth regulated protein (GRO), CXCL1/GRO-α, CXCL-8/IL-8, CCL-1/I309, CCL-15/macrophage inflammatory protein (MIP)1-delta, and stem cell factor (SCF) (**FIGURE 5C**). In contrast, transforming growth factor (TGF)-β2 is significantly lower in EVs from these patients compared to healthy controls. Further, EVs isolated from fibroblasts from severe asthmatics induce cell proliferation in primary human bronchial epithelial cells, while EVs isolated from fibroblasts from healthy subjects dampen such proliferation. Interestingly, when TGF-β2 was overexpressed in the fibroblasts from severe asthmatics, the proliferation in the bronchial epithelial cells was reduced. When TGF-β2 was knocked down in the fibroblasts from healthy subjects, bronchial epithelial cell proliferation increased (82). These observations suggest that fibroblast-produced EVs and immune cell-derived EVs may also regulate the airway remodeling observed in asthma.

2.1.4. Body fluid-derived EVs.

EVs can be isolated locally from the airways by nasal lavage fluid (NLF), BALF, or sputum or systemically from blood. EVs from NLF can induce the migration of several immune cells, such as monocytes, neutrophils, and natural killer cells. Furthermore, the NLF-derived EVs contain

functional inducible nitric oxide synthase 2 (NOS2) (83). Nitric oxide is part of the innate immune system and participates in the initial responses to bacterial infection, and a family of nitric oxide synthase enzymes such as NOS2 synthesizes it, increasing exhaled NO in some patients. Additionally, a subgroup of asthma patients suffers worsening symptoms after exposure to nonsteroidal anti-inflammatory drugs (NSAIDs). EVs isolated from the sputum of such patients induce the transcription of inflammatory host defense proteins and cytokine production in the negative regulation of type 2 immune responses in macrophages to a lesser degree than sputum EVs from healthy controls. Furthermore, sputum EVs from these asthmatics have been shown to stimulate the airway epithelial production of cytokines and chemokines involved in granulocyte recruitment (51). This suggests that airway EVs from asthma patients affect multiple cellular events that may favor T2 inflammation and that this activity may be altered in inflammatory airway diseases. The pathological functions of EVs from human body fluids and animal models are summarized in **FIGURE 5*D***.

Similarly to immune cell-derived EVs, active LTA_4H, LTC_4S, FLAP, and 15-LO-1 are also present in EVs isolated from human plasma (79) and BALF (84). Furthermore, BALF EVs from asthma patients induce significantly higher levels of leukotriene and IL-8 release when coincubated with bronchial epithelial cells as compared to BALF EVs from healthy controls, and this biological function is inhibited by the cysteinyl leukotriene (CysLT) 1 receptor antagonist montelukast (84). This further suggests that BALF EV-induced IL-8 release in bronchial epithelial cells is at least partly leukotriene dependent.

Adding plasma-derived EVs from asthmatics to mouse tracheal rings results in increased bronchial smooth muscle contractions when subjected to bronchoconstrictive or relaxing stimuli compared to plasma EVs from healthy controls (85). This suggests that asthma patient EVs, even in blood, may facilitate the production of bronchoconstrictive stimuli, and this is supported by the presence of the leukotriene machinery in BALF EVs from asthmatics. However, this study did not explore which molecules in the EVs from asthmatics are causing the increased sensitivity of mouse airway tissues.

Together, these data imply that EVs isolated either locally from the airways or systemically from the circulation can have proinflammatory effects in asthmatics. The relative role of different cargo in the EVs that may participate in these effects, including miRs and proteins, requires further study.

2.1.5. Animal models.

Common models for studying asthma are OVA or HDM sensitization to induce allergic airway inflammation in mice or rats. A study in rats using the OVA model showed increased CD63 expression in lung tissue and increased acetylcholinesterase activity in BALF compared to control rats, which the authors argue is a sign that EVs are released in the inflamed lung (86). However, it has more recently been suggested that acetylcholinesterase activity is not a generic marker for EVs (87), and therefore, these results would need validation with alternative analytical techniques. Nevertheless, in two additional studies using the OVA allergen model in mice, where EVs were isolated directly from the lung tissue and from BALF, more EVs could be isolated from either sample from the animals sensitized and challenged with OVA compared to the control PBS mice (42, 88). GW4869 is a drug that reduces EV secretion, and in mice, this has been shown to reduce the numbers of EVs in BALF in parallel with a reduction in the numbers of infiltrating monocytes and macrophages in the lung, reduced bronchial hyperresponsiveness, reduced mucus production, reduced serum levels of IgE, and reduced inflammatory cytokines (42). Furthermore, a quantitative proteomics study showed that mitochondrial proteins are decreased in lung tissue EVs during allergic inflammation, whereas immune cell and inflammation-related proteins are increased after allergen challenge (88). Interestingly, the immunomodulatory protein uteroglobin, which is primarily produced by nonciliated bronchial epithelial cells called club cells, is among the most clearly decreased proteins in OVA-lung-derived EVs. These may imply that the club cells release fewer EVs during inflammation while the infiltrating immune cells are responsible for the increased numbers of EVs (88), although such conclusions need further detailed single-vesicle analytics to be confirmed. One supportive piece of data has been produced by Pua and colleagues (89), who showed in naïve mice that the miRs in cell-free BALF mostly correlated with miRs from airway brushings, which harvest airway epithelial cells, compared to the cell pellet from BALF, which primarily consists of hematopoietic cells. When mice were immunized and challenged with OVA, almost all of the upregulated miRs in BALF were also expressed at higher levels in the hematopoietic cell pellet compared to the epithelial brushings. It was later shown that EV membranes protected the miRs in BALF. Interestingly, transmission electron microscopy suggested that most (~90%) of the particles were likely to represent disruption of the surfactant lipid lining of the lung, as only 10% showed the morphology of EVs. Furthermore, an experiment using transgenic mice and a single EV flow analysis was performed to trace the origin of EVs during OVA stimulation. It was shown that 80% of the BALF EVs were of bronchial epithelial origin, while no bronchial epithelial signal could be detected in serum EVs from

the same mice. In BALF, 15% of the EVs were of hematopoietic cell origin, which is in contrast to serum, where the majority of the EVs were of hemopoietic origin. After OVA exposure, the hematopoietic cell-derived EVs increased with a 2.7-fold change in BALF, which may be a sign of disruption of the local airway endothelium, allowing the influx of blood EVs into the tissues (89). This implies that primarily the immune cell-derived EVs reach the circulation, while the bronchial epithelial cell-derived EVs stay mostly in the airways.

Several studies have also shown increased numbers of EVs in BALF from HDM-stimulated mice compared to PBS control mice (44, 90). Furthermore, 85 miRs have been suggested to be altered in BALF EVs from HDM-stimulated mice compared to PBS mice, with the majority of them significantly increased. Computational analysis revealed that several of the putative target genes for these miRs are related to T2 inflammation.

When GW4869 was used in the murine HDM asthma model, the number of EVs, as well as several of the previously identified miRs, were decreased in BALF after HDM exposure. Furthermore, when mice were exposed to HDM, the influx of cells such as eosinophils was increased in the BALF; however, if the mice had been treated with the GW4869 fewer eosinophils were detected in the BALF (44, 45, 90). Additionally, in a mouse model that used both HDM and RSV to establish a mouse model of acute asthma exacerbation, GW4869 reduced the number of infiltrating Th2 cells and neutrophils but not Th1 cells (45). Furthermore, several inflammatory cytokines, such as IL-4, IL-5, and IL-13, were decreased in the lung when HDM mice were treated with GW4869. At the same time, conflicting results were observed for IFN-γ depending on whether RSV was included in the model (44, 45, 85), again implying the involvement of EVs in regulating allergic airway inflammation. Furthermore, levels of serum IgE, peribronchial leukocyte infiltration, goblet cell hyperplasia, and mucus production were reduced after GW4869 treatment. This indicates that inhibition of EV release may reduce inflammation in these models. Additionally, HDM has also been used in an allergic asthma model in guinea pigs. In that model, serum EVs from HDM-exposed animals could stimulate bronchial epithelial cells to release IL-6 and nerve growth factor (NGF), while control EVs could not. Furthermore, it was suggested that heat shock protein 70 expressed on the surface of EVs is responsible for the epithelial cell reaction, triggering inflammation via the TLR4-NF-kB pathway (91).

Animal asthma models may help explain the in vivo function of EVs in this disease. However, it becomes crucial to determine from which cell the EVs originate, to which cell they signal, and how that signal is conveyed. Focusing only on one molecule identified in an EV population might be insufficient to understand the EV-mediated biology in asthma fully. In this process, identifying specific surface markers on EVs from certain cells may be helpful and can ideally be supported by single-EV analytical methods.

2.1.6. Summary of pathological functions of EVs in asthma.

In asthma, the EV-mediated interaction between epithelial cells and immune cells, including monocytes, macrophages, neutrophils, and eosinophils, has been studied (**FIGURE 5**). Generally, airway epithelial cells that take up EVs, produce and release proinflammatory cytokines, chemokines, and leukotrienes, which are involved in immune cell recruitment and activation. Furthermore, both apoptosis and proliferation were induced. Immune cells receiving EVs have been shown to respond by increased proliferation, migration, and activation. EVs reaching smooth muscle cells induced increased contractions, while endothelial cells responded by increasing microvascular permeability. All these features have also been observed in vivo in different rodent models of asthma. Together, these results suggest that EVs can contribute to the airway remodeling observed in asthma and can initiate and enhance inflammation in asthma at several levels of pathophysiology and via communication among several types of cells.

We suggest that future studies of the regulatory effects of EVs in asthma pathophysiology should ideally focus on studies of well-described phenotypes and endotypes of disease. By doing that, future research may have the power to dissect similarities and differences in EV-mediated mechanisms both at the cellular and molecular levels, also beyond T2-type inflammation.

2.2. COPD

COPD is a heterogeneous lung disease that presents with cough, increased sputum production, shortness of breath, and nonreversible airflow obstruction. COPD is associated with chronic bronchitis and is often combined with pulmonary emphysema, with some differences in inflammation patterns having been described in different groups of patients. The main cause of COPD is tobacco smoking followed by smoke exposure during cooking over an open fire (92).

2.2.1. Airway epithelial cell-derived EVs.

As tobacco smoking is the main cause of COPD, extensive research has been performed on the effect of cigarette smoke on airway cells and their released EVs, and emerging evidence suggests that EVs from cigarette

smoke-exposed airway epithelial cells can regulate various processes in immune cells and fibroblasts (**FIGURE 6*A***). EVs isolated from cultured airway epithelial cells exposed to cigarette smoke extract (CSE) can induce macrophages to release IL-8 and granulocyte-macrophage colony-stimulating factor (GM-CSF; also known as CSF2), especially when the macrophages are isolated from patients with alpha-1 antitrypsin deficiency (AATD). AATD is the most common genetic risk factor for COPD, especially for individuals who smoke. Interestingly, macrophages from healthy individuals do not respond in this way. Thus AATD patients might have an inflammatory-prone macrophage phenotype that results in an exaggerated inflammatory response to cigarette smoke-induced EVs (93). Furthermore, EVs isolated from CSE-stimulated airway epithelial cells have also been shown to stimulate the polarization of macrophages into mainly type M1 via lncRNA maternally expressed gene 3 (MEG3), miR-21-3p, miR-27b-3p, miR-125a-5p, and miR-221-3p (94–97).

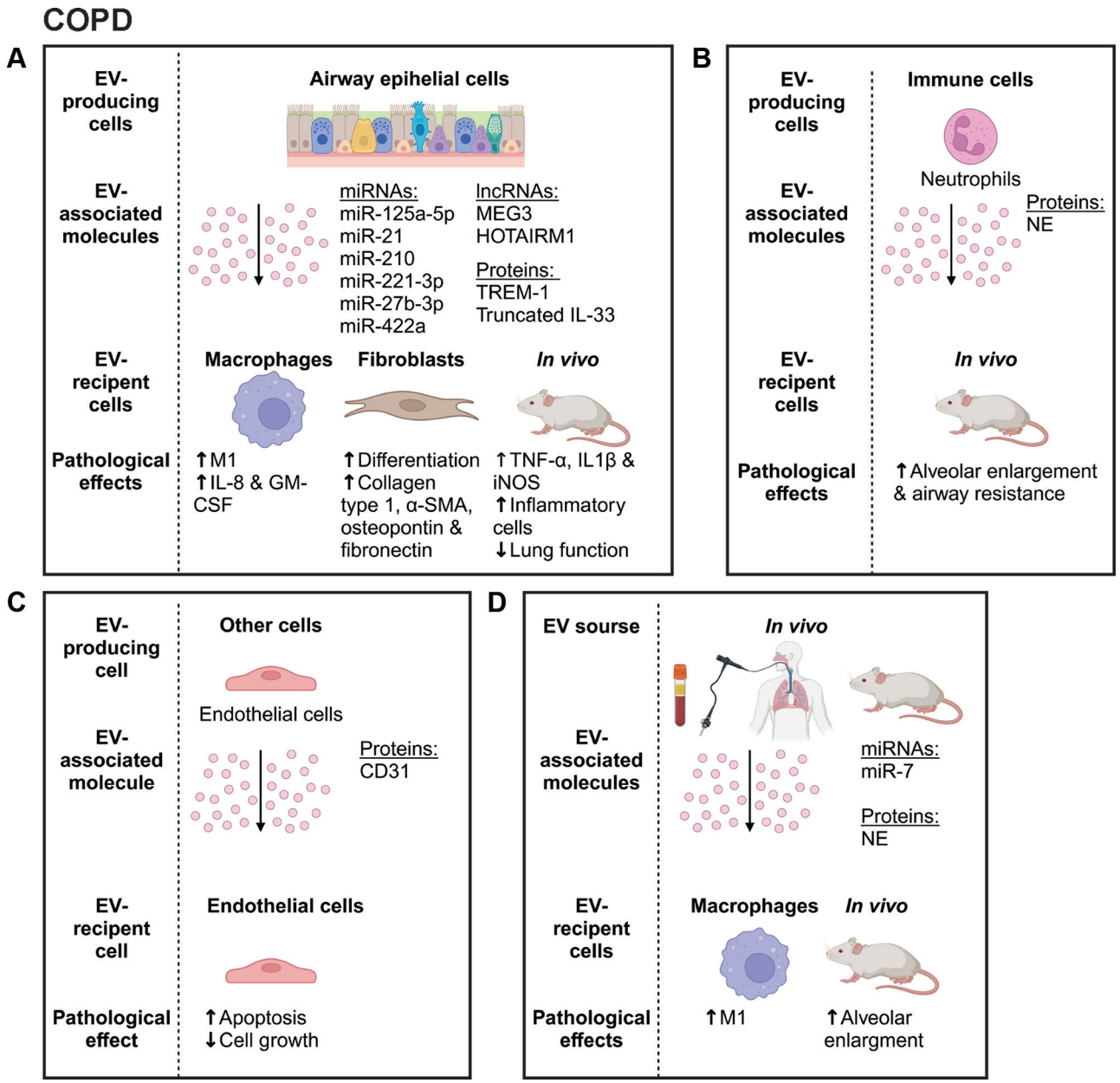


FIGURE 6. The pathological functions of extracellular vesicles (EVs) in chronic obstructive pulmonary disease (COPD). The pathological functions of epithelial cell-derived EVs (*A*), immune cell-derived EVs (*B*), EVs derived from other cells (*C*), and EVs derived from in vivo samples such as body fluids or tissues from either humans or rodent disease models of COPD (*D*). GM-CSF, granulocyte-macrophage colony-stimulating factor; HOTAIRM1, HOX antisense intergenic RNA myeloid 1; IL, interleukin; iNOS, inducible nitric oxide synthase; MEG3, maternally expressed 3; NE, neutrophil elastase; SMA, smooth muscle actin; TNF, tumor necrosis factor; TREM, triggering receptor expressed on myeloid cells. The image was created by BioRender.com with permission.

EVs isolated from CSE-exposed airway epithelial cells have also been suggested to induce differentiation in fibroblasts and to increase the expression of collagen type 1 and alpha-smooth muscle actin (α-SMA) (98–100), and several mechanisms for this have been proposed. Xu et al. (98) suggested that EV-associated miR-21 mediates this activity, and they showed that treatment with anti-miR-21 in a COPD mouse model was associated with reduced airway inflammation. Fujita et al. (99) suggested that miR-210 plays a role in this mechanism, which is interesting because this miR was found to be upregulated in the lungs of smokers. Finally, Dai et al. (100) suggested that EV-associated miR-422a is reduced after exposing epithelial cells to CSE, leading to higher levels of secreted phosphoprotein-1 (SPP1; also called osteopontin) and collagen type 1 and α-SMA in the recipient fibroblasts.

Further, there is some evidence that EVs from tobacco-exposed cells can influence lung biology in vivo. Wang and coworkers (101) showed that EVs isolated from cultured epithelial cells exposed to cigarette smoke increased lung TNF-α, IL-1β, and inducible nitric oxide synthase (iNOS) in a cigarette smoke-induced COPD mouse model. It was further shown that this partly depended on triggering receptors expressed on myeloid cells-1 (TREM-1) (101). TREM-1 is an innate immune receptor that can amplify TLR-mediated inflammation and is constitutively expressed in monocytes, macrophages, and neutrophils. Together, these studies suggest that cigarette smoke alters the cargo and function of epithelial-derived EVs, activating macrophages and fibroblasts.

Also, small particulate matter ($PM_{2.5}$) found in environmental air pollution can contribute to COPD, and EVs isolated from airway epithelial cells exposed to $PM_{2.5}$ stimulate fibroblasts to express α-SMA, collagen type 1, and fibronectin. It has been suggested that the lncRNA HOXA transcript antisense RNA myeloid-specific 1 (HOTAIRM1) in EVs is responsible for the activation of fibroblasts. When these EVs are administered to a $PM_{2.5}$-induced COPD mouse model, reduced lung functions and enhanced inflammatory cell infiltration are observed, and this observed effect is diminished by treatment with a siRNA blocking HOTAIRM1 (102).

Finally, IL-33 is an alarmin that plays a central role in initiating and amplifying type 2 immune responses, and its truncated form has been shown to bind to the surface of EVs derived from airway basal cells. This is interesting because IL-33 is increased in lung tissue from COPD patients, which might reflect a subphenotype of the disease. In an *Alternaria*-induced mouse model of airway inflammation, blocking of neutral sphingomyelinase with the pharmacological blocker GW4869 reduced both EVs and IL-33 in BALF, as well as downstream type 2 inflammatory pathways, suggesting a potential role for the EV/IL-33 axis in COPD (103).

Together, these studies suggest that cigarette smoke has a profound effect on EVs released by airway epithelial cells and that epithelial EVs may intricately regulate COPD-related pathological processes and influence other cells in the vicinity of the epithelium.

2.2.2. Immune cell-derived EVs.

Many immune cells present in the airways, including macrophages, dendritic cells, and granulocytes, can release large quantities of EVs. In COPD, primarily EVs released from neutrophilic granulocytes have been studied (**FIGURE 6*B***). Genschmer et al. (104) demonstrated that EVs released by granulocytes stimulated with a bacterial formylated peptide have more neutrophil elastase (NE) on their surface. The EV-bound NE can cleave type 1 collagen, a process resistant to alpha-1 antitrypsin (α1AT) inhibition. When these NE-containing EVs were administered intranasally to mice, they caused hallmarks of COPD, such as alveolar enlargement and increased airway resistance. The same investigators also evaluated EVs isolated from BALF from COPD patients, showing that they contained more NE. When administered to mouse airways, the patient-derived BALF EVs could also cause alveolar enlargement (104). Additionally, Margaroli et al. (105) showed that mice treated with LPS, which resulted in neutrophil-dominant pulmonary inflammation, had more EVs carrying NE in their BALF. Similar to humans, mouse EV-bound NE was resistant to α1AT. Furthermore, when BALF-EVs from LPS-stimulated mice were administrated to naïve mice, they induced alveolar enlargement. When neutrophil-derived EVs were removed from the BALF by capture of specifically neutrophil-released EVs, this effect was reduced (105). These studies argue that NE on the neutrophil EV surface may affect COPD pathophysiology.

2.2.3. EVs derived from other cells.

It has been suggested that mitochondrial dysfunction is a feature of COPD and that smooth muscle cells release more mitochondria-containing EVs when exposed to cigarette smoke (106). Also, it has been shown that CSE-stimulated pulmonary microvascular endothelial cells release more $CD31^+$ EVs. These $CD31^+$ EVs induce apoptosis and decrease cell growth when incubated with nontreated pulmonary microvascular endothelial cells, suggesting a toxic function for these EVs (**FIGURE 6*C***) (107). These studies all suggest that cigarette smoke affects not only the EVs released by epithelial cells but also those released by other cells participating in the pathophysiology of COPD, highlighting the influence of cigarette smoke-induced EV-associated pathological processes in the lung.

2.2.4. Body fluid-derived EVs.

Nieri et al. (108) showed that plasma-derived EVs from COPD patients during acute symptom exacerbation induce airway epithelial cells to release significantly more IL-8 and CCL-2 than from stable COPD patients or healthy controls. Furthermore, this effect was reduced when EVs were isolated from plasma 8 weeks after the exacerbation of symptoms (108). It is unclear whether these observations can fully be attributed to EVs, as also other plasma components may have been coisolated with the EVs, thereby contributing to the observed effects.

2.2.5. Animal models.

Studying the biological functions of EVs in vivo is methodologically difficult, and only a few provide conclusive results from in vivo COPD studies. A mouse model of COPD using porcine pancreatic elastase to induce emphysema showed elevated levels of EVs in BALF (109). Furthermore, Jiang et al. (110) showed that serum EV-associated miR-7 is upregulated in a cigarette smoke-induced mouse model of COPD. These EVs can induce M1 macrophage polarization both in vitro and in vivo, and it was subsequently suggested that miR-7 affects macrophages through the regulation of phosphorylation in recipient cells (110). The pathological functions of EVs from human body fluids and from animal models are summarized in **FIGURE 6*D***.

2.2.6. Summary of pathological functions of EVs in COPD.

In COPD, the primary effects of EVs on macrophages and fibroblasts have been described (**FIGURE 6**). Macrophages can respond to EVs by differentiating into the M1 phenotype, which may result in differential release of chemoattractants for neutrophils or other granulocytes. Fibroblasts can respond to EVs by differentiating into myofibroblasts and increasing the release of collagen type 1, α-SMA, osteopontin, and fibronectin. It was clear that when EVs were administrated in vivo to rodents, they could induce signs of COPD, such as alveolar enlargement and enhanced inflammatory cell infiltration into the lung. As with asthma, it is imperative to consider the COPD phenotype when studying EV-associated pathophysiological mechanisms.

2.3. Cystic Fibrosis

CF is a genetic disorder associated with reduced mucus clearance from the airways and is caused by mutations in the cystic fibrosis transmembrane conductance regulator gene (CFTR), which translates to a chloride channel important in maintaining normal mucus viscosity. This disease, therefore, is associated with increased mucus viscosity and reduced mucus clearance, which increases the risk of colonization of the lungs and airways by different bacteria, including *Pseudomonas aeruginosa*, *Haemophilus influenzae*, and *Staphylococcus aureus*, and sometimes fungi such as *Aspergillus fumigatus*. CF can lead to long-term reduction of lung function, associated with chronic pulmonary inflammation and severe infectious exacerbations.

2.3.1. Airway epithelial cell-derived EVs.

Human bronchial epithelial cells with mutations in the CFTR gene release more EVs than bronchial epithelial cells with wild-type (WT) CFTR. Importantly, when the CFTR mutated cells were treated with kinase inhibitors to restore CFTR expression, the EV release was reduced to similar levels as wild-type cells. Furthermore, EVs released by cells with mutant CFTR express more proteins associated with immune pathways and integrin signaling, such as epithelial cellular adhesion molecule (EPCAM), VCAM, and S100 A12. These EVs also induce the migration, transmigration, and activation of neutrophils. When S100 A12 was knocked down in the EV-producing cells, and thus the expression was reduced in the released EVs, a significant reduction in EV-induced neutrophil chemotaxis was observed (111). Additionally, Szul and coworkers (112) showed that LPS stimulation of bronchial epithelial cells results in increased release of EVs in a TLR4-dependent manner. These EVs contain the enzyme prolyl endopeptidase, a serine peptidase that generates the neutrophil chemoattractant tripeptide Pro-Gly-Pro from collagen. This is interesting because prolyl endopeptidase-containing EVs have also been seen in sputum from CF patients (112). Finally, Lozano-Iturbe et al. (113) showed that the protein and miR cargo of airway epithelial-derived EVs are altered upon *Pseudomonas aeruginosa* exposure and that this response is different in epithelial cell lines with CF mutations compared to healthy epithelium.

2.3.2. Immune cell-derived EVs.

Forrest and colleagues (114) have observed that neutrophil-derived EVs in sputum from CF patients carry caspase-1, a protease that activates proforms of proinflammatory interleukins in the IL-1 family by cleaving them. Furthermore, it was shown that these EVs can activate naïve neutrophils isolated from blood, leading to upregulation of caspase-1. In a follow-up experiment, these investigators isolated EVs from activated neutrophils and added these to airway epithelial cells, which resulted in the release of IL-1α, IL-1β, and

IL-18 (114). This suggests that neutrophil-derived EVs in the CF airways can activate newly recruited neutrophils and bronchial epithelial cells to sustain inflammasome activation, leading to chronic inflammation.

2.3.3. EVs derived from other cells.

Some studies suggest that CFTR inhibition can reduce endogenous anti-inflammatory responses. Zulueta et al. (115) treated MSCs isolated from the lung and expanded them in vitro in the presence of a CFTR inhibitor. The untreated MSC-derived EVs had anti-inflammatory function, but EVs from CFTR inhibitor-treated MSCs failed to show any anti-inflammatory activity. Thus these EVs failed to reduce proinflammatory cytokines such as IL-1β, IL-8, and IL-6 released by bronchial epithelial cells from a CF patient (115). Furthermore, it was shown that the lipid content was altered as the EVs contained more proinflammatory ceramide. The authors argued that this means that EVs released under CFTR dysfunction can contribute to maintaining the chronic inflammatory status observed in CF patients.

2.3.4. Body fluid-derived EVs.

Al-Humiari et al. (116) have suggested that EVs isolated from the BALF of pediatric CF patients, as well as EVs from asthma patients, can increase epithelial sodium channel activity in small airway epithelial cells. This is interesting because CF patients are known to have epithelial sodium channel hyperactivity. However, as it is deemed unethical to collect BALF from healthy children as sedation is required, the experiment, unfortunately, lacks EVs from healthy controls, which makes it difficult to determine if this activity is increased in CF. In addition, EVs from sputum from CF patients activate inflammasome signaling in human naïve neutrophils (114) and induce neutrophil infiltration in mouse lungs to a similar degree as the LPS controls, while EVs from sputum from patients with primary ciliary dyskinesia did not, and instead, they induced infiltrating macrophages (117).

Experiments using body fluid-derived EVs in CF to learn about their potential involvement in the disease can currently primarily be performed in adults, which is a later stage of this genetic disease. To learn about EV-associated biological processes early in the disease, in vitro models, or potentially animal models, will need to be the primary source of information.

2.3.5. Animal models.

Mao and colleagues have used a transgenic mouse overexpressing sodium channel nonvoltage-gated 1, beta subunit (Scnn1b) in the airways, which leads to increased transepithelial Na(+) absorption and dehydrates the airway surface, resulting in a lung phenotype similar to CF patients. Proteomic analysis of BALF EVs from this model revealed that 127 proteins were significantly upregulated, and 30 proteins were downregulated in *Scnn1b* transgenic mice as compared to WT mice. The proteins upregulated were associated with macrophage activation and mucoinflammatory process (118). However, future mechanistic studies will have to validate whether these EVs can also activate macrophages and affect mucosal inflammation.

2.3.6. Summary of pathological functions of EVs in CF.

Relatively few studies have investigated the role of EVs in CF. However, interactions between epithelial cells and neutrophils via EVs have been described. Neutrophilic inflammation is observed in many CF patients. Most of the studies discussed here suggest that EVs contribute to sustaining inflammation by passing signals between recruited neutrophils and epithelial cells in CF airways (**FIGURE 7, *A–D***). Taken together, these studies suggested that EVs isolated under CF conditions (CTFR mutations or CTFR inhibitors) resulted in the release of more proinflammatory EVs. Specifically, more EVs are produced by bronchial epithelial cells in CF and may participate in the neutrophil recruitment observed in this disease. Furthermore, upon bacteria stimulation, the bronchial epithelial cells in CF release EVs with altered miR and protein cargo, with some proteins suggested to also be able to stimulate an increased neutrophil chemoattractant capacity. Additionally, neutrophil-derived EVs and sputum-derived EVs activate both neutrophils and bronchial epithelial cells. It would be interesting to see mechanistic studies in animal models to further elucidate EV-mediated cross talk in CF. One consideration for future EV studies of CF is that the patient's airways often have bacterial overgrowth, and thus, their BALF can contain EVs from these bacteria. Therefore, some of the results described here could potentially be explained by the mixture of bacterial and airway cell EVs acquired from BALF from CF patients.

2.4. Pulmonary Fibrosis

Pulmonary fibrosis is presented with fibrotic scarring of the lung parenchyma, excessive accumulation of extracellular matrix (ECM), and remodeling of the lung. These processes progressively reduce lung function, resulting in respiratory failure and eventually death. In most cases, the causes of pulmonary fibrosis are unknown, and therefore, the term idiopathic pulmonary fibrosis (IPF) is often

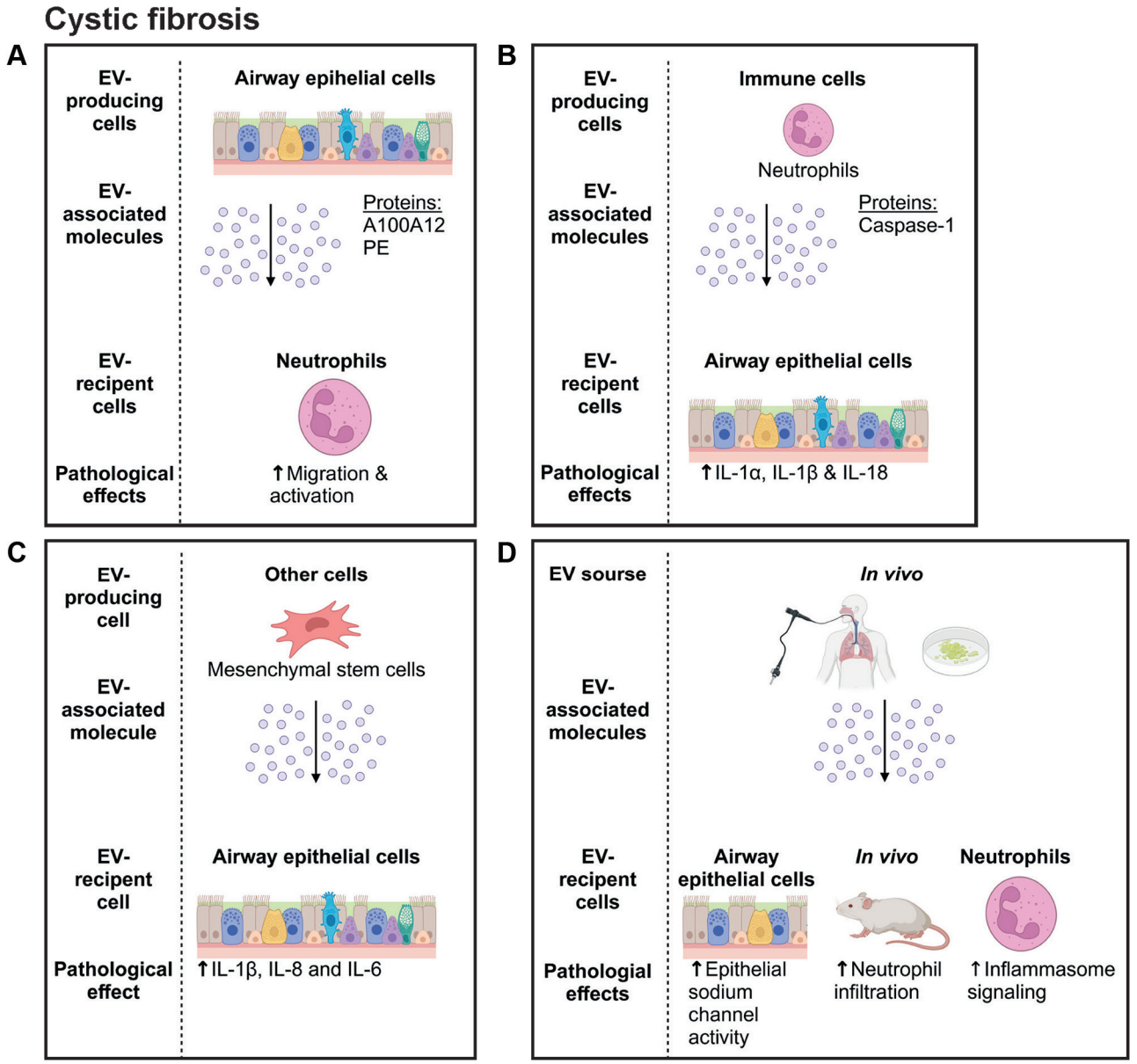


FIGURE 7. The pathological functions of extracellular vesicles (EVs) in cystic fibrosis. The pathological functions of epithelial cell-derived EVs (*A*), immune cell-derived EVs (*B*), EVs derived from other cells (*C*), and EVs derived from in vivo samples such as body fluids or tissues from either humans or rodent disease models of cystic fibrosis (*D*). IL, interleukin; PE, propyl endopeptidase. The image was created by BioRender.com with permission.

used. Known causes can otherwise be occupational exposure to metals or silica dust or inhalation of dust contaminated with bacterial materials. Cigarette smoking is known to increase the risk of pulmonary fibrosis. In patients with pulmonary fibrosis, the lung parenchyma becomes progressively fibrotic, and fibroblasts are generally considered to be important contributors to the disease.

2.4.1. Airway epithelial-derived EVs.

Only one study has so far compared EVs from airway epithelial cells from IPF patients and EVs from epithelial cells from healthy controls (119). In contrast, other studies have used different models to induce an IPF phenotype in the bronchial cells. Furthermore, most studies looking at EVs from airway epithelial cells in pulmonary fibrosis have investigated their effect on fibroblasts. Activated fibroblasts differentiate into myofibroblasts, release extracellular matrix components, and show upregulation of α-SMA, fibronectin, and collagen type 1. This process leads to wound healing, but when the process becomes unregulated, it can lead to fibrosis. Bronchial and/or alveolar epithelial cells exposed to CSE, hypoxic conditions, radiation, or the IPF-inducing pollutant arsenite, all release EVs that promote fibroblast differentiation and activation (120–123). It has been suggested that CSE-stimulated epithelial cells downregulate circRNA_0026344, which functions as a sponge for miR-

21. Therefore, the miR-21 levels in cells are increased, and this miR is then loaded into EVs. It has been suggested that EV-associated miR-21 induces lower levels of mothers against decapentaplegic homolog 7 (SMAD7) in fibroblasts, leading to their differentiation and ECM production (120). The EVs from arsenite-treated bronchial epithelial cells also carry miR-21 (123). These EVs promoted glycolysis and fibroblast differentiation. Furthermore, EVs released by alveolar epithelial cells under hypoxic conditions contain more of the lncRNA HOTAIRM1. HOTAIRM1 was also upregulated in a bleomycin-induced pulmonary fibrosis mouse model, and several of the clinical features were further increased when HOTAIRM1 was overexpressed but decreased when sh-HOTAIRM1 silencing RNA was given. Importantly, when HOTAIRM1-containing EVs isolated from alveolar epithelial cells were injected into mice, they reduced lung compliance and increased lung resistance. The investigators, therefore, argued that EV-associated HOTAIRM1 promotes the proliferation and differentiation of lung fibroblasts, enhancing extracellular matrix remodeling and accelerating IPF development (121). Finally, radiation-damaged alveolar epithelial cells release EVs that promote proliferation, migration, and ECM synthesis in fibroblasts (122). Together, these studies suggest that EVs released by airway epithelial cells under the influence of fibrosis-triggering stimuli activate the fibroblasts to increase their ECM production, an important hallmark of pulmonary fibrosis.

In addition, the autocrine/paracrine effect of EVs from bronchial epithelial cells during pulmonary fibrosis has also been studied. Specifically, bronchial epithelial cells from IPF patients release significantly more EVs than epithelial cells from healthy controls, and these EVs contain increased levels of miR-411, miR-137, miR-195, and miR-7 and can induce senescence in naïve epithelial cells (119). Polyhexamethylene guanidine is a biocidal disinfectant, and when used to cause an IPF-like bronchial epithelial cell phenotype, these cells release EVs with altered miR cargo. Specifically, miR-451a, miR-15a, miR-30a-3p, let-7g-5p, and miR-27b-3 were found to be decreased, while the expression of miR-6813, miR-4492, and miR-483 were upregulated compared to control EVs. Low levels of miR-451a result in epithelial-mesenchymal transition and fibrogenesis in naïve epithelial cells (124).

Together, these studies suggest that EVs released by epithelial cells under fibrotic conditions stimulate fibroblast differentiation but can also influence naïve epithelial cell functions (**FIGURE 8*A***). The majority of the studies have so far focused on the EV RNA cargo. Still, it is important to determine whether other EV molecules, such as cargo proteins, surface proteins, or lipids, also can participate in the influence on any recipient cells in IPF.

2.4.2. Immune cell-derived EVs.

EVs from angiotensin II-, IL-4 (M2 macrophages)-, and silica-exposed macrophages can stimulate and activate fibroblasts, leading to increased levels of production of collagen type 1 and α-SMA (125–129). Furthermore, macrophages exposed to silica secrete EVs that can affect the T1/T2 and T17/regulatory T (Treg) balance (128). Specifically, Th1 cells decreased, while Th2, Th17, and Treg increased. The EVs released by silica-treated macrophages contain miR-125a-5p and miR-107, while the EVs from IL-4-stimulated macrophages contain miR-328 (126, 128, 129). The authors of these studies have argued that some of the observed effects in fibroblasts may be attributed to these RNAs (**FIGURE 8*B***). Another study using silica-activated macrophages suggests that the EVs activate the fibroblast by inducing endoplasmic reticulum stress in the fibroblasts but did not determine which EV-associated molecule(s) may be responsible for the observed effect (127). Similarly to epithelial-derived EVs, immune cell-derived EVs can influence and activate fibroblasts, implicating them in IPF pathogenesis. Additionally, it was suggested that EV mediate an imbalance in Th1/Th2/Th17/Treg distribution, which may further promote fibroblast activation.

2.4.3. Fibroblast-derived EVs.

Fibroblasts isolated from IPF patients' lung tissue release more EVs than those isolated from non-IPF lungs (130, 131). Also, senescent fibroblasts release significantly more EVs than nonsenescent fibroblasts (131). Interestingly, the IPF fibroblast-derived EVs induce increased senescence and accumulation of dysfunctional mitochondria in airway epithelial cells compared to non-IPF-derived EVs (**FIGURE 8*C***). Several miRs are upregulated in IPF fibroblast-derived EVs, and it has been suggested that miR-23b-3p and miR-494-3p facilitate EV-mediated mitochondrial damage and cellular senescence (130). Additionally, fibroblast-derived EVs have fibronectin on their surface, which induces an invasive phenotype in recipient fibroblasts (131). Inactive fibroblasts are called fibrocytes, and there is evidence that EVs isolated from cultured fibrotic tissue-derived fibrocytes from a rat fibrosis model also had altered miR cargo compared to EVs isolated from fibrocytes from control lungs. Specifically, miR-21 was evaluated and shown to be upregulated in EVs from fibrocytes from fibrotic tissue and in fibrocytes during mechanical stretching. Furthermore, these EVs induce a profibrotic effect in fibroblasts (132).

Together, these results suggest that not only airway epithelial cells and immune cells release EVs that activate fibroblasts, but even fibroblast-derived EVs can

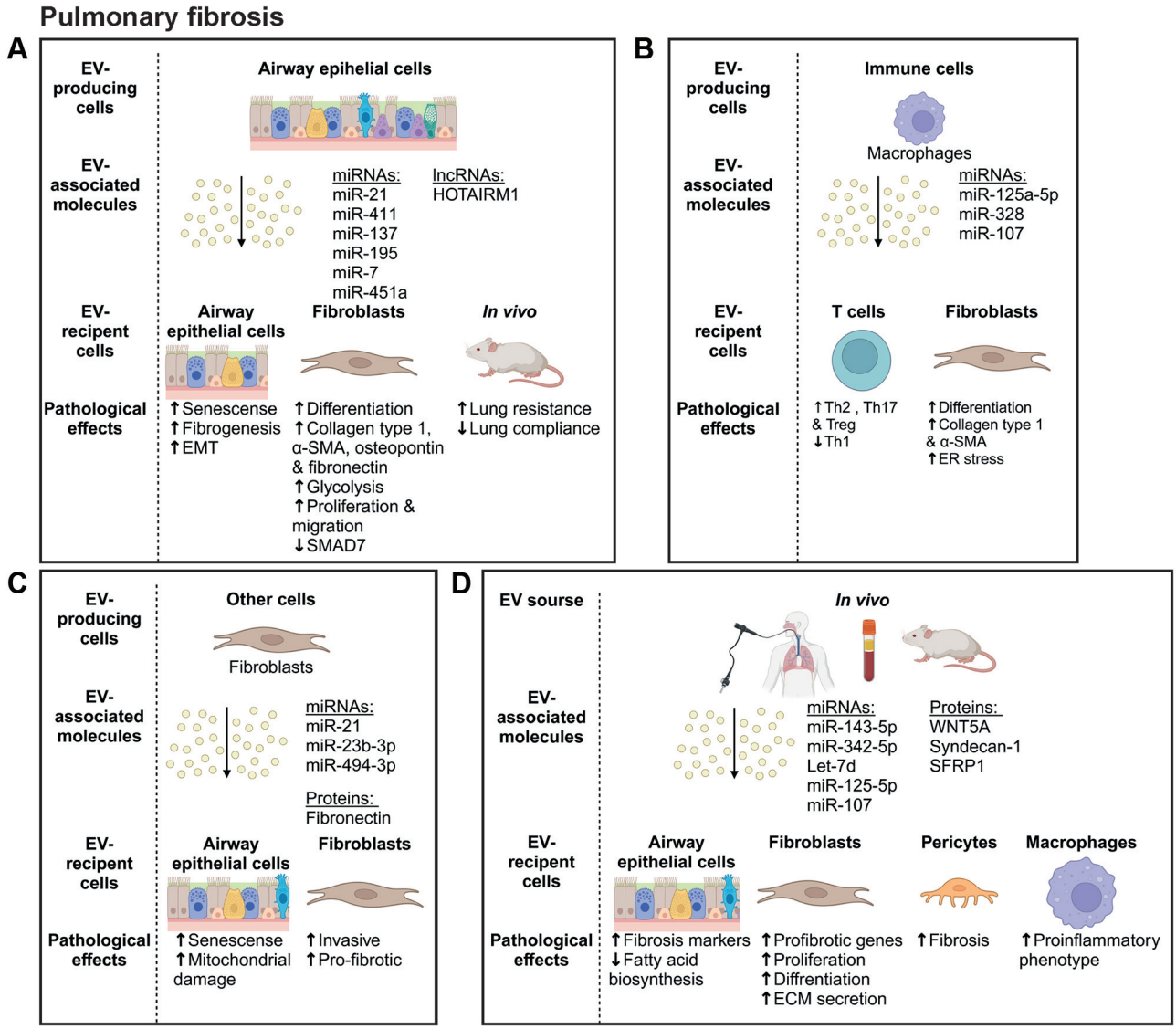


FIGURE 8. The pathological functions of extracellular vesicles (EVs) in pulmonary fibrosis. The pathological functions of epithelial cell-derived EVs (*A*), immune cell-derived EVs (*B*), EVs derived from other cells (*C*), and EVs derived from in vivo samples such as body fluids or tissues from either humans or rodent disease models of pulmonary fibrosis (*D*). ECM, extracellular matrix; EMT, epithelial-mesenchymal transition; ER, endoplasmic reticulum; HOTAIRM1, HOX antisense intergenic RNA myeloid 1; SFRP1, secreted frizzled-related protein 1; SMA, smooth muscle actin; SMAD7, mothers against decapentaplegic homolog 7; Th, T helper; Treg, regulatory T cell; WNT5A, Wnt family member 5 A. The image was created by BioRender.com with permission.

induce fibroblasts to become more invasive in an autocrine and/or paracrine manner. Additionally, fibroblast-derived EVs can induce senescence in lung epithelial cells, and as lung epithelial cell senescence is a crucial step for the initiation and progression of fibrosis in IPF, it suggests that fibroblast EVs can also contribute to this pathology.

2.4.4. Body fluid-derived EVs.

EVs isolated from lung tissue from IPF patients upregulate profibrotic genes in fibroblasts and downregulate fatty acid synthase (FASN), achaete-scute-like protein-4 (ASCL-4), and surfactant protein-C (SP-C) mRNA in alveolar type II cells leading to decreased fatty acid biosynthesis and senescence (**FIGURE 8*D***). It has also been suggested that miR-143-5p and miR-342-5p in the EVs are responsible for the effect on recipient cells. The levels of EV-associated miR-143-5p and miR-432-5p and their effects on recipient cells are related to the severity of fibrosis (133). Interestingly, lung tissue-derived EVs from IPF patients taking the antifibrosis drug nintedanib had decreased profibrotic response in fibroblasts and induced increased expression of FASN and SP-C in alveolar type II cells as compared to EVs from naïve IPF patients. This indicates the critical role of EVs in modulating alveolar type II cell function in this disease.

2.4.5. Animal models.

Several different rodent models of pulmonary fibrosis exist, with bleomycin-induced fibrosis being the most commonly used. In a mouse bleomycin-induced IPF model, it was found that BALF EVs had less let-7d, and these EVs can induce fibrosis phenotype in lung pericytes (**FIGURE 8*D***). However, transmission electron microscopy images clearly illustrate contaminants in the EV isolates in this study. Therefore, it is uncertain whether the observed biology depends on the EVs or other components coisolated with them (134). Martin-Medina et al. (135) demonstrated that BALF-derived EVs in the bleomycin-induced IPF mouse model also contain more Wnt family member 5A (WNT5A). The investigators argued that fibroblasts released the WNT5A EVs and could induce proliferation in other fibroblasts. Furthermore, TGF-β stimulated increased WNT5A in the released fibroblast EVs (135). Parimon et al. (136) also used the bleomycin-induced mouse model and isolated EVs from BALF, and they found that EVs isolated from fibrotic lungs did not induce fibrosis in naïve mice but did augment existing mild fibrosis. Using syndecan-1-null mice, it was suggested that syndecan-1 controls the packing of antifibrotic miRs into EVs, and when these EVs were given to mice with fibrosis, they did not augment the existing fibrosis. The investigators argued that syndecan-1 can drive lung fibrosis in vivo through the differential regulation of EV cargo (136). Finally, Burgy et al. (137) isolated BALF EVs from the bleomycin model several times after the bleomycin (day 3 to day 56) to cover lung fibrosis initiation, progression, and resolution. They showed that EVs were consistently increased in BALF samples at all time points after the bleomycin challenge compared with controls, with a peak at day 14. Furthermore, the amount of EVs was related to lung function as it was most severe on day 14. Notably, the BALF EVs from day 14 in the bleomycin model induced expression of extracellular matrix and fibrosis markers in alveolar epithelial type 2 (AT2) stem cell-based organoids compared to the control mice EVs. Furthermore, the protein cargo in BALF EVs in the bleomycin model was altered, and bioinformatics suggested an association with fibrosis-inducing processes. The authors suggested that fibroblast is the primary source of these EVs and that secreted frizzled-related protein 1 (SFRP1) was suggested to be responsible for the profibrotic effects on the alveolar epithelial cells. Further, the authors also isolated EVs from primary lung fibroblasts and BALF from IPF patients and verified that SFRP1 was increased in EVs from IPF patients compared to healthy controls (137).

The less commonly used silica- and radiation-induced IPF mouse models, primarily EV miRs, have been studied. In the silica-induced mouse fibrosis model, it was suggested that serum-derived EVs contain miR-107 and miR-125-5p and can contribute to lung fibrosis by triggering fibroblast trans-differentiation (129, 138). In the radiation-induced pulmonary fibrosis rat model, BALF EVs contain higher levels of miR-214-3p and miR-143-3p and lower levels of miR-542-3p and miR-1306-5p (122). Future studies should determine the specific roles of these EV-associated miRs in patients.

Tang et al. (139) reported that in a mechanical ventilation-induced pulmonary fibrosis mouse model, BALF EVs activate fibroblasts and increase the extracellular matrix production, which was suggested to occur via the c-Jun NH2-terminal kinase (JNK) signaling pathway in the fibroblasts. Finally, in a polyhexa-methylene guanidine phosphate-induced pulmonary fibrosis mouse model, BALF EVs have altered protein cargo, specifically increased levels of proteins associated with coagulation and fibrinolysis. Furthermore, the BALF EVs induce a proinflammatory phenotype in recipient macrophages, which was suggested to be mediated by EV-associated fibrinogen (140).

Interestingly, GW4869 treatment, which reduces EV production in all cells, has been shown to attenuate pulmonary fibrosis in the bleomycin-induced IPF mouse model, the mechanical ventilation-induced pulmonary fibrosis mouse model, and the silica-induced mouse model (125, 127, 139). This and the previous results suggest BALF EVs alter their RNA and protein cargo upon IPF triggers in vivo. Further, these EVs may activate fibroblasts and induce fibrosis. Significantly, inhibiting EV release could alleviate pulmonary pathologies in these models, further emphasizing the importance of studying EV function in respiratory diseases.

2.4.6. Summary of pathological functions of EVs in pulmonary fibrosis.

In pulmonary fibrosis, the majority of studies have determined the EV-mediated interactions between airway epithelial cells and fibroblasts (**FIGURE 8**). EVs promote proliferation and differentiation of fibroblasts, leading to increased release of collagen type 1, α-SMA, osteopontin, and fibronectin. This suggests that EVs can induce fibroblast-to-myofibroblast transition, epithelial-to-mesenchymal transition, and the extracellular matrix deposition observed in pulmonary fibrosis. Pulmonary fibrosis has been suggested to be caused by an imbalance in alveolar epithelial cells, whereas many studies have utilized bronchial epithelial cells. More studies on alveolar epithelial cell-derived EVs would be recommended to understand how EVs may contribute to the initiation of IPF.

2.5. Lung Injury

Acute lung injury (ALI) and its more severe form, acute respiratory distress syndrome (ARDS), describe clinical

syndromes of acute decreases in lung gas exchange followed by respiratory failure. The mortality of ALI/ARDS is high, and for the individuals who survive, substantial long-term morbidity remains an issue. ALI/ARDS are disorders of acute inflammation that cause disruption of the lung endothelial and epithelial barriers. Cellular characteristics of ALI/ARDS include loss of alveolar-capillary membrane integrity, inflammation with excessive transepithelial neutrophil migration, and activation of macrophages, combined with increased vascular permeability and pulmonary edema. ALI/ARDS can be caused either by direct injury to the lung, for example, from pneumonia, trauma, mechanical ventilation, and aspiration, or by indirect causes, such as sepsis, severe burns, blood transfusions, and pancreatitis (141).

2.5.1. Airway epithelial cell-derived EVs.

Most of the studies in this lung injury section have utilized LPS exposure to mimic a sepsis-induced ALI/ARDS phenotype in EV-producing cells. Liu and colleagues (142) demonstrated, for example, that LPS-treated rat alveolar epithelial cells release EVs with increased levels of miR-92a-3p, a molecule previously shown to regulate inflammation. Administering these EVs intratracheally to rats can induce inflammatory cell infiltration into the lungs and increase the extent of lung injury (**FIGURE 9*A***). The investigators suggested that these EVs could activate macrophages via EV-associated miR-92a-3p activation of NF-κB signaling by targeting phosphate and tension homology deleted on chromosome ten (PTEN) in the recipient macrophages (142). Gong et al. (143) showed that LPS-treated alveolar epithelial cells have increased levels of TN-C in their EVs and suggested that EV-associated TN-C promotes M1 macrophage polarization and pyroptosis via DNA damage response pathways.

2.5.2. Immune cell-derived EVs.

Multiple studies have stimulated macrophages with LPS to determine the effect of their released EVs on airway epithelial cells (**FIGURE 9*B***) (144–147). Briefly, LPS stimulation of macrophages leads to increases in EV-associated TNF-α, IL-6, IL-1β, and interferon-induced guanylate-binding protein 2 (GBP2). When these EVs are administered to mice, they cause lung injury (144–148). Furthermore, EVs released by LPS-stimulated macrophages increase ICAM-1 expression on epithelial cells (144) and reduce expression of alpha-epithelial sodium channel (α-ENaC), gamma-epithelial sodium channel (γ-ENaC), and Na^+/K^+-ATPase-α1 and -β1 in epithelial cells, which was suggested to be induced in a TNF-α-dependent manner (146). This mechanism may contribute to the dysregulation of the alveolar lining fluid, which may contribute to the progression of lung injury and pulmonary edema. Huang and coworkers (147) found that EVs from LPS-treated macrophages promote inflammation by targeting the NOD-like receptor protein 3 (NLRP3) inflammasomes, resulting in alveolar epithelial cell dysfunction and cell death through pyroptosis. Ye et al. (148) also suggested that EVs from LPS-treated macrophages induced pyroptosis in alveolar epithelial cells; however, they indicated that miR-2137 in the EVs was responsible for this via targeting of Wnt9a. Additionally, Sui et al. (145) propose that EVs from LPS-stimulated macrophages induce the release of TNF-α, IL-6, and IL-1β in naïve macrophages. Further, $PM_{2.5}$-stimulated macrophages release EVs that contain TNF-α, which can activate epithelial cells to increase the expression levels of surfactant proteins SP-A, SP-B, SP-C, and SP-D. Also, these EVs induce ALI when administered to naïve mice, which confirms their proinflammatory biological function (149). Xu and coworkers (150) described that hypervirulent *Klebsiella pneumoniae* stimulated macrophages to release EVs that contain higher levels of miR-155-5p, a miR intricately involved in regulating inflammation. This was associated with macrophage M1 polarization and ALI when administered to mice in vivo. Wu et al. (151) demonstrated that bleomycin-stimulated macrophages release ficolin B-containing EVs and that these EVs induce autophagy and ferroptosis in lung epithelial cells. When these EVs are administrated to a bleomycin-induced ALI mouse model, they induce additional lung injury and concomitant tissue fibrosis. Finally, Wang et al. (152) described that hyperoxia-exposed neonatal macrophages impaired endothelial function via EV-associated miR-23a-3p. Together with in vivo experiments, the authors therefore suggested that macrophage-derived EVs play a role when premature infants requiring mechanical ventilation develop bronchopulmonary dysplasia due to loss of endothelial progenitor cells and impaired vascularization.

In addition, other immune cells have been stimulated with LPS. For example, EVs isolated from LPS-stimulated $CD4^+$ T-cells, or serum of patients with sepsis-induced ALI, both enhance the progression of lung injury in a sepsis mouse model. Furthermore, the $CD4^+$ T cell-derived EVs induce apoptosis and oxidative stress in airway epithelial cells in vitro (153). Additionally, Fang et al. (154) described that EVs from LPS-treated mast cells induce ferroptosis and inflammation in bronchial epithelial cells and suggested that reduced EV-associated miR-744 after LPS treatment was responsible for this, as anta-miR-744 modulated ferroptosis, inflammation and lung injury in a mouse model of LPS-induced ARDS. This was also supported by a decline in miR-744 expression in plasma EVs in patients with ARDS as compared to healthy controls.

Finally, one study has shown that neutrophils stimulated with TNF-α also release EVs that induce lung injury

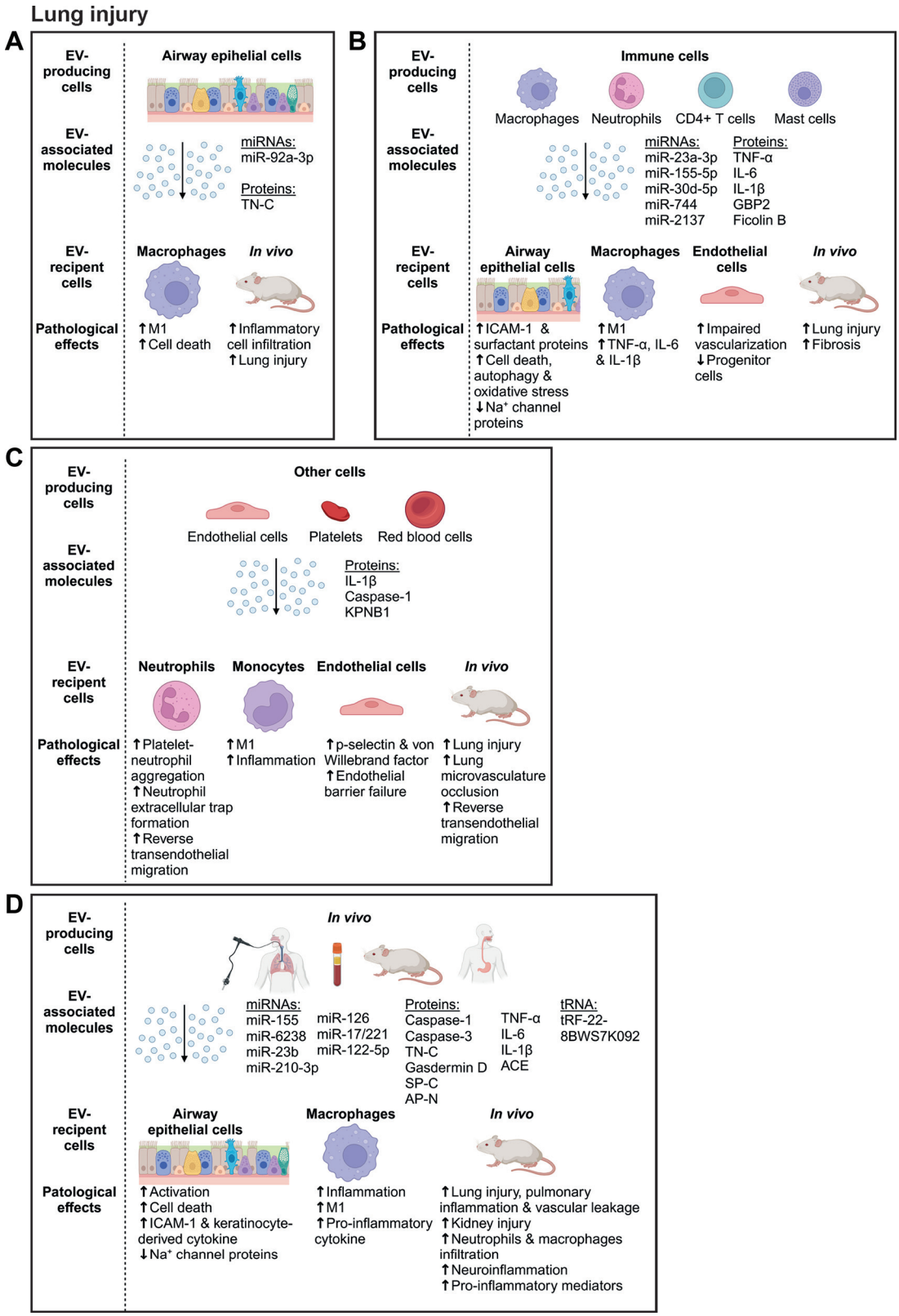


FIGURE 9. The pathological functions of extracellular vesicles (EVs) in lung injury. The pathological functions of epithelial cell-derived EVs (*A*), immune cell-derived EVs (*B*), EVs derived from other cells (*C*), and EVs derived from in vivo samples such as body fluids or tissues from either humans or rodent disease models of lung injury (*D*). ACE, angiotensin-converting enzyme; AP-N, adiponectin; GBP2, interferon-induced guanylate-binding protein 2; ICAM, intercellular adhesion molecule; IL, interleukin; KPNB1, karyopherin subunit beta-1; SP-C, surfactant protein-C; TN-C, glycoprotein tenascin-C; TNF, tumor necrosis factor; tRF, tRNA fragments. The image was created by BioRender.com with permission.

when administered to naïve mice. The investigators suggested that this is induced by miR-30d-5p in the EV-activated macrophages (155), although other molecules may also contribute to the observed effects.

Together, these studies imply that immune cells release EVs that induce cell death in epithelial cells, thus affecting the epithelial barrier, in parallel with having proinflammatory properties.

2.5.3. EVs from other cells.

Several studies have investigated the role of platelet-derived EVs in lung injury (**FIGURE 9C**) (156–158). Briefly, in sickle cell disease, platelet EVs contain IL-1β and caspase-1 that can cause lung injury by promoting platelet-neutrophil aggregation and occlusion of the microvasculature in the lung (156). Furthermore, Jiao and coworkers (158) suggest that platelet-derived EVs promote excessive neutrophil extracellular trap formation in sepsis, which can contribute to subsequent organ injury. Finally, plasma transfusion by itself can induce ALI, most likely via endothelial barrier damage induced by platelet EVs. Importantly, the number of platelet EVs increases if plasma is stored for long periods of time and can be more tissue damaging after the plasma is frozen (157, 159).

Additionally, endothelial EVs can induce ALI in mice and rats (160–164), and LPS and cyclic stretching of cultured endothelial cells (a model for ventilator-induced lung injury) increases the release of EVs (163, 165). Furthermore, Wang and coworkers (163) demonstrated that LPS-treated endothelial cells release EVs that induce M1 macrophage polarization and facilitate monocyte-mediated proinflammation via the interaction between VCAM1 on the EVs with integrin subunit alpha 4 (ITGA4) on the monocytes. It was indeed suggested that the EVs affect monocyte differentiation among the resident macrophages in the lung, while Zi and coworkers (164) showed that LPS-treated endothelial cells release EVs that induce reverse transendothelial migration in neutrophils both in vitro and in vivo, which contributed to lung injury. It was suggested that karyopherin subunit beta-1 (KPNB1) in the EVs upregulated NE in the neutrophils that caused the reverse transendothelial migration. Neutrophils that undergo reverse transendothelial migration are implicated in the systemic dissemination of inflammation.

Finally, red blood cell-derived EVs can also induce ALI in vivo in mice (166). Specifically, when the red blood cells have been stored for an extended period, their EVs then can induce greater release of P-selectin and von Willebrand factor from endothelial cells (167).

Overall, these studies imply that the EVs convey communication between a multitude of cells in ALI, beyond airway epithelium and immune cells. Thus disentangling the trafficking of EVs between a multitude of cells in tissues will be an important future field of research.

2.5.4. Body fluid-derived EVs.

Although many studies have determined the role of EVs in animal models, only one has evaluated the effect of EVs isolated from ALI patients. Qian et al. (168) showed that plasma EVs isolated from severe COVID-19 patients, and then instilled intratracheally into mice, induce the release of proinflammatory cytokines and infiltration of inflammatory cells into the lung. This is observed in parallel with an induction of the M1 polarization of macrophages by these EVs (168).

2.5.5. Animal models.

Multiple studies have used rodent models of ALI to evaluate the role of EVs in the pathophysiology of lung injury (**FIGURE 9*D***). The most common model is LPS-induced ALI, but other models induced by mechanical ventilation, hyperoxia, cecal ligation and puncture, acid exposure, ischemia/reperfusion, hemorrhage-resuscitation, bacteria, and particulate matter have also been utilized.

In the LPS-induced ALI model, it has been demonstrated that BALF and blood-derived EVs have increased levels of TNF-α, IL-6, IL-1β, angiotensin-converting enzyme (ACE), and miR-155, all proinflammatory molecules (144, 146, 169–171). Importantly, when these EVs are administered to naïve mice or rats, they induce lung injury (170, 172, 173). Furthermore, the EV-release-reducing molecule GW4869 also reduces lung injury and increases the survival rate, supporting the concept that EVs play a crucial role in the pathophysiology of ALI/ARDS (145).

The origin of EVs released in the LPS-induced ALI model has been studied in detail. One hour after LPS exposure alveolar macrophage-derived EVs dominate in BALF. The epithelial-derived EVs increase slowly over 4 hours. In comparison, neutrophil-derived EVs are primarily observed after 4 hours, which is logical, as the neutrophils need to be recruited before they can release EVs locally (144, 169). In the circulation, the situation seems different; however, Tan et al. (172) showed that neutrophil and monocyte-derived EVs increase 1 hour after LPS exposure, while macrophage EVs are undetectable in this body fluid. Platelet, endothelial, and erythrocyte-derived EVs were not found to be altered during the 4 hours they were monitored in this study (172). Furthermore, one study suggested that the primary EV subpopulation present in and induced after LPS exposure is large EVs isolated at 2,000 × *g*, as compared to medium-sized or smaller EVs isolated at 12,000 and 100,000 ×*g*, respectively (169). In contrast, another study found that the release of small

EVs is increased (174). This is in contrast to what was observed in a hyperoxia-induced model and a hydrochloric acid-induced model, where medium-sized EVs were the most prevalent (175, 176).

Several functions have been proposed for EVs released in the LPS-induced rodent ALI models. Most studies suggest that BALF-derived EVs are primarily of macrophage origin (146, 169). For example, EVs from BALF that are released in a caspase-1-dependent manner can activate epithelial cells, induce vascular leakage, and recruit neutrophils to the airways (169). Soni et al. (144) demonstrated that TNF-α-containing BALF EVs stimulate epithelial cells to upregulate ICAM-1 and keratinocyte-derived cytokines. Furthermore, TNF-α-containing BALF-derived EVs decrease α-ENaC, γ-ENaC, and Na^+/K^+-ATPase α1- and β1-levels in epithelial cells (146). Jiang and coworkers (170) showed that proinflammatory miR-155 is present in serum EVs and can induce both TNF-α and IL-6 release from macrophages in vitro and induce pulmonary inflammation when injected into naïve mice. Finally, it has also been demonstrated that tRNA fragments (tRFs) and lipid mediators are differentially expressed in BALF EVs (174, 177). Specifically, the upregulation of tRF-22-8BWS7K092 was suggested to promote cell death in alveolar epithelial cells by targeting Wnt5B (177). tRFs are 14- to 32-base fragments derived from tRNAs and have been proposed to regulate gene expression similarly to miRs.

Some studies have compared EV functions in different ALI mouse models. Lee et al. (178) have compared EVs from four ALI mouse models, including two with noninfectious causes, including hyperoxia-induced and acid-induced ALI, and two with infectious causes, including LPS-induced and bacteria-induced ALI. The investigators suggest that epithelial EVs increase most in BALF after the noninfectious causes, while macrophage-derived EVs increase the most after ALI induced by infectious causes. The isolated BALF EVs from all four causes induced macrophage infiltration in the lung when administered intranasally to naïve mice. However, neutrophil recruitment was only observed for EVs from the bacteria-induced model. It was suggested that under noninfectious conditions, the lung epithelium is damaged and releases EVs that activate macrophages and induce pulmonary inflammation, while during infection, macrophages are the first to release proinflammatory EVs and thus propagate lung inflammation (178). Park et al. (179) also compared noninfectious stimuli ($PM_{2.5}$) and infectious-like stimuli (LPS). While $PM_{2.5}$ induced epithelial cells to release EVs with miR-6238, LPS induced macrophages to release EVs with miR-155 (179). Together, these studies suggest that depending on the cause of ALI, the origin of EVs participating in inflammation differs.

Mechanical ventilation can induce ALI, and rodent models are used to study this. Thus BALF EVs from rodents exposed to such pulmonary stress contain TNF-α and IL-1β and can induce lung injury and inflammation in a cofilin-dependent way when administered to the airways of naïve animals (180). Additionally, in this model, plasma EVs have increased caspase-1 expression, which can induce neuroinflammation when administered to normal newborn rats (181). It is well known that oxygen therapy can cause both lung and brain damage in premature infants. In a hyperoxia-induced neonatal rat model, plasma EVs containing surfactant protein C and gasdermin D (a molecule that can create membrane pores) can induce lung inflammatory injury and brain inflammation when injected into normal newborn rats (182). These findings suggest that EVs produced in ALI can contribute to disease elsewhere, including causing brain injury and poor neurodevelopmental outcomes in preterm infants with ventilation-induced lung injury. Also, in a hyperoxia model, it was shown that BALF epithelial-derived EVs contain caspase-3, which can induce inflammatory lung responses and activated macrophages (175).

Cecal ligation and puncture (CLP) is a model for septic lung injury in rats and mice. In this model it has been shown that EVs in general, and endothelial cell-derived EVs specifically, are *1*) increased in plasma (163, 164, 171, 183); *2*) BALF and serum EVs have higher levels of TN-C (143); *3*) lung tissue-derived EVs have changed miR, mRNA, and lncRNA cargo during sepsis-induced ALI (184); *4*) plasma EVs have increased levels of miR-23b and miR-210-3p; and *5*) plasma EVs have increased levels of aminopeptidase N. These EVs can enhance macrophage inflammation and airway epithelial cell apoptosis/necroptosis and induce vascular leakage and lung injury when injected into naïve rats (183, 185, 186). Furthermore, like in several other disease models, GW4869 treatment leads to reduced airway epithelial-derived EVs in BALF, decreased sepsis scores, and increased survival in the ALI model (142).

Kojima et al. (187) have used a trauma/hemorrhagic shock-induced ALI model in both rats and mice. Mesenteric lymph-derived EVs from these animals, suggested by the authors to be of gut epithelial origin, induce proinflammatory cytokine production in macrophages. When injected into naïve animals, these EVs cause lung injury in a TLR4-dependent manner (187). Additionally, Zhang and colleagues (188) have used two different ischemia/reperfusion-induced lung injury models in rats, including abdominal aorta occlusion- and a hemorrhage/transfusion-induced model. The origin of the plasma EVs was evaluated, and it was shown that endothelial-derived and platelet-derived EVs were increased and could induce pulmonary vascular leakage and lung injury when

injected into naïve mice. Furthermore, proinflammatory miR-155 is increased in endothelial-derived EVs, and miR-126 is increased in platelet-derived EVs, and the authors have suggested that the EV-associated miRs explain observed effects (188).

Yet another way to induce ALI is using acids such as hydrochloric acid. Lee et al. (176) have suggested that epithelial cells are the primary origin of BALF EVs in this model and that they increase after acid exposure. The investigators suggested that miR-17/221 in the acid-induced EVs modulates macrophage recruitment and migration in the lung, leading to upregulation of integrin-β1 on macrophages (176).

ALI is a common postoperative complication after liver transplantation. Therefore, serum EVs were isolated from a hepatic ischemia-reperfusion (HIR) rat model and administrated to naïve or HIR rats. It was shown that HIR EVs induce lung injury and increase the expression of proinflammatory mediators, including TNF-α, iNOS, IL-6, and IL-1β. Similarly to other rodent ALI models, GW4869 treatment reduces lung injury and suppresses proinflammatory mediators. Furthermore, the serum EVs can induce M1 polarization in macrophages, and miR-122-5p has been suggested to be the effector molecule in EVs (189).

Also, serum EVs from a *P. aeruginosa*-induced pneumonia and sepsis mouse model induce lung injury and kidney injury when injected into naïve mice, suggesting the released EVs can mediate inflammation (190). It should be noted, however, that the circulation in these mice may contain EVs released by *P. aeruginosa*, which could contribute to the observed lung and kidney injury in recipient mice. Finally, exposure to $PM_{2.5}$ induces higher numbers of macrophage-derived EVs in BALF in mice (149).

Although some studies have suggested that the levels of residual LPS, endotoxins, bacterial EVs, or bacteria in isolated EVs are low (143, 174, 187), residual bacterial components can be coisolated with EVs. They may, therefore, be at least partly responsible for some of the effects attributed to tissue or blood EVs in this section.

2.5.6. Summary of pathological functions of EVs in lung injury.

Multiple studies have reported on the intricate contribution of EVs precipitating lung injury. Still, the results are based on diverse test systems and multiple disease models, which may explain the diverse findings reported in this review (**FIGURE 9**). Mostly, the recipient cells studied are macrophages and airway epithelial cells but, in some reports, also endothelial cells. Macrophages were reported to respond to EVs by differentiating into a proinflammatory M1 phenotype. Furthermore, several of the referred studies have suggested that the EVs themselves can convey lung injury when administered to the lungs in rodents. This may suggest that they can be directly detrimental to health by causing tissue injury and inflammation, depending on the status of the producing cell.

2.6. Overall Conclusions of Pathological Functions of EVs in Respiratory Diseases

The mechanistic involvement of EVs in the pathogenesis of respiratory diseases has been investigated both in vitro and in vivo in multiple studies (**FIGURES 5–9**). Overall, these results strongly suggest that EVs can contribute to the pathogenesis of several respiratory diseases. However, we highlight some general limiting factors in the published literature that can influence the conclusiveness of published results. These concerns might not necessarily be just limited to EV-specific methodology but also to certain cellular or in vivo models.

When evaluating EV biology in respiratory disease, it is well accepted that many of the in vivo and in vitro models fail to recapitulate the chronic and progressive nature in patients. For example, adding proinflammatory cytokines, allergens, LPS, or tobacco smoke extract to a single type of cell in vitro may only identify a limited portion of pathways in disease-associated pathophysiology. Further, in mechanistic studies of EVs, it is crucial to determine dose relationships, as we are not yet aware of the concentrations of these EVs present within tissues or how they diffuse through tissues to traffic between cells.

Additionally, lung injury can have diverse causes, either direct or indirect, and by infectious or noninfectious agents. Further, pulmonary fibrosis pathogenesis is largely unknown, and asthma and COPD are widely heterogeneous diseases, which, of course, adds challenges when creating models to study EVs in these diseases. CF, despite its hurdles, is probably one of the more homogeneous respiratory diseases. Although it has the fewest scientific reports, following future progress in appropriate models will be interesting.

Another consideration for the future is to understand the role of EVs in the pathophysiology of respiratory disease, which could include coculture of epithelial, immune, and structural cells instead of monoculture of one cell to produce EVs for further experimentation. Also, cocultures may better represent the microenvironment in the tissues. Further, considering the use of more appropriate controls in EV studies in the future will allow for firmer conclusions to be drawn.

The molecule GW4869 has been reported to attenuate EV release from many cells and has been used as a tool to conclude whether EVs are involved in certain biological observations. However, it is unclear whether this molecule only influences the release of certain

subpopulations of EVs or whether it is a general EV-release inhibitor. While data emerging from the use of this molecule can provide some information, the provided data cannot fully and conclusively determine the role of EVs in the reported results.

It is encouraging that many studies have identified which molecules in the EVs may be responsible for the biological effects in a recipient cell. However, it should be acknowledged that EVs deliver far more than single molecules to a recipient cell, and intravesicular proteins, RNA species, membrane proteins, or other molecules present in the EVs may together mediate the observed biological effects. Thus, more than one molecule is likely contributing to an observed biological response in a recipient cell or animal.

3. EV-ASSOCIATED BIOMARKER CANDIDATES IN RESPIRATORY DISEASES

It has, over the last decade, become evident that diseased cells release EVs with different molecular cargo than healthy cells, which has encouraged a vast amount of biomarker discovery studies in multiple disease areas, including respiratory diseases. During pathological conditions, cells will express different proteins and RNAs than cells during homeostasis, a process associated with the disease's pathophysiology. These changes are mirrored in the molecular cargo of EVs released from the diseased cells, which can provide an opportunity to identify EV-associated disease biomarker candidates. We have identified over 100 articles showing changes in EV cargo in respiratory diseases, and these are sometimes claimed to be potential biomarker candidates. These candidates have been identified in both asthma in humans and heaves in horses. Still, we have refrained from discussing biomarker candidates based on experiments in small animal or cell models of any respiratory diseases, as these are far from being validated in patients. As described in **FIGURE 10**, all but one of the biomarker studies have been published after 2010, with the majority published in the last 5 years (**FIGURE 10*A***). The majority of the discussed studies have focused on asthma or COPD.

Blood has been an easily accessed biofluid for many years for biomarker discovery studies. However, their EVs may primarily evolve from blood components such as red blood cells or platelets. Thus EVs from the lungs or airways, or indeed any organ, will be extensively diluted in plasma or serum, and disease-specific EVs will thus be rare and difficult to identify in this body fluid. Therefore, it may be more appropriate to characterize EVs in local samples such as NLF, BALF, or sputum to identify EV-diagnostic candidates. Of the articles included here, EVs from blood are more commonly used in studies of asthma, COPD, pulmonary fibrosis, and lung injury, while for CF, fluids from the airways are more commonly used (**FIGURE 10*B***). When searching for EV-associated biomarkers in blood, most investigators have studied plasma (**FIGURE 10*C***), while BALF has been the primary source for local biomarker studies (**FIGURE 10*D***).

An important consideration when looking for EV-based biomarker candidates is that every cell produces multiple subtypes of EVs, and the relative concentration of any of these subpopulations may change under disease conditions, as has been implicated in cancer (191). Another hurdle is to identify one or several unique molecules as biomarker candidates in EVs harvested from a complex biofluid that includes EVs from multiple cellular sources and high quantities of non-EV components. Overall, biomarker candidate discoveries for respiratory diseases have utilized samples from NLF, BALF, sputum, exhaled breath condensate, lung tissue, and pulmonary edema fluid as well as blood and urine (**FIGURE 10, *B–D***). Thus, in all biofluids, the disease-associated EVs are mixed with other components, which might influence the sensitivity of any applied analysis. Depending on the EV-associated molecules analyzed, non-EV entities in the biofluid can contaminate downstream analysis to a significant degree. The types of molecules that are often referred to as EV biomarkers include EV proteins or EV miRs, but some investigators have used cruder quantification measurements such as the number of EVs or the total EV proteins. In asthma, pulmonary fibrosis, and lung injury, the most studied EV-associated molecule is RNA, while in COPD and CF, the EV concentration is the most common measurement, which may not be disease specific (**FIGURE 10*E***).

All studies summarized below are divided by type of disease, and we report whether EV-associated RNA, protein, lipids, DNA, or EV concentration have been evaluated.

3.1. Asthma

3.1.1. EV concentration.

Studies describing the numbers of EVs in different biofluids in asthmatics versus healthy controls are conflicting. For example, the concentration of EVs in BALF quantified as particle numbers is increased in human asthmatic individuals, but this could not be confirmed when EV protein was quantified in another study (84, 192). Further, the EVs in BALF are not increased in horses with heaves (193). Wagner et al. (194) reported that the EV concentration is increased in the plasma from allergic versus healthy subjects. At the same time, Torregrosa Paredes and coworkers (84, 194) found that BALF EVs expressing mucin 1 (MUC1) are present in much

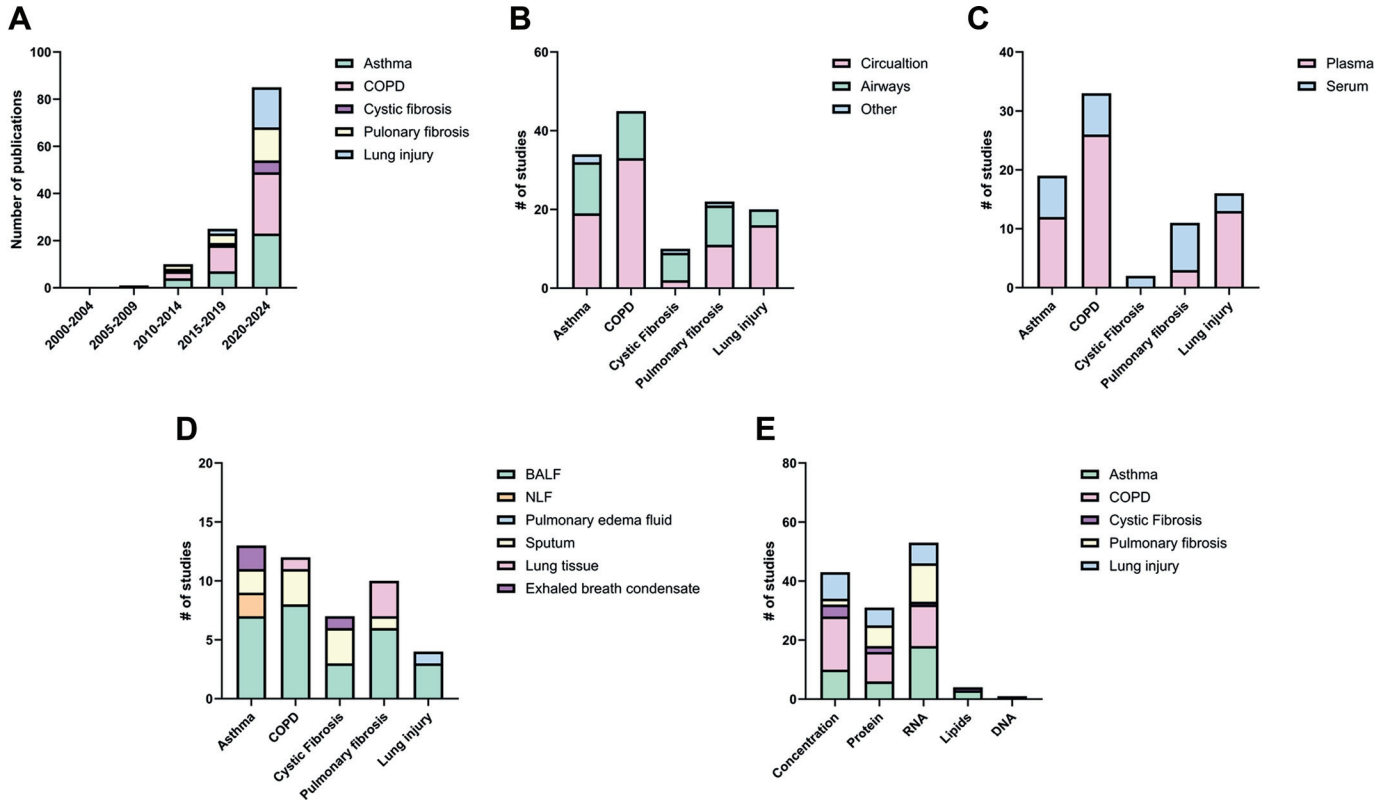


FIGURE 10. Overview of the extracellular vesicle (EV)-associated biomarker candidates in respiratory diseases. *A*: EV-associated biomarker studies per 5-year interval. *B*: EV-associated biomarker candidates per disease, divided based on being identified in blood, body fluids, or tissue from the airways or urine (other). *C*: EV-associated biomarker candidate studies identified in blood divided into plasma and serum. *D*: EV-associated biomarker candidate studies identified in samples from the airways. *E*: EV-associated biomarker molecule. BALF, bronchoalveolar lavage fluid; COPD, chronic obstructive pulmonary disease; NLF, nasal lavage fluid.

lower numbers after allergen provocation. Similarly, the results are conflicting when the EVs are quantified with the help of different membrane proteins. Briefly, BALF EVs expressing CD81, CD63, CD36, HDL-DR, or CD54 have been described as either increased or unchanged in asthmatics versus healthy subjects (81, 84, 192). Nevertheless, the concentration of EVs in BALF was found to be correlated with blood eosinophilia and IgE titers in one study (192). Additionally, $CD9^+$ EVs were found to be increased in the saliva of children with acute asthma exacerbations as compared to children with chronic persistent asthma and healthy controls (195).

Lucchetti and coworkers (196) have acquired EVs from exhaled breath condensate and sputum from patients with asthma or COPD and healthy controls. In that study, differential ultracentrifugation was used to isolate two different types of EVs, one at $17{,}000 \times g$ and one at $120{,}000 \times g$. The $120{,}000 \times g$ EVs were significantly increased in exhaled breath condensate in asthma patients compared to healthy controls and COPD patients. By contrast, the $17{,}000 \times g$ EVs were increased in exhaled breath condensate from patients with COPD compared to healthy controls and asthma patients. No differences were observed in either EV population in sputum between asthma and COPD; for this biofluid, there was no comparison with healthy controls (196).

Although very common for studies of COPD, only a few studies have analyzed the number of blood EVs in relation to asthma. However, one study has suggested that platelet-derived EVs ($CD31^+$ $CD42b^+$) in plasma are increased in asthmatics versus healthy controls (197). Similarly, endothelial-derived EVs ($CD31^+/CD41^-$) have been suggested to increase plasma in asthmatics during periods of high air pollution (85).

Overall, few conclusions can currently be drawn for the concentration of EVs in different biofluids in asthma. This is most likely due to the fact that the studies so far have used different body fluids and different isolation methods but have also chosen to focus on EVs released from different cells. Further, it may be more efficient to use an EV-based molecule as a biomarker rather than an elusive measure such as EV concentration.

3.1.2. EV-associated proteins.

As the molecular cargo is altered in EVs released during inflammation, an attractive approach has been taken to determine the protein cargo of EVs in asthma patients.

Quantitative proteomics of NLF-derived EVs from asthmatics with or without chronic rhinosinusitis (CRS) and healthy controls showed that mucins and serum proteins are enriched in EVs from both subjects with asthma and CRS versus healthy controls, while several presumed antimicrobial proteins and barrier-associated proteins are decreased in NLF-derived EVs from subjects with asthma and CRS. The increased expression of mucin and serum-associated proteins in NLF EVs observed in subjects with airway diseases may reflect an ongoing inflammatory process, while the decreased expression in barrier and antimicrobial proteins could possibly contribute to increased susceptibility to infections, which has important clinical implications for disease progression (83). Additionally, mucin-5B, together with heterogeneous nuclear ribonucleoprotein U (HNRNPU), has also been shown to be enriched in saliva EVs in asthmatic children as compared to healthy children (198). Furthermore, Park et al. (39) observed that the procoagulant molecule "tissue factor" is enriched in BALF EVs from asthmatics versus healthy controls.

The protein cargo of serum- and sputum-derived EVs from patients with eosinophilic or noneosinophilic asthma has been evaluated by proteomics analysis (199, 200). While Yoshimura et al. (200) described in total, five proteins, namely galectin 10 (Gal10), eosinophil peroxidase (EPO), major basic protein 1 (MBP1), eosinophil-derived neurotoxin (EDN), and arachidonate 15-lipoxygenase (ALOX15), to be significantly upregulated in serum EVs from eosinophilic asthmatics compared with noneosinophilic asthmatics and healthy controls. Gil-Martinez et al. (199) instead described four proteins; valosin-containing protein (VCP), EH domain containing protein 1 (EHD1), proteasome subunit beta type-1 (PSMB1), and TSPAN9 as enriched in serum-derived EVs in eosinophilic asthmatics compared with noneosinophilic asthmatics. Additionally, they identified five proteins, copine-1 (CPNE1), FYN-binding protein 1 (FYB1), filaggrin (FLG), cysteine-rich secretory protein 3 (CRISP3), and ras-related protein Rap-2b (RAP2B), as increased in serum-derived EVs in noneosinophilic asthmatics compared to eosinophilic asthmatics. The majority of the proteins in the Yoshimura study correlated with blood eosinophil count, exhaled nitric oxide (FeNO), mucus score, forced expiratory volume in 1 second (FEV1), and wall thickening but not with serum IgE levels (200). Furthermore, Gal10 and EPO were increased in serum EVs from patients with chronic rhinosinusitis with nasal polyps (CRSwNP) as compared with patients with chronic rhinosinusitis without nasal polyps (CRSwoNP). These proteins were also enriched in lung tissue from asthmatics as compared to healthy controls and in nasal tissues from CRSwNP patients as compared to CRSwoNP. Gil-Martinez and coworkers (199) also compared the protein cargo of sputum-derived EVs in the eosinophilic asthmatics and noneosinophilic asthmatic. Interestingly, while they could only identify and quantify 403 proteins in the serum EVs, they could identify and quantify 1,934 proteins in the sputum-derived EVs. This together with the conflicting results between different studies again highlights the difficulties in identifying proteins in EVs in serum and plasma samples. In sputum EVs, 92 proteins were unique to eosinophilic asthma and 113 proteins to noneosinophilic asthma (as compared to 4 and 5, respectively for serum-derived EVs). Interestingly, several of the proteins that the study of Yoshimura et al. (200) found to be upregulated in serum-derived EVs in eosinophilic asthma, such as MBP1, EPO, EDN, and Gal10, the study of Gil-Martinez found (199) upregulated in sputum-derived EVs but not serum-derived EVs in eosinophilic asthma compared to noneosinophilic asthma. Additionally, allergic individuals have significantly different cytokine profiles in their plasma EV isolates. Specifically, they have an increase in TNF-α, IL-4, IL-5, IL-6, IL-17F, CCL2, and CCL17 but a reduction in IL-11, IL-27, and CCL20 (194). Vázquez-Mera and colleagues (201) performed proteomic analysis of EVs isolated from eosinophils, neutrophils, Th1, Th2, and Th17 cells. A proof-of-concept study was designed to verify some of the EV proteins identified to be upregulated in the EVs from the different cell types. EVs were isolated from serum from a small number of T2-high and T2-low asthmatics, and ITGA4 and integrin subunit beta 2 (ITGB2), which had been shown to be upregulated in T2 EVs, were validated to be enriched in serum EVs from T2-high patients compared to T2-low patients.

These findings suggest that both circulating and airway-derived EV protein content may reflect disease phenotype in asthma. However, when analyzing EVs from soluble protein-rich biofluids, care should be employed in interpreting data for nonmembrane proteins, as they may be contaminants and not directly EV-associated. However, when a combination of isolation methods has been used to better separate EVs from non-EV material and soluble proteins, such conclusions are more likely to be correct. The probability for proteins such as TNF-α, tissue factor, ITGA4, and ITGB2 to be truly EV associated is higher because they are integral membrane proteins, whereas many other bioactive molecules are not. Future studies would benefit from using, for example, density gradients and/or SEC to verify that identified proteins are truly EV-associated. Many of the studies reported have utilized different types of isolation kits, differential UCF, or affinity capture, and may, in some instances, coisolate soluble proteins together with the EVs. It should be noted that a recent publication of in vitro cell cultured-derived EVs showed that a combination of density gradient and SEC is effective in separating soluble cytokines from EVs (202).

3.1.3. EV-associated RNAs.

Multiple miRs in EVs have been implicated as biomarkers for asthma, both in EVs isolated from blood or airways (Supplemental Table S2). Currently, the most-studied EV biomarker candidates for asthma are miRs in plasma and serum-derived EVs, with miR-21/miR-21-5p being the most commonly explored. Although this miR is upregulated in blood EVs in asthmatics versus healthy controls, its relationship to disease severity is debated (47, 48, 50). Additionally, EV-associated miR-21 was correlated with FEV1 and with the FEV1/FVC ratio in one study (50). In sharp contrast, a fourth study implied that miR-21-5p is downregulated in sputum EVs in asthmatics with NSAID-exacerbated respiratory disease compared to healthy controls (51), which may suggest differential expression of miR-21 in EVs in the airways versus the circulation. Another miR that has attracted attention is miR-126/miR-126-3p, which in two studies was shown to be increased in serum EVs in asthmatics versus healthy controls and in moderate-to-severe asthma versus mild asthma (47, 203). However, Wang et al. (49) did not detect any differences for miR-21-5p or miR-126-3p in serum EVs from allergic mild asthmatics compared to healthy controls. Importantly, these investigators observed that both miRs were upregulated in serum-derived EVs in asthmatics after a rhinovirus challenge compared to healthy controls (49). Finally, another study did not at all detect miR-126 in plasma EVs of allergic asthmatics or healthy controls (48). MiR-125b has been described to be upregulated in plasma and serum EVs from patients with asthma versus healthy controls, and the expression level of miR-125b is associated with serum levels of C-reactive protein (CRP), IgE, and asthma severity (204, 205). Additionally, while Vázquez-Mera and colleagues (47) showed that miR-146a-5p is significantly upregulated in serum EVs in moderate-to-severe asthma versus mild asthma, Rostami Hir et al. (48) did not see differences in miR-146 in allergic asthmatics versus healthy controls. Another miR with contradicting evidence is miR-122-5p. While one study showed that miR-122-5p is increased in plasma EVs in asthmatics versus healthy controls and that EV miR-122-5p positively correlates with blood eosinophil and neutrophil count (206), another study did not detect any differences for miR-122-5p in serum EVs from allergic mild asthmatics versus controls (49). However, these investigators did describe that rhinovirus exposure downregulates miR-122-5p in allergic mild asthmatics versus healthy controls (49). Additionally, miR-122-5p and miR-191-5p are enriched in sputum-derived EVs from asthmatics versus healthy controls (206). Furthermore, let-7a/let-7a-5p are upregulated in serum EVs in asthmatics after rhinovirus stimulation, in serum EVs in patients with severe asthma versus healthy controls, and in sputum EVs in asthmatics with NSAID-exacerbated respiratory disease versus healthy controls (49–51). Additionally, EV-associated let-7a has been shown to be related to FEV1 and the FEV1/FVC ratio (48). Further, let-7f-5p, let-7b-5p, and let7c-5p are also upregulated in sputum EVs in asthmatics with NSAID-exacerbated respiratory disease versus healthy controls (51). Levänen and coworkers (207) found that the miR content of BALF EVs is also significantly changed in patients with asthma, and even 24 miRs were suggested to be altered in mild intermittent asthmatics compared to healthy controls. In contrast to the other studies, this included the downregulation of several of the let7 miRs as well as miR-21.

Finally, miR-223 has been suggested to be increased in moderate asthma versus controls in plasma EVs and to be further increased in severe asthma versus mild-to-moderate asthma versus healthy controls in serum EVs (48, 50). EV-derived miR-223 has also been shown to correlate with lower FEV1 (50).

Many additional miRs have been detected in blood-derived EVs in single studies, which obviously suggests that these findings still need further validation. For example, miR-215-5p was found to be upregulated in serum EVs from moderate-to-severe asthma versus mild asthma (47). Furthermore, the expression of miR-215-5p together with miR-21-5p and the anti-inflammatory miR-146a correlate, with blood IgE and IL-6 levels and are also differentially expressed when the patients are further divided into T2 high atopic, T2 high nonatopic, and T2 low asthma and into IL-6 high and IL-6 low asthma phenotypes (47). Additionally, let-7i-5p is increased in plasma EVs in children with asthma versus healthy controls (64) and miR-129-2-3p is enriched in plasma EVs from subjects with asthma versus healthy controls (63).

Multiple studies have also reported on miRs that are downregulated in EVs in asthmatics, including miR-124, miR-130a, and miR-133b in plasma EVs from patients with severe asthma versus healthy controls (204). Additionally, miR-3168 was reported to be reduced in plasma EVs in asthmatics versus healthy controls (65, 206). Further, miR-3168, together with miR191-5p and miR-122-5p, have also been found related to levels of blood eosinophils, neutrophils, and lymphocytes, as well as to lung function ($FEV1_{\%pred}$) (206). Children with mild-to-moderate or severe asthma have less miR-542-5p in their NLF-derived EVs than healthy children. In addition, miR-92b, miR-210, and miR-34a levels significantly correlate with clinical physiological parameters such as FEV1, $FVC_{\%pred}$, and $FEF_{25\text{-}75\%pred}$ (35). Finally, mature miRs are significantly reduced in BALF EVs from severe asthmatics versus healthy controls, while rRNAs are significantly more abundant (208).

While many studies have used polymerase chain reaction (PCR) of perhaps 1–5 miRs, four studies have more broadly sequenced miR from plasma, serum, exhaled

breath condensate, and BALF-derived EVs (49, 208–210). The first study detected no altered miRs in serum EVs in allergic mild asthmatics versus healthy controls at baseline (49). However, after the rhinovirus challenge (RV16), 26 miRs were differentially expressed between asthmatics and healthy controls. Two miR clusters were identified, 1 with 12 upregulated miRs (including the previously mentioned miR-21-5p and miR-126-3p) and 1 with 11 downregulated miRs (including the previously mentioned miR-122-5p) after rhinovirus challenge in asthmatics versus healthy controls (49). Several clinical asthma parameters, including lung function measurements (FVC%, FEV1%, and PEF%), inflammatory biomarkers (FeNo, eosinophil percent, and neutrophil percent), and NLF cytokines [IL-13, IL-17a, IFN-γ, interferon γ-induced protein (IP)-10, and IL-10 in NLF] correlated with these two miR clusters (49). Alhamdan et al. (209) also sequenced small RNAs and showed that 706 and 636 miRs were differentially expressed in plasma EVs from obese and nonobese T2 low asthmatic patients versus healthy controls. When the 2 asthma groups were compared, 679 differentially expressed miRs were identified. Importantly, miRs can be divided into clusters if cotranscribed from physically neighboring genes. Two such miR clusters were enriched in obese T2 low asthmatics compared to healthy controls, namely miR-17-92 and miR-106-363, which are present on chromosomes 13 and X. The expression of the miR-17-92 cluster was also enriched in obese T2 low asthmatics compared to nonobese T2 low asthmatics, and correlated with several clinical parameters such as FeNO, CRP, blood immune cells, and lung function parameters (209). In addition, 90 miRs were significantly lower in BALF EVs from severe asthmatics versus healthy controls, while no miRs were enriched (208). The downregulated miRs include miR-125b-5p and miR-223-3p, which are upregulated in asthmatics' serum and plasma EVs (48, 204, 205). This suggests that these miRs are downregulated in the airways by being shed into the circulation in EVs. Additionally, 11 EV-associated miRs, miR-9-5p, miR-10b-5p, miR-151a-3p, miR-151a-5p, miR-202-5p, miR-202-3p, miR-224-5p, miR-568, miR-581, miR-615-3p, and miR-625-3p, were found to correlate with FEV1, atopy, and percentage of eosinophils and neutrophils in BALF (208). Finally, one study that isolated EVs from exhaled breath condensate and BALF could only detect significant miR differences in asthmatics versus healthy controls when capturing EVs with anti-Clara cell secretory protein (CCSP) and anti-SP-C but not when capturing EVs with anti-CD9, anti-CD63, and anti-CD81 antibodies. CCSP and SP-C are believed to be lung-specific proteins expressed by bronchial Clara cells and alveolar type II cells, respectively (210).

One study analyzed RNAs other than miR and described that the lncRNA $PM_{2.5}$-associated exosomal transcript (PAET) was increased in plasma EVs in children with asthma versus healthy controls (65).

Several of the miRs identified in more than one asthma study, such as miR-21, miR-126, mir-223, miR146a, and let-7a, have been suggested to participate in asthma pathophysiology potentially (211). However, it is clear that at this point, the results for these molecules as EV-associated biomarkers conflict with those of many of the specific miRs. Overall, our understanding of the biology and function of EV-associated miR is complicated and sometimes even conflicting, and such data need to be interpreted with caution.

3.1.4. EV-associated lipids.

EV-associated lipids are overall much less studied compared to RNA or protein cargo. However, Hough et al. (192) identified 10 significantly altered lipids in BALF EVs from asthmatics versus healthy controls, and there was a significant relationship between several of the EV lipids and exposure to second-hand tobacco smoke. Thus sphingomyelin 34:1 was significantly increased in BALF EVs in those asthmatics exposed to second-hand smoke versus healthy controls exposed to second-hand smoke. Surprisingly, no difference was observed between asthmatics not exposed to second-hand smoke versus healthy controls also not exposed to second-hand smoke. However, phosphatidylglycerol 34:2 was significantly downregulated in BALF EVs in asthmatics versus healthy when both groups were not exposed to second-hand smoke (192). A more recent study evaluating metabolomics in plasma EVs reported that the lipids PS[18:1(9Z)/18:2(9Z,12Z)], PC[18:1(9Z)e/2:0], PS[24:1(15Z)/22:2(13Z,16Z)], PE[22:4(7Z,10Z,13Z,16Z)/22:5(4Z,7Z,10Z,13Z,16Z)], and PE[16:0/20:3(8Z,11Z,14Z)] were enriched in asthmatics as compared to healthy controls (212).

Additionally, Höglund and colleagues (213) evaluated fatty acid profiles in BALF EVs in horses with heaves. During the acute phase of inflammation, fatty acids are hydrolyzed from body lipids and converted into proinflammatory lipid mediators, for example, prostaglandins and leukotrienes. Thus BALF EVs from horses with severe heaves had significantly lower proportions of palmitic acid 16:0 but significantly increased proportions of dihomo-γ-linolenic acid 20:3n-6, eicosapentaenoic acid 20:5n-3, and lignoceric acid 24:0 versus horses with mild/moderate heaves and healthy control horses, implying a role of EVs in contributing to lipid mediators in equine heaves. Furthermore, several of these fatty acids were positively or negatively related to the number of macrophages, neutrophils, and mast cells in BALF (213).

Although EV lipids are less studied than RNA, proteins, and EV concentrations, this limited number of published papers suggests that this field may provide novel and interesting, potentially novel insight into EV function in asthma, as well as novel biomarker candidates.

3.1.5. EV-associated DNA.

Only one study has suggested that BALF EVs from asthmatics contain more mitochondrial DNA compared to healthy controls (81), and there is room for additional studies of EV-associated DNA in asthma specifically and respiratory disease in general.

3.1.6. Summary of EV-associated biomarker candidates in asthma.

The multitude of biomarker candidates in asthma span over RNA, proteins, lipids, and even DNA, although the results from different studies sometimes lack full validation and may sometimes be conflicting. Even more importantly, the phenotype/endotype of asthma is not always fully considered, which ideally should be a starting point for any biomarker discovery studies. Diagnosing asthma is performed by physiological parameters combined with clinical history, whereas identifying subgroups of this disease could be further improved by adding biomarkers that distinguish different disease mechanisms. Further, the proposed biomarker candidates should be confirmed in multiple studies before being considered for translation to clinical practice.

3.2. COPD

3.2.1. EV concentration.

COPD and cardiovascular morbidity are closely related (214), and an extensive body of literature on endothelial microparticles/circulating vesicles primarily evaluates endothelial function. However, most of these studies have not isolated EVs from the blood compartment but instead have mainly used direct antibody labeling of plasma and serum EVs followed by flow cytometry analysis. Typically, endothelial and immune cell marker combinations have been analyzed, but different studies have detected different epitopes. Briefly, endothelial EVs have been defined in the different studies as either single or double positive for $CD31^+$, $CD61^+$, $CD144^+$, $CD146^+$, ULEX lectin$^+$, $CD51^+$, $CD54^+$, $CD62E^+$, and annexin V^+ or single positive for one of the above in combination with being negative for one of $CD42^-$, $CD45b^-$, $CD41^-$, or $CD42b^-$. For simplicity, the EVs will be referred to as just endothelial EVs in this text, but all studies and the markers used are summarized in Supplemental Table S3.

Several studies have described that endothelial EVs are enriched in the plasma of COPD patients versus healthy controls, even though different markers have been used to identify them (107, 215–218). However, it is also important to note that in one of the studies, endothelial EVs were increased in current smokers versus nonsmokers (217), suggesting that smoking status and smoking history might affect the number of circulating endothelial EVs. The number of endothelial EVs in plasma has also been described to relate to multiple clinical parameters, including FEV/FVC ratio, FEV1, predicted FEV1%, loss in FEV1 per year, percent emphysema, pulmonary microvascular perfusion, the LAA score (low attenuation area according to lung computer tomography), body mass index (BMI) and diffusion lung capacity for carbon monoxide (DL_{CO}), and DL_{CO}/alveolar volume (DL_{CO}/VA) (107, 215, 216, 218, 219). Additionally, endothelial EVs in sputum correlate with FEV1 (220). However, some studies could not detect any differences in some endothelial EV types, specifically when $CD146^+$ EVs were analyzed (216, 217, 221–224).

Endothelial EV concentrations in blood have been found to correlate with COPD severity [global initiative for chronic obstructive lung disease (GOLD) classification], although with some conflicting results. Three studies found that plasma endothelial EVs are the highest in COPD patients with severe COPD (215, 216, 225, 226), while another study showed that plasma endothelial EVs are upregulated in GOLD 0–2 patients and downregulated in GOLD 3–4 patients versus healthy nonsmokers (227). Therefore, whereas relationships between COPD severity and circulating endothelial EVs may exist, the data from clinical trials are not always conclusive.

Finally, endothelial EVs are increased in COPD patients with exacerbation versus stable COPD patients (216, 218) but are decreased in COPD patients during exercise. In contrast, in healthy controls, no such changes have been observed (228).

Several studies have also evaluated immune and blood cell-derived EVs in COPD patients. Briefly, red blood cell EVs are enriched in plasma from stable COPD patients versus healthy controls, and in an uncontrolled observational patient study, red blood cell EVs in sputum had a positive correlation with dyspnea index and a negative correlation with BMI (218, 220). For platelet EVs, the results are conflicting because they have been found to be both increased and not changed in plasma from COPD patients versus healthy controls (218, 222). However, platelet EVs are correlated with disease severity, FEV1/FVC, and the body-mass index, airflow obstruction, dyspnea, and exercise score (BODE index) (218, 222). Interestingly, granulocyte EVs in sputum and neutrophil EVs in BALF also correlate with the BODE index (220, 221). Two studies collecting EVs from both the airways and the circulation have observed that while EVs from neutrophils, macrophages, epithelial cells, and activated endothelial cells were enriched in BALF from COPD patients versus healthy controls and correlated with FEV%, exercise tolerance, symptoms score, hyperinflation, and lung parenchymal damage,

the EVs in the blood did not correlate with any of the clinical parameters (221, 223).

Similar to endothelial EVs, monocyte EVs in plasma are increased in plasma from stable COPD patients versus healthy controls and further increased in COPD patients with acute exacerbation versus stable COPD patients (218).

Furthermore, monocyte EVs have been suggested to be associated with COPD severity and lung function (218, 225). Macrophage EVs were suggested to be significantly increased in BALF in smokers with COPD versus BALF in smokers without COPD and nonsmokers. Importantly, macrophage EVs are also significantly higher in smokers with COPD versus healthy (224, 229). Furthermore, the number of macrophage-derived EVs has been found to correlate positively with pack years of smoking and negatively with the FEV1% (224).

Also, leukocyte and lymphocyte EVs have been suggested to be enriched in plasma from stable COPD patients versus healthy controls (218). However, another study did not detect any relationship between EVs from leukocytes, neutrophils, or monocytes in blood with clinical markers (221).

Two studies analyzed the commonly used EV markers CD9 and CD81, meaning that the cell of origin of the EVs is unknown, as these are considered common markers of EVs. However, it was found that $CD81^+$ EVs in serum are increased in COPD patients versus healthy controls (230) and that $CD9^+$ EVs are increased in plasma in both stable COPD patients and in COPD patients with acute exacerbation versus healthy controls (231). Furthermore, the concentration of $CD9^+$ EVs correlates with CRP, sTNFR1, and IL-6 levels in plasma (231).

Thus researchers have correlated different types of EVs in blood or airways with multiple clinical parameters, which does not imply causality but might suggest that some EVs change in concentration in parallel with disease progression or severity. Importantly, the findings that multiple types of immune-cell-derived EVs are being increased in COPD may be related to ongoing chronic inflammation.

3.2.2. EV-associated proteins.

Kotsiou et al. (232) found that sputum EV-associated proteins were increased in patients with COPD versus healthy controls. However, measuring only the amount of protein is a blunt measurement, as non-EV content can also contribute to this measure. However, determining the EV protein cargo can provide alternative biomarker opportunities in diseases including COPD. Nine studies have pursued such an analysis, and the first captured plasma EVs on slides coated with 40 different EV surface antibodies and quantified them using anti-CD81, anti-CD63, and anti-CD9 antibodies. This bulk analysis of EVs suggested that CD45, CD28, cytotoxic T-lymphocyte associated protein 4 (CTLA4), TNF-R-II, and CD16 could distinguish COPD patients with acute exacerbation from patients with community-acquired pneumonia (233). The second study took a nontargeted and targeted mass spectrometry approach and suggested that fibulin-1, fibulin-3, tripeptidyl-peptidase 2 (TPP2), and soluble scavenger receptor cysteine-rich domain-containing protein (SRCRL) were significantly upregulated in serum EVs from COPD patients versus healthy controls. Additionally, the concentration of these proteins correlated with lung function (234). Furthermore, Feller and colleagues (235) have demonstrated that serum EVs isolated from COPD patients contain more proinflammatory proteins and cytokines such as Wnt5a, IL-8, IL-1β, IL-6, TNF, and IL-12p70 compared to healthy subjects. However, this study used an unspecific kit to isolate EVs, recently it was shown that cytokines can be separated from the EV preparation by density gradient (202), illustrating the need for validation of results from studies using EV-isolation kits. Additionally, Sundar and coworkers (236) also analyzed the proteome of plasma EVs in COPD patients using a kit for isolation. They could identify a few EV-associated proteins being altered between healthy smokers and COPD patients, but not between healthy nonsmokers and COPD patients. Nieri and coworkers (108) showed that the tissue factor and phosphatidylserine membrane components are increased in plasma EVs from COPD patients during acute exacerbation versus stable COPD patients and healthy controls. Furthermore, tissue factor in plasma EVs is significantly reduced after recovery from acute exacerbations (108). Tao et al. (237) found that F-box/WD repeat-containing protein 1A (BTRC), transcription intermediary factor 1-beta (TRIM28), and CD209 are increased in plasma EVs in COPD patient's versus healthy controls. In contrast, nuclear receptor coactivator 3 (NCOA3) and translocon-associated protein subunit gamma (SSR3) were found to be downregulated.

Two studies have evaluated EVs from the airways. First, Liu et al. (238) suggested that IL-6, IL-8, and MUC1 are increased in sputum and BALF EVs from COPD patients with hyperuricemia, a parameter that has been associated with increased mortality in COPD, compared with COPD patients without hyperuricemia, while antitrypsin is decreased. Secondly, Bartel et al. (239) performed proteomic analysis on BALF EVs and showed that 284 proteins were upregulated, and three proteins were downregulated in COPD patients versus healthy controls. The top three most upregulated proteins were lactotransferrin (LTF), deleted in malignant brain tumors 1 (DMBT1), and apolipoprotein A1 (APOA1). As these all are nonmembrane proteins common in mucosal secretions, they should ideally have to be validated in further

isolated EVs, such as purified by density gradient, which would confirm whether they are genuinely EV-associated or not contaminants.

Interestingly, several studies have reported increased neutrophil elastase on EVs from plasma, sputum, and BALF in COPD patients versus healthy controls and in COPD patients with hyperuricemia versus COPD patients without hyperuricemia (99, 233, 234). Again, this finding may be explained by the prominent neutrophilic inflammatory component present in most cases of COPD.

Importantly, in several of these studies, some of the identified proteins are not integral membrane proteins but are either considered extracellular or intracellular proteins. Therefore, it is still possible that some of these biomarker candidates are present in non-EV protein contaminants, especially when EVs have been isolated from complex body fluids such as plasma. MUC-1, CD209, and tissue factor are examples of integral membrane proteins, suggesting that their detection is linked to isolated membrane-carrying EVs.

3.2.3. EV-associated RNAs.

Although most COPD EV biomarker candidate studies have focused on quantifying endothelial EVs in blood and sometimes BALF, some studies have also analyzed EV miR content. All of the EV-RNA studies in COPD are summarized in Supplemental Table S4. A few specific miRs have been identified in more than one study; for example, miR-221/miR-221-5p have been shown to be enriched in plasma EVs from COPD patients versus healthy controls, which may reflect a compensatory mechanism, as miR-221/miR-221-5p can have anti-inflammatory properties (240, 241). Interestingly, another study found that miR-221-3p in BALF EVs was enriched in smokers without COPD versus smokers with COPD, which, in theory, could be related to the anti-inflammatory effects of this miR in smokers without COPD. However, no difference was observed between smokers with COPD and nonsmokers without COPD (229). Two of the above studies have also shown that miR-320b and miR-22-3p are significantly upregulated in BALF- and plasma-derived EVs from COPD patients versus healthy nonsmokers and smokers (241, 242). The same two studies also found that miR-423-5p and miR-122-5p were altered. However, while miR-423-5p was significantly downregulated in BALF EVs, it was upregulated in plasma EVs from COPD patients versus smokers and nonsmokers (241, 242). miR-122-5p was found to be downregulated in lung tissue-derived EVs from COPD patients versus both nonsmokers and smokers. However, for plasma EVs, miR-122-5p was upregulated in COPD patients versus smokers but lower in COPD patients compared to nonsmokers (241, 242).

Both miR-223-3p and miR-106b-3p have been shown to be altered in COPD patients (243, 244). O'Farrell and coworkers (243) showed that plasma EVs from acute exacerbation COPD patients contain higher levels of miR-92b-3p, miR-223-3p, miR-374a-5p, and miR-106b-3p but lower levels of miR-34a-5p versus stable COPD patients. Furthermore, these miRs are connected to disease severity, pack years, lung function parameters, the inflammatory marker fibrinogen, and COPD assessment test (CAT) scores. The second study identified a total of six miRs, miR-361-5p, miR-27a-3p, miR-223-5p, miR-193a-5p, miR-423-3p, and miR-106b-3p, that are downregulated in plasma EVs from stable COPD patients compared to lung cancer patients. Another miR, miR-543, was shown to be upregulated in COPD patients (244). Thus, while the first study found that miR-223-3p and miR-106b are upregulated in plasma EVs in COPD patients with acute exacerbation, the other study found that they are downregulated in stable COPD patients versus lung cancer patients. MiR-21 levels have also been shown to be upregulated in serum EVs from COPD patients versus healthy controls (98, 245) and to be correlated with FEV1/FVC (%) (98). However, miR-21 was also upregulated in serum EVs in smokers without COPD compared to nonsmokers (98).

A broader picture of EV RNA cargo can be drawn by utilizing RNA sequencing, which, for example, can provide information on miRs as well as mRNAs, tRNAs, lncRNAs, piwi-interacting RNAs (piRNAs), and circular RNAs (circRNAs). Shen et al. (240) identified 39 upregulated and 20 downregulated miRs in plasma EVs in COPD patients using miR sequencing. Of these, five miRs were validated with PCR to be enriched in COPD patients (miR-23a, miR-1, miR-574, miR-152, as well as the already mentioned miR-221), and four miRs were validated to be decreased in COPD patients (miR-3158, miR-7706, miR-6850, and miR-144). The expression levels of miR-23a, miR-221, and miR-574 correlated negatively with FEV1/FVC (240). Sundar et al. (241) showed that plasma EVs from COPD patients contain more miRs and fewer tRNAs and mRNAs compared to nonsmokers and smokers. Furthermore, 45 and 39 miRs were significantly altered in plasma EVs from COPD patients versus nonsmokers and smokers (including the previously mentioned miR-221-5p, miR-320b, miR-22-3p, miR-423-5p, and miR-122-5p), respectively. Additionally, seven tRNAs and three piRNAs were altered in COPD patients compared to smokers and nonsmokers. A third RNA sequencing study found that 1,578 lncRNAs and 3,071 mRNAs were differentially expressed in plasma EVs in stable COPD patients versus healthy controls. While the majority of the lncRNAs were downregulated, the majority of the mRNAs were upregulated (246). Finally, another

study utilizing RNA sequencing identified 135 mRNAs, 132 lncRNAs, and 359 circRNA altered in EVs in COPD patients versus healthy subjects (247). Additionally, Circ_0040929 has been shown to be increased in serum EVs from smokers versus nonsmokers and in COPD patients versus both smokers and nonsmokers (248).

When comparing BALF-derived EVs from COPD patients, miR-100-5p was significantly upregulated compared to smokers (242). Additionally, when the BALF-derived EVs from COPD patients were compared to IPF patients, one miR, miR-375-3p, was shown to be downregulated in COPD patients. In addition, when lung tissue-derived EVs from COPD patients were compared to IPF patients, 67 significantly differentially regulated (31 upregulated; 36 downregulated) miRs were identified (242).

Three muscle-specific miRs, miR-206, miR-133a-5p, and miR-133a-3p, have been shown to be enriched in plasma EVs from GOLD group B versus groups A, C, and D (226).

MiR-1258, a presumed proapoptotic miR, levels have been found to be higher in serum EVs from COPD patients with acute exacerbation versus stable COPD patients and healthy controls, and miR-1258 is also higher in stable COPD patients versus healthy controls. Furthermore, miR-1258 correlates with biomarkers such as neutrophil count, white blood cells, CRP, lactate dehydrogenase, and the ratio between neutrophils and lymphocytes (249). Additionally, one study has shown that miR-191-5p in plasma EVs in COPD patients correlates positively with the inflammation marker IL-6 in plasma and negatively with the functional marker DL_{CO} (250). However, no healthy controls were included in this study, and therefore, the data may not be fully conclusive.

3.2.4. Summary of EV-associated biomarker candidates in COPD.

There is a massive amount of data published about EV-associated miR biomarker candidates in many diseases, including COPD. However, no clear picture has emerged on which miRs will be useful clinically, as larger and more targeted clinical trials are required. Further, for proteins, many of the identified EV proteins are associated with inflammatory cells, including neutrophils. Whether such biomarkers will add value to the arsenal of currently available clinical tests used in COPD is questionable. However, if EV biomarker discovery studies were performed in distinct COPD phenotypes, perhaps in parallel with large clinical trials, there may be additive and useful information being produced that can help teach us about COPD severity and progression.

3.3. Cystic Fibrosis

3.3.1. EV concentration.

CF patients have been found to have significantly higher numbers of EVs in their sputum compared to healthy controls and primary ciliary dyskinesia patients (112, 251). Specifically, $CD11a^+$ (a leukocyte marker), $CD66b^+$ (a granulocyte marker), and $CD63^+$ (a general EV marker) EVs are increased, while no difference is seen for $CD11b^+$ EVs (a monocyte/macrophage marker) (112, 251). These results argue that the EVs reflect the chronic ongoing inflammation in the airways of CF patients. Cruder measurements, such as particle counting without staining for any EV surface proteins, provide conflicting results. Trappe et al. (252) found no difference in either serum or BALF EV numbers in pediatric CF patients versus healthy controls. In contrast, Useckaite et al. (111) observed an increase in BALF EVs in patients with homozygous F508del mutations in the *CTFR* gene compared to non-CF controls. These results suggest that well-controlled CF, with little or no ongoing inflammation, might be associated with "normal" EV concentrations in different biofluids, whereas ongoing inflammation increases the EV numbers. This is supported by a study showing increased EV numbers and changes in EV protein cargo as CF patients age (111). Finally, one study found no difference in the concentration of BALF EVs between children with CF and asthmatic children (116).

3.3.2. EV-associated proteins.

Changes in EV composition have also been reported in nonlung biofluids from CF patients. For example, one study showed that the urine EV protein cargo is different in CF patients not treated with CFTR-modulating drugs compared to healthy controls. Specifically, 118 proteins involved in oxidative stress control, phagosome maturation, and endosomal sorting complex were downregulated, while 17 proteins, including epidermal growth factor receptor (EGFR) and transglutaminase 1/3 (TGM1/3), were upregulated. However, this study did not identify differences in urine EV proteins in untreated versus treated CF patients (253). Another study attempted to compare protein cargo in serum EVs, but they identified primarily non-EV-associated serum proteins, which makes their results less conclusive (252).

3.3.3. EV-associated RNAs.

Stachowiak et al. (254) using next-generation sequencing observed no differences in EV-associated miRs in sputum, serum, or exhaled breath condensate between stable pediatric CF patients and patients during exacerbations.

However, when focusing on a limited number of miRs, a PCR analysis of the presumed anti-inflammatory miR-16-5p was upregulated in EVs in exhaled breath condensates but not in sputum or serum EVs. This again illustrates the difficulties in identifying miR biomarker candidates in different disease states.

3.3.4. EV-associated lipids.

Only one study has attempted to analyze lipids in EVs in CF so far. In this study, the total lipid concentration was twice as high in BALF EVs from pediatric CF patients compared to pediatric asthma patients. Specifically, the lipid classes phosphatidylcholines, phosphatidylethanolamine, and phosphatidylinositol were significantly enriched in EVs from the CF patients, while phosphatidylserine was enriched in EVs from the asthma group. A total of 62 of the 328 lipid species analyzed were statistically different between the 2 groups, and of these 42, were significantly higher in the CF group (116).

3.3.5. Summary of EV-associated biomarker candidates in CF.

A very limited number of studies have aimed at identifying EV biomarkers in CF. Importantly, similar to COPD, the protein EV biomarker candidates are seemingly associated with inflammatory processes, including neutrophilic inflammation prominent in this disease. The miR and lipid biomarker candidates in CF will require extensive additional experimental validation, and the presented data should be considered preliminary.

3.4. Pulmonary Fibrosis

3.4.1. EV concentration.

Only two studies have evaluated the EV numbers in pulmonary fibrosis. Martin-Medina and coworkers (135) have presented data indicating that IPF patients have significantly more EVs in their BALF versus patients without interstitial lung disease (ILD), and Adduri and coworkers (255) have suggested that IPF patients have more EVs in plasma versus healthy subjects. However, EVs were also elevated to a similar degree in plasma in patients with other ILDs, including chronic hypersensitivity pneumonitis (CHP) and nonspecific interstitial pneumonia (NSIP), and this observation may thus be similar to previous findings.

3.4.2. EV-associated proteins.

Several studies have evaluated proteins in EVs isolated from pulmonary fibrosis patients. The first study used a bead-based flow cytometry technique and found that CD19, CD69, CD8, CD86, CD133/1, CD209, MCSP, and ROR1 were significantly increased in serum EVs in IPF patients compared to healthy controls. Only CD42a (a platelet-associated molecule) was lower in IPF patients (256). Tomoto and coworkers (257) performed mass spectrometry analysis of serum EVs from IPF patients, and they constructed a Bayesian network based on medical records, blood tests, and protein analysis. This study suggested that networks involving TGF-β, fibrosis, complement, tubulin, and serological markers were relatively IPF-specific (257). Adduri et al. (255) demonstrated with proteomics and ELISA that high mobility group box protein 1 (HMGB1), surfactant protein B (SFTPB), aldolase A (ALDOA), calmodulin-like 5 (CALML5), and talin-1 (TLN1) are enriched in plasma EVs in IPF patients as compared to other ILDs patients that had CHP and NSIP instead of IPF. Among ILDs, IPF shows a progressive phenotype. However, some of the non-IPF-ILDs may also show a progressive pulmonary fibrosis (PPF) phenotype similar to IPF. Enomoto and coworkers (258) set out to identify EV-associated PPF biomarkers to predict non-IPF-ILD progression. They showed that pulmonary surfactant-associated protein B (SFTPB) was enriched in serum EVs from ILD patients compared to healthy controls. Still, notably, SFTPB was significantly increased in PPF patients versus non-PPF patients and predicted ILD progression better than known biomarkers such as serum Krebs von den Lungen-6 (KL-6) and SP-D (258). However, proteomics analysis of blood EVs is often complicated to interpret because of significant non-EV protein contaminations in the isolates, including lipoproteins, albumin, and other abundant serum proteins.

By focusing on EVs locally in the lung, Novelli et al. (259) showed that the EVs containing the procoagulant tissue factor (TF) are increased in BALF from pulmonary fibrosis patients compared to patients with other diseases. When the PF patients were further divided into IPF and non-IPF groups, TF-containing EVs were more enriched in IPF patients than non-IPF patients. Furthermore, the TF-containing EVs were related to lung function (259). Martin-Medina et al. (135) showed that WNT5A, a protein upregulated in IPF fibroblasts, is increased in BALF EVs from IPF patients versus non-IPF patients. Finally, Burgy et al. (137) demonstrated that $CD63^{+}$ $SFRP1^{+}$ EVs were enriched in BALF from IPF patients compared to healthy controls. This is particularly interesting as the authors demonstrated the profibrotic effects of $SFRP1^{+}$ EVs in an IPF mouse model.

3.4.3. EV-associated RNAs.

Several studies have analyzed RNA in EVs from blood, BALF, sputum, and urine in IPF and silicosis patients

(Supplemental Table S5), and three miRs have been consistently shown to be changed in multiple studies. Specifically, the antifibrotic miR let-7d is downregulated in EVs from serum, BALF, urine, and sputum in IPF patients versus healthy controls (260–263), which may also reflect a pathogenetic mechanism. Additionally, let-7d-5p in sputum EVs positively correlates with lung function (262). Let-7d is expressed in epithelial cells in normal lungs, but it is downregulated in lung tissue from IPF patients. The downregulation of let-7d induces epithelial-to-mesenchymal transition and can activate profibrotic processes such as increased collagen production from epithelial cells (264). Thus let-7d in EVs mirrors its downregulation in lung tissue in IPF, and reduced levels of let-7d might be an appropriate indicator for the profibrotic effects. Another EV-miR analyzed in more than one study is anti-inflammatory miR-21-5p, which is upregulated in serum and BALF EVs from IPF patients compared to healthy controls (261, 265). However, this miR was found to be downregulated in BALF EVs from IPF patients versus non-IPF patients (266), which clouds any firm interpretation. This study also found that BALF EV miR-92a is increased in IPF patients versus non-IPF patients. Additionally, miR-92a was more expressed in nonsmoking IPF patients compared to smoking IPF patients (266). Silicosis is caused by inhaling silica particles, leading to pulmonary fibrosis. Two studies of silicosis patients from a research group in China suggest that miR-125a-5p is upregulated in serum EVs from patients with silicosis (128, 129) and is related to disease severity (128).

Some individual studies have indicated that several other miRs may be up- or downregulated in body fluid-derived EVs in IPF and silicosis patients. In serum EVs, the antifibrotic miR-16 was found to be downregulated in IPF patients compared to healthy controls (260). In BALF EVs a total of 69 miRs were shown to be significantly altered in IPF patients compared to healthy controls. Of these 69 miRs, miR-374b-5p, miR-128-3p, miR-125b-5p, miR-100-5p, miR-140-3p, and miR-21-5p were increased by more than 2-fold, and miR-103-3p, miR-30a-5p, miR-27b-3p, and Let-7d were downregulated by more than 2-fold in IPF patients (261). Several of these miRs are associated with fibrosis or with fibroblast functions. Furthermore, when comparing BALF-derived EVs from IPF patients with nonsmokers, four miRs were found to be upregulated (miR-320b, miR-22-3p, miR-320a-3p, and miR-24-3p), and five were found to be downregulated (miR-200a-3p, miR-141-3p, miR-375-3p, miR-200b-3p, and miR-423-5p). Additionally, when the BALF-derived EVs from IPF patients were compared to those from COPD patients, one miR that has broad regulatory functions, miR-375-3p, was shown to be increased in IPF patients (242). This study also evaluated lung tissue-derived EVs and found that 55 miRs were altered in IPF patients compared to nonsmokers (26 upregulated and 29 downregulated). Additionally, when the lung tissue-derived EVs from IPF patients were compared to those from COPD patients, 67 significantly differentially regulated miRs were identified (31 downregulated and 36 upregulated) (242). Hayek and colleagues (133) analyzed the miR cargo of lung tissue-derived EVs and found that miR-143-5p and miR-342-5p were increased, while miR-155-5p, miR-449b-5p, and miR-10a-5p were decreased in IPF patients versus healthy controls. Additionally, this study also evaluated these miRs in plasma EVs from the same patients and found that all five of them as well as miR-142-5p were increased in IPF patients (133). The discrepancy in EVs between airways and blood is not unique to IPF, and this has also been observed in studies of immune cell-derived EVs in COPD (221, 223). Thus EVs in blood do not always mirror disease progression, and this might be explained by the blood EVs having totally different cellular origins than lung EVs.

In sputum EVs, seven miRs were reported to be upregulated and 14 miRs were described as downregulated in patients with IPF compared to healthy controls (262). The levels of mir-142-3p and the previously mentioned Let-7d-5p are related to lung function in IPF. In serum EVs, miR-107-3p, miR-122-5p, miR-126-5p, and miR-335-5p are increased in silicosis patients versus healthy controls (129). Finally, in urine EVs, miR-29a-5p, miR-181b, and the previously mentioned Let-7d were downregulated in IPF patients compared to healthy controls, while miR-199a-3p was upregulated (263).

Two studies have analyzed circRNAs in IPF and silicosis patients. Gan et al. (267) showed that circ_0044226, circ-0004099, and circ_0008898 are increased in plasma EVs from IPF patients compared to healthy controls. Additionally, circ_0044226 has been shown to have an inverse correlation with FEV1 and FVC and is increased in IPF patients with acute exacerbation compared to stable IPF patients (267). Zhang et al. (268) suggested that circRNA11:120406118|12040782 is upregulated in serum EVs in silicosis patients versus healthy controls. However, despite these findings, the biological function of circRNA in EVs remains to be fully elucidated.

3.4.4. Summary of EV-associated biomarker candidates in pulmonary fibrosis.

The molecules that are up or downregulated in different pulmonary fibrosis diseases include a plethora of proteins and RNA species, many of which may participate in different ways in disease processes. Some of these molecules are potential biomarker candidates, whereas others may mirror disease processes. Specifically, each miR can have broad functions and regulate many

cellular pathways. Further, any specific miRs may be up or downregulated in multiple diseases, and any changes observed in fibrotic disease may not be disease specific. Therefore, identifying and validating a specific biomarker candidate for pulmonary fibrosis in general, or any pulmonary fibrosis subgroups, remain challenges.

3.5. Lung Injury

3.5.1. EV concentration.

Most studies have suggested that EVs are increased in numerous different biofluids in ALI/ARDS patients. Specifically, ARDS patients have increased numbers of EVs in pulmonary edema fluid compared to controls with hydrostatic pulmonary edema (269), and they have more $VCAM1^+$ endothelial EVs in plasma versus non-ARDS patients and versus healthy controls (163). Gong et al. (183) showed that the number of crude EVs was increased in plasma in sepsis patients with ALI versus healthy controls. Furthermore, angiotensin-converting enzyme $(ACE)^+$ EVs were significantly increased in the serum of septic patients who subsequently developed ARDS versus those who did not. This suggests that increased EVs released from pulmonary microvascular endothelial cells expressing ACE might be an early sign of ARDS development (171). Endothelial EVs are also increased in plasma from patients who have undergone cardiac surgery and subsequently developed ARDS compared to those who did not (162). Additionally, endothelial EVs are enriched in plasma in ARDS patients with sepsis compared to healthy controls and patients with sepsis but without ARDS. Furthermore, the concentration of these EVs correlated with the proportion of "reverse transepithelial migrating neutrophils in blood"; thus neutrophils are considered to have returned to circulation from the diseased lung (164). In BALF, autophagosome marker-expressing EVs (specifically $LC3B^+$ EVs) are elevated in ARDS patients versus non-ARDS controls. LC3B in EVs might reflect ongoing tissue damage in ARDS because LC3B is involved in the degradation of cytoplasmic contents. Also, $CD68^+$ EVs are increased in BALF in ARDS, and this is a macrophage marker and might reflect increased numbers of these cells in the airways of ARDS patients (270). One study has suggested that reduced numbers of microparticles/EVs in plasma are associated with patients who eventually develop ARDS, but this at least partly contradicts other studies (271). Differing results between studies indicate the complexity of quantifying EVs in blood as well as the potential differences in EV concentrations at different stages of ARDS development.

Several studies have also suggested that the number of EVs is associated with survival from ARDS. Thus the total EV concentration in pulmonary edema fluid on the day of admission in ARDS patients is higher in those who later died compared to those who survived (264). In addition, autophagosome marker-positive ($LC3B^+$) EVs in BALF were shown to be enriched on day 3 of admission in nonsurvivors versus survivors (270).

Finally, the number of EVs has also been associated with the cause and the type of ARDS. Briefly, circulating EVs were increased in patients who later developed ARDS due to infectious causes versus noninfectious causes (271). Additionally, ACE^+ EVs were increased in indirect ARDS compared to direct ARDS (171).

An additional study implicated that EVs are increased in plasma in COVID-19 patients compared to healthy controls when protein was measured (168). However, this is a crude measurement, as many non-EV proteins could also be increased in COVID-19 patients, and these could be coisolated with EVs without being truly EV associated.

3.5.2. EV-associated proteins.

EVs pulmonary edema fluid contain more TFs and receptor for advanced glycation end products (RAGE) in ARDS patients versus patients with hydrostatic pulmonary edema, which suggests that procoagulatory processes are mediated by these EVs (269). $CD63^+$ EVs are elevated in plasma from COVID-19 patients compared to healthy controls, and these EVs had increased TF activity and procoagulant properties in the patients who died compared to those who survived (272).

Significantly increased levels of aminopeptidase-N, tenascin-C, and diacylglycerol kinase kappa in plasma and serum EVs have been reported in patients with sepsis-induced ALI versus healthy controls (143, 153, 183). Furthermore, EV-associated tenascin-C is also significantly higher in nonsurvivors versus survivors (143).

The arachidonic acid cascade-associated enzyme secretory phospholipase A_2 ($sPLA_2$-IIA) protein and phospholipase A_2 group IIA (*PLA2G2A*) mRNA are upregulated in BALF EVs from patients with early ARDS versus patients with late ARDS and non-ARDS patients (273). The presence of sPLA2-IIA might be associated with increased inflammatory mediator production in the airway, including prostaglandins and leukotrienes.

3.5.3. EV-associated RNA.

For lung injury, only one miR, miR-122-5p, is upregulated in more than one study, specifically in plasma and serum EVs in patients with ALI/ARDS versus healthy controls

(189, 274). For the rest of the data describing changes in lung-injury-associated EV-miRs, observations have been made only in single studies (Supplemental Table S6). One study using small RNA sequencing suggested that Let-7d-3p, miR-24-3p, miR-130-3p, miR-98-3p, miR-221-3p, miR-193a-5p, and miR-1273b are upregulated in plasma EVs from ARDS patients versus healthy controls, while let-7a-5p is downregulated (274). Another study using a targeted PCR approach suggested that miR-223 is significantly upregulated in plasma EVs in ARDS patients versus healthy controls. Additionally, miR-223 was found enriched in plasma EVs in ARDS patients with an infectious cause compared to ARDS patients with a noninfectious cause. Furthermore, miR-223 was higher in nonsurvivors at day 30 versus survivors (275). Li et al. (185) showed that miR-210 is increased in plasma EVs in sepsis patients with a risk of ALI versus healthy controls. Fang et al. (154) identified a downregulated miR in plasma EVs in ARDS patients as compared to healthy controls, specifically miR-744. Finally, one study showed that one EV-associated miR was upregulated (miR-485-5p) and one miR was downregulated (miR-206) in plasma from patients with acute type A aortic dissection (ATAAD) and ALI (276).

One study evaluating circRNA by microarray found 430 EV-associated circRNAs were upregulated and 199 were downregulated in BALF EVs from ARDS patients caused by infection compared to healthy subjects. circRNA_042882 was validated in a larger cohort to be downregulated in both plasma and BALF EVs in ARDS patients compared to healthy controls, while circRNA_104034 was found to be upregulated in both plasma and BALF EVs. The BALF EVs were $CD86^{+}$, which suggests that macrophages most likely released them (277).

3.5.4. Summary of EV-associated biomarker candidates in lung injury.

The EV biomarker discovery studies in lung injury have identified multiple EV-associated molecules that most likely are participating in the pathophysiological process of the disease and may even be released by inflammatory cells participating in the injury process. These results include immune-cell-associated proteins, as well as miRs that may have broad effects in cells, in both health, lung injury as well as other pathological conditions. Translating such data to validate a proper biomarker candidate specific for lung injury or pulmonary lung injury subgroups remains a major scientific hurdle. That said, some promising EV-biomarker candidates could potentially be predictive of whether a milder lung injury process will shift over to ARDS, for example, ACE-carrying EVs.

3.6. *Overall Conclusions of EV-Associated Biomarker Candidates in Respiratory Disease*

More than 100 studies have explored EVs as biomarker candidates in the discussed respiratory diseases, and the most commonly studied are asthma and COPD followed by pulmonary fibrosis and lung injury (**FIGURE 11** and Supplemental Figure S3). EV concentrations have often been shown to differ in disease states versus healthy states, but miRs are even more commonly analyzed (**FIGURE 10*E***). A concern is that the results are sometimes contradictory among publications, especially those related to EV-RNA. There are likely multiple explanations for these inconsistent results, possibly related to different EV and RNA isolation approaches or RNA analysis platforms. However, it should also be noted that all of these diseases are highly heterogeneous in themselves, with several subgroups of patients presenting different clinical phenotypes, which may reflect different disease mechanisms and/or molecular endotypes (278). It is crucial to be cautious when interpreting data based on plasma- or serum-EV studies because most such analyses have included significant quantities of non-EV material, which can skew any protein, RNA, or particle concentration analysis. Further, serum and plasma have, per se, very different EV contents, especially in terms of the number of platelet-derived EVs (279, 280). Furthermore, as described in Supplemental Tables S2 and S4–S6, many EV-miR studies have used different types of relatively crude kits to isolate EVs and/or EV-associated RNA, which could explain differences in the results between studies. It is known that the technical reproducibility of, for example, RNA microarray profiling is very low for some EV isolation kits (281). Furthermore, different kits use different isolation methods and could potentially isolate different EV subpopulations with different cargo and non-EV contaminants. Studies have also shown that different RNA isolation methods can result in very different RNA yields and RNA patterns in EVs. This suggests that the RNA isolation method influences downstream analyses and can thereby skew data (282). Finally, the correlation between RNA sequencing, microarray, and PCR analyses of miRs can be poor, reducing the conclusiveness of many RNA-focused EV-biomarker candidate studies (283).

In COPD, the most common EV-associated biomarker candidate identification studies have been performed on plasma or serum samples, mainly without any prior EV separation from non-EV material or characterization of the isolates. Thus many of these studies call the studied blood components “microparticles,” and these data may not always represent circulating EVs. Further, there

have been some discrepancies in the endothelial and immune cell-derived EV concentration measurements, for example, in patients with COPD. This might be explained by some investigators using standard flow cytometer equipment to quantify particles in plasma or serum. In contrast, flow cytometers that can identify nano-sized structures have only recently become available. Conventional flow cytometers are generally unable to detect the smallest EVs, and importantly, different flow cytometers may have different lower cutoff detection limits. Most likely, studies using conventional flow cytometers may primarily study larger EVs, whereas identifying and characterizing smaller EVs require more specialized instruments with higher resolution.

Proteomics analyses of EV isolates can potentially be helpful for EV-biomarker candidate identification. However, many body fluids have large amounts of non-EV-associated proteins, which might contaminate the sample and skew the analysis. This is especially important for EVs isolated from blood and EVs isolated by precipitation, which coisolates many non-EV components, such as extracellular proteins. Potentially, this hurdle could be somewhat overcome if the biomarker candidate is an integral membrane protein because these are much more likely to be truly associated with EVs.

Another important observation in the biomarker candidate literature in respiratory disease is that active tobacco smoking can influence both EV numbers and their molecular cargo. This is especially important in studies of COPD, thus emphasizing the importance of appropriate control groups in these studies.

Another observation is that several of the identified EV biomarker candidates, such as miR-21, miR-223, and tissue factor, or immune cell proteins, may simply reflect ongoing disease processes and enrichment of certain cells in the diseased lung. Some potential molecular markers, such as miR-122-5p and tissue factor, have also been implicated in several diseases covered in this review (**FIGURE 11, *A–C***). This has several implications, one being that these specific biomarker candidates might reflect overlapping disease processes, including endothelial injury and inflammation present in multiple diseases. Importantly, any biomarker candidate should be reasonably specific for a specific disease if it is to be used for diagnostic purposes. However, biomarker candidates overlapping multiple diseases could potentially be utilized to monitor disease progression or treatment responses when the diagnosis is undisputed.

Although fewer studies have been conducted about EV-associated biomarkers for CF, pulmonary fibrosis, and lung injury, some emerging data appear interesting. For example, Let-7d is downregulated in EVs from serum, BALF, urine, and sputum from IPF patients versus healthy controls. However, for analytical purposes, it may be easier to clinically interpret a biomarker that is upregulated in a disease, and not reduced, under pathological conditions.

It is also important to emphasize that biomarker discovery in any disease needs to consider the statistical power of differences between cohorts. Furthermore, an identified biomarker candidate must be validated in several cohorts of appropriate sizes to be considered clinically useful. Notably, many of the studies reported in this section are small, and changes in molecules in disease should primarily be considered hypothesis-generating observations.

We do not have any clinical need for EV biomarkers to diagnose diseases such as asthma or COPD, as physiological assessments and measurements such as FEV1 and the FEV1/FVC ratio, together with clinical presentation, are the gold standard diagnostic tools. However, there is a significant need to understand and identify subgroups of asthma and COPD (phenotypes or endotypes; Ref. 278) and their underlying molecular mechanisms. For example, biomarker candidates will likely differ in diverse asthma phenotypes such as allergic asthma, late-onset asthma, and obesity-associated asthma. These differences could potentially also be important in COPD, CF, pulmonary fibrosis, and lung injury. Thus we propose that future mining of EV-biomarker candidates in respiratory diseases should ideally focus on samples from distinct disease phenotypes.

4. THERAPEUTIC EVs IN RESPIRATORY DISEASE MODELS

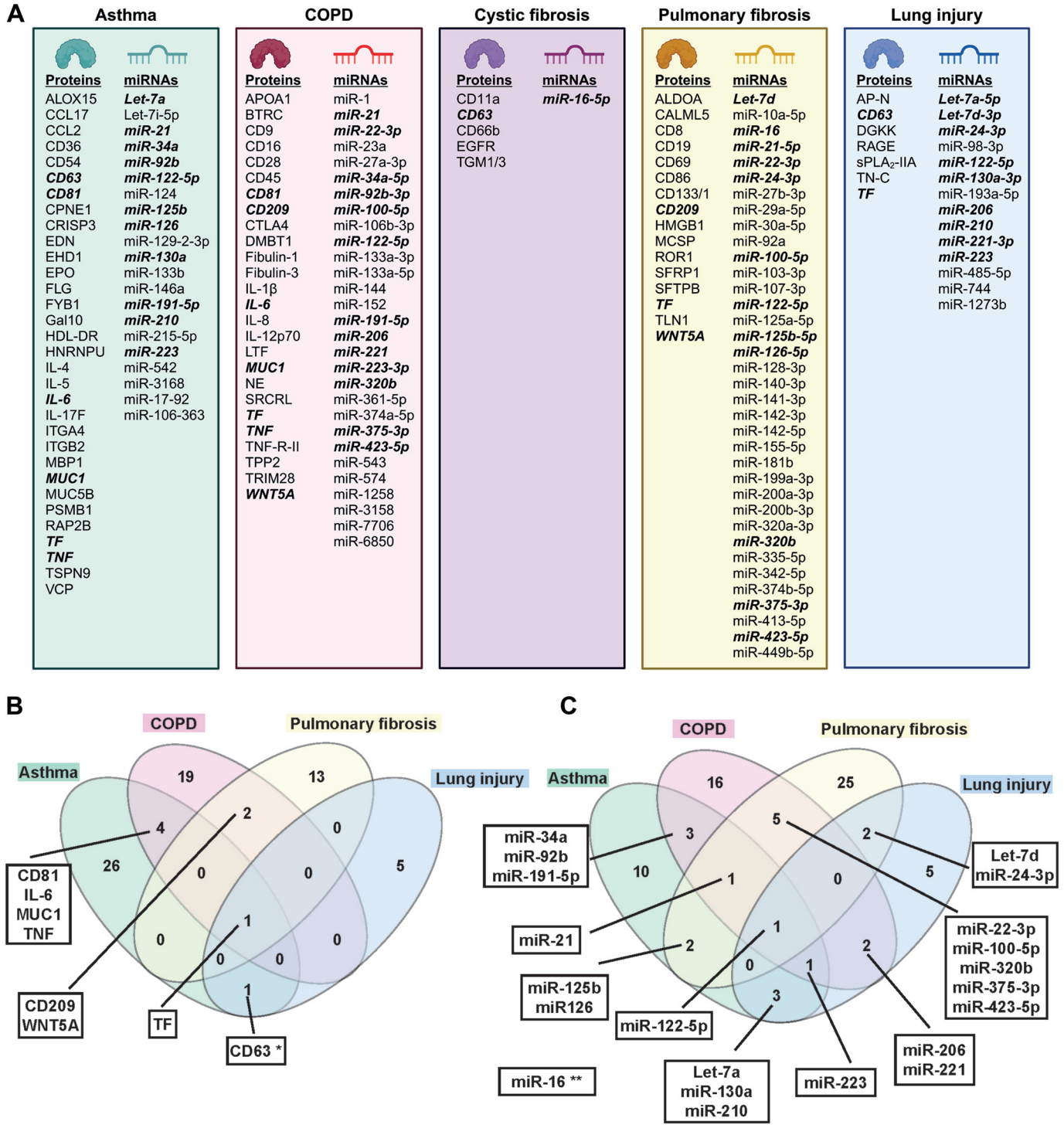
The unique biological and functional properties of EVs suggest that they potentially can be developed as advanced therapeutics in multiple diseases, including inflammatory diseases and cancer. Their ability to induce signaling pathways through membrane interactions and by delivering intravesicular cargo molecules to a recipient cell cytoplasm may allow them to function as advanced biologics with multiple functions in various diseases. It is today well established that EVs released from MSCs have intrinsic anti-inflammatory functions, mimicking the therapeutic potential of the MSCs themselves. Below, we will describe the potential therapeutic effects of EVs derived from *1*) MSC, *2*) other stem cells, *3*) epithelial cells, *4*) endothelial cells, and *5*) immune cells.

Further, EVs can be engineered to carry specific molecules, enhancing their therapeutic potential across a broad range of diseases, also beyond pulmonary disease. Emerging data for lung diseases indicate that engineered anti-inflammatory EVs hold clinically significant therapeutic promise, demonstrating the potential to

mitigate severe inflammation in the lung, thereby improving disease outcomes.

4.1. MSC-Derived EVs

MSCs have shown potent therapeutic benefits in animal models of inflammatory diseases and in clinical trials, also in human disease. The observed therapeutic effects of these cells are considered to be mediated via direct cell-to-cell contact as well as via paracrine functions (19, 284). Indeed, MSCs have recently been approved by the Food and Drug Administration for the treatment of graft versus host disease in children (https://www.fda.gov/drugs/resources-information-approved-drugs/fda-approves-remestemcel-l-rknd-steroid-refractory-acute-graft-versus-host-disease-pediatric). However, for future development, there is a strong trend of shifting focus to utilizing the EVs derived from MSCs as therapeutics, given their equivalent



efficacy as the cells themselves (285), and simplified manufacturing and distribution. MSC-EVs have been shown to have therapeutic activities in experimental models of multiple respiratory diseases, including asthma, COPD, pulmonary fibrosis, and lung injury (20, 286–298). The MSC-EVs administered intravenously also home efficiently to lung tissues during inflammation (299). In this context, we here highlight how MSC-EVs that deliver proteins and RNAs as bioactive therapeutics can convey therapeutic efficacy in different lung diseases (**FIGURE 12**).

4.1.1. Asthma.

MSC-EVs harbor many molecules, including hundreds of integral membrane proteins, thousands of cytosolic proteins, and many RNA species. Noncoding RNAs constitute a significant portion of the transcriptome, and while they do not encode proteins, they play crucial roles in various biological processes related to lung pathophysiology (300). Among noncoding RNAs, the biology and function of miR have been intensely investigated and repeatedly verified in many nonclinical studies. Some recent studies have implied that the therapeutic effects of MSC-EVs in models of asthma may at least partly be mediated by EV-cargo miR. Feng et al. (301) suggested that miR-301a-3p from adipose-derived MSCs was able to regulate airway smooth muscle cells in an OVA-induced asthma model by targeting signal transducer and activator of transcription 3 (STAT3), an intracellular inflammatory cascade molecule. Vesicular miR-221-3p from bone barrow-derived MSCs may be important in alleviating asthma progression by targeting fibroblast growth factor 2 (FGF2) and further downstream signaling pathways (302). In addition, MSC-EVs have been reported to contribute to the reduction of proliferation in bronchial smooth muscle cells by regulating the jumonji and AT-rich interaction domain containing 2 (JARID2)/Wnt/β-catenin axis (303). However, the downregulation of miR-188 blocks the protective potential of MSC-EVs on the bronchial smooth muscle cells. The anti-inflammatory function of miR-146a-5p has been described by Zhou et al. (304) and Fang et al. (305), who showed that MSC-EVs can prevent the differentiation of Th2 cells and can inhibit the function of group 2 innate lymphoid cells and thus may play a role in the pathophysiology of asthma, respectively. Vesicular miR-223-3p can significantly suppress airway remodeling by targeting the NLRP3 inflammasome-related signaling pathway (306). Moreover, miR-1470 from MSC-EVs has been implicated in differentiating regulatory T-cells in asthma patients, maintaining immunological homeostasis (307). Interestingly, the circular noncoding RNA mmu_circ_0001359 is enriched in MSC-EVs and has been suggested to improve airway remodeling by promoting M2-like macrophages (308).

The MSC-EV composition can be altered by exposing the cells to different external stimuli, which sometimes can improve the therapeutic efficacy of the MSC-EVs (309). It has been suggested that activating MSCs with LPS or other inflammatory cytokines may enhance their therapeutic potential per se but can also result in increased release of EVs with increased anti-inflammatory efficacy (310–313). Recently, EVs produced from MSCs under hypoxic or inflammatory conditions have attracted increasing attention in lung diseases. Several studies showed that culturing MSCs in a hypoxic environment produces higher numbers of EVs (314, 315). Further, EVs released under hypoxia are more potent than normal EVs in suppressing chronic allergic airway remodeling in mice, together with reduced expression of profibrogenic markers. Also, airway administration of hypoxic MSC-EVs can significantly reduce cytokine levels in BALF (316) and can significantly attenuate allergen-specific IgE production (317).

Overall, these studies show that MSC-EVs can have a beneficial therapeutic effect in asthma and imply that multiple molecules are involved in that process, including miRs, but potentially other molecules such as cargo or surface proteins. The anti-inflammatory effects observed reach ∼50%, which suggests that MSC-EVs are unlikely to replace well-established asthma treatments such as

FIGURE 11. Summary of the extracellular vesicle (EV)-associated protein and microRNA biomarker candidates in the included studies. *A*: molecules are listed in alphabetical order per disease, and the molecules highlighted in bold and italics are biomarker candidates suggested in more than one disease. *B* and *C*: Venn diagram of proteins (*B*) and microRNAs (*C*) that have been suggested as biomarkers in asthma, chronic obstructive pulmonary disease (COPD), pulmonary fibrosis, and lung injury, respectively. Cystic fibrosis was excluded from the Venn diagrams due to too few molecules identified. *Also a biomarker candidate for cystic fibrosis. **Biomarker candidate in both pulmonary fibrosis and cystic fibrosis. ALDOA, aldolase A; APN, adiponectin; ALOX15, arachidonate 15-lipoxygenase; APOA1, apolipoprotein A1; BTRC, F-box/WD repeat-containing protein 1A; CALML5, calmodulin like 5; CPNE1, copine-1; CRISP3, cysteine-rich secretory protein 3; CTLA4, cytotoxic T-lymphocyte-associated protein 4; DGKK, diacylglycerol kinase kappa; DMBT1, deleted in malignant brain tumors 1; EDN, eosinophil-derived neurotoxin; EGFR, epidermal growth factor receptor; EHD1, EH domain-containing protein 1; EPO, erythropoietin; FLG, filaggrin; FYB1, FYN-binding protein 1; HMGB1, high mobility group box protein 1; HNRNPU, heterogeneous nuclear ribonucleoprotein U; IL, interleukin; ITGA4, integrin subunit alpha 4; ITGB2, integrin subunit beta 2; LTF, lactotransferrin; MCSP, chondroitin sulfate proteoglycan 4; MPB1, eosinophil granule major basic protein 1; MUC1, mucin 1; MUC5B, mucin 5B; NE, neutrophil elastase; PSMB1, proteasome subunit beta type-1; RAGE, receptor for advanced glycation end products; SFRP1, secreted frizzled-related protein 1; SFTPB, surfactant protein B; sPLA2-IIA, secretory phospholipase A2; SRCRL, soluble scavenger receptor cysteine-rich domain-containing protein; TF, tissue factor; TGM, transglutaminase; TLN1, talin-1; TN-C, tenascin-C; TNF, tumor necrosis factor; TPP2, tripeptidyl-peptidase 2; TRIM28, transcription intermediary factor 1-beta; VCP, valosin-containing protein; WNT5A, wnt family member 5A. Image was created by BioRender.com with permission.

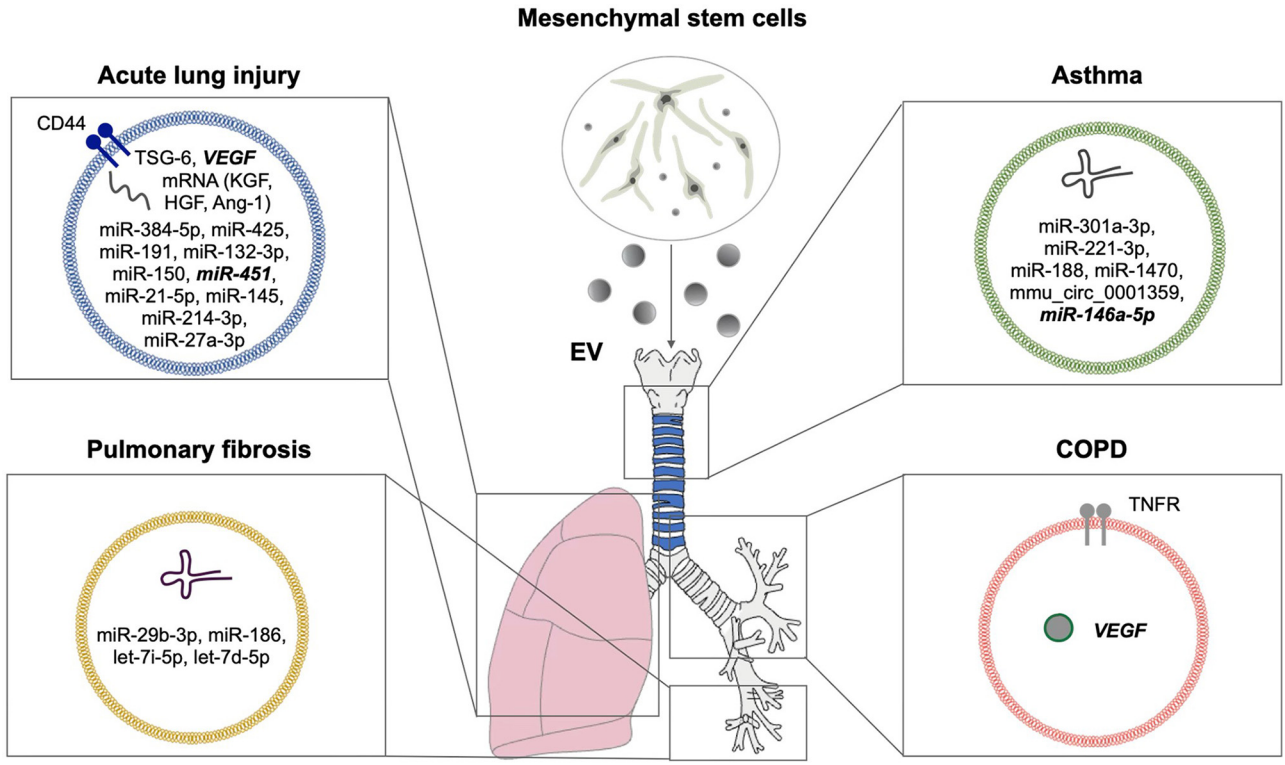


FIGURE 12. The vesicular factors that have been suggested to contribute to the therapeutic activity of mesenchymal stem cell (MSC)-derived extracellular vesicles (EVs) in various lung diseases. Specifically, MSC-derived EVs demonstrate anti-inflammatory, antioxidant, or regenerative effects in conditions such as asthma, chronic obstructive pulmonary disease (COPD), pulmonary fibrosis, and acute lung injury. Bold and italic text indicates some key molecules in MSC EVs that may be important and associated with therapeutic activity. Ang-1, angiopoietin-1; EV, extracellular vesicle; HGF, hepatocyte growth factor; KGF, keratinocyte growth factor; MSC, mesenchymal stem cell; TNFR, tumor necrosis factor receptor; TSG-6, tumor necrosis factor-stimulated gene-6; VEGF, vascular endothelial growth factor. Image was created by BioRender.com with permission.

inhaled glucocorticoids. However, these studies may provide a deeper understanding of how inflammation is regulated and support the development of MSC-EVs in other inflammatory respiratory diseases, as they at least are not detrimental to asthma.

4.1.2. COPD.

MSC-EVs express many common EV-associated proteins such as tumor susceptibility gene 101 (TSG101), Alix, tetraspanins, and even mitochondrial membrane proteins (318). Regarding lung diseases, MSC-EV proteins have been experimentally shown to modulate various inflammatory pathways, such as those active via VEGF, TNF-stimulated gene-6 (TSG-6), and CD44. Previous studies have confirmed the therapeutic effect in COPD models of protein components within MSC-EVs. Chen et al. (319) reported that EVs isolated from human umbilical cord-derived MSCs can protect against papain-induced emphysema by preventing apoptosis through VEGF-mediated pathways. Also, MSC-EVs contain large numbers of immunomodulatory molecules that can attenuate cigarette smoke-induced COPD by inducing soluble TNF receptors and IL-1 receptor antagonists (320). Additional experimental studies should ideally be performed to explore the potential beneficial effects of COPD, as there are still clinical unmet needs in this disease, especially to treat more severe disease and severe exacerbations of COPD.

4.1.3. Pulmonary fibrosis.

Recent evidence suggests that vesicular MSC-associated noncoding RNA can modulate the progression of pulmonary fibrosis. For example, miR let-7i-5p from umbilical cord MSCs can attenuate fibroblast activation in silicosis via regulation of the TGF-beta receptor 1(TGFBR1)/Smad3 signaling pathway (321). Gao et al. (322) have suggested that adipose MSC-EVs have similar effects by transferring let-7d-5p into target cells, resulting in reduced pulmonary fibrosis. Also, MSC-EVs carrying miR-30b play a crucial role in suppressing apoptosis, inflammation, and fibrosis by downregulating sprouty-related EVH1 domain containing 2 (Spred2) transcription (323). The presence of miR-148a-3p and miR-26a-5p in EVs has been shown to significantly decrease collagen synthesis and secretion in silica-induced pulmonary fibrosis (324, 325), and vesicular miR-223-3p, miR-218, and miR-17-5p have been shown to mediate the suppression of lung inflammation and fibrosis

(326–328). It also has been found that miR-29b-3p and miR-186 delivered by bone marrow-derived MSC-EVs can suppress the activation of pulmonary fibroblasts by targeting specific genes, thus suggesting that these are promising therapeutic molecules for the treatment of idiopathic pulmonary fibrosis (329, 330). In theory, MSC-EVs can potentially serve as a treatment for pulmonary fibrosis. However, prolonged treatment may be expensive due to the likely high costs associated with producing MSC-EVs in sufficient quantities. If their potency can be enhanced, MSC-EVs could form the basis for innovative therapeutic approaches to pulmonary fibrosis.

4.1.4. Lung injury.

Most prominently, MSC-EV proteins have been reported to mediate therapeutic effects in treating acute lung injury. When adipose-derived MSC-EVs are injected into LPS-induced acute lung injury mice, they provide significant protection against lung injury, accompanied by restored macrophage mitochondrial function (331). In particular, MSC-EVs can effectively donate mitochondrial proteins, such as mitochondrial membrane proteins, improving macrophage mitochondrial integrity and immune homeostasis. In a similar study, Morrison et al. (332) showed that MSC-EVs transfer mitochondrial proteins and that alveolar macrophages pretreated with these vesicles reduce lung inflammation in LPS-exposed mice. In addition, MSC-EVs have been suggested to attenuate experimental bronchopulmonary dysplasia, partly via EV-associated TSG-6. The recovery was abrogated by a TSG-6-neutralizing antibody, suggesting that TSG-6 in MSC-EVs could play an important role in EV-mediated lung repair (333). The CD44 receptor is known to be involved in the uptake of MSC-EVs into injured monocytes and alveolar epithelial cells (334), and inhibition of CD44 on EVs reduces their trafficking to the alveolus and reduces their anti-inflammatory activities in *P. aeruginosa*-induced pneumonia in mice (335). Like the aforementioned COPD study, the therapeutic role of VEGF in MSC-EVs has also been investigated in lung injury models, and it has been shown that MSC-EVs from VEGF knockdown cells are not able to attenuate the effects of hyperoxic lung injury, including cell death, impaired alveolarization, and proinflammatory cytokine profiles (336). In addition, the serum amyloid A1 and TGF-β pathways can contribute to MSC-EV-mediated protection in sepsis-induced lung injury (337, 338), and Ye et al. (339) have confirmed that MSC-EVs restore lung permeability through the sphingosine-1-phosphate signaling pathway.

Beyond delivering proteins to recipient cells, evidence suggests that MSC-EVs can modulate immune responses by delivering packaged mRNA (340). Zhu et al. (341) confirmed that MSC-EV treatment protects against endotoxin-induced acute lung injury via EV-mediated delivery of keratinocyte growth factor (KGF). In that study, siRNA against KGF was given to MSCs as a pretreatment, resulting in a partial reduction of the therapeutic effect of EVs, suggesting that KGF expression contributes to the underlying therapeutic mechanism of the MSC-EVs. Another group reported that MSC-EVs contain angiopoietin-1 (Ang-1) mRNA, which can be essential in vascular stabilization in LPS-induced acute lung injury (342). Ang-1 mRNA-deficient MSC-EVs increase the infiltration of neutrophils and macrophages in BALF. The role of Ang-1 mRNA has also been investigated in a similar study on lung microvascular endothelial cells (343). Specifically, Ang-1 siRNA pretreatment eliminates EV-induced recovery of vascular permeability among the lung endothelial cells. Several previous studies have demonstrated that MSC-EVs can stabilize endothelial barrier function in acute lung injury, and hepatocyte growth factor gene knockdown reduces the therapeutic effects of EVs in terms of transcellular permeability and endothelial cell apoptosis (344). Also, Wharton's Jelly-derived MSCs (harvested from the umbilical cord) have been shown to release bioactive EVs carrying hepatocyte growth factor mRNA, and the EVs from these cells can ameliorate acute lung injury at least partly mediated by this mRNA (345).

Numerous studies have provided evidence suggesting that noncoding RNAs in MSC-EVs can contribute to their therapeutic effects in acute lung injury (346–349). Recently, several reports have suggested that miR-384-5p, miR-27a-3p, and miR-132-3p alleviate LPS-induced acute lung injury by negatively regulating Beclin-1, NF-κB1, and tumor necrosis factor receptor-associated factor 6 (TRAF6), respectively (350–352). Also, miR-191, miR-223, miR-223-3p, miR-26a-3p, miR-21-5p, miR-148a-3p, miR-335-5p, miR-7704, and miR-125b-5p in MSC-EVs have been proposed to suppress acute lung injury by regulating the status of alveolar macrophages, epithelial cells, and endothelial cells (353–362). EV-mediated miR-150, miR-150-5p, and miR-130b-3p are involved in the attenuation of the same acute lung injury model, perhaps by regulating microvascular endothelial cells and the MAPK pathway (363, 364) as well as the TGF-β pathway (365). Also, miR-22-3p in EVs can reduce apoptosis, inflammation, and oxidative stress (366). Furthermore, MSC-EVs are known to be able to reprogram macrophages and neutrophils in acute lung injury models, potentially through vesicular miR-181a or miR-127-5p (367–369), and these EVs might also modulate epithelial sodium channel expression and oxidative stress through vesicular miR-199a-3p, miR-199a-5p, and miR-34c (370– 372). In another LPS-induced acute lung injury study, miR-377-3p delivered by MSC-EVs could ameliorate severe lung damage by

targeting RPTOR (regulatory associated protein of MTOR complex 1) to induce autophagy (373). In addition, Pei et al. (374) showed that EVs can deliver miR-146a-5p, which contributes to anti-inflammation in sulfur mustard-induced acute lung injury.

Wu et al. have evaluated the therapeutic efficacy of bone marrow-derived MSC-EVs in the treatment of hyperoxia-induced lung injury (375), and their work suggests that MSC-EVs promote miR-425 expression in the lungs and inhibit H_2O_2-induced lung cell injury by targeting the intracellular phosphatase PTEN. A similar study found that adipose-derived MSC-EVs carry miR-21-5p and thus can support the recovery of pathological lung structures and severe inflammation in a mouse model of hyperoxia-induced lung injury (376). Systemic administration of umbilical cord-derived MSC-EVs in burn-induced acute lung injury was shown to have anti-inflammatory effects based on inflammatory cytokine profile changes, possibly through the delivery of miR-451 to recipient cells in several studies (377–379). The therapeutic effect of MSC-EVs has also been assessed in a radiation-induced acute lung injury rat model. The authors proposed that MSC-EVs can reduce pulmonary radiation injury by the transfer of miR-214-3p (380). Another group reported that vesicular miR-466f-3p may have antifibrotic potential and that it suppresses radiation-induced lung injury through the protein kinase B (AKT)/glycogen synthase kinase-3 beta (GSK3β) pathway (381). In addition, MSC-EVs have shown immunosuppressive activity in a bacterial pneumonia model, potentially mediated by vesicular miR-145, thereby resulting in enhanced antimicrobial activity through the LTB_4/BLT_1 signaling pathway (382). Wharton's Jelly-derived MSC-EVs have been shown to release vesicular miR-100, which may enhance autophagy and suppress acute lung injury (383). Interestingly, adipose-derived MSC-EVs carry large numbers of circRNAs such as Circ-Fryl, which can promote autophagy activation through the sirtuin 3 (SIRT3)/AMP-activated protein kinase (AMPK) signaling pathway, resulting in the suppression of sepsis-induced lung injury (384).

Further, MSC-EVs isolated under hypoxic conditions have been tested in a mouse model of acute lung injury, and administration of the stimulated MSC-EVs reduced the infiltration of neutrophils and chemoattractants into the BALF of endotoxin-injured mice, compared to non-hypoxic MSC-EVs (385). For ischemic preconditioning, the MSCs were subjected to hypoxia over different periods, and the ischemic conditions may potentiate the therapeutic activity of the MSC-EVs. Also, there is considerable interest in using MSC-EVs produced under inflammatory conditions. Sui et al. (386) found that LPS can upregulate lncRNA-p21 expression in MSCs in a dose-dependent manner, resulting in the packaging of the RNA molecule into EVs. The EVs carrying this lncRNA were able to reduce pulmonary cell apoptosis and prevent LPS-induced lung injury (386). Another group suggested that EVs from TNF-α, INF-γ, or IL-1β-primed MSCs attenuate sepsis-induced lung injury more effectively than EVs from normal MSCs (387–391). Also, the vesicles can markedly suppress the NF-κB signaling pathway by controlling the miR-199b-5p and Aftiphilin, a protein involved in vesicular trafficking (389). Hwang et al. (392) utilized TLR3- and TLR4-primed MSCs to produce more effective EVs. Furthermore, a specific enriched growth medium can make MSCs produce more EVs with an increased level of neurotrophic and immunomodulatory factors (393). The primed EVs are superior to normal EVs in suppressing LPS-induced physiological lung damage and proinflammatory cytokines. Another group has overexpressed nuclear factor-like 2 proteins and heat shock factor 1 on MSC-EVs and confirmed the enhanced beneficial effect in acute lung injury (394, 395).

4.1.5. Summary MSC-derived EVs in pulmonary disease models.

Overall, there is strong evidence that EVs derived from MSC have anti-inflammatory efficacy in respiratory diseases. The protein and RNA cargos of MSC-EVs exhibit broad immunomodulatory properties, making them potentially beneficial for the treatment of various respiratory conditions. These functional molecules are likely contributors to the disease-modulating effects of MSC-EVs, and possibly an array of EV molecules synergize to mediate the anti-inflammatory effects. Given that multiple MSC-EV components appear to mediate anti-inflammatory effects, more than one molecule likely plays a role in their therapeutic potential. Further dissection of the specific roles of individual MSC-EV components could enhance the development of more effective EV-based therapies for lung diseases in the future.

Further, vesicular miRs and other noncoding RNAs likely facilitate communication between MSC-EVs and recipient cells, potentially influencing the progression of respiratory diseases. However, no evidence has yet supported the therapeutic effects of miRs within MSC-EVs in clinical lung diseases, although such considerations may be evaluated in future studies.

Despite the enhanced therapeutic effects of EVs from prestimulated MSCs, it remains unclear how specific biochemical signals can further potentiate the therapeutic function of MSC-EVs. Some studies have demonstrated significant changes in the anti-inflammatory miR profiles of preconditioned MSC-EVs, suggesting that external stimulation may alter the composition of MSC-EV cargo, thereby enhancing their immunomodulatory activity (312, 396). Thus the detailed dissection of EV composition after priming

MSCs may help explain which molecular mechanisms may be behind the observed enhanced therapeutic effects.

4.2. Other Stem Cells-Derived EVs

Apart from MSC-EVs, other types of stem cells have also been shown to release EVs that may have a therapeutic potential in lung diseases (397). Induced pluripotent stem cells (iPSCs) are derived from somatic cells through reprogramming by several transcription factors, and these cells have shown therapeutic potential in various diseases (398). Additionally, iPSCs can produce EVs containing bioactive cargo, suggesting the therapeutic potential of iPSC-EVs (399). Furthermore, EVs derived from stem cells of a less mature phenotype than MSCs, such as amnionic epithelial cells (AECs) or embryonic stem cells, have recently been recognized as an alternative EV source to treat pulmonary inflammation because they, under some conditions, exhibit comparable properties to MSCs (400).

4.2.1. Pulmonary fibrosis.

EVs can be purified from iPSC conditioned culture medium and may inhibit M2-type macrophages in pulmonary fibrosis in mice (401). Also, the vesicles are enriched in miR-302a-3p, which may help mitigate pulmonary fibrosis. Tan et al. (402) showed that AEC-EV treatment can reduce lung inflammation in bleomycin-induced pulmonary fibrosis through increased macrophage phagocytosis, suppressed T-cell activity, and reduced neutrophil myeloperoxidase activity. In a bleomycin-induced pulmonary fibrosis model, AEC-EVs suppress airway fibrosis, and a significant therapeutic synergism is observed in combination with serelaxin, an antifibrotic agent (403).

Yang et al. (404) generated MSC-like immune and matrix regulatory cells (IMRCs) from embryonic stem cells to improve the homogeneity of cell quality. Treatment with IMRC-EVs was highly effective against bleomycin-induced pulmonary fibrosis. Interestingly, the therapeutic mechanism of EVs is different depending on the administration route. Additionally, menstrual blood-derived endometrial stem cells (MenSCs) have also been tested as a stem cell therapy, and human MenSCs can protect the lung from pulmonary fibrosis by reducing the expression of collagen and antiapoptotic genes (405). Based on the characteristics of these cells, Sun et al. (406, 407) confirmed that MenSCs produce bioactive EVs containing miR-Let-7 and the vesicles can mitigate pulmonary fibrosis through the regulation of mitochondrial DNA damage and reactive oxygen species. Also, EVs derived from antler stem cells, a newly described type of adult stem cell, can inhibit the infiltration of active macrophages in the pulmonary fibrosis model (408).

4.2.2. Lung injury.

Recently, iPSC-EVs have also been proposed to mitigate sepsis-induced acute lung injury by delivering miR-125b-5p (409), thereby attenuating hyperoxia-induced lung injury (410). Ju et al. (411) reported that iPSC-EVs can be taken up by pulmonary microvascular endothelial cells. The group also introduced siRNA into the vesicles to gain selective gene silencing in the endothelial cells, inhibiting ICAM-1 expression in an acute lung injury model. Enhancing the effects of any anti-inflammatory EV, in this case, by loading them with siRNA, may significantly potentiate their potency and efficacy and can help facilitate the clinical translation of EV therapeutics.

AEC-EVs have shown exhibited protective effects in experimental bronchopulmonary dysplasia. AEC-EVs significantly improve the tissue-to-airspace ratio and septal crest density in mice, potentially because they display a high density of surface epitopes CD105 (soluble VEGF receptor), CD326 (epithelial cell marker), CD142 (tissue factor), and CD133 (prominin-1, a stem cell marker) (412). Moreover, these vesicles can reduce hyperresponsiveness and pulmonary hypertension; however, the therapeutic efficacy is not confirmed with EVs isolated from preterm AECs, suggesting the importance of the impact of donor criteria and suggesting that differences in EV molecular cargo can influence efficacy. Furthermore, the anti-inflammatory activity of amniotic fluid-derived EVs has been tested in the same bronchopulmonary dysplasia model. Intratracheal administration of the EVs maintained alveolar development and attenuated pulmonary inflammation by suppressing proinflammatory cytokines and inhibiting macrophage infiltration (413).

4.2.3. Summary of other stem cells-derived EVs in pulmonary disease models.

These findings indicate that EVs derived from stem cells beyond MSCs demonstrate promising therapeutic potential for treating lung diseases. Notably, most studies consistently show reliable therapeutic effects of stem cell-derived EVs in modulating immune responses. However, the exact source and apparent differences in EV molecular cargo can influence efficacy. This highlights the need to explore their potential clinical application across various lung conditions, with care taken on characterizing sources and molecular EV cargo.

4.3. Airway Epithelial Cell-Derived EVs

An increasing body of evidence has revealed a functional role and potentially therapeutic effect of EVs from lung-tissue-derived cells in lung diseases, as described in **FIGURE 13**. Specifically, airway epithelial cell-derived

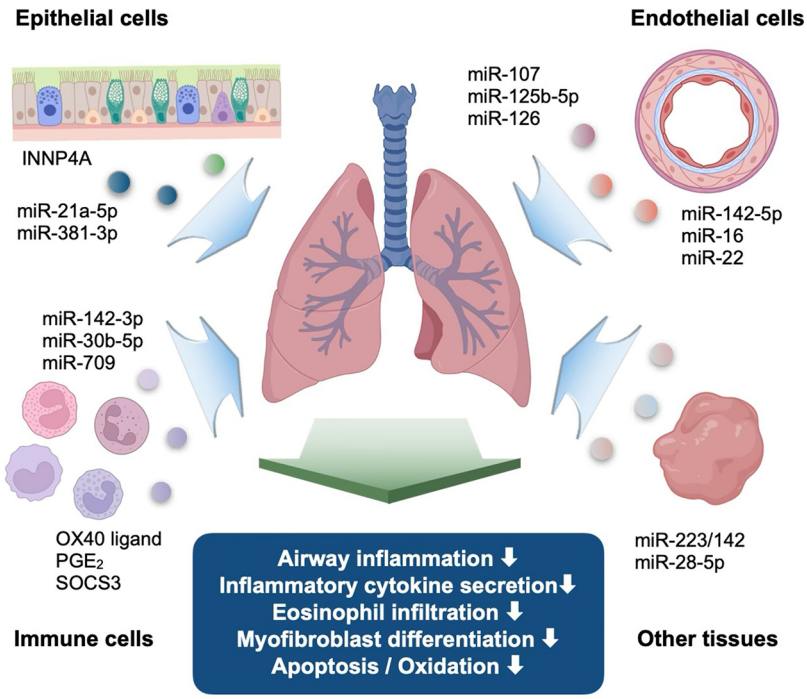


FIGURE 13. Schematic representation of extracellular vesicles (EVs) secreted from various lung cells and other tissues involved in EV-mediated therapeutic effects. EVs can be released from epithelial cells, endothelial cells, different immune cells, and other tissues such as adipose tissues. Some vesicles can reduce airway inflammation by promoting a shift from an inflammatory to a noninflammatory state. Also, EVs are involved in regulating pulmonary fibrosis and oxidative stress, which is crucial in preserving lung health. INNP4A, inositol polyphosphate 4-phosphatase type IA; PGE_2, prostaglandin E2; SOCS3, suppressor of cytokine signaling 3. The image was created by BioRender.com with permission.

EVs have been suggested to play important roles in the regulation of airway biology, including roles in innate defense and homeostasis (414).

4.3.1. Asthma.

In the case of asthma modeling, it has been reported that EVs isolated from stimulated bronchial epithelial cells significantly inhibit the proliferation of bronchial smooth muscle cells (415). Moreover, the same group identified miR-381-3p as the most upregulated miR in the EVs and suggested that this can mediate the suppression of TGF-β3, further indicating the therapeutic potential of epithelial cell-derived EVs for treating asthma. In another study, cultured epithelial cells were found to release EVs carrying enzymatically active inositol polyphosphate 4-phosphatase type IA (INNP4A), which is known to be genetically associated with asthma (416). Their study confirmed that INNP4A can be transferred to fibroblasts via EVs, where it acts as an important regulator of the phosphoinositide 3-kinase (PI3K) pathway, leading to the inhibition of airway inflammation and remodeling. Also, Hu et al. (417) confirmed that OVA-induced $CD4^+$ T-cell imbalance can be controlled by epithelial cell-derived EVs.

4.3.2. COPD.

The therapeutic benefits of epithelial cell-derived EVs have also been documented in the field of COPD. For example, Benedikter et al. (418) reported that exposure to CSE significantly augments the release of EVs from a bronchial epithelial cell line. Still, thiol-antioxidants prevent CSE-induced EV production, implying the regulation of EV secretion. In a similar study, naringenin, a natural flavanone, could induce CSE-treated bronchial epithelial cell lines to release EVs with an anti-inflammatory phenotype (419). Also, these EVs can modulate growth factors, leading to attenuation of elastase-induced emphysema (420). Specifically, the released EVs suppress M1 macrophage polarization by inhibiting CD86 and CD80 expression and subsequent proinflammatory cytokine secretion. In another in vitro study, Neri et al. (421) showed that tiotropium, a muscarinic receptor antagonist in COPD, can regulate the production of proinflammatory EVs from bronchial epithelial cells. Interestingly, another study described EV-mediated communication between injured epithelial cells and MSCs (422). Briefly, EVs from the injured alveolar epithelial cells were able to promote the proliferation and migration of MSCs, implying that the EVs can improve the therapeutic efficacy of MSCs for treating COPD.

The results of using epithelial cell-derived EVs as therapeutics in airway remodeling may not result in a clinical drug. Still, they can at least emphasize how EVs from different cells may regulate homeostasis or pathophysiology within lung tissues.

4.3.3. Cystic fibrosis.

The effects of epithelial cell-derived EVs on CF have also been explored. Koeppen et al. (423) reported that EVs released by airway epithelial cells can regulate macrophage responses. In particular, mutations in CFTR can

attenuate the increased secretion of proinflammatory cytokines in macrophages. Also, the role of EVs from the respiratory epithelium has been investigated concerning viral-bacterial interactions in a CF model (424), and the vesicles were suggested to promote either antiviral or proviral effects during respiratory syncytial virus-associated polymicrobial infections. Moreover, bronchial epithelial cell-derived EVs have been shown to influence the TGF-β-mediated differentiation of myofibroblasts to exhibit even more potent activity than MSC-EVs (425). This attenuation may be mediated by various miRs enriched in EVs, specifically those able to reduce Wnt signaling (Wingless and Int-1). Sarkar et al. (426) showed that EVs from primary human bronchial epithelial cells inhibit *P. aeruginosa* biofilm formation by decreasing the levels of specific essential biofilm proteins in a CF model.

4.3.4. Pulmonary fibrosis.

EV-mediated procoagulant activity from human alveolar epithelial cells can be modulated by Pirfenidone, which is a therapeutic drug for treating pulmonary fibrosis (427). Gu et al. (428) have confirmed that airway basal cell-derived EVs can inhibit pulmonary fibrosis by preventing the epithelial-mesenchymal transition of lung cells. Moreover, EVs from senescent epithelial cells can modulate the interaction between epithelial cells and pulmonary fibroblasts, thus protecting against pulmonary fibrosis (429).

4.3.5. Lung injury.

In an acute lung injury model, EVs can be found in the supernatants of epithelial cells, which are involved in maintaining homeostasis in the lung tissue (430, 431). MiR-21a-5p is upregulated in the EVs and might regulate M2 macrophage polarization. Another group has reported that pneumolysin-treated lung epithelial cells release increased numbers of EVs that can carry mitochondrial content (432). These vesicles can inhibit the ability of neutrophils to release reactive oxygen species, resulting in the attenuation of oxidative bursts.

4.3.6. Summary epithelial cell-derived EVs in pulmonary disease models.

Although airway epithelial cell-derived EVs exhibit therapeutic-like effects in pulmonary models, their clinical translation is unlikely due to the complexity of culturing epithelial cells and the exceptionally high production costs associated with such an EV-based therapeutic. Nevertheless, these experimental models are valuable for advancing our understanding of how epithelial cell-derived EVs regulate airway processes. They also provide insights into the EV cargo and the biological effects they impart on recipient cells.

4.4. Blood or Endothelial Cell-Derived EVs

4.4.1. Asthma.

EVs in the circulation are mostly derived from endothelial cells, erythrocytes, platelets, and blood immune cells (433). In particular, endothelial cells play a key role in modulating vascular inflammation and coagulation, and their EVs have been associated with the regulation of vascular homeostasis (434). A significant amount of EVs is found in the serum of antigen-fed mice. Interestingly, these vesicles prevent allergic sensitization in a mouse model of asthma mice and were referred to as "tolerosomes." The treated animals exhibit significantly reduced numbers of airway eosinophils and reduced OVA-specific IgE levels (435). Matsuda et al. (436) confirmed that EVs from serum in allergen immunotherapy-treated mice inhibit type 2 allergic inflammation by reducing IL-5 secretion.

4.4.2. COPD.

Endothelial cell-derived EVs can improve airway remodeling by inhibiting ferroptosis of lung epithelial cells in COPD (437), and it has been confirmed that the EVs modulate lung inflammation by transferring miR-126 (438).

4.4.3. Pulmonary fibrosis.

EVs released from pulmonary vascular endothelial cells alleviate pericyte-induced pulmonary fibrosis (439). In particular, EV-delivery of miR-107 has been suggested to antagonize the profibrotic phenotypes of pericytes by targeting complex intracellular pathways. In this case, the authors have suggested that pathways affected include hypoxia-inducible factor-1α (HIF-1α)/neurogenic locus notch homolog protein 1 (Notch1)/platelet-derived growth factor receptor-β (PDGFRβ)/yes-associated protein 1 (YAP1)/Twist-related protein 1 (Twist1) signaling.

Further, the therapeutic activity of serum-derived EVs has been investigated in bleomycin-induced pulmonary fibrosis. Kuse et al. (440) found that miR-22 in EVs may modulate fibroblast-to-myofibroblast differentiation through inhibition of the extracellular signal-regulated kinase 1/2 (ERK1/2) signaling pathway. The same group also reported the role of miR-16 from serum-derived EVs (441). The expression of vesicular miRs was analyzed through extensive miR array analysis, indicating that miR-16 is significantly increased in bleomycin-treated mice. Moreover, the vesicle-mediated transfer of miR-16

appears to ameliorate lung fibrosis via the regulation of mTOR and TGF-β1 in recipient fibroblasts.

4.4.4. Lung injury.

A majority of studies evaluating the therapeutic effects of endothelial cell-derived EVs have primarily focused on acute lung injury. In lungs injured by LPS, EVs isolated from pulmonary microvascular endothelial cells ameliorate lung edema and inflammation (442). In particular, the vesicles carry syndecan-1, which can inhibit proinflammatory cytokines, suggesting that they might be potentially therapeutic. Similarly, the activity of endothelial cell-derived EVs has been tested in LPS-induced acute lung injury, and Wu et al. (443) confirmed that the vesicles can restore pulmonary capillary integrity, reduce pulmonary inflammation, and reduce edema seemingly through the transfer of miR-126. The miR delivery by endothelial-derived EVs has also been confirmed in a recent study by Jiang et al. (444), who verified that endothelial cell-derived EVs are able to deliver miR-125b-5p to protect from sepsis-induced acute lung injury via inhibition of DNA topoisomerase II alpha (TOP2A). Also, these EVs play a crucial role in protecting against ventilation-induced lung injury (445).

In acute lung injury in vitro and in vivo models, serum-derived EVs have been studied for their protective roles against lung inflammation (446). For example, EVs can be isolated from the serum of healthy subjects and used to modulate the responses of lung alveolar cells to particle- exposure (447). These EVs inhibit lung cell apoptosis, seemingly through protein kinase B (known as AKT) phosphorylation. In another work by Zhu et al. (448), serum-derived EVs were shown to protect against LPS-induced acute lung injury. Specifically, miR-142-5p encapsulated by the vesicles can reduce pulmonary edema, neutrophil infiltration, and proinflammatory cytokine production through the downregulation of PTEN. Interestingly, EVs isolated from the plasma of recovered COVID-19 patients display high levels of SARS-CoV-2 spike peptides and other immunomodulatory molecules, which may either suggest that SARS-CoV-2 viruses we coisolated with the EVs, or these molecules were indeed expressed on host EVs (449). Also, Yu et al. (450) confirmed that simvastatin can suppress acute lung injury potentially through modulating endothelial EV levels in the blood.

4.4.5. Summary of blood or endothelial cell-derived EVs in pulmonary disease models.

Blood or endothelial cell-derived EVs may have some therapeutic effects in respiratory disease models, mediated by their protein and/or RNA cargo molecules being delivered to recipient cells. To us, it seems unlikely that these EVs will be possible to translate to become clinical drug candidates in humans, in view of the variability in response, the potential procoagulative effects of these EVs, and the complexity in isolating such EVs for treatment purposes. However, these studies shed some light on understanding the potential biological functions of blood or endothelial cell EVs in respiratory diseases.

4.5. Fibroblast and Immune Cell-Derived EVs

Immune cell-derived EVs have garnered growing research interest due to their distinct characteristics and potential for bioinspired applications, especially focusing on macrophages or DCs (451). Mostly, the therapeutic effects of macrophages or DCs have been explored in respiratory disease models.

4.5.1. Asthma.

Macrophages are known to be major sources of EVs in the lungs (42). For example, M2 macrophages have the capacity to release high quantities of EVs, which can attenuate OVA-induced remodeling and inflammation in a mouse asthma model (452). That study also confirmed that the EVs carry miR-370, which can alleviate asthma progression by regulating the FGF1/MAPK/STAT1 axis. In a similar study, Tang et al. (453) treated macrophages with centipede venom (SC) to enhance the release of M2 macrophage-derived EVs by promoting the polarization of the cells. The authors suggested that EV-shuttling of miR-30b-5p participated in the attenuation of the asthma phenotype by preventing airway epithelial cell pyroptosis. The group also sequenced the vesicular miR, hypothesizing that these molecules are involved in the underlying therapeutic effect of SC treatment in asthma. They found that 328 vesicular miRs were differentially expressed in EVs from SC-treated asthmatic mice versus controls, and importantly, miR-147-3p, miR-98-5p, and miR-10a-5p were enriched in Wnt and MAPK inflammatory signaling (454). Moreover, another group reported that resident alveolar macrophage-derived EVs suppress allergic inflammation through transcellular delivery of suppressor of cytokine signaling 3 (SOCS3) (455).

DC-derived EVs are also involved in allergic inflammation through the modulation of T and B cells (456). In an asthma model, thymic stromal lymphopoietin-activated DCs EVs regulate T2 differentiation seemingly via vesicular OX40 ligand (457). Hemin is a molecule that has antioxidant and anti-inflammatory effects, and Wu et al. (458) found that hemin-treated DCs release EVs that suppress allergic airway inflammation. Specifically, mice were treated with EVs in parallel with HDM to induce allergic responses, and then the changes in inflammatory cells

and cytokines were analyzed in the lung and BALF. As a result, hemin-induced EVs were able to reduce eosinophil infiltration and mucus secretion and reduce the levels of IL-4, IL-5, and IL-13, suggesting these EVs convey immune-regulatory functions.

4.5.2. COPD.

Lung fibroblasts are the most abundant cells in the lung interstitial space, and these cells produce the extracellular matrix that provides the structural integrity of the lung and is strongly involved in wound healing activity (459). van der Koog et al. (460) isolated EVs from lung fibroblasts and demonstrated that these EVs can improve elastase-induced emphysema, resulting in reduced lung injury and increased expression of alveolar epithelial cell markers of parenchymal regeneration. Also, fibroblast-derived EVs were found to exert beneficial effects in COPD via the promotion of macrophage polarization (461).

4.5.3. Pulmonary fibrosis.

The role of macrophage-derived EVs has been investigated in clinical samples of patients with pulmonary fibrosis (462, 463). Guiot et al. (463) showed that vesicular miR-142-3p is significantly upregulated in the sputum and plasma of patients, and the source of miR-142-3p is mainly from macrophages, suggesting the therapeutic activity of macrophage-derived EVs through the delivery of anti-fibrotic miRs. Macrophage-derived EVs can also suppress fibroblast activation mediated by vesicular long-noncoding RNAs (464).

Also, EVs from proinflammatory cytokine-activated fibroblasts can inhibit TGF-β-induced myofibroblast differentiation by antifibrotic activity (465). Moreover, these EVs contain high levels of antifibrotic prostaglandin E2 (PGE_2), which may contribute to the biological effects. Ibrahim et al. (466) activated dermal fibroblasts to produce more functional EVs with tissue repair properties. Intratracheal administration of these vesicles showed a favorable safety profile with no significant changes in body or lung weight.

4.5.4. Lung injury.

In LPS-induced acute lung injury, macrophages release more EVs upon LPS stimulation, and the release of vesicles is mostly regulated by epithelial cell-derived IL-25, indicating cross talk between macrophages and epithelial cells via EVs (467). Also, M2 macrophage-derived EVs exert a protective effect in sepsis-induced acute lung injury, and this is suggested to be mediated by miR-709 (468) and/or PGE_2 (469). Other immune cells such as neutrophils and platelets have been confirmed to secrete EVs and contribute to the protection against lung diseases by modulating EV secretion (470, 471). Interestingly, natural killer (NK) cell-derived EVs can improve bacteria-induced acute lung injury by promoting M1 lung macrophage polarization (472).

4.5.5. Summary fibroblast and immune cell-derived EVs in pulmonary disease models.

EVs derived from fibroblasts, macrophages, and DCs can have therapeutic effects in respiratory diseases. The therapeutic benefits are thought to be mediated by the protein and RNA cargo within these EVs. We suggest that neither of these EV types will become clinically translated as medicines. Still, these experiments are furthering the understanding of how a multitude of cells that can release EVs in the lung also regulate disease pathways.

4.6. Other Sources of EVs

In this section, we summarize therapeutic studies involving EVs isolated from various sources, including BALF, adipose tissue, and rare cells such as telocytes. Overall, the volume of publications confirming their therapeutic potential remains limited.

4.6.1. Asthma.

There are high quantities of EVs in BALF, and these vesicles may contribute to lung homeostasis and lung repair (473, 474). It has been shown that EVs from BALF can improve mitochondrial activity, allowing suppressed airway inflammation in a neutrophilic asthma mouse model (475), and it was suggested that miR-223/142 is involved in BAL EV-mediated functionality in lung inflammation (476).

4.6.2. Lung injury.

A telocyte is a unique cell type found in the interstitium of various organs, and these cells have been proposed as an alternative therapy for tissue injury and repair (477), as implanted telocytes can relieve lung inflammation and edema induced by LPS challenge (478). Tang et al. (479) isolated and purified EVs from lung telocytes and showed that these EVs directly can regulate the expression of target genes, similar to what the telocytes do themselves. Furthermore, telocyte-derived EVs partly reduce the severity of lung tissue edema and induce epithelial cell proliferation and differentiation. Also, EVs secreted from telcocytes with elevated integrin levels promote endothelial cell proliferation and suppress apoptosis in LPS-induced inflammation (480).

Xu et al. (473) isolated EVs from the BALF of rats after the induction of phosgene-mediated acute lung injury, and these vesicles had a similar shape and size as normal EVs. Moreover, the isolated EVs can increase the secretion of IL-10, resulting in enhanced immunoregulatory properties of the MSCs. Also, vesicular miR-28-5p was considered a possible contributing factor to lung EV-induced immunomodulation. The same group also confirmed that miR-34c-3p is downregulated in MSCs treated with BALF-derived EVs, suggesting that EVs contribute to the communication between the lung environment and MSCs (481).

Adipose tissue has also been used as a direct source of therapeutic EVs, because of their protective effect on pulmonary endothelial cells in acute respiratory distress syndrome (482). Yu et al. (474) obtained a high yield of vesicles from adipose tissue by performing tangential flow filtration from isolated tissues, and in their in vivo study the generated EVs were able to attenuate lung injury in mice and increase the expression of the adhesion link-protein VE-cadherin, thus showing a protective effect against lung barrier damage. Further, adipose tissue from obese animals was used to isolate EVs, which were subsequently shown to exhibit potent attenuation of acute lung injury (483). Also, extracellular matrix-derived EVs can contribute to lung repair by protecting alveolar epithelial cells from damage (484). Interestingly, Li et al. (485) showed that milk-derived EVs can suppress oxidative lung injury seemingly by delivering circRNAs that target pathological changes. Further, milk-derived EVs have the potential to modulate lung inflammation by changing the phenotype of macrophages (486). Moreover, EVs from cardiopulmonary progenitor cells can prevent acute lung injury by restoring endothelial function (487).

4.6.3. Summary of other sources of EVs in pulmonary disease models.

Vesicles harvested from animals, for example, adipose tissue or BALF, can convey therapeutic functions in some models of respiratory disease. Further, novel cellular sources of EVs such as telocytes can provide EVs with therapeutic function. Overall, it will be important to carefully assess the safety of EVs from any new source, which also may be a hurdle for the clinical translation of these EVs.

4.7. Engineered EVs

Through physical, chemical, or genetic bioengineering methods, EVs can be adapted to include therapeutic substances such as proteins, lipids, and nucleic acids. Given their unique biocompatibility and stability, EVs may be optimal nanocarriers for delivering bioactive components that trigger signal transduction, immunomodulation, or other therapeutic outcomes in recipient cells. Importantly, EVs can potentially also be designed to target specific cell types, which may provide a significant advantage over other nano-drug carriers. Moreover, EVs are nonreplicating, making them safer and easier to transport and distribute than cell-based therapy products. Additionally, EVs can traverse several biological barriers essential for drug delivery function. These capabilities can allow for targeted delivery of therapeutic agents, thus improving their bioavailability and reducing the required doses for therapeutic efficacy without or with reduced systemic side effects. In the following sections, we will review how these unique properties of EVs have been harnessed to treat various lung diseases (**FIGURE 14**).

4.7.1. Asthma.

Exaggerated T2 responses play a critical role in the pathogenesis of allergic asthma (488). To kill antigen-specific T2 cells, Zhang et al. (489) have developed EVs called “CARsomes,” which carry the anti-DEC205 antibody, the scFv, the epitope of Dermatophagoides farinae-1, and the perforin molecule. These CARsomes are 60–100 nm in size and present fusion proteins on their surface. Adding CARsomes to cell culture leads to the apoptosis of antigen-specific T2 cells. Similarly, administration of CARsomes induces antigen-specific T2-cell apoptosis in the lungs of sensitized mice. CARsomes can also promote the generation of antigen-specific regulatory T cells, suggesting that these vesicles potentially can treat allergic asthma. Additionally, the allergen OVA can be loaded into EVs, and sublingual or intranasal treatment with these vesicles results in significant reductions of IgE levels and IL-4 secretion in an asthma model (490–492). Moreover, the intrinsically anti-inflammatory miR-146a-5p has been engineered into EVs to enhance their efficacy in treating allergic airway inflammation (493).

Polymorphonuclear neutrophils can, in some cases, contribute to allergic asthma (494). Zhao et al. (495) engineered EVs specifically to target these neutrophils, suppressing the neutrophilic inflammation in the asthma model through apoptosis. The modified EVs display a complex of anti-CD64 antibody and Fas ligand, allowing the vesicles to target neutrophils expressing CD64. Also, a small RNA-loaded EV approach has been used to mitigate neutrophilic asthma (496), caused by the proinflammatory miR-155-induced extracellular release of double-stranded DNA, leading to severe allergic lung inflammation (497). In this approach, the miR-155 inhibitor is loaded into serum-derived EV using modified calcium-mediated transfection, and the loaded vesicles lead to a reduction in the miR-155 level in the lung and a decrease

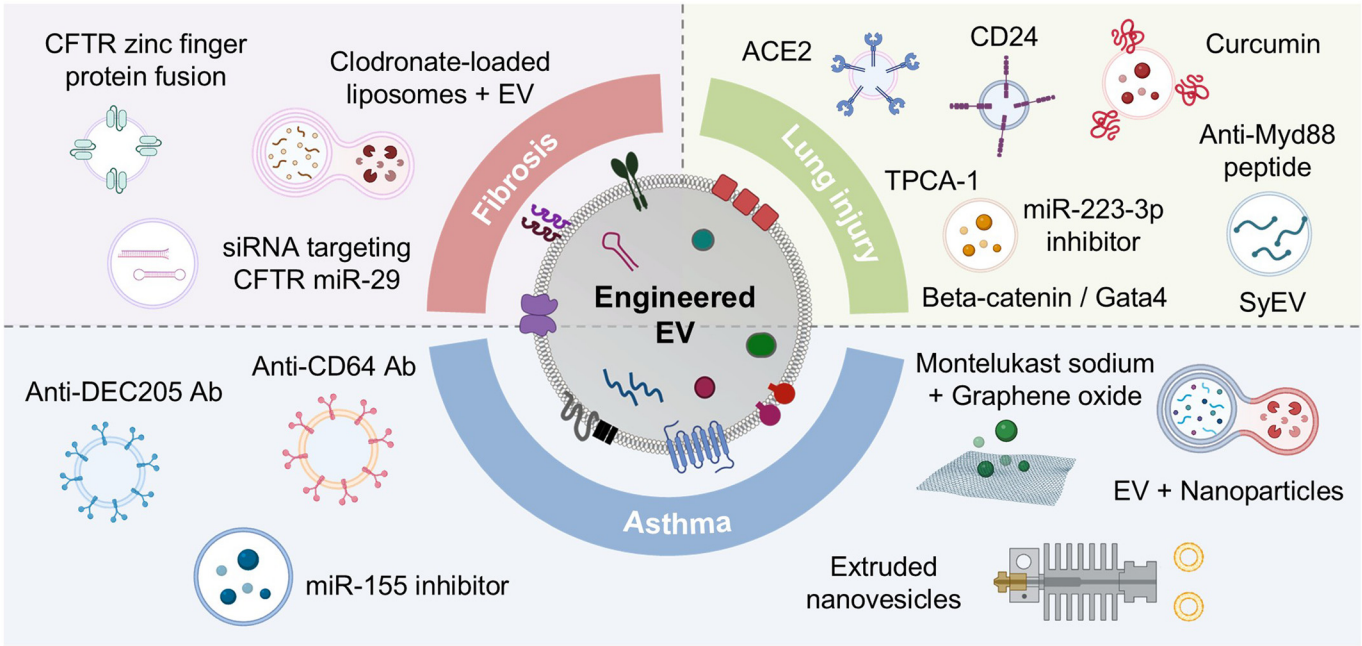


FIGURE 14. Various strategies for engineering extracellular vesicles (EVs) as therapeutics for lung diseases. Genetic modification of EV-producing cells can be employed to overexpress specific proteins or other therapeutic molecules that are then automatically packaged into EVs. Introducing genes that encode targeting proteins on the EV surface can provide enhanced targeting efficiency to specific recipient cells. Moreover, loading therapeutic cargoes into EVs allows for increased therapeutic efficacy, which may be provided by the fusion of EVs with other nanoparticles such as liposomes. Ab, antibody; ACE2, angiotensin-converting enzyme 2; CFTR, cystic fibrosis transmembrane conductance regulator; Gata4, GATA binding protein 4; Myd88, myeloid differentiation primary response 88; SyEV, synthetic eukaryotic vesicle; TPCA-1, [5-(p-fluorophenyl)-2-ureido]thiophene-3-carboxamide. The image was created by BioRender.com with permission.

in neutrophil infiltration into the BALF, all while showing no signs of toxicity.

Interestingly, various other types of non-EV nanoparticles have been utilized in conjunction with EVs. One study indicated combining montelukast sodium with graphene oxide nanomaterials can reduce allergic airway inflammation (498). Additionally, these alternative nanocarriers can facilitate the delivery of coloaded drugs to penetrate and enrich the pulmonary inflammation site, thus enabling effective targeting of the mitochondria for drug delivery. This may enhance the suppression of antiapoptotic proteins, leading to apoptosis of inflammatory cells. Other groups have conducted studies in which they have modified synthetic nanoparticles by incorporating the essential characteristics of EV membranes (499, 500). These modifications aim to create an effective delivery system for different drugs that may not penetrate membranes easily to reach the intracellular space. In this study, investigators developed vesicles by combining EV membranes from M2 macrophages with PLGA nanoparticles encapsulating the DNA methyltransferase 3A silencer (Dnmt3aos). The engineered vesicles were intravenously administered to a mouse asthma model to determine their therapeutic effects. The results showed that these vesicles were distributed in various organs, including the lungs, and remained present for over 48 hours. Moreover, they effectively targeted M2 macrophages, significantly reducing M2 macrophages and inflammatory cytokines in the airway. Notably, these vesicles were able to significantly suppress the overall immune function of the host, suggesting their potential as a safe delivery vehicle for therapeutic applications.

Several research studies have developed EV-mimetic nanovesicles, manufactured from different types of cells by serially extruding them through filters, which creates nanovesicles (501, 502). This approach is utilized to overcome the low yield of naturally occurring EVs harvested from cell cultures. Recently, these nanovesicles have been applied to a mouse model of allergic airway inflammation (503). Bandeira et al. (503) demonstrated that extruded nanovesicles derived from MSCs exhibit comparable morphological and molecular features to naturally released EVs. These nanovesicles have shown therapeutic immunomodulatory properties in a mouse model of airway inflammation induced by OVA sensitization and challenge, with similar efficacy as naturally released MSC-EVs. Notably, these vesicles effectively reduce excessive airway inflammation in mice by significantly decreasing eosinophil infiltration and cytokine production in BALF and lung tissues. This suggests that EV-mimetic nanovesicles hold promise for clinical translation due to their high yields.

Overall, engineered EVs represent a promising advancement in the targeted delivery of therapeutic agents for

asthma. Their unique properties enable innovative engineering strategies for the targeted immunomodulation of specific cell types, paving the way for safer and more effective treatments. To maximize their therapeutic potential, future research should focus on optimizing production methods to enhance yield, targeting efficiency, and comparing engineered EVs with traditional drug delivery systems.

4.7.2. COPD.

Several reports have explored the therapeutic use of MSC-derived EVs for treating COPD, as mentioned in the previous section. However, no studies have yet focused on engineering EVs to enhance their therapeutic potential in this disease. Engineered EVs that carry anti-inflammatory agents or modified surface vesicular proteins could provide a novel approach to COPD treatment, potentially offering targeted therapy with improved efficacy, which may be especially helpful in managing severe COPD exacerbations. Therefore, it is essential to unlock the full potential of EVs by employing multiple specific engineering approaches in the context of COPD.

4.7.3. Cystic fibrosis.

Small molecule treatments designed to correct CFTR genes have demonstrated clinical benefits but are only effective for a small percentage of CF patients with specific CFTR mutations (504). To address this limitation, Villamizar et al. (505) engineered MSCs and HEK293 cells to produce EVs containing a CFTR zinc finger protein fusion with transcriptional activation domains that target the CFTR promoter. Treatment with these vesicles resulted in strong activation of CFTR transcription in vitro in bronchial epithelial cells derived from patients, indicating that EVs can be employed as delivery vehicles for a packaged zinc finger activator in the context of next-generation genetic therapy. Vituret et al. (506) engineered EVs to efficiently deliver CFTR mRNA to correct genetic defects in recipient cells. Another approach for correcting CFTR function involves the delivery of siRNA via EVs. The conventional method of transfecting siRNA with viral-based vectors has been used to introduce genetic material into human airway epithelial cells, which, however, is complicated, costly, and time-consuming to implement. By contrast, it has been suggested that EV-based siRNA delivery can offer an alternative and potentially more convenient approach for delivering small RNAs to treat CF (507). Specifically, an antisense oligonucleotide directed to the BGas region of the CFTR locus can be loaded into EVs (508). The delivery of this antisense oligonucleotide by EVs successfully restores CFTR function in nasal cells by elevating CFTR protein levels. This implies that this approach can be further explored by utilizing patient-derived EVs to customize targeting to recipient cells that specifically need increased CFTR expression while minimizing unintended off-target effects. Notably, however, these studies have shown that the therapeutic effectiveness of encapsulated nanoparticles is reduced compared to EV-mediated delivery.

In summary, engineered EVs hold significant promise for advancing therapeutic strategies in treating cystic fibrosis through their ability to deliver therapeutic cargoes. This targeted delivery can facilitate the correction of genetic defects and address the limitations of current small-molecule treatments. Personalized therapy is a consideration for individual cystic fibrosis patients, as the disease involves specific mutations in the CFTR gene. Therefore, future studies should focus on tailoring EV therapies to the unique genetic profiles of individual cystic fibrosis patients, potentially increasing the precision and effectiveness of treatments.

4.7.4. Pulmonary fibrosis.

The therapeutic potential of engineered EVs has also been investigated in the context of pulmonary fibrosis. Clodronate is commonly employed to induce apoptosis for the depletion of macrophages (509), and Sun et al. (510) used clodronate-loaded liposomes in an attempt to reduce macrophages and thereby reduce macrophage clearance of the EVs, thereby enhancing the targeting efficiency of EVs to fibrotic lung tissue. However, when loaded with clodronate, these liposomes were found to be inadequate for the effective delivery of anti-fibrotic drugs to the lungs primarily due to the limited ability of nanoparticles to penetrate the extracellular matrix (510). A hybrid system has been developed to address this limitation by combining clodronate-loaded liposomes with EVs derived from fibroblasts. This hybrid approach successfully induces apoptosis in Kupffer cells through passive targeting after intravenous injection, leading to a significantly reduced accumulation of intravenously injected EVs in the liver. Interestingly, this hybrid system primarily accumulates in fibrotic lung tissue and significantly improves penetration within pulmonary fibrotic tissue through targeted delivery. The hybrid system has also been confirmed to specifically target lung myofibroblasts to deliver antifibrosis drugs (511). Another research group has harnessed EVs for the therapeutic delivery of miR to address pulmonary fibrosis (512). They designed a functional biomaterial consisting of miR-29-loaded EVs and extracellular matrix. This EV-containing engineered biomaterial successfully inhibited the TGFβ1/Smad3 signaling pathway and effectively reduced the accumulation of pulmonary fibrosis in mice, thus showing promise

as a platform for gene therapy in the treatment of pulmonary fibrosis. Also, other groups have loaded antifibrotic factors such as nintedanib, pirfenidone, glycyrrhetinic acid, TGF-β1 inhibitor, or SMAD4 inhibitor into EVs to suppress pulmonary fibrosis and achieve improved pulmonary function (513–517). Moreover, miR-486-5p was engineered into MSC-EVs to improve the survival rate and pathological changes in pulmonary fibrosis (518). Interestingly, matrix metalloproteinase-19 can be engineered on the surface of EVs to efficiently degrade extracellular matrix around the fibrotic lung (519). Long et al. (520) engineered EVs to display CD38 antigen receptors, which can support targeting senescent alveolar epithelial cells and attenuate pulmonary fibrosis.

Collectively, engineered EVs have shown potential in treating pulmonary fibrosis by enhancing targeting to fibrotic lung tissue, with systems like clodronate-loaded liposomes combined with fibroblast-derived EVs improving drug delivery. Research also focuses on loading EVs with antifibrotic agents or miRs, and engineering them to degrade extracellular matrix or target senescent cells, offering promising therapeutic approaches.

4.7.5. Lung injury.

In the context of attenuating acute lung injury, much of the research has focused on engineering the protein composition of EVs to provide an efficient anti-inflammatory drug candidate. In the search to identify novel approaches against various strains of SARS-CoV-2, which enters host cells via the ACE2 receptor, El-Shennawy et al. (521) isolated circulating EVs that express ACE2 proteins and compared the inhibitory effectiveness of these EVs with vesicle-free recombinant human ACE2. Notably, the vesicles exhibited significant efficacy in preventing SARS-CoV-2 infections. Moreover, these vesicles could provide protection to transgenic mice against lung injury and mortality induced by a wide range of SARS-CoV-2 strains. In a related study, a specialized type of EVs authors called activated specialized tissue effector extracellular vesicles (ASTEX) was created using genetically enhanced skin fibroblasts to counteract SARS-CoV-2 infection (522). These vesicles expressing beta-catenin and gata4 are able to reduce viral replication, mitigate virus-induced cell damage, and prevent the shedding of ACE2, which potentially inhibits the dysregulation of the renin-angiotensin-aldosterone system. Notably, these effects are specific to the ASTEX and cannot be fully replicated by other types of therapeutic EVs. For the development of a potent EV-based vaccine candidate against viruses, Tsai et al. (523) have demonstrated that mRNA encoding SARS-CoV-2 spike proteins loaded into EVs can induce a long-lasting cellular and humoral response against the spike protein. Moreover, these vesicles have been shown to be safer compared to lipid nanoparticle-based mRNA vaccines (523). Additionally, engineered EVs containing the virus receptor-binding domain have effectively promoted virus clearance in hamsters, presenting a promising vaccine candidate for preventing COVID-19 in this case (524).

Also, EVs have been modified to overexpress CD24, which acts as an immune modulator in infection by binding to sialic acid-binding Ig-like lectin 10 (Siglec 10) on inflammatory cells (525–527), and these CD24 expressing EVs have even been tested clinically in intensive care (see section 5.3), resulting in significantly reduced cytokine and chemokine secretion from human immune cells. Moreover, Kim et al. (528) conducted research involving the engineering of EVs for the targeted pulmonary delivery of a RAGE-binding peptide (RBP), which is an anti-inflammatory peptide. To efficiently incorporate the peptide into EVs, the group used an integral membrane protein, Lam2b, and also introduced additional curcumin to enhance the anti-inflammatory effects. Another anti-inflammatory peptide targeting the programmed cell death receptor 1 (PD-1) was loaded into EVs, resulting in therapeutic efficacy in sepsis-induced acute lung injury (529). Additionally, Park et al. (530) developed EV-mimetic vesicles derived from MSCs, called synthetic eukaryotic vesicles (SyEVs), characterized by high yield and purity. Importantly, the SyEVs have maintained anti-inflammatory effects, similar to EVs released by MSCs. The authors demonstrated that loading of the SyEV with an anti-inflammatory peptide targeting myeloid differentiation primary response 88 (Myd88) results in synergistic suppression of lung injury, where the MSC-SyEVs and the loaded therapeutic peptide together have more than twice the efficacy of each component alone (530).

The curcumin-loaded EVs demonstrated superior intracellular curcumin delivery efficiency compared to curcumin on its own or curcumin loaded into unmodified EVs. Furthermore, vesicles from fibroblasts can be engineered by loading IL-4 and IL-10, and these EVs exhibit enhanced anti-inflammatory effects in the lungs of an acute lung injury model (531). Chen et al. (532) generated polymeric nanoparticles with a hybrid membrane from platelet-derived EVs and showed that the vesicles can eliminate a specific subset of activated neutrophils.

He et al. conducted a study based on screening data of miR levels in BALF-derived EVs (533), and they found that miR-223-3p is significantly downregulated in response to LPS. Subsequently, they loaded a specific inhibitor targeting miR-223-3p into EVs and assessed their therapeutic efficacy in mice with LPS-induced lung injury. The results showed that these loaded vesicles enhanced cell viability and reduced apoptosis and inflammation in alveolar macrophages, ultimately

reducing acute lung injury. Specific inhibitors targeting miR-125a-5p or miR-155 were also loaded into therapeutic EVs, significantly alleviating the inflammatory response (534, 535). Han et al. (536) used antisense oligo-loaded EVs to specifically downregulate long noncoding RNAs related to bacteria-induced lung injury. Another research group has made efforts to develop engineered EVs loaded with anti-inflammatory cargoes such as [5-(p-fluorophenyl)-2-ureido]thiophene-3-carboxamide (TPCA-1), Club cell protein 16, or resveratrol/celastrol (537–539). These vesicles can be sourced from platelets, which naturally possess an affinity for sites of inflammation (540). Importantly, these vesicles can provide significant therapeutic benefits by inhibiting the infiltration of inflammatory cells into the lungs and mitigating local cytokine storms when compared to those treated with the free drug, suggesting that these vesicles have the potential to be a versatile platform for selectively targeting various inflammatory sites. Another group has engineered vesicles to deliver a specific noncoding RNA for inducing therapeutic efficacy in lung injury (541, 542). For example, Ma et al. (543) loaded miR-182-5p into EVs to suppress acute lung injury through modulation of NADPH oxidase 4 (NOX4)/dynamin-related protein-1 (Drp-1)/NLRP3 signaling.

These findings indicate that engineered EVs are emerging as powerful tools for treating acute lung injury, particularly in the context of viral infections, including COVID-19. The ability of the engineered EVs to carry various therapeutic agents and overexpress certain molecules paves the way for innovative treatments that can effectively mitigate inflammation and promote viral clearance. Continued exploration of EV engineering and its underlying mechanisms may lead to significant advancements in managing acute lung injury.

4.7.6. Summary engineered EVs in pulmonary disease models.

Engineered EVs are increasingly being used to deliver therapeutic substances such as proteins and nucleic acids for treating various lung diseases. EVs offer advantages over traditional drug delivery systems due to their biocompatibility, stability, and ability to target specific cell types, enhancing drug efficacy and reducing side effects. The development of engineered EVs represents a significant advancement in personalized and targeted medicine, offering a new approach to treating a range of pulmonary diseases. Their ability to transport bioactive compounds with minimal side effects marks a shift towards more efficient therapies, especially in conditions like asthma, cystic fibrosis, and acute lung injury. Future research should focus on optimizing EV production processes and targeting mechanisms to fully harness their potential in a clinical setting.

4.8. Overall Conclusions of Therapeutic EVs

The therapeutic potential of EVs derived from various cellular sources, particularly MSCs, presents a promising avenue for treating respiratory diseases characterized by inflammation and fibrosis. In most cases, EVs can effectively mimic their parent cells' therapeutic actions while overcoming some logistical and safety concerns associated with direct cell therapies. MSC-derived EVs demonstrate significant anti-inflammatory properties and modulate various signaling pathways through their diverse cargo, which includes proteins, mRNAs, and noncoding RNAs. Additionally, the ability to engineer these EVs enhances their therapeutic efficacy, allowing for targeted treatments tailored to specific lung pathologies.

This research highlights that EVs from either stem cells or nonstem cells, such as immune cells, can significantly alleviate inflammation and promote tissue repair in various lung disease models. The mechanisms of action involve complex interactions between EV cargos and recipient cells, suggesting a multifaceted approach to therapy. Since the comparability of the therapeutic activity between stem cell-derived EVs and nonstem cell-derived EVs has yet to be clearly defined, systematic comparisons of their activity and the specific vesicular cargoes involved are essential. Also, additional efforts are needed to enhance the therapeutic activity of nonstem cell-derived EVs, positioning them as potentially valuable therapeutic entities alongside MSC-derived EVs.

Further research is essential to unravel the specific mechanisms of action of EVs, optimize their production and application, and conduct clinical trials to establish their safety and efficacy in human patients. Investigating vesicular proteins and noncoding RNAs as potential therapeutic components within EVs warrants further exploration, as this could lead to innovative strategies for managing chronic respiratory diseases and severe cases of acute lung injury or COPD exacerbations. Additionally, it is crucial to address other key challenges, including the standardization of EV isolation and characterization methods, ensuring the consistency and purity of EV preparations, understanding the pharmacokinetics and dynamics of therapeutic EVs, and establishing robust clinical trial designs.

Moreover, engineered EVs are emerging as a transformative technology in treating various chronic lung diseases and acute lung injury. Their distinct properties, such as biocompatibility, stability, ability to cross biological barriers, and potential for targeted delivery, position them as optimal nanocarriers for therapeutic agents. By adapting EVs to deliver proteins, nucleic acids, peptides,

or other bioactive substances, researchers leverage their capacity to selectively target specific cell types, thereby enhancing therapeutic efficacy and minimizing side effects.

To fully realize the capabilities of EVs as delivery vehicles for therapeutic payloads, it is essential to optimize several key characteristics. Depending on the specific clinical application and intended route of administration, this optimization may involve modifying the biodistribution and pharmacokinetics of EVs and enhancing the loading of therapeutic cargo either on the vesicle's surface or within its internal space. Achieving these modifications will require extensive research focused on identifying and understanding the roles of molecules enriched in and associated with EVs. This pursuit could lead to breakthroughs in targeted therapies and personalized medicine for patients with complex lung conditions.

5. CLINICAL STUDIES WITH THERAPEUTIC EVs

The initial clinical indications of the therapeutic potential of MSC-EVs, in general, have sparked a recent surge in clinical testing of EV-based therapies also in the respiratory field. Over 15 registered clinical trials (www.clinicaltrials.gov) currently focus on disorders such as severe viral or bacterial pneumonia and ARDS. Among these trials, five have been registered as completed, and studies with published results are detailed in **TABLE 1**. Moreover, we here review the clinical trials that utilized therapeutic EVs from nonstem cells to treat lung injury specifically.

5.1. MSC-EV COPD

While MSC-based therapies have been evaluated in clinical trials for COPD (548), the clinical application of MSC-EVs remains in its early stages. Interestingly, Harrell et al. (320) have investigated the therapeutic effects of an MSC-derived product they termed exosome-derived multiple allogeneic protein paracrine signaling (Exo-d-MAPPS) in COPD patients. This clinical trial is not registered on the "www.clinicaltrials.gov" website, limiting the availability of information on study design details. However, according to their published findings, administration of EVs by inhalation to thirty COPD patients resulted in significant pulmonary improvements in all participants, with no reported adverse effects. Unfortunately, the study did not have a control group that received vehicle/placebo inhalation. Still, these findings support the notion that MSC-EVs hold promise as a therapeutic option for COPD patients, warranting further validation in larger and better-controlled clinical trials.

5.2. MSC-EVs for Lung Injury

EVs derived from various tissue sources of MSCs, including bone marrow, adipose tissue, umbilical cord, and amniotic fluid, are being clinically tested as therapeutics against lung injury. Shi et al. (549) have investigated the safety and tolerance of nebulized adipose-derived MSC-EVs in healthy volunteers. The nebulization of MSC-EVs was well tolerated in all volunteers, with no serious adverse events observed from the initiation of nebulization up to 7 days postadministration, suggesting that MSC-EVs have short-term safety after administration via inhalation.

Based on these safety evaluations, Zhu et al. (544) have provided insights into the safety and efficacy of aerosol inhalation of EVs derived from adipose-derived MSCs in patients with acute pulmonary inflammation induced by COVID-19 in the MEXCOVID trial. This trial enrolled seven eligible patients who received a daily dose of inhaled MSC-EVs (2×10^8 particles) for 5 consecutive days. Notably, all patients exhibited good tolerance to the treatment, with no evidence of adverse events during the nebulization period. Additionally, varying degrees of resolution of pulmonary lesions were observed in the patients, with noteworthy improvement observed in four out of seven individuals. Nevertheless, further and larger trials are needed to comprehensively assess both the safety profile and efficacy of inhaled MSC-EVs. A parallel interventional trial conducted in Russia investigated the safety and efficacy of aerosol inhalation of MSC-EVs in treating 30 patients with severe COVID-19-induced lung disease (NCT04491240). Participants in this study received EVs ($0.5–2 \times 10^{10}$ particles) twice a day for 10 days. The clinical outcomes of this trial similarly reported no occurrences of adverse events. However, neither of these studies included a placebo control arm, which reduces their conclusiveness.

MSC-EVs are also undergoing evaluation as intravenously administered therapeutics in various other clinical trials for virus-induced lung disease. Sengupta et al. (545) have isolated bone marrow-derived MSC-EVs in FDA-registered facilities that meet the criteria for Current Good Manufacturing Practices. COVID-19 patients received a single intravenous dose of MSC-EVs, and safety and efficacy were assessed from days 1 to 14 posttreatment. The study has successfully met all safety endpoints, with no adverse events confirmed within 72 h of EV administration. Based on the results of this study, the company Direct Biologics has completed a phase 2 clinical trial (NCT04493242) in hospitalized adult COVID-19 patients with moderate-to-severe ARDS (546). This is the first reported multicentered, randomized, double-blind, and placebo-controlled trial performed with EVs for the treatment of COVID-19 or any other respiratory disease and

Table 1. *Current clinical trials of EVs for the treatment of lung diseases*

Source of EVs/ Disease	Status	Study Design (n = Sample Size)	Route of Administration	Country/ Registration Year	Study Identifier
MSCs					
ARDS	Ongoing (phase I and II)	Placebo-controlled, randomized, double-blinded	Inhalation	China/2020	NCT04602104
COVID-19 Pneumonia	Ongoing (phase II)	Placebo-controlled, randomized, double-blinded	Inhalation	Russia/2020	NCT04602442
COVID-19 ARDS	Ongoing (phase I and II)	Randomized, double-blinded	Intravenous	US/2021	NCT04798716
COVID-19	Ongoing (phase II and III)	Placebo-controlled, randomized, double-blinded	Intravenous	Indonesia/2022	NCT05216562
COVID-19 Lung fibrosis	Ongoing (observational)	Ex vivo study	Ex vivo study	Germany/2022	NCT05191381
COVID-19 ARDS	Ongoing (phase I and II)	Placebo-controlled, randomized, double-blinded	Intravenous	Brazil/2023	NCT06002841
Healthy	Completed (phase I)	Open label ($n = 24$)	Inhalation	China/2020	NCT04313647 (Ref. 549)
COVID-19 Pneumonia	Completed (phase I and II)	Placebo-controlled, randomized, double-blinded ($n = 30$)	Inhalation	Russia/2020	NCT04491240
COPD	Completed	($n = 30$)	Inhalation		Ref. 320
Adipose tissue MSCs					
COVID-19	Completed (phase I)	Open-label ($n = 24$)	Inhalation	China/2020	NCT04276987 (Ref. 544)
Umbilical cord MSCs					
COVID-19 ARDS	Ongoing (phase I)	Placebo-controlled, randomized, double-blinded	Intravenous	US/2022	NCT05387278
COVID-19	Ongoing (phase I)	Placebo-controlled, randomized, single-blinded	Inhalation	China/2023	NCT05787288
COVID-19	Ongoing (phase I)	Single-blinded	Inhalation	China/2023	NCT05808400
Bone marrow MSCs					
COVID-19 Dyspnea	Ongoing (phase I and II)	Placebo-controlled, randomized, double-blinded	Intravenous	US/2021	NCT05116761
ARDS	Ongoing (phase I and II)	Placebo-controlled, randomized, double-blinded	Intravenous	US/2021	NCT05127122
ARDS	Ongoing (phase III)	Placebo-controlled, randomized, double-blinded	Intravenous	US/2022	NCT05354141
COVID-19 ARDS	Completed (phase II)	Placebo-controlled, randomized, double-blinded ($n = 102$)	Intravenous	US/2020	NCT04493242 (Ref. 546)

Continued

Table 1.—*Continued*

Source of EVs/ Disease	Status	Study Design (n = Sample Size)	Route of Administration	Country/ Registration Year	Study Identifier
T cells					
COVID-19 Pneumonia	Ongoing (phase I)	Open-label	Inhalation	Turkey/2020	NCT04389385
CD24-expressing T-REx-293 cells					
COVID-19	Ongoing (phase I)	Open label	Inhalation	Israel/2021	NCT04747574
COVID-19	Ongoing (phase II)	Placebo-controlled, randomized, double-blinded	Inhalation	Israel/2021	NCT04969172
COVID-19	Completed (phase II)	Randomized, single-blinded ($n = 91$)	Inhalation	Greece/2021	NCT04902183 (Ref. 551)

ARDS, acute respiratory distress syndrome; COPD, chronic obstructive pulmonary disease; EV, extracellular vesicle; MSC, mesenchymal stem cell.

showed evidence of clinical efficacy without any serious adverse events being reported. In a subgroup analysis of participants aged 18 to 65 years experiencing respiratory failure, the intravenous administration of EVs resulted in a significant reduction in 60-day mortality compared to the placebo treatment group, further emphasizing the importance of these observations.

Another recently published study by Zamanian et al. (547), studied the effect of intravenously injected placenta-MSC-derived EVs in 45 patients with severe COVID-19 and ARDS. Twenty-one patients were given active treatment, and 24 were given the control infusion. The study reports a significant reduction in mortality ratio in the intervention arm versus the control arm, with 19% versus 54% mortality, respectively. This was observed even though the patient's oxygen saturation at admission was lower in the intervention arm versus the control arm (74 vs. 86%). The dose was 2×10^9 of MSC EVs per kilogram of body weight infused intravenously over 30 min on 2 consecutive days. Thus the dose was high compared to many other studies, reaching 10^{11} for a 70-kg patient.

Thus several studies have now been published showing that MSC-EVs are safe to give intravenously or inhaled to patients with ARDS. Of course, it is possible that MSC-EVs could also be effective in other causes of ARDS beyond COVID-19, which would require additional clinical testing that is currently ongoing.

5.3. Other Therapeutic EVs for Lung Injury

EVs released by immune cells can potentially hinder the biogenetic synthesis of viral particles by targeting viral RNA polymerases, resulting in the failure of viral replication (550). One clinical trial specifically focuses on the safety and efficacy of inhaled EVs derived from virus-specific T cells for inhibiting virus infection (NCT04389385). T cells obtained from donors are activated in vitro and expanded by exposure to viral peptides together with immune-stimulatory cytokines. These specific virus peptides selectively activated T cells to secrete potent EVs that carry IFN-γ. This EV-based therapy is expected to exhibit potential efficacy in treating the early stages of pneumonia in COVID-19 patients; however, the current progress of this trial is unknown as per the website (www.clinicaltrials.gov).

As mentioned above, Shapira et al. (525) engineered HEK293 EVs to overexpress surface molecule CD24, a molecule with broad anti-inflammatory potential also known as heat-stable antigen (HSA). CD24 inhibits NF-κB signaling and the production of inflammatory cytokines from immune or inflammatory cells. Specifically, CD24 binds to Siglec-10, expressed on many immune and inflammatory cells. This group has tested the therapeutic efficacy of CD24-EVs in various lung disease models (asthma, pulmonary fibrosis, sepsis, and ARDS), with significant therapeutic effects in most models, arguing that CD24-expressing EVs could be developed as drugs (527). The therapeutic efficacy of inhalation of EVs carrying CD24 has been clinically tested in phase I (NCT04747574) and phase II trials (NCT04969172). The CD24-EVs are given by inhalation through a standard nebulizer. Subsequently, patients with lung injury induced by COVID-19 are treated with various concentrations of the engineered EVs once daily for 5 days. No adverse events were observed, but cytokine and chemokine production are significantly suppressed in all patients receiving active treatment. Interestingly,

CD24-EVs are able to specifically suppress the host response to damage-associated molecular pattern (DAMP) without affecting pathogen-associated molecular pattern (PAMP) recognition, which might be essential for effective inhibition of excessive inflammatory processes that can be damaging to the body (526). Additionally, another phase II study was performed in Greece to assess the safety and efficacy of the inhaled CD24-expressing EVs in COVID-19 patients (NCT04902183). In this study, patients were randomized to receive either 10^9 or 10^{10} CD24-EVs for 5 consecutive days by inhalation and were then monitored for 28 days. Significant reductions in inflammatory markers, including C-reactive protein, lactate dehydrogenase, and proinflammatory cytokines, were observed. By contrast, the levels of the anti-inflammatory cytokine IL-10 increase following EV treatment (551). Moreover, the CD24 vesicles have exhibited a favorable safety profile without drug-drug interactions, which further suggests that inhaled CD24 EVs may be helpful in treating lung injury and/or ARDS.

5.4. *Overall Conclusions of Clinical Studies with Therapeutic EVs*

The expanding field of therapeutic EVs, particularly those derived from MSCs, has demonstrated promising clinical potential in treating respiratory conditions such as COPD, severe lung injury, ARDS, and viral or bacterial pneumonia. More clinical trials are required for COPD, and EVs are most likely suitable for short-term treatment of, for example, exacerbations of COPD. They are, however, less likely to be suitable for regular treatment of chronic respiratory diseases, primarily because of the probable high cost of the treatment itself. Regarding asthma, there is, unfortunately, relatively limited information available regarding the clinical role of EVs or the therapeutic effects of different types of EVs. However, we suggest that the MSC-EV therapeutics effective in COVID-19-induced lung injury may also be effective in other types of lung injury, asthma, or even COPD exacerbations.

Current clinical trials indicate that both inhaled and intravenously administered MSC-EVs are well-tolerated and exhibit encouraging efficacy, notably in reducing mortality rates among patients with severe respiratory infections such as COVID-19. Additionally, other engineered EVs, such as those from immune cells and modified HEK293 cells expressing anti-inflammatory molecules, further underscore the diverse therapeutic possibilities of EVs in managing lung diseases.

While EVs show promise as novel therapeutic tools for modulating the lung cell environment and downstream signaling, several limitations should be addressed regarding their clinical application. One significant challenge is the lack of a comprehensive understanding of the mechanisms underlying EV function in physiological settings, particularly for MSC-EVs. Additionally, the techniques employed for the isolation and purification of EVs for large-scale production have yet to achieve standardization, although substantial progress is being made. Ultracentrifugation, a commonly used method to isolate EVs, has significant drawbacks, primarily due to its limited capacity for producing sufficient quantities for wide clinical distribution (552). In contrast, alternative methods, such as tangential flow filtration, are considered superior for providing more reproducible EV batches from large culture volumes (553).

Another limitation of EV therapeutics is the lack of a consensus on appropriate storage conditions that preserve the EV products' intact structure to maintain therapeutic function. While freezing conditions at −80°C have been shown to stably maintain RNA and protein profiles over long periods (347), repeated freeze-thaw cycles can lead to aggregation and a reduction in particle numbers (554). Employing lyophilization for more stable and manageable storage of EV-based medicinal products could address this challenge and potentially extend the shelf life of these products. Therefore, alternative strategies to fully control the handling, maintenance, and stable transportation of EVs need to be considered.

Detailed pricing for EV-based therapeutics is currently unavailable, as no widely approved EV medicinal products exist. EVs are generally produced in comparably limited quantities, making it challenging to obtain low-cost products. However, a growing body of research focuses on developing novel strategies to achieve higher EV yields, hopefully reducing the cost of EV therapeutics manufacturing (530, 555, 556). Also, the cost of EV products can vary significantly depending on factors such as the EV source and production scale. Additionally, preclinical and clinical development expenses and the regulatory process should be considered, making the total cost likely to be high and comparable to other advanced biologic medicines.

6. BACTERIAL OUTER MEMBRANE VESICLES AND LUNG DISEASES

All bacteria release extracellular vesicles, which can contribute to human disease or even be used for therapeutic or preventive treatments. Outer membrane vesicles (OMVs) are nanosized proteoliposomes originating from the outer membrane of Gram-negative bacteria and naturally carry PAMPs, for example, endotoxin. These vesicles are produced ubiquitously in culture and organs during infection, and their significant roles in host-microbe interactions are now widely acknowledged. Emerging data

strongly indicate that bacterial OMVs play important roles in the pathogenesis of bacterial infection-mediated pulmonary inflammatory responses. Here we review preclinical studies showing the pathogenic role of OMVs in lung diseases, and we discuss the diagnostic and therapeutic potential of these vesicles (**FIGURE 15**).

6.1. Pathological Function

Indoor dust contains OMVs, which can trigger severe inflammation and worsening inflammatory pulmonary disorders such as asthma (557, 558). Kim et al. (559) detected LPS^{+} OMVs in the indoor dust collected from a bed mattress, and the vesicles could activate human airway epithelial cells and mouse macrophages in vitro. Also, the intranasal administration to mice of low doses of dust-derived vesicles over four weeks induced a neutrophilic respiratory inflammation phenotype. This phenotype was associated with the infiltration of Th1 and Th17 cells into the lungs, which is reversed upon treating the dust with polymyxin B which neutralizes endotoxin. The same group reported that *Escherichia coli* OMVs induce emphysema in mice through IL-17A-mediated neutrophilic inflammation (560). Exposure of airways to OMVs leads to an elevation in the production of proinflammatory cytokines, including TNF-α and IL-6. Moreover, repeated inhalation of *E. coli* OMVs over a 4-wk period induces emphysema, accompanied by increased elastase activity, in the mice. The emphysema and enhanced elastase activity induced by OMVs are mitigated in the absence of the IL-17A gene, suggesting that IL-17-dependent neutrophilic inflammation is involved in this disease model. *H. influenzae* also secrete OMVs, which can contribute to neutrophilic asthma through induction of IL-17 (561). The dust-derived OMVs have also been investigated in the field of lung injury. Meganathan et al. (562) reported that vesicles present in organic dust can even cause lung injury. Specifically, OMVs have been observed to release inflammatory mediators from lung epithelial cells and immune cells. Also, intranasal administration of dust OMVs in mice results in significantly elevated inflammatory cytokine levels together with enhanced deposition of collagen around the airways (562).

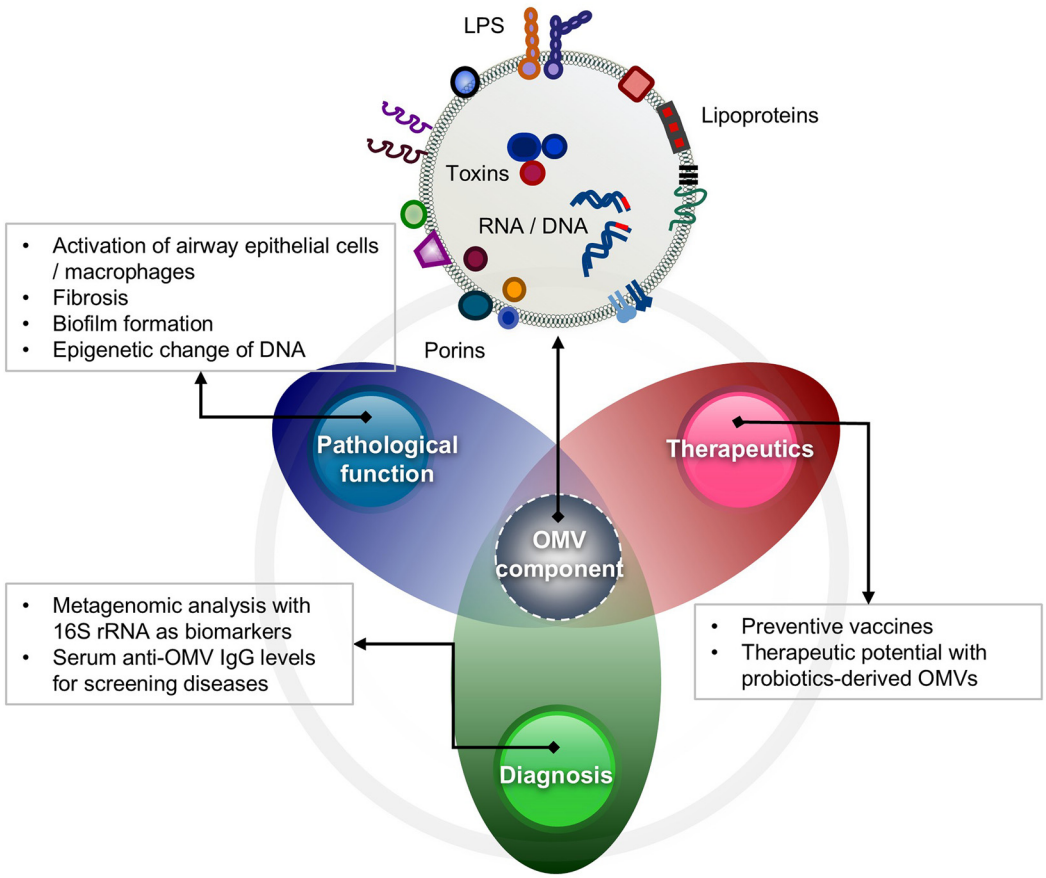


FIGURE 15. The composition of outer membrane vesicles (OMVs) and their contributions to pathological processes and their potential biomedical applications. OMVs comprise various pathogen-associated molecular patterns such as lipopolysaccharide (LPS), lipoproteins, porins, and genes, which can overactivate host cells through cytokine induction. OMVs contain specific 16S rRNA that can be used to elucidate the microbial composition related to lung diseases. Moreover, multiple outer membrane proteins and other molecules on OMVs can make the vesicles efficient adjuvants for specific pathogen vaccines.

P. aeruginosa is well known to contribute to severe lung diseases such as CF or COPD. This bacterial species has the ability to release OMVs, which can induce inflammatory responses in the lungs (563, 564). Lee et al. (565) found that *P. aeruginosa* OMVs can induce extensive epigenetic changes in DNA methylation in human macrophages, and these changes are associated with a dysfunctional innate immune response. Also, the vesicles downregulate MHC molecules in human lung macrophages, potentially leading to decreased pathogen clearance (566). *Pseudomonas* quinolone signal (PQS)-regulated biofilms are considered to be a crucial virulence factor in the pathogenesis of *P. aeruginosa*-induced CF (567). Interestingly, the PQS signal can induce OMV production via a biophysical mechanism, in which the OMV production is maximally enhanced during biofilm dispersion (568). Further, *P. aeruginosa* OMVs can actively degrade extracellular proteins, lipids, and DNA, and this capability enables the coordinated and controlled degradation of biofilm matrix components, particularly in chronic lung infections.

Mycobacterium abscessus, a common species in CF patients, has been shown to significantly release OMVs. These vesicles can contribute to resistance against the antibiotic clarithromycin treatment by carrying increased levels of ribosomal subunits, which can target clarithromycin (569). Moreover, Yang et al. (570) showed that lung commensal bacteria can regulate a profibrotic inflammatory cytokine network through OMVs, leading to severe pulmonary fibrosis. Specifically, OMVs have been intranasally administered into bleomycin-challenged mice and shown to induce IL-17A and IL-17B cytokines as well as immune cell infiltration.

6.2. OMVs as Diagnostics

Bacterial OMVs are considered to be a fingerprint of the parent bacterial cells due to their species-specific cargos, including proteins, RNAs, and DNAs, and they can be detected in multiple body fluids for diagnostic purposes. Samra et al. (571) isolated OMVs from urine samples from allergic asthmatics, extracted 16S ribosomal RNA, analyzed the bacterial composition, and reported associations between these data and disease parameters. As a result, the group suggested that bacterial OMVs in urine may exhibit distinctive features in asthma diseases, and they argued that there is a significant relationship between total IgE and eosinophils. The same group has also reported that urine-derived OMVs potentially can be used as biomarkers for monitoring asthma diseases in children (572). Further, a unique pattern of bacterial diversity in allergic asthma has been confirmed, with a notable increase in the *Actinobacteria phylum* and the *Sphingomonadaceae* families in asthmatic children. In a similar study, Lee et al. (573) observed consistent alterations in OMVs found in the urine of children with asthma, including specific changes in bacterial diversity and functional profiles. However, these studies would need to be extensively expanded before the findings can be used in the clinical management of asthma.

Interestingly, isolated OMV concentration in blood has been suggested to reflect the progression of asthma by identifying a distinct microbiota. Thus Lee et al. (574) investigated the bacterial composition of blood-derived OMVs from 190 adults with asthma and healthy controls. At the genus level, differences in relative abundance were observed in 24 bacterial genera between asthmatic patients and controls. Additionally, at the phylum level, *Bacteroidetes* exhibited higher abundance in asthmatics, whereas *Actinobacteria*, *Verrucomicrobia*, and *Cyanobacteria* were more prevalent in healthy controls. Also, An et al. (575) isolated OMVs from exhaled breath condensates, which are comprised of aerosol and volatile compounds, thus providing a noninvasive means to analyze and gain insights into the bacteria present within the lungs. The group confirmed significant differences between the lung microbiome of individuals with asthma and healthy controls by metagenomic analysis with OMV-derived 16S ribosomal RNA, and this suggests the usefulness of OMV-based microbiome analysis for understanding different asthma phenotypes. Since bacterial OMVs can be detected in indoor dust and cause neutrophilic pulmonary inflammation (559), serum IgG antibody levels against dust-derived OMV were evaluated in asthmatic patients and controls (576). That study revealed a significant upregulation of antidust OMV IgG levels in the serum of asthmatic patients with a noneosinophilic phenotype. In the same context, indoor dust OMVs were isolated from mattresses, and specific antibodies against dust OMVs in the serum were used to discriminate asthma patients from healthy controls (577). Quantitative ELISA indicated that anti-indoor dust bacterial OMV IgG, IgG1, and IgG4 antibody titers in serum are significantly increased in asthmatic patients versus healthy controls.

In the context of the diagnosis of COPD, Kim et al. (578) focused on the microbiome of lung tissue-derived OMVs. They extracted OMVs from the lungs from age- and sex-matched COPD patients, nonsmokers, and healthy smokers using numerous enzymatic treatments combined with ultracentrifugation, followed by 16S ribosomal RNA gene sequencing. In the COPD group, lung tissue-derived OMVs exhibited higher species diversity, with a notable prevalence of *Firmicutes* compared to the other groups. Also, the microbiomes of OMVs are distinct from those of the whole lung tissue with regard to diversity, dominant organisms, and principal component analysis, highlighting the potential utility of OMVs as

unique diagnostic tools in COPD. Furthermore, a proteomics study of the biofilm matrix including OMVs proteins was performed to screen for the bacterial pathogenic proteins related to CF (579). Specifically, the biofilm-growing *Burkholderia multivorans* C1576 strain was selected to isolate and characterize OMVs. The proteomic analysis of OMVs revealed the presence of proteins implicated in host tissue invasion and defense against immune responses. This suggests that the identified OMV proteins might serve as potential biomarkers for bacterial overgrowth in the lungs in patients with CF.

6.3. OMVs as Therapeutics or Vaccines

Ding et al. (580) published data suggesting that low-dose LPS may prevent the development of allergic sensitization in an animal model of allergic asthma. Based on this finding, their group speculated that bacterial OMVs, which harbor LPS, might mediate the protective effects of bacteria on asthma sensitization. They successfully isolated vesicles from *P. aeruginosa* and then evaluated the immunomodulatory capacity of these OMVs in a mouse model induced by OVA. Their results showed that pretreatment with *P. aeruginosa*-derived OMVs reduced airway hyper-responsiveness, perivascular inflammation, and serum OVA-specific IgE levels in experimental asthma models, suggesting that mice pretreated with *P. aeruginosa*-derived OMVs may be protected against subsequent sensitization with OVA. Also, *Micrococcus luteus*-derived OMVs can suppress neutrophilic asthma by modulating miR expression in airway epithelial cells (581).

Additionally, the potential protective role of *P. aeruginosa* OMVs has been investigated in CF and lung injury models (582–585). OMVs not only mitigated tissue injury and respiratory dysfunction but also led to a decrease in lung wet-to-dry weight ratio and total protein concentration. Following OMV preconditioning, the numbers of macrophages, neutrophils, and lymphocytes are significantly reduced in the lung injury model. Moreover, OMVs significantly regulate the balance between regulatory T cells and Th17 cells. Furthermore, the protective effect of *P. aeruginosa* OMVs has been assessed in a bacteria-infected mice model (586, 587). OMVs from *P. aeruginosa* were first isolated, then formulated with an aluminum phosphate adjuvant, and administered as a vaccine in a mouse model of acute lung infection. The results demonstrated that active immunization with OMVs leads to decreased bacterial colonization, reduced cytokine secretion, and reduced tissue damage in the lung tissue, thereby protecting mice against lethal challenges with bacteria. Furthermore, cytokine assays confirmed that immunization with OMVs efficiently induces a mixed cellular immune response in mice. In a similar study, *P. aeruginosa* OMVs were conjugated with alginate, a substance known to protect *P. aeruginosa* against host immune defenses (588), and were evaluated to assess the immunogenic response in mice (589). The synthesized alginate-OMV conjugate vaccine protected mice against *P. aeruginosa* infection by inducing high titers of opsonic and anti-alginate antibodies. Another study demonstrated that intranasal administration of *Clostridium butyricum*-derived OMVs alleviated LPS-induced lung injury by suppressing the TLR4/MyD88 signaling pathway (590).

The therapeutic potential of probiotics-derived OMVs has also been proposed in asthma (591, 592). Specifically, Lee et al. (591) isolated OMVs from *Lactococcus latis* and evaluated their function against airway hyperresponsiveness and inflammation in mice. Intranasal treatment with OMVs resulted in a significant reduction in airway hyperresponsiveness, eosinophil numbers, cytokine secretion, and mucus production. Moreover, *L. lactis*-derived OMV treatment shifted immune responses from a T2 to T1 profile by inducing dendritic cells to produce IL-12, thus suggesting a potential benefit for treating allergic asthma. Also, oral administration of OMVs isolated from *Lactobacillus paracasei*, which is known to reduce lung inflammation, has been shown to decrease airway resistance and inflammation in mice with neutrophilic asthma (593).

6.4. Overall Conclusions of OMVs

Bacterial OMVs play a multifaceted role in lung diseases, demonstrating both pathogenic and therapeutic potentials. Preclinical studies have highlighted the detrimental effects of OMVs derived from Gram-negative bacteria, such as *E. coli* and *P. aeruginosa*, in exacerbating inflammatory responses and conditions like asthma and cystic fibrosis. However, the unique species-specific cargo of OMVs also positions them as promising diagnostic tools, enabling the identification of microbial compositions in various body fluids and offering insights into disease states. Furthermore, recent research emphasizes the therapeutic potential of OMVs, demonstrating their ability to modulate immune responses and function as vaccine candidates against bacterial infections due to their enrichment with pathogen antigens. Notably, four licensed OMV-based vaccines are currently available (594, 595), including the meningococcal group B OMV vaccine (Bexsero), which contains multiple antigens providing broad protection against meningococcus infections (596). Although OMV-based vaccines can prevent severe pulmonary diseases in the future, their toxicity profile may hinder the active development of these therapeutic platforms in clinical settings. Significant efforts are currently being made to address this challenge (597–599), aiming to treat various lung diseases more effectively while minimizing severe adverse events and ensuring prolonged immunity.

7. CONCLUSIONS AND FUTURE PERSPECTIVES

EVs have intrigued the biomedical scientific community for over five decades and are currently recognized for their potential in lung disease diagnosis and therapy. In this review, we have discussed the pathophysiological function, diagnostic biomarkers, and therapeutic application of EVs in the treatment of various lung diseases, which involves the transfer of a specific subset of vesicular proteins and RNA species leading to the regulation of biological pathways in a recipient cell. Because the clinical translation of EVs is in its early stages, several aspects of the EV field still need further technical development to ensure correct, successful, and timely translation to patient care. For example, even though we can track the biodistribution of whole EVs to certain organs, the subsequent specific cells that take up the EVs in the organ are less clear, and understanding the intracellular traffic of the EV cargo molecules requires further research. Therefore, basic research explaining such processes is still needed, in parallel with the performance of diligent clinical investigations.

To increase the quality of EV research in the future, high-quality methods need to be employed to improve EV isolation and characterization. There are ambitious efforts from the EV research community to achieve this goal, such as the MISEV guidelines and EV-Track (28–31). Of the EV articles covered in this review, only slightly more than 30 have been included in EV-TRACK, a platform that quantifies the extent to which methods are reported in a study. These articles achieved an average metric score of just 14 out of 100. We, therefore, encourage the respiratory EV field to be much more rigorous and transparent in future scientific publications of EV research, which will increase the likelihood that the proposed functions, biomarker candidates, and therapeutic effects described are truly EV associated. Furthermore, we can facilitate and increase reproducibility in the field by reporting methods and data in detail, in line with EV-TRACK (31). EV heterogeneity is also important to understand, and advanced techniques should be considered to identify different EV subpopulations, thereby enhancing the potential success of EV diagnostics and therapeutics. For example, nanoflow cytometry techniques are able to analyze EVs at the single particle level, thus enabling accurate and quantitative analysis of separated EV subpopulations (279).

As EV cargos can reflect disease processes, they have been extensively explored in biomarker discovery studies. We must also remember that asthma, COPD, and pulmonary fibrosis exist in many subgroups (phenotypes/endotypes) of respiratory disease. Therefore, it is likely that a diagnostic biomarker may be specific for a subgroup of these respiratory diseases. Further, biomarker candidates may alternatively reflect disease stage or disease progress, which may or may not be related to the disease subgroup. Therefore, we strongly suggest that future EV-biomarker discovery studies base their analysis on the very careful characterization of the patients. Further, some EV biomarker candidates may overlap between diseases, which similar active pathophysiological mechanisms in multiple diseases may explain. In these cases, the biomarker candidates may not be sufficiently specific to warrant clinical use.

As we discussed, evidence is strong that EVs play specific roles in regulating inflammation, immune responses, and lung tissue repair. In particular, MSC-derived EVs can carry anti-inflammatory molecules, growth factors, and miRs that modulate cellular signaling processes and can convey anti-inflammatory and potentially healing functions. Moreover, EVs have natural properties that make them attractive as delivery vehicles for therapeutic drugs. EVs can cross over biological barriers, including the lung epithelium, and deliver bioactive molecules to specific cells. Despite these advantages, EV-based diagnostics and therapeutics necessitate a distinct regulatory process compared to other synthetic nanoparticles, due to their biological origin and inherent complexity (600). Notably, several studies have shown that EVs from MSC, or EVs overexpressing CD24, can be effective in treating severe inflammation in the lung, when given either by inhaled our intravenous route. Extensive characterization data and evidence of safety and efficacy from both preclinical and clinical studies will support the clinical translation of EV-based drugs for lung disease. We strongly suggest that double-blind placebo-controlled approaches are utilized to provide convincing results regarding the suitability of any EV therapeutics candidate in patient care.

In summary, the future of studying and using EVs in lung diseases shows significant promise across various aspects, including biomarker discovery, disease monitoring, and innovative therapeutic interventions. Continued research is crucial to fully understand the therapeutic capabilities of EVs and overcome challenges in their clinical application. With ongoing advancements in EV biology and technology, these extracellular structures have great potential to transform the diagnosis, treatment, and management of diverse lung diseases, ultimately enhancing patient outcomes and quality of life.

SUPPLEMENTAL MATERIAL

Supplemental Figures S1–S3 and Supplemental Tables S1–S6: https://doi.org/10.5281/zenodo.14992199.

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DISCLOSURES

K.S.P. and J.L. have filed multiple patents for developing mammalian and bacterial vesicles for therapeutic purposes. K.S.P., C.L., and J.L. own equity in Exocure Sweden AB and its subsidiaries. J.L. consults for ExoCoBio Inc., South Korea.

AUTHOR CONTRIBUTIONS

K.S.P. and C.L. prepared the figures; K.S.P., C.L., and J.L. drafted the manuscript; K.S.P., C.L., and J.L. edited and revised the manuscript; and K.S.P., C.L., and J.L. approved the final version of the manuscript.

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